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Preparation, Characterization and Optimization of Oromucosal Clotrimazole Emulgel Formulation

By

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This is to certify that the thesis prepared by Mahilet Mengesha, entitled Preparation, characterization and Optimization of oromucosal Clotrimazole Emulgel formulation and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Pharmaceutics complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Contents

List of Abbreviations

ANOVA	Analysis of Variance
API	Active Pharmaceutical Ingredient
CCD	Central Composite Design
CYP	Cytochrome P
DoE	Design of Experiment
EPHARM	Ethiopian Pharmaceuticals Manufacturing Sh. Co.
2FI	Two Factor Interaction
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
HLB	Hydrophilic Lipophilic Balance
HPLC	High Pressure Liquid Chromatography
HPMC	Hydroxypropylmethyl cellulose
MIC	Minimal Inhibitory Concentration
Na CMC	Sodium Carboxymethyl Cellulose
NF	National Formulary
PAA	Poly Acrylic Acid
PRESS	Predicted Residual Sum of Squares
PROB	Probability
RH	Relative Humidity
RSM	Response Surface Method
SEPPIC	Société d'exploitation pour les produits de l'industrie chimique
SD	Standard Deviation
UAE	United Arab Emirates
UK	United Kingdom
USP	United States Pharmacopoeia
WHO	World Health Organization

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Abstract

The topical applications of a drug to the oral mucosa offer the potential advantages of delivering the drug directly to the site of action and possibly delivering the drug for an extended period of time. The environment of the oral cavity and the continual secretion and swallowing of saliva are unique problems which need to be considered to ensure successful delivery of a drug via this route. Recently, emulgel has emerged as one of the most interesting topical preparations in the field of pharmaceutics. The use of emulgel as a delivery system has several advantages such as ease of administration, increased residence time of the drug at the applied site, and better drug release and diffusion.

The objectives of this study were to formulate Clotrimazole, a practically insoluble imidazole antifungal, as oromucosal emulgel formulation, characterize, optimize, and to study the release profile and storage stability of the formulation.

Different oils were compared for their solubilizing capacity of clotrimazole and phase diagrams were constructed using Tween 80 and ethanol as an emulsifier and co-emulsifier, respectively. Two of the commonly used polymers, sodium carboxymethyl cellulose (Na CMC) and hydroxypropylmethyl cellulose (HPMC), were studied for their gelling capacity. A systematic experimental design and optimization approach was carried out using central composite design (CCD) to select study points and generate polynomial equations which describe the relationship between the dependant and independent variables. The formulated emulgels were characterized by their physical behavior, content, pH, apparent viscosity, *in vitro* release, relative *in vitro* residence time, and spreadability using modified and standard methods. The mean globule size, release profiles, mechanisms, and kinetics of the optimized formulations were compared and studied.

Based on relative solubility study oleic acid and virgin olive oil were chosen as oil phase and it was found that Tween 80 and ethanol at a ratio of 2:1 managed to emulsify the oil phase with greater area of transparent emulsions in the phase diagram. The emulsions were jellified using Na CMC without disrupting their phase behavior. Rheological studies revealed that the clotrimazole emulgels exhibited a shear-thinning behavior which is desirable for ease of administration. Based on the predicted responses and their desirability indices, three

formulations were selected as the optimum formulations with oil phase concentration of around 12.4%, surfactant solution concentration of 30%, and Na CMC concentration of 4%. The optimized clotrimazole emulsions exhibited 139-146 nm and 0.16-0.26 of mean globule size and polydispersity, respectively. When *in-vitro* drug release data were fitted to five commonly employed release kinetic models, zero order release model showed the best fit with high linearity for all the optimized formulations. The polymer relaxation and erosion were found to be the rate controlling steps when the first 60% drug releases were fitted to the Korsmeyer-Peppas model.

From this, it can be concluded that clotrimazole can be formulated as an emulgel which can possibly enhance its release in aqueous media with acceptable spreadability and residence time at the target site.

Keywords: Emulgel; Clotrimazole; Oral *candidiasis*; Optimization; Shear thinning.

1. Introduction

1.1. Diseases of the mouth cavity and treatment approaches

There are many kinds of diseases and disorders that affect the oral cavity. From these the most common are dental cavities, periodontal (gum) disease, oral cancer, trauma from injuries, hereditary lesions, and oral infectious diseases (WHO, 2012). There are also more than 120 specific systemic diseases such as diabetes, arthritis, osteoporosis, and AIDS, as well as therapies for systemic diseases, which can directly or indirectly compromise oral tissues.

Worldwide, almost half (40–50%) of people who were HIV positive on 2012 has oral infections, which included oropharyngeal candidiasis (WHO, 2012). In Europe, North America, Mexico, Thailand, and South Africa, oral candidiasis is observed in 50–67 % of children, and is considered the most common oral manifestation of HIV infection. Oropharyngeal candidiasis occurs in up to 55 % of people with HIV infection and in over 90 % of those with advanced disease. This implies the crucial need of prevention of this superficial oral infection in order to improve the quality of life as well as to prevent the possible development of systemic fungal infection (Dhake, *et al.*, 2011).

Treatment approaches for disorders in the oral cavity include mechanical means, enhanced oral hygiene procedures, surgical procedures, the use of systemic or local antibiotics or antifungal agents, steroids and analgesics, and the use of saliva substitutes.

One of the first choices of management of oral candidiasis is clotrimazole. The conventional clotrimazole formulation is in lozenge form. However, because of the rapid removal of the active constituent from the target site, primarily through movement and copious salivary flow (Smart, 1993) and relatively small surface area (0.01sq m) it has a high frequent (five times a day for fourteen days) dosing regimen which may be the main reason for poor patient compliance and quick relapses (Albougy & Naidoo, 2002). The other reasons for incomplete eradication of *candidiasis* may be due to the short residence time of the antifungal agent in the oral cavity and degradation in salivary fluid (Harish, *et al.*, 2009).

1.2. Barriers to local drug delivery to the oral mucosa

Continuous salivary flow which ranges 1-7 ml $min^{-1}$and the mechanical movements of the tongue are the major physical barriers of drug delivery to the oral mucosa (Petelin, *et al.*, 2004). Components of saliva are adsorbed onto the surface of the oral mucosa to form a salivary pellicle 0.1-0.7 mm thick, which act as unstirred layer. This pellicle coats all surfaces in the mouth and is a multilayered structure. These molecules maintain hydration, provide lubrication, concentrate protective molecules such as secretory *immunoglobulins*, and determine the attachment and adhesion of microorganisms which are contained in the oral cavity. The salivary pellicle also concentrates protective molecules (e.g. *IgA*) at the mucosal surface and may act as a selective barrier to macromolecules (Smart, 1993; Petelin, *et al.*, 2004; Shravan, *et al.*, 2012). Because of the presence of sialic acids and ester sulfates, mucus is negatively charged at physiological salivary pH of 6.2–7.4. Saliva contains enzymes namely α-amylase (breaks 1–4 glycosidic bonds), *lysozyme* (protective, digests bacterial cell walls) and lingual lipase (break down the fats). Consequently, the major part of a successful application of local drug delivery systems in the oral cavity is a proper selection of vehicles, which prevent the washout and swallowing of the medicament, protect the drug substance from enzymatic degradation, facilitate the dissolution and diffusion of the drug through the mucus, and maintains the therapeutic properties of the constituents.

1.3. Drug delivery systems to the oral mucosa

Drug delivery systems to the oral mucosa can be used either for systemic absorption or for local treatment of the oral cavity. Historically, lozenges and troches, sprays and mouthwashes, gels, creams, and ointments, chewable tablets, medicated chewing gums (e.g. Vitaflo CHX®), and other solid or semi-solid preparations that are intended to be chewed or dispersed in the mouth with or without water before being swallowed were the traditional means for delivering drugs to the oral cavity. In these systems the major fraction of the drug will eventually be swallowed with the saliva and transported along the GIT where the drug may be subsequently absorbed.

More recently mucoadhesive gels, films (strips), tablets and patches, phase changing polymeric solutions, liposomal formulations (Erjavec, *et al.*, 2006; Franz-Montan, *et al.*, 2015), varnish to be coated on the teeth (Czerninski, *et al.*, 2010), oral lyophilisates and fast dissolving tablets,

periodontal fibers (Shifrovitch, *et al.*, 2009) and drug delivery devises have broadened the design of oral mucosal drug delivery dosage forms. The primary delivery route for many of these novel oral dosage forms is not by swallowing but rather, the drugs are targeted to the mouth's mucosal membranes for local action or systemic absorbtion. These systems have been shown to give the advantages of patient compliance, less invasive treatment approaches, quick onset of action of the drug, lowered required dosage, and prolonged release.

1.4. Emulgels

Gels are currently receiving increasing attention, especially hydrogel formulations, for topical application of drugs since they have an attractive appearance and develop pleasant cool feeling. They are easy to apply and remove and generally provide faster drug release compared with ointment and cream. Gels for topical use have several favorable properties such as being thixotropic, greaseless, easily spreadable, easily removable, emollient, non-staining, compatible with several excipients, and water-soluble or miscible. The pharmacological activity of gel formulations may not change as rapidly as the solution form (Jain, *et. al.,* 2010; Yassin, 2014). Gel dosage forms are successfully used as drug delivery systems to control drug release and protect the medicaments from a hostile environment (Khunt, *et al.,* 2012).

In spite of many advantages of gels a major limitation is in the delivery of hydrophobic drugs. Drugs which are practically insoluble in water can only be dispersed in gel formulation because greater than 90% of the composition is aqueous or hydroalcoholic vehicle. Therefore, they have a higher aqueous component that limits the dissolution of drugs, and also easy migration of the drug through a vehicle that is essentially a liquid. To overcome this limitation, emulgels are prepared and used so that even a hydrophobic therapeutic moiety can enjoy the unique properties of gels (Patel, *et al.,* 2014).

Eumlgels are emulsion gels which are hydrogels containing randomly distributed oil microdroplets (Jain, *et. al.,* 2010; Deveda, *et al.,* 2010). They are emulsions either of oil-in-water or water-in-oil type, which are gelled by mixing with gelling agent. They have been recently used as vehicles to deliver various drugs to the skin (Sabri, *et. al.,* 2009; Jain, *et. al.,* 2010; Baibhav, *et al.,* 2011; Bhanu, *et al.,* 2011; Panwar, *et al.,* 2011; Singla, *et al.,* 2012; Haneefa, *et al.,* 2013) and vagina (Nair, *et al.,* 2010).

There are two emulgel formulations available on the market: Voltaren emulgel (Novartis Pharma, Switzerland), containing diclofenac diethylamine (Bhanu, *et al.*, 2011) and Miconaz-H emulgel (Medical Union Pharmaceuticals, Egypt), containing miconazole nitrate and hydrocortisone (Jain, *et. al.*, 2010).

Nifedipine is a poorly water soluble drug which presents several challenges to maintain potency, uniformity, and usability characteristics when used for extemporaneously compounded preparation (McCluskey & Brunn, 2011). Recently, Alishech VMC Ltd. has developed Lido-Nifedipine emulgel which is one of the currently most effective methods for non-operational treatment of the hemorrhoids and fissures. It heals hemorrhoids and fissures fast and effectively (Alishech VMC Ltd., 2014). It is also safe and avoids surgical procedures in the great majority of cases, avoids complications and does not require hospitalization (Klin, *et al.*, 2012).

1.5. Rationale for using emulgels

Emulsified gels are stable and better vehicles for hydrophobic or poorly water soluble drugs**.** They have the advantages of emulsions and gels. They are used to deliver various drugs to the mucosa due to enhanced drug absorption through the mucosa since the drug substance is in solution. They show higher aqueous release of hydrophobic drugs than conventional gel formulations.

When indomethacin was formulated as an emulgel, it showed better drug release, up to 90.12%, as compared to standard gel prepared which had only 55.67% drug release at 12 hrs. The xanthan gum based formulation showed better anti-inflammatory activity and skin permeation through rat skin (Mulye, *et al.*, 2013). Chlorphenesin emulgel exhibited higher drug release and antifungal activity than the commercially available topical powder where percentage inhibition reached up to 25.3% for emulgel preparation and only 8.4% for the powder (Mohamed, 2004).

Microemulsions have several advantages such as enhanced drug solubility, good thermodynamic stability, ease of manufacturing and enhancement effect on permeation ability over conventional emulsions. However, it is also essential to have a dosage form which adheres to the mucosa and increases the residence time of the drug at the site of treatment in contrast to the low viscosity of microemulsion which restrains its application in the pharmaceutical industry. This can be

impacted by formulating microemulsion based emulgels (Nair, *et al.*, 2010; Lee, *et al.*, 2010; Baboota, *et al.*, 2011).

The release controlling ability of terbinafine hydrochloride loaded microemulsion was significantly improved in comparison to commercial cream when it contains gel formulation. The microemulsion was composed of cottonseed oil (oil phase), Tween 80 (surfactant) and ethanol (cosurfactant) which increased the solubility and bioavailability of terbinafine hydrochloride (Dabhi, *et al.*, 2011). The permeability enhancement factor for microemulsion based metronidazole emulgel when compared with marketed gel formulation Metrogyl was also found to be 3.65 (Rao, *et al.*, 2013).

The twisted matted strands in gels which are tied together by *van der Waals* forces to form crystalline and amorphous regions throughout the system create interlacing three dimensional networks restricting the movement of dispersion medium. This makes an emulgel as an attractive candidate for topical drug administration where systemic absorption of drugs is not desired. It has been shown that even though microemulsion based hydrogels showed lower drug permeation profile they facilitated the skin deposition and penetration of itraconazole compared to their corresponding microemulsion. This was proved by the absence of itraconazole in the blood samples collected from mice at all time points and higher drug content of skin (8-10 $\mu g/cm^2$) from 2 to 8 hrs after administration. The reaction between the surfactants and stratum corneum of the skin and the presence of higher amount of surfactant could be the reason for the higher skin drug content at 2 hrs (Lee, *et al.*, 2010).

A statistically significant improvement has been achieved when using an emulgel as a coupling medium during ultrasound wave treatment of localized traumatic and rheumatic painful conditions against the regular gel. Treatment was discontinued due to complete cure in 60% of patients using Voltaren Emulgel® compared with only 15% of those using regular gel and complete relief of pain was also statistically significant in favor of the emulgel throughout the trial period (El-Hadidi & El-Garf, 1991).

Emulgels have been used to overcome the unwanted effects caused by drug substances making the formulation more tolerable. Different vegetable oils with emollient properties have been used as oil phase to alleviate the considerable skin dryness and irritation caused by benzyl peroxide,

an anti-acne agent, which sometimes leads to the discontinuation of the treatment. It was found that Carbopol 940 based emulgel with sesame oil showed no sign of redness or dryness as compared with the marketed cream and gel formulations (Thakur, *et al.,* 2012*).*

1.6. Formulating an emulgel

Preparation of emulgels comprises of simple and short steps which increase feasibility of the production. There are no specialized instruments needed for the production of emulgels. Moreover, materials used are easily available and cheap. All these; decrease the production cost of emulgels. The rheological properties and the breakdown behavior of gels filled with emulsions droplets can be varied by changing the interactions between oil droplets and gel matrix, the oil content and the oil droplet size (Jain, *et. al.,* 2010).

Basically emulgel preparation consists of two steps; emulsification and incorporating the emulsion into a gel base (Figure 1.1). Important constituents of the emulgel are the aqueous phase, the oil phase and the gelling agents. Commonly used aqueous agents are water and alcohols.

Gelling agents are agents used to increase the consistency of any dosage form that can also be used as thickening agents. The selection of polymer for preparing gel is normally based on the character of external phase (Baboota, *et al.,* 2011). The choice of the polymer as a gelling agent depends on its ability to obtain desired release profile and its muco-adhesive property. In order to achieve optimized drug release characteristics and good retention time combination of polymers with different chemistry can be used.

Many emulsions for topical use contain oils that are present as carriers for the active ingredient. Therefore the oily phase preferably has to be a good solvent for the active ingredient which also ensures a large drug loading capacity of the formulation. Widely used oils in oral preparations are non-biodegradable mineral and castor oils that provide a local laxative effect, and fish liver oils or various fixed oils of vegetable origin (e.g., olive, arachis, cottonseed, and maize oils) as nutritional supplements (Panwar, *et al.,* 2011).

It must also be realized that the type of oil used may have an effect on the stability of the final product. The high stability makes jojoba oil a useful vehicle for cosmetic applications

Clotrimazole emulgel containing Jojoba oil together with a combination of Pemulen TR1 and TR2 showed excellent stability as well as high rate of drug release. Jojoba oil is characterized by its resistance to oxidation, ability to withstand high temperatures and pressures without breakage, and low rancidity potential (Shahin, *et al.*, 2011a).

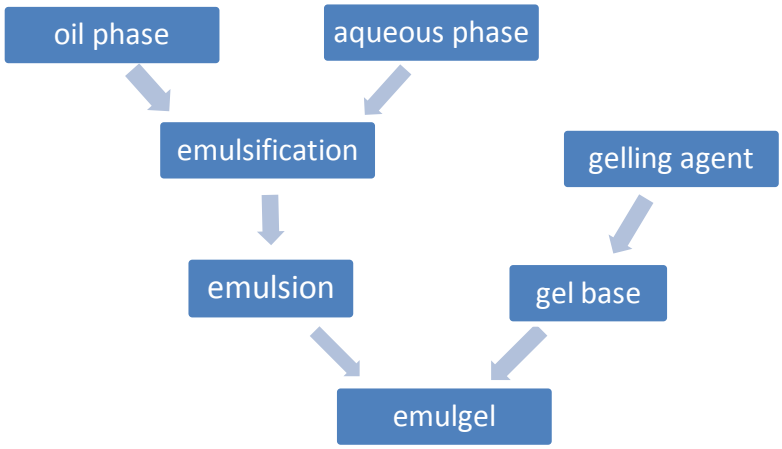

Figure 1.1: Processes of emulgel preparation (Singla, *et al.*, 2012).

1.6.1. Selection of oil phase

Lipophilic drugs are preferably solubilized in o/w emulsions. Drug loading per formulation is a very critical design factor in the development of emulsion systems for poorly soluble drugs, which is dependent on the drug solubility in various formulation components. Solubility of the drug in the oil phase is an important criterion for the selection of oils. This is important as the ability of emulsion to maintain the drug in solubilized form in aqueous media is greatly influenced by the solubility of the drug in the oil phase. If other components in the formulation are contributing to a great extent to drug solubilization, there could be a risk of precipitation, as dilution of emulsion in the aqueous media will lead to lowering of the solvent capacity of these mediators. Thus, an understanding of factors influencing drug loading capacity is essential to the design of stable and appropriately low-volume emulsion systems for drug delivery applications. This has to be done without impinging on the capability of the system to undergo monophasic dilution with water and minimized tendency for drug precipitation or crystallization in diluted

systems As of late, novel semisynthetic medium chain derivatives, which can be defined as amphiphilic compounds with surfactant properties, are being preferred (Azeem, *et al.,* 2009).

1.6.2. Emulsification

Emulgel helps in the incorporation of hydrophobic drugs into the oil phase and then oily globules are dispersed in aqueous phase resulting in o/w emulsion. And this emulsion can be mixed into gel base. Therefore, one has to pay attention to the emulsion components there of the dissolution of the hydrophobic component in aqueous media.

In order to create an oil in water emulsion (one that remains stable for a long enough time), work must be done to overcome the interfacial tension between the two phases. This can be achieved by mixing; however mixing even at very high rates is not enough to provide long term stability. An emulsifier or combination of emulsifiers is needed to stabilize droplets of the dispersed phase. Generally, the introduction of an emulsifying agent will lower the interfacial tension of the two phases and decreasing the coalescence of dispersed droplets.

Percentage of internal phase volume, emulsifier type and concentration affects the particle size of the internal phase, apparent viscosity, and sedimentation volume of an emulsion which determines its stability (Sepulveda, *et al.,* 2003). The smaller the particle size the more stable the emulsion will be (Lee, *et al.,* 2010).

Studies have shown that the emulsifying agent concentration has the most pronounced effect on the drug release from the emulgels, followed by the oil phase concentration, which has a retardation effect (Mohamed, 2004; Deveda, *et al.,* 2010; Patel, *et al.,* 2014). Indomethacin emulgel formulation prepared with the oil phase concentration in its low level (5%w/w) and emulsifying agent concentration in its high level (2.5%w/w) had the maximum percent drug release and spreadability (Mulye, *et al.,* 2013). This could be due to an increase in the hydrophilicity of the emulgel, which in turn facilitates penetration of the release medium into the emulgel (Mohamed, 2004; Rao, *et al.,* 2013).

Sometimes more emulsifier is not necessarily better to accommodate the large percentage of oil used. As the amount of oil increases, the amount of emulsifier required for its successful emulsification tends to be reduced. For example, the increase of Pemulen TR1 and TR2 two

times for the emulsification of clotrimazole containing heavy liquid paraffin made the formulation less stable (Shahin, *et al.*, 2011a). The *in-vitro* drug diffusion is also influenced primarily by the solubility of drug in vehicle. If the vehicle can significantly raise the solubility of the drug, then the drug itself would be retained in the vehicle after placing at the membrane surface, which resulted in reduced diffusion into the receiving compartment (Dabhi, *et al.*, 2011). Lower drug diffusion of metronidazole was obtained at higher level of oil and surfactant concentration (Rao, *et al.*, 2013).

The sequence of addition and degree of agitation of the two phases also determines the surface area of any given internal phase volume, and consequently the stability of the system (Sepulveda, *et al.*, 2003). The indirect method used in the preparation of Clotrimazole emulgels gave a relatively stable emulsion with Isopropyl myristate, jojoba oil, and liquid paraffin as oil phase in Pemulen based emulgels (Shahin, *et al.*, 2011a).

Due to the low water solubility of hydrophobic drugs, a quantity of short chain alcohols is needed to dissolve the drug. These alcohols incorporated into the emulsion system not only make the lipophilic drugs soluble in the system but also reduces the interfacial tension between the oil phase and the aqueous phase (Lee, *et al.*, 2010*)*. The presence of alcohol decreases the bending stress of the interface and allows an interfacial film with sufficient flexibility to assume different curvatures over a wide range of compositions and it also adjusts the HLB value of the formulation by making the polar solvent less hydrophilic. Alcohols may also increase miscibility of the aqueous and oily phases due to its partitioning between these phases (Azeem, *et al.*, 2009). It was seen that when the surfactant (Tween 20) was used alone, oil phase was solubilized to a lesser extent implying that surfactant alone was not able to reduce the interfacial tension of the oil droplets to sufficiently low level and thus was not able to reduce the free energy of the system to ultra low level (Baboota, *et al.*, 2011). The smaller particle size and more stable of itraconazole microemulsion was also obtained with 1:1 ratio of surfactant to cosurfactant (Lee, *et al.*, 2010).

1.6.3. Selection of gelling agents

A major difficulty for the successful eradication of infections of the oral cavity is the dilution and rapid elimination of topically applied drugs due to the flushing action of saliva. The delivery system in which the drug is incorporated is therefore an important consideration and should be formulated to prolong retention of the drug in the oral cavity (Abdul Rasool, *et al.,* 2010).

The incorporation of highly hydrophilic polymeric thickener considerably increases the viscosity of the external aqueous phase in case of O/W emulsions thereby retarding the probability of collisions and subsequent merging of dispersed internal phase globules. Such polymeric additives have shown an excellent potential in terms of sustained release of incorporated oil phase and also the sustained release of pharmaceutically active excipients in dispersed oil phase (Kumthekar, 2012). Polymers play major role in various characterization parameters of topical gel such as *in vitro* release and rheological properties. The formulation of an effective gel requires the use of an appropriate gelling agent, usually a polymer. The preferred characteristics of such polymer include the inertness, safety, and biocompatibility with other ingredients, good adhesion to mucous membrane, permission of drug permeation while not being absorbed into the body, irritation-free, and preferably biodegradable (Garg, *et al.,* 2002).

Bioadhesive polymers that have been used to adhere to mucosal surfaces for extended periods of time provide controlled release of drugs. These are widely used in the delivery of drugs to the oral cavity. They have the ability to form intimate contact with the mucosal membrane and release the drug at the absorption site in a controlled manner. One of the oral mucosal adhesive delivery systems-"Orabase R" consists of finely ground pectin, gelatin and sodium carboxymethylcellulose ("Orahesive" Powder) dispersed in a poly(ethylene) and a mineral oil gel base, which can be maintained at its site of application for 15-150 min. This has been used for the local application of steroids for the treatment of mucosal ulceration. Other commercially available bio-adhesive gels are Daktarin oral gel (Miconazole) and Pansoral gel buccal (Choline salicylate) (Patel, *et al.,* 2011).

Some of the most extensively studied mucosal adhesives are the poly (acrylic acids), e.g. Carbopol 934 and polycarbophil. The high concentration of carboxyl groups in the formulation of poly (acrylic acid) would be predicted to generate a low surface pH on moistening, and pH

values of between 2 and 3. A low pH would be expected to damage a contacting mucosal surface, and this has been reported in an *in vivo* study. Thus, the ultimate suitability of poly (acrylic acid) for use as a bio-adhesive component in a pharmaceutical formulation may be questioned (Smart, 1993).

From the traditional non-specific first-generation mucoadhesive polymers, anionic polymers are the most widely employed mucoadhesive polymers within pharmaceutical formulation due to their high mucoadhesive functionality and low toxicity. Such polymers are characterized by the presence of carboxyl and sulphate functional groups that give rise to a net overall negative charge at pH values exceeding the pKa of the polymer. Typical example is sodium carboxymethyl cellulose (pKa = 4.30) which forms a strong hydrogen bonding with mucin (Singh, *et al.*, 2013).

The type of gelling agent and concentration affects drug diffusion through the gel matrix. When chlorphenesin was prepared as an emulgel lower drug release from Carbopol-based than the drug release from HPMC-based was observed in phosphate buffer of pH 5.5 since carbopol-based formulations possessed considerably higher viscosities than the HPMC-based formulations (Mohamed, 2004).

The type of the gelling agent should be that which doesn't disrupt the stability of the emulsion. It has also been suggested that the interaction of Pemulen emulsifier molecules with the aqueous phase (responsible for the three dimensional build up) was interfered with the lipophilic nature of paraffin oil resulting in lower degree of hydration, lower viscosity, and lower stability than those prepared with isopropyl myristate (Shahin, *et al.*, 2011a).

1.7. Emerging excipients for emulgel dosage forms

In recent years, there has been great interest in the use of polymers with complex functions as emulsifiers and thickeners because the gelling capacity of these compounds allows the formulation of stable emulsions and creams by decreasing surface and interfacial tension and at the same time increasing the viscosity of the aqueous phase. Several polymers are available with such emulsion stabilizing characteristics, such as carboxymethylcellulose sodium, guar gum, hydroxyl propyl cellulose, hydroxypropyl methyl cellulose, and sodium alginate (Khullar, *et al.*, 2011).

Pemulens are novel oil-in-water (o/w) emulsifier polymers. They are hydrophobically modified co-polymers of acrylic acid (acrylates/C10-C30 alkyl acrylates) and cross-linked with allyl pentaerythritol (Figure 1.2). A Pemulen emulsifier could act both as a primary emulsifier and viscosity enhancing agent. The lipophilic portion of Pemulen (long-chained methacrylate) adsorbs at the oil–water interface and the hydrophilic portion swells in the water forming a gel network around oil droplets to provide exceptional emulsion stability to a broad range of oils, regardless of the oil phase HLB or temperature of emulsification. Thus, when two oil droplets approach each other, a physical repulsive force is generated by the presence of these adsorbed gel layers. They have numerous benefits to emulsions prepared with them including low usage level (as small a concentration as 0.4%), low irritancy, simple preparation procedures, and fast release of the oil phase. This rapid release is mainly due to the deswelling of the acrylic hydrophilic portion of the Pemulen emulsifier hydrogel upon contact with the salts commonly present on skin surface. Interestingly, Pemulen could also control the release of drugs having high log P values. There are two types of Pemulens: TR1 and TR2 and the main difference between them is the more hydrophobic modifications of TR2 type and therefore it is able to emulsify a greater amount of the oily phase (Shahin, *et al.*, 2011a). Pemulen TR-1 holds up to 20% oil in an emulsion while Pemulen TR-2 holds up to 50% (Ravenel, 2010).

Figure 1.2: Structure of Pemulen emulsifier (Shahin, *et al.*, 2011a).

Sepineo P 600 is a ready to use 3 in 1 new range emulsifier and thickener polymer. It is a concentrated droplet dispersion of acrylamide/sodium acryloyldimethyl taurate (a viscous liquid at room temperature) in isohexadecane as the oily dispersing phase. The presence in this dispersion of polysorbate 80 is important for keeping the resultant dispersion stable. When water is added, based on the concept of droplet hydroswelling, these polymer droplets disappear

because the polymer molecules interact with it strongly, instantly forming a stable semisolid system (Figure 1.3) (Malandain, 2007). This polymer emulsifies and stabilizes almond oil in the aqueous phase with minimal changes to the elastic character of the gel (Bonacucina, *et al.*, 2009).

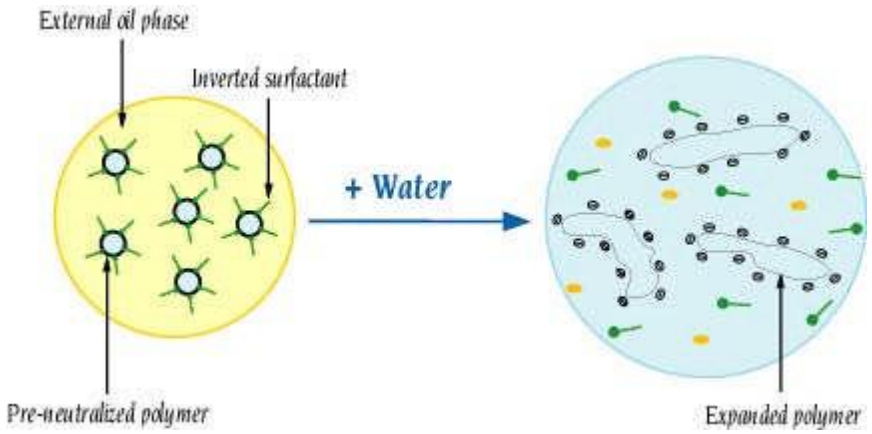

Figure 1.3: SEPINEO^{TM}, Hydro swelling droplets polymer (SEPPIC S.A., 2008).

1.8. Clotrimazole

Clotrimazole is a white or pale yellow, tasteless, crystalline powder which is practically insoluble in water (water solubility of less than 0.49 μg/ml). Clotrimazole has pH dependant solubility where solubility increases with decreasing pH. It is due to presence of ionizable imidazole group present in its structure (Figure 1.4). The nitrogen atoms are protonated in acidic pH and impart polar nature to clotrimazole, making it more soluble in lower pH excipients. Clotrimazole also hydrolyzes in acidic medium at higher temperature to (2-chlorophenyl) diphenylmethanol and imidazole (Hoogerhaide & Wyka, 1982; Bachhav & Patravale, 2009; Hashem, *et al.*, 2011).

It is a well-tolerated fungistatic drug with anticandidal and antistaphylococal activity (Ellepola & Samaranayake, 2000). It alters the permeability of the fungal cell wall and inhibits the activity of enzymes within the cell. It specifically interferes with sterol synthesis via inhibition of CYP-dependent C-14 α demethylase, a fungal CYP enzyme important in converting lanosterol to ergosterol. Studies show minimal concentrations of clotrimazole cause leakage of intracellular

phosphorus compounds into the ambient medium, along with the breakdown of cellular nucleic acids and an accelerated K^+ efflux. This leads eventually to the cell's death. Minimal inhibitory concentration (MIC) for clinical isolates of *C. albicans* is between 1μg/ml and 4μg/ml (Torres-Rodriguez, *et al.*, 1999; Boushra & Hayam, 2010).

Figure 1.4: Chemical structure of Clotrimazole (BP 2009).

Clotrimazole was introduced as a topical agent in 1969. It is used for the treatment of superficial candidiasis, and in the skin infections pityriasis versicolor and dermatophytosis. Clotrimazole may also be used occasionally for symptomatic relief of the protozoal infection trichomoniasis when other drugs are contraindicated. For the treatment of oropharyngeal candidiasis, one 10-mg lozenge of clotrimazole (a common brand is Mycelex® troche, Miles Pharma, USA) is dissolved in the mouth five times daily for 14 days (Singh, *et al.*, 2008). It is also effective for prophylaxis in patients undergoing chemotherapy, myeloablative treatment, and transplant recipients and for patients with solid malignant neoplasms (Czerninski, *et al.*, 2010).

Clotrimazole is safer (especially in pregnant women) than other antifungals currently listed on the WHO Essential Drugs List. It is available without prescription in most countries and this regulatory approval status indicates that the drug is generally recognized as safe. Reported adverse effects have been rare and not serious. United Kingdom data from the British Medical Association and Royal Pharmaceutical Society suggest that the medicine is reasonably priced (Bero, 2005).

1.9. The present study

The low water solubility of clotrimazole (0.49 μg/ml) presents a hindrance for its local availability and limits its effective antifungal therapy (Bachhav & Patravale, 2009; Hashem, *et al.,* 2011) by impeding drug release from its device (Reddy, *et al.,* 2011). And because of the rapid removal of the active constituent from the target site, primarily through movement and copious salivary flow (Smart, 1993) it has a high frequent (five times a day for fourteen days) dosing regimen which may be the main reason for poor patient compliance and quick relapses (Albougy & Naidoo, 2002).

Many attempts have been made to improve the bioavailability of clotrimazole and to reduce the dosing frequency and improve the patient compliance for an effective and safe therapy of oral *candidiasis*, such as pH triggered *in situ* gel system (Harish, *et al.*,2009) and formulating a bioadhesive tablet of the β-cyclodextrin complex (Dhake, *et al.,* 2011). However, there is still the need for alternate dosage forms which can enhance the solubility and residence time of this medicament. So far, no work has been reported on the formulation of clotrimazole oromucosal emulgel for oral *candidiasis.* Thus, the aim of the present research work was to investigate the potential of emulgel in enhancing the local release of clotrimazole, in the oral cavity for the treatment of oropharyngeal *candidiasis*. Clotrimazole emulgel 1%w/w was formulated and optimized by performing *in-vitro* diffusion studies and spreadability.

1.10. Objectives of the study

1.10.1. General Objective

To formulate, characterize, and optimize oromucosal Clotrimazole emulgel preparation.

1.10.2. Specific objectives

- To formulate Clotrimazole emulgels;
- To evaluate the rheological properties and physical characteristics of the formulations;
- To compare the physical characteristics of the formulations with marketed oromucosal gels;
- To optimize Clotrimazole emulgel formulations;
- To study the in vitro drug release properties and globule size of the optimized formulations; and
- To study the release stability of the formulated emulgels.

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2. Materials and Methods

2.1. Materials

Clotrimazole powder (China Associate Co., Ltd. China) was received as a generous gift from Ethiopian Pharmaceuticals Manufacturing Sh. Co. (EPHARM). Sodium carboxy methyl cellulose (NaCMC) (Hengshui Taocheng Chemicals Auxiliary Co., Ltd. China), Hydroxypropylmethyl cellulose (Shandong Head Co., Ltd. China), Polysorbate (Tween 80) (China Associate Co., Ltd. China), Ethanol, oleic acid (Merck & Co., Inc., Germany), and mineral oil (Horst G.F. Von Valtier GmbH & Co. KG, Germany) were also donated by EPHARM. Virgin olive oil (Angel Camacho Alimentacion, Spain), refined olive oil (Angel Camacho Alimentacion,, Spain), refined sun flower oil (Hatun®, UAE), and refined soya bean oil (Tena, Ethiopia) were purchased from local market and used as received. Mycoheal® oral gel (Dar Al Dawa, Na'ur-Jordan) and Decozol® oral gel (Hoe Pharmaceuticals Sdn. Bhd., Malaysia) were also purchased from local pharmacy and used as received. All other chemicals used were of analytical grade, such as methanol (Carlo Erba Reagents, Italy), anhydrous acetic acid (Carlo Erba Reagents, Italy), sodium hydroxide (Carlo Erba Reagents, Italy), monobasic potassium phosphate (Fisher Scientific LTD., UK), agar powder (Fisher Scientific LTD., UK) absolute ethanol (Carlo Erba Reagents, Italy), and dipotassium hydrogen phosphate (Merck & Co., Inc. Germany), were of analytical grade.

2.2. Methods

2.2.1. Preliminary studies

2.2.1.1.Solubility studies

An excess of clotrimazole was added individually to the oils (4 g each) (Mineral oil, Virgin olive oil, Refined olive oil, Sun flower oil, Soya bean oil, Oleic acid) in screw capped tubes. The mixture was then sonicated (Bandelin Sonorex RK 514 ultrasonic baths, BANDELIN electronic GmbH & Co. KG, Germany) for 15 min with intermittent shaking (3 min) with a mechanical shaker (Edmund Buhler 7400 Laboratory Multi-Flask Shake, Edmund Bühler GmbH, Tübingen, Germany) at 200 rpm in order to facilitate proper mixing of drug with the vehicles. After 72 h, each sample was centrifuged at high speed for 15 min (Shafiq, *et al.,* 2007; Borhade, *et al.*,

2012). The supernatants (1 gm) from triplicate tests were passed through 0.45 µm syringe driven cellulose membrane filter units and put in water bath (1092 Shaking Water Bath, GFL Gesellschaft für Labortechnik mbH, Germany) at 50 ^{o}C for 5 min in absolute ethanol and cooled. The mixture was then promptly centrifuged and the supernatant was collected to determine the amount of clotrimazole present by validated HPLC method (Section 2.2.3.1.viii).

2.2.1.2.Phase study

For the phase study, different combination of oil (30:70, 40:60, 50:50, & 60:40) were prepared. Surfactant and ethanol in each group then were mixed in different weight ratios (2:1, 1:1, & 1:2) (Shafiq, *et al.,* 2007).

Pseudo-ternary phase diagrams were constructed using water titration method, where slow titration (5% w/w increments) with aqueous phase was done to 5 ml of oil and surfactant solution prepared in different volume ratios (1:19, 1:5.7, 1:3, 1:1.9, 1:1.2, 1:0.8, 1:0.5, 1:0.3, 1:0.2, & 1:0.05). The concentration of water represented the percentage in the total mixture, i.e., the total amount of lipid, surfactant and water. Therefore, as the combined weight of lipid and surfactant was kept constant at 5 ml, the weight of water added increased with the increased concentration of water in the mixture. Following each addition, the mixtures in flasks were stirred for 2 to 3 min and were allowed to equilibrate at room temperature for 30 min (Bachhav & Patravale, 2009; Patela, *et al.,* 2012). After equilibration, the physical state of the resulting emulsion was marked on a pseudo-three-component phase diagram, using Chemix® school software 3.00, with one axis representing water, the other representing oil and the third representing a mixture of surfactant and ethanol at fixed weight ratios.

The emulsion was considered as ‘transparent’ and ‘cloudy’ when the emulsion was easily flowable and clear, and non-clear but translucent, respectively. Meanwhile, the emulsion was classified as viscous emulsion (gel) when longer time is required for emulsifying and equilibration and did not show a change in the meniscus after being tilted to an angle of 90°. The emulsion was considered as unstable when there was poor or no formation of emulsion and immediate coalescence of oil droplets when gentle agitation was stopped (Baboota, *et al.,* 2011).

2.2.1.3.Selection of oil phase and emulsifier composition

A pseudoternary phase diagram was constructed based on four different types of oleic acid: virgin olive oil volume combinations (30:70, 40:60, 50:50, and 60:40) at room temp. and were assigned A, B, C, and D, respectively. The term pseudoternary is used because the constituents are mixtures and not a single component.

Short to medium chain length alcohols (C_2–C_7) are commonly added, in the formation of emulsions, which further reduce the interfacial tension and increase the fluidity of the interface. Ethanol (HLB = 8) which is one of these alcohols (Narang, *et al.,* 2007) is also a good solubilizer for clotrimazole (95 mg/ml) (Hoogerhaide & Wyka, 1982) and was chosen to enhance the emulsification ability of Tween 80. Surfactant (Tween 80) was blended with ethanol in the weight ratios of, 1:1, 1:2, and 2:1. And each surfactant ratio was mixed thoroughly with each oil mixture in ratios of 1:19, 1:6, 1:3, 1:1.9, 1:1.2, 1:0.8, 1:0.5, 1:0.3, 1:0.2 and 1:0.05.

A water titration method was employed to construct phase diagrams by adding a small amount of distilled water drop wise up to 45 ml to 5 ml of the oil surfactant mixture. Phase changes caused by each increment of water were observed visually and recorded.

2.2.1.4.Selection of gelling agent

High-viscosity grade Sodium Carboxymethylcellulose (pKa = 4.3) and Hydroxypropyl Methylcellulose (Methocel K4M) were evaluated for their ability to gel the selected emulsion up to a concentration of 6% w/w and 12% w/w, respectively. Different combinations of both gelling agents (0:1, 1:0, 1:1, 1:2, & 2:1) were prepared by dispersing in hot (~70 ^{o}C) (Sabri, *et al.,* 2009) aqueous citrate buffer pH~6.8 (Trisodium citrate 0.3% w/w, Citric acid q.s) (El-Houssieny & Hamouda, 2010). After complete hydration, the gels were combined with the chosen emulsion system in 1:1 ratio. The emulgels were then assessed visually for any effect on the phase behavior of the emulsion, feel, and ease of spreadability after 24 h (Bachhav and Patravale, 2009; Rowe, *et al.,* 2009).

2.2.2. Clotrimazole emulgel preparation

The gel was prepared as described above, after adding methylparaben (0.18% w/w) and propylparaben (0.02% w/w) (previously dissolved in propylene glycol, 2% w/w) as preservatives and sodium sulphite (0.1% w/w) as an antioxidant, it was left overnight. The oil phase of the

emulsion was prepared by dissolving clotrimazole (2% w/w) in the oil, Tween 80, and ethanol mixture. Both the oily and aqueous phases were separately heated to 70 to 80 °C; then the water phase was added slowly to the oil phase while stirring continuously with magnetic stirrer (Stuart Scientific Magnetic stirrer SM5, Bibby Scientific Limited, UK), at 2,000 rpm and stirring was continued for 5-10 min until cooled to room temperature (Soliman, 2010). The emulsion obtained was then mixed with the gel in 1:1 ratio with spatula to obtain a uniform emulgel (Mohamed, 2004; Sabri, *et al.*, 2009). 200 g of emulgel was prepared for each formulation.

2.2.3. Analytical methods

2.2.3.1.Characterization of clotrimazole emulgels

i. Physical examination

The prepared emulgel formulations were inspected visually for their optical clarity, color, homogeneity, fluidity, and phase separation after 24 h of preparation (Mohamed, 2004; Singh, *et al.*, 2014).

ii. pH

The pH values of 1% aqueous solutions of the prepared emulgels were measured by a calibrated pH meter (SevenEasy^{TM} Mettler Toledo 8603, Mettler-Toledo AG Analytical, Switzerland) (Mohamed, 2004).

iii. Viscosity

Viscosity determinations of the prepared Emulgels (~80 g) were carried out using a rotational viscometer (FUNGILAN S.A. HELDAL UNIT, Barcelona, Spain) with 'T' shaped special spindles (PA, PB, PC, PD, and PE) exerting minimal disturbance to sample at room temperature. A typical run will comprise a velocity from 0.3 to 12 rpm with 10 sec between each two successive speeds. Equilibration of the sample for 5 min was made following loading of the viscometer. The reading for each viscosity stage was done at least after 20 sec of run. The average of three readings was used to calculate the viscosity (El-Houssieny & Hamouda, 2010).

iv. Spreadability

The spreadability of the emulgel formulations was determined 48 h after preparation, by measuring the spreading diameter of 0.5 g emulgel which was placed within a circle of 1 cm

diameter pre-marked on a glass plate over which a second glass plate (75 gm) was placed (Eq. 2.1). A weight of 425 g was allowed to rest on the upper glass plate for 5 min where no more spreading was expected (El-Houssieny & Hamouda, 2010, Soliman, 2010). The increase in the diameter due to spreading of the gels was noted. The spreadability ($g.cm.min^{-1}$) was calculated by using the formula:

$$S = m.\ l/t \quad \text{..... Eq. 2.1}$$

Where: S is spreadability, m is the weight of the upper plate and rested on it (g), l is the diameter of the spreading emulgel (cm), and t is the time taken (min) (Jelvehgari, *et al.,* 2007; Prajapati and Patel, 2010; Nair, *et al.,* 2010).

v. *In vitro* release study

In vitro release study was conducted using a glass tube with an internal diameter of 1.5 cm (effective release area of approximately 7.5 cm^2) filled with 5 g of the emulgel, covered with a cellulose acetate membrane (0.45 µm) (which was previously soaked in phosphate buffer of pH 6.8 for about 24 hr), sealed with a rubber band (to make water tight), and inverted under the surface (Mohamed, 2004; Sabri, *et al.,* 2009; Hashem, *et al.,* 2011) of 500 ml of phosphate buffer of pH 6.8, containing 1% Polysorbate 80:ethyl alcohol (1:1), at 37 ± 0.5 ℃ in a *United States Pharmacopeia (USP)* dissolution tester apparatus II, with stirring speed of 100 rpm (El-Houssieny & Hamouda, 2010). Samples of 5 ml was withdrawn at 30, 60, 120, 180, 240, 300, 360, 420 and 480 min and replaced with an equivalent amount of fresh receptor medium (Ueda, *et al.,* 2009). The cumulative percent drug release was determined in with validated HPLC method (Section 2.2.3.1.viii).

vi. Relative *in vitro* residence time

The residence time of the emulgel was evaluated using a locally modified disintegration apparatus (Figure 2.1). An agar solution (1.5%, w/w) was prepared in pH 6.8 phosphate buffer by boiling in water bath (~100 ^oC). Then, 10 ml of the solution was poured on 85 mm diameter Petri dish and swirled so that it completely covers the bottom. The agar solution was allowed to solidify for two hrs. Test sample, 50 mg was placed at the center of the plate and after 5 min, the agar plate was attached to a USP disintegration test apparatus (ERWEKA® Gmbh, D-63150 Heusenstamm, Germany) and moved up and down in pH 6.8 phosphate buffer (~800 ml) at

37±2.0 ^{o}C. The sample on the plate was immersed into the solution at the lowest point and will be out of the solution at the highest point. The time necessary for complete erosion of the emulgel from the plate was noted visually and recorded (mean of triplicate determinations) (Nafee, *et al.,* 2003; Bachhav & Patravale, 2009; Nair, *et al.,* 2010; Reddy, *et al.,* 2011).

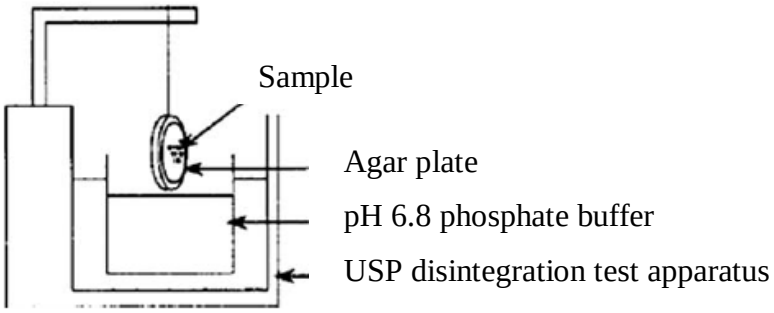


Figure 2.1: Schematic representation of apparatus used for relative *in vitro* residence time study (Bachhav & Patravale, 2009).

vii. Content of clotrimazole emulgel

Randomly ten samples of emulgel from different sides of the mixing container were taken and mixed (Singh, *et al.,* 2014); the equivalent weight of 10 mg of Clotrimazole was taken and transferred to a 50 ml screw capped centrifuge tube. 10 ml of absolute ethanol was added and heated at 50 ^{o}C for 5 min in water bath (1092 Shaking Water Bath, GFL Gesellschaft für Labortechnik mbH, Germany) and cooled. The mixture was then promptly centrifuged and the supernatant was collected and analyzed in triplicate (USP30-NF25, 2007) with validated HPLC method (Section 2.2.3.1.viii).

viii. HPLC method

The HPLC system consisted of a Shimadzu (Shimadzu Corporation, Japan) chromatographic system equipped with Shimadzu LC-10 AD vp pump, Shimadzu SPD-10A vp UV/Visible detector, DGU-12A degasser and SCL-10A vp system controller. Samples were injected using an injector at injection volume of 20 µl and 1.0 ml/ min flow rate. The analytical column was a C18 column (Shim-pack vp-ODS, 4.6 × 300 mm) with C8 (2 x 60 mm) guard column. A mobile phase consisting of a mixture of methanol/dipotassium hydrogen phosphate (0.025 M) at a ratio of 75:25 *v*/*v* was used in the analysis that was conducted in an isocratic elution mode. Prior to use, the mobile phase was sonicated and filtered through 0.45 µm cellulose membrane under vacuum.

Data acquisition and integration were carried out using Shimadzu Class VP software (version 6.12 SP4). The detection wavelength was 254 nm. Averages of triplicate peak areas (three injections) were recorded and compared with the area of the standard. The sensitivity and linearity of the assay were checked over a concentration range from 20 to 120 µg/ml ($y = 14410 x$; $r^2 = 0.9993$). Inter-day examinations ($n = 3$) at the concentration of 60, 80, and 100 µg/ml had shown insignificant variations ($p > 0.05$) in peak area responses (Annex 1).

2.2.3.2.Experimental design and optimization method

A systematic experimental design and optimization was carried out using a standard RSM design called a central composite design (CCD) for estimating the effect of independent variables (concentration of gelling agent, surfactant and oil phase) on dependent variables (*in vitro* 1st hr and 8th hr release, residence time, and spreadability) using Design-Expert ® software (Version 6.0.8, Stat-Ease Inc., Minneapolis, MN, USA). The experiments were conducted over a three-day period, in three blocks with the default setting of rotatable design with the axial (star) points set at 1.68179 ($\alpha = 2^{k/4}$) coded units from the center:

Block 1. Six runs: composed of four factorial points, plus two center points.

Block 2. Six runs: composed of four factorial points, plus two center points.

Block 3. Eight runs: composed of six axial (star) points, plus two center points.

In this full CCD, each numeric factor was varied over five levels: plus and minus alpha (axial points), plus and minus 1 (factorial points) and the center point (Table 2.1). The star points were let to exceed the operating limits.

The responses of the 20 experiments were analyzed numerically by fitting linear, two-factor interaction (2FI), and quadratic polynomials (models) to the responses. The highest order polynomial was selected where the additional term is significant ($P < 0.05$), has insignificant lack of fit, exhibits low standard deviation (SD), high "R-Squared" values, and a low "PRESS.". The general form of the model is represented as in the following:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 AB + \beta_5 AC + \beta_6 BC + \beta_7 A^2 + \beta_8 B^2 + \beta_9 C^2 \quad \text{Eq. 2.2}$$

Table 2.1: Experimental levels of the independent variables for optimizing Clotrimazole emulgel.

Variables (%w/w)	**Levels**				
	-α	-1	0	+1	+α
Gelling agent (Na CMC)	0.65	1.5	2.75	4	4.85
Surfactant (Tween 80 : ethanol)	3.18	10	20	30	36.82
Oil Phase (olive oil : oleic acid)	1.59	5	10	15	18.41

α = 1.68179

where β_0, the intercept, is the arithmetic average of all quantitative outcomes of twenty runs, β_1 to β_9 are the coefficient computed from the observed experimental values of Y, and A, B and C are the coded levels of the independent variable(s).The terms AB, AC, AB and A^2, B^2 and C^2 are the interaction and polynomial terms, respectively. The main effects (A, B and C) postulate the average result of changing one factor at a time from its low to high value. The interaction term (AB, AC and BC) show how the response changes when two factors are changed accordingly. The polynomial terms (A^2, B^2, and C^2) symbolize nonlinearity. The polynomial equation was used to draw conclusion after considering the intensity of coefficient and the mathematical sign it carries, that is, positive or negative. A positive sign signifies synergism (Chakraborty, *et al.*, 2013).

And the model was used to generate the response surface plots, three-dimensional displays of the response surface, and predicted response(s) for any set of factors. The adequacy of the model equations to represent the relationship between the responses and the measured components were statistically validated using ANOVA provision in the software. After feasibility and grid searches, a multiple response optimization was done by setting factor ranges to the actual levels and the "Goal" at maximum values for spreadability and residence time, targeted to 100% for release at the 8th hr, and in range between 12.5% and 20% for drug release at the 1st hr. The lowest acceptable value (the lower limit) and the higher limit were set (Table 2.2) to get the desirability equation. Equal emphasis to upper or lower bounds was given for each response with a weight of 1 where desirability (di) was varied from 0 to 1 in linear fashion. And all responses were given medium setting of importance level as compared to one another.

Table 2.2: Lower and upper limits of responses for the predicted optimum formulation(s).

Responses	Lower limit	Upper limit
Release at 1st hr (%)	12.5	20
Release at 8th hr (%)	85	observed higher extreme
Residence time (min)	15	20
Spreadability ($g.cm.min^{-1}$)	285	observed higher extreme

The software was also used to optimize the process graphically to see a broader operating window, with the same requirements as in the numerical optimization. It produced the "overlay" plot of response contours for each response shaded out regions not meeting the specifications, leaving an operating window or "sweet spot" (colored yellow) where the factors was set to satisfy the requirements on all the responses.

2.2.3.3.Characterization of the optimized formulations

i. Globule Size Analysis of the emulsion

The average globule size and polydispersity index of the optimized clotrimazole emulsions were determined in triplicate by the photon correlation spectroscopy (Brookhaven Instruments Corporation, USA). Measurements were carried at an angle of 90° at 25°C. A diluted suspension of the emulsion was prepared to a concentration of 0.1% v/v using distilled water. Digital autocorrelator software (90Plus particle sizing software, USA) was used to calculate the equivalent spherical particle size and polydispersity.

ii. Release profile comparisons

The release profile comparison was carried out with model independent methods using a difference factor (f_1) and a similarity factor (f_2). The difference factor (f_1) was used to calculate the percent (%) difference between the two curves at each time point and is a measure of the relative error between the two curves (Eq. 2.3).

$$f_1 = \{[\Sigma_{t=1}^{n} | R_t - T_t |]/[\Sigma_{t=1}^{n} R_t]\} \times 100 \quad \text{..... Eq. 2.3}$$

where n is the number of time points, R and T are the release values of the first and the second formulations respectively at time t.

The similarity factor (f_2) is a logarithmic reciprocal square root transformation of the sum of squared error and was used to measure the similarity in percent (%) release between the two curves (Eq. 2.4).

$$f_2 = 50 \text{ x } \log \{[1+(1/n)\Sigma_{t=1}^{n} (R_t - T_t)^2]^{-0.5} \text{ x } 100\} \quad \text{Eq. 2.4}$$

For curves whose f_1 values are close to 0 and f_2 values close to 100 were considered similar. Generally, f_1 values up to 15 (0-15) and f_2 values greater than 50 (50-100) ensure similarity of the two curves and, thus, of the performance of the two preparations or same preparation over different times (U.S. Department of Health and Human Services, 1997).

iii. Drug release kinetics and mechanism of drug release

The kinetics of drug release, from the optimized clotrimazole emulgels, were investigated by fitting the *in vitro* release studies into model dependent methods such as zero order (Eq. 2.5), first order (Eq. 2.6), Higuchi (Eq. 2.7), and Hixson Crowell (Eq. 2.8).

Zero-order model

$$Q_t = Q_0 - K_0 t \quad \text{Eq. 2.5}$$

Where Q_t is the amount of drug dissolved in time t, Q_0 is initial concentration of drug, and K_0 is the zero order release constant expressed in units of concentration/time.

First order model

$$\log Q_t = \log Q_0 - Kt / 2.303 \quad \text{Eq. 2.6}$$

Where Q_t is the amount of drug dissolved in time t, Q_0 is the initial concentration of drug, K is the first order rate constant.

Higuchi model

$$M_t / M_\infty = K_H t^{1/2} \quad \text{Eq. 2.7}$$

Where K_H is the Higuchi dissolution constant and Mt / M_∞ is a fraction of drug released at time t.

Hixson Crowell model

$W_o^{1/3} - W_t^{1/3} = \kappa t$ …… Eq. 2.8

Where W_0 is the initial amount of drug in the pharmaceutical dosage form, W_t is the remaining amount of drug in the pharmaceutical dosage form at time t and κ (kappa) is a constant incorporating the surface-volume relation.

In order to find out the mechanism of drug release from the optimized Clotrimazole emulgels, drug release data were fitted to the Korsmeyer-Peppas model (Eq. 2.9):

Korsmeyer-Peppas model

$Mt / M_\infty = Kt^n$ ……..... Eq. 2.9

Where Mt / M_∞ is a fraction of drug released at time t, K is the release rate constant and n is the release exponent that is used to characterize different release mechanisms.

iv. Stability study

The optimized clotrimazole emulgels were filled in the collapsible tubes and stored at different temperature condition: 30 ± 2 ^{0}C/65 ±5% RH, and 40 ± 2 ^{0}C/75 ± 5% RH for a period of three months. Samples were withdrawn at 30-days intervals and evaluated for *in vitro* drug release. The profiles were compared with initial results using f_1 and f_2 (Eq. 2.3 & 2.4).

2.2.3.4.Statistical analysis

Results were treated statistically, whenever appropriate, using Microsoft Office Excel 2007. In all cases, individual differences between relevant data were evaluated by one-way analysis of variance (ANOVA) using GraphPad InStat 3.1 software (GraphPad Software, Inc, California).P value of less than 0.05 was considered to be evidence of a significant difference.

3. Results and Discussion

3.1. Preliminary studies

3.1.1. Clotrimazole solubility study and selection of oil phase

The solubility tests were performed to determine the best oil to use as the internal oil phase for emulsion preparation. From the edible oils, literature has shown that olive oil and soy bean oil have been shown to have good dissolving capacity (Kassem, *et. al.,* 2010; Borhade, *et al.,* 2012) for clotrimazole. Oleic acid also shows reasonably high clotrimazole dissolving capacity (Kassem, *et. al.,* 2010; Hashem, *et. al.,* 2011). Therefore, these oils and mineral and sunflower oil were chosen for solubility study. Clotrimazole was found to be highly soluble in oleic acid (154.03 mg/g). Virgin olive oil showed relatively better dissolving capacity than refined olive oil, sun flower oil, and soya bean oil (Table 3.1).

Table 3.1: Solubility of Clotrimazole in different oils.

Oils	Solubility (mg/gm)
Mineral oil	0.82±0.01
Virgin olive oil	63.32±0.12
Refined Olive oil	42.59±0.07
Sunflower oil	29.52±0.003
Soya bean oil	40.99±0.02
Oleic acid	154.03±0.22

Due to its relatively higher ability to solubilize clotrimazole, oleic acid was chosen to be the oil phase of the formulation. However, oleic acid with hydrophilic lipophilic balance (HLB) of 17, according to Griffin's HLB method for optimizing the HLB of the oil phase with the emulsifier, the surfactant choice of this study, Tween 80 (HLB = 15), could not solely stabilize the system. Therefore, olive oil with an HLB requirement of around 7-8 can be used to even out the high HLB requirement of oleic acid (Gibson, 2007).

3.1.2 Phase study

During the construction of phase diagrams (Figures 3.1 - 3.4) emulsion was considered as cloudy (red) and transparent (green) when the droplets can spread easily in water and produce fine emulsion. Meanwhile, the emulsion was classified as gel (blue) when longer time was required in emulsifying and producing emulsion. The emulsion was considered as unstable (black) when there was poor or no formation of emulsion and immediate coalescence of oil droplets took place when gentle agitation was stopped.

It is clear from the comparison between diagrams (Figures 3.1 - 3.4), that the ternary phase diagram system with oil (olive oil : oleic acid) ratio of 1:1 and Tween 80 : ethanol ratio of 2:1 (Figure: 3.3.a) managed to produce minimal phase separation area and maximum transparent emulsion area at higher water concentration. The transparency of the emulsion could be due to lower dispersed phase size (≤200 nm) which is the size range of micelles and microemulsions, which in contrast to coarse emulsion do not show the physical instability in terms of agglomeration or separation of the dispersed phase (Tenjarla, 1999). Kumar *et al.* (2010) also found that Tween 80 and ethanol at 2:1 ratio resulted in greater microemulsion region during emulsification of oleic acid and castor oil. Therefore, this system was chosen for further study.

The use of alcohol in this system is believed to affect the incorporation of large amount of oil phase. The penetration of the alcohol into the interfacial film reduces the repulsion of the long hydrophobic tails of the surfactant at the interface, favoring its dissolution in the oil phase. The presence of the alcohol also affects the physical properties of the water. The alcohol disrupts the water structure, creating an increase in the lipophilic character of the Tween 80. Furthermore, the alcohol provokes a decrease in the mixture viscosity; therefore, the big molecules such as Tween 80 can reach the interface faster (Prieto & Calvo, 2013).

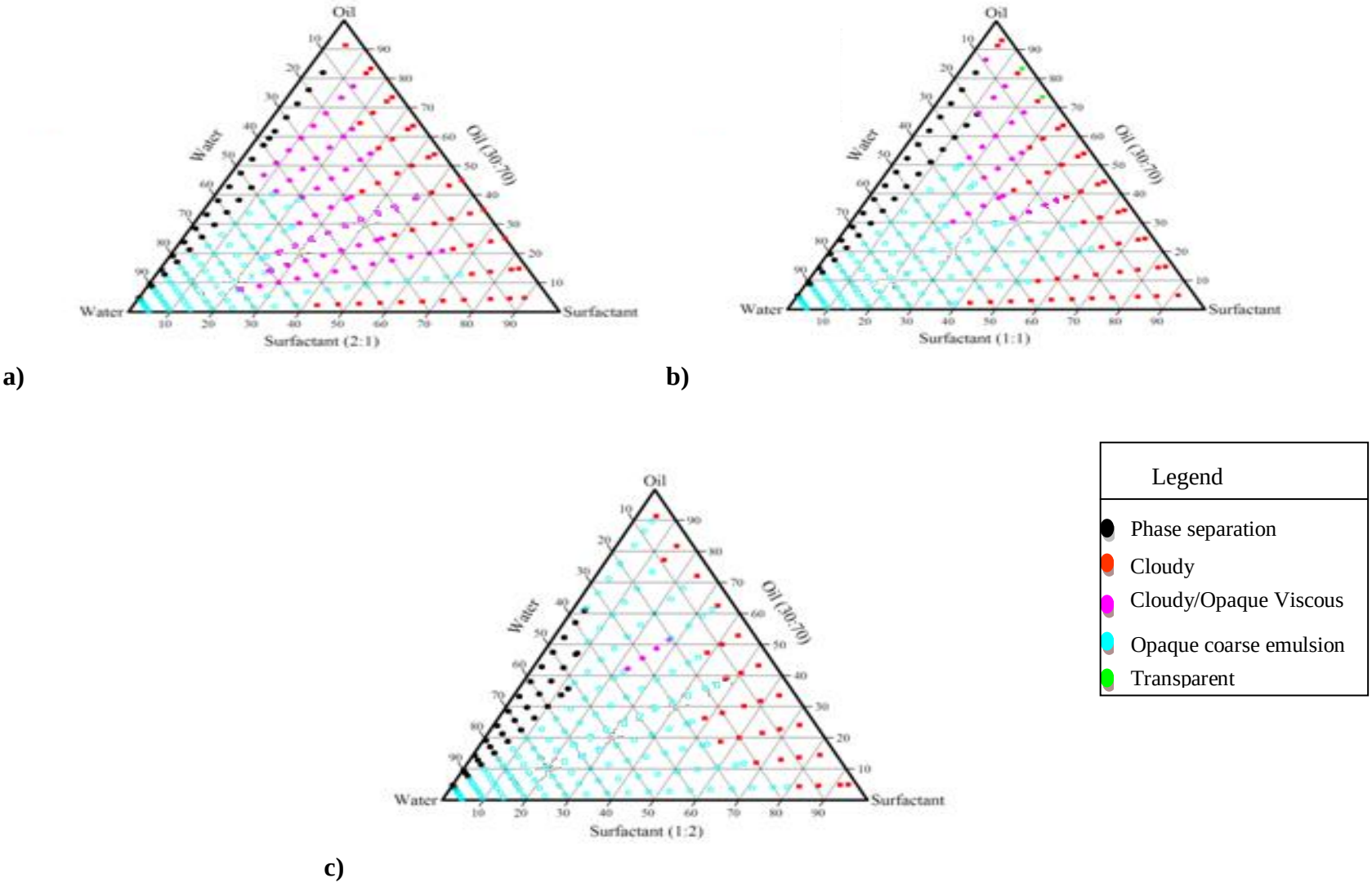
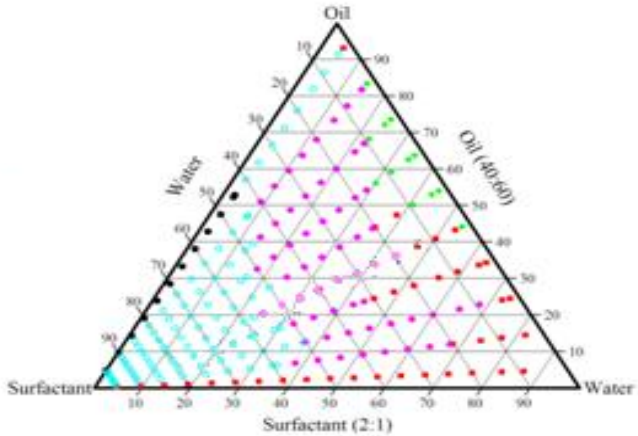
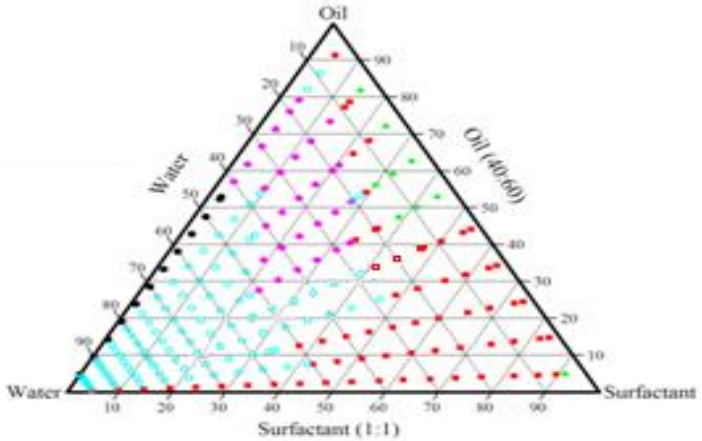
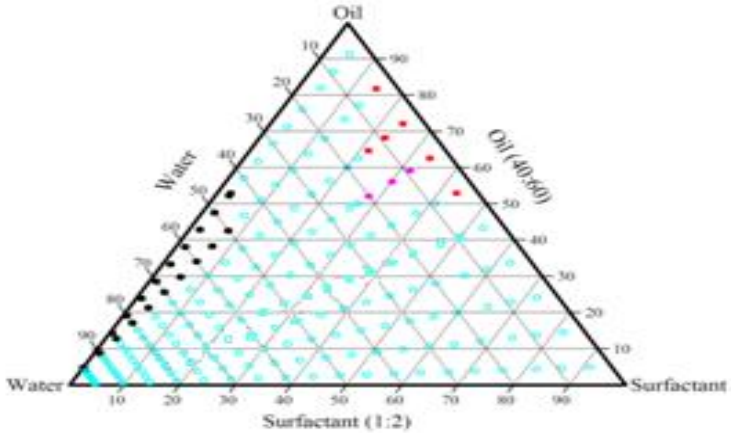


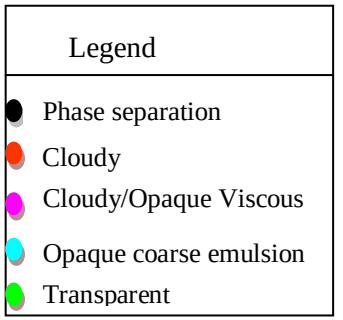
Figure 3.1: Phase diagram for oil phase A (30:70) where Tween80/ethanol is a) 2:1, b) 1:1, and c) 1:2.





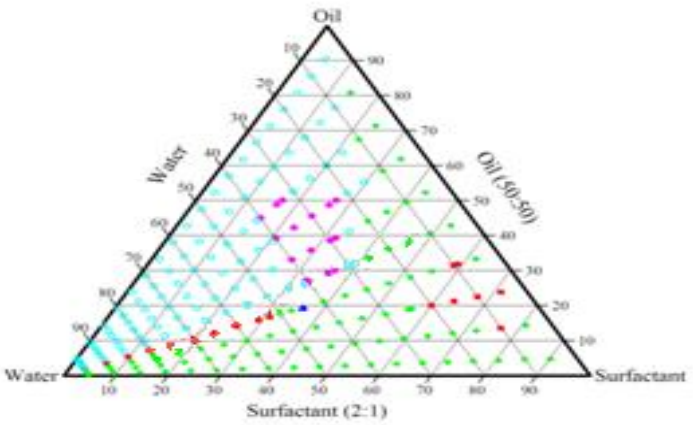
b)



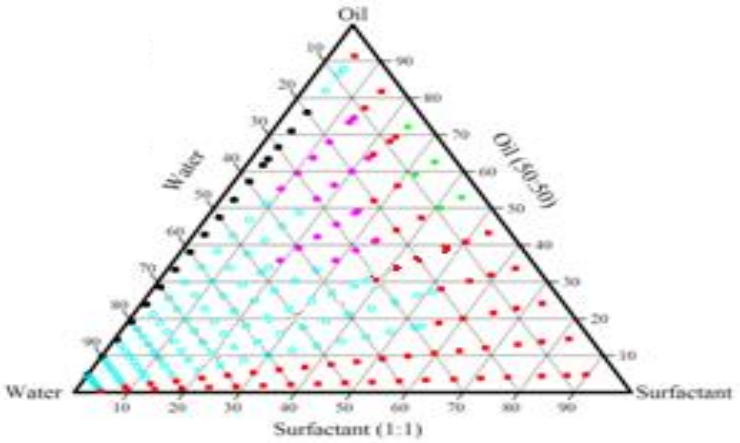


c)

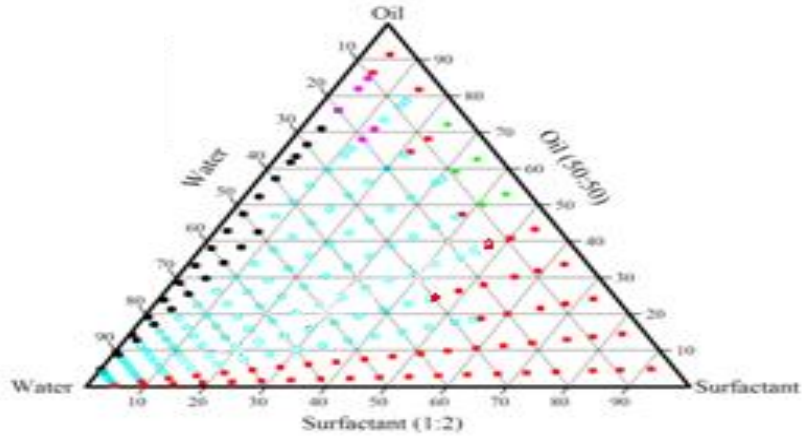
Figure 3.2: Phase diagram for oil phase B (40:60) where Tween80/ethanol is a) 2:1, b) 1:1, and c) 1:2.



a)



b)



c)

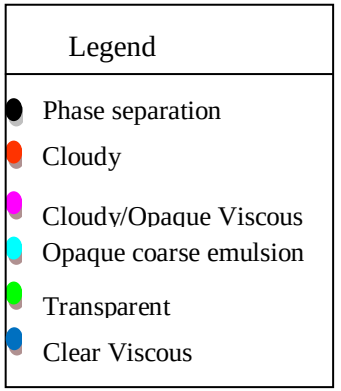


Figure 3.3: Phase diagram for oil phase C (50:50) where Tween80/ethanol is a) 2:1, b) 1:1, and c) 1:2.

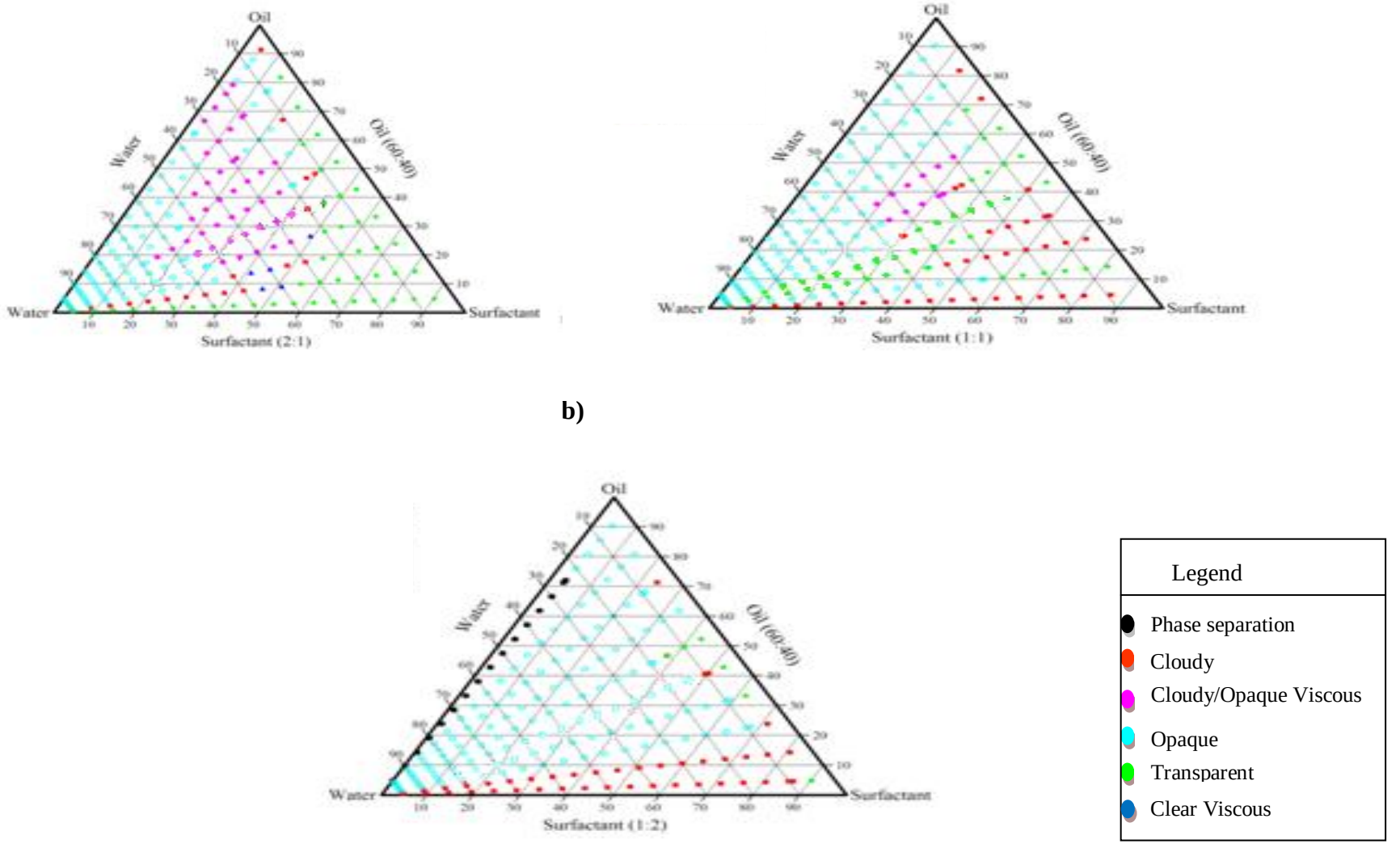


Figure 3.4: Phase diagram for oil phase D (60:40) where Tween80/ethanol is a) 2:1, b) 1:1, and c) 1:2.

3.1.3 Selection of gelling agent

Park and Robinson (1984) have conveniently divided polymers that are commonly employed in the manufacture of mucoadhesive drug delivery into three broad categories which include polymers that adhere through non-specific, non-covalent interactions that is primarily electrostatic in nature (although hydrogen and hydrophobic bonding may be significant). Of these, anionic polymers are the most widely employed mucoadhesive polymers within pharmaceutical formulation due to their high mucoadhesive functionality and low toxicity. Such polymers are characterized by the presence of carboxyl and sulphate functional groups that give rise to a net overall negative charge at pH values exceeding the pKa of the polymer. Typical examples include poly (acrylic acid) (PAA) and its weakly cross-linked derivatives and sodium carboxymethylcellulose (Na CMC) (Singh, *et al.*, 2013). However, PAA has been shown to induce severe irritation of the buccal mucosa (Bottenberg, *et al.*, 1991; Mohammed & Khedr, 2003). Na CMC has been used in the marketed Triamcinolone acetonide adhesive preparation rather known as Kenalog® in Orabase® (Bristol-Myers Squibb, New Zealand) for temporary relief of symptoms associated with oral inflammatory lesions and ulcerative lesions with no known side effects associated with it (Al-Na'mah, *et al.*, 2009). Therefore, Na CMC was chosen as a mucoadhesive polymer for the formulation of clotrimazole emulgel which can be used as treatment to oral candidiasis.

Na CMC possesses excellent mucoadhesive characteristics due to the formation of strong hydrogen bonding with mucin. Such forces have been considered the most important in the adhesive interaction phenomenon because, although they are individually weak, a great number of interactions can result in an intense global adhesion (Singh, *et al.*, 2013).

The actual amount of drug available to the site of action, which is responsible for the therapeutic efficacy, not only depends on the extent of the residence time but is also influenced by release properties of the formulation. Mixing of two gelling agents at different ratios allowed modulation of the release of the active ingredient. Sodium carboxymethylcellulose (Na CMC) has been reported to have synergistic hydrogen-bonding interactions with HPMC. This combination is popular for the formulation of sustained release matrices in order to achieve zero-order release kinetics (Devi, *et al.*, 1989; Conti, *et al.*, 2007; Contreras, *et al.*, 2012).

From the chosen emulsion system, i.e. Olive oil : oleic acid in the ratio of 1:1 and Tween 80 : ethanol ratio of 2:1, an Oil : surfactant ratio of 1:4, which is transparent and stable over a wide range of water concentration, was prepared for the selection of gelling agent. Two gelling agents namely Na CMC and HPMC were chosen for this study. HPMC K4M up to a concentration of 12% w/w and high viscosity grade Na CMC up to a concentration of 8% w/w were prepared and combined with the emulsion in 1:1 ratio and kept at room temperature for 24 h. The addition of Na CMC caused no visually detectable distinction from the original emulsion system except the increase of viscosity. However, HPMC showed phase separation at all concentration level and when it is in the ratio of 1:1 and above with Na CMC. This may be due to the formation of micelles on the polymer chain. This changes the conformation of the polymer chain in solution resulting in polymer-polymer association and caused phase separation (Slvadasan and Somasundaran, 1990; Shahin, *et al.*, 2011b; Tomas & Penfold, 2014).

This result indicates Na CMC as a potential polymer to gel the clotrimazole emulsion without affecting much of its structure. This may be due to the enhancement in hydrophilic character of Na CMC, which built-up the consistency index (an indication of apparent viscosity) of external aqueous phase and thereby preventing the successive inter-globule collisions responsible for creaming and subsequently for emulsion oiling. Na-CMC, an ionic polymer in aqueous solution behaves as a polyelectrolyte.

3.2 Clotrimazole emulgel preparation and characterization

3.2.1 Clotrimazole emulgel preparation

Clotrimazole emulgel was optimized based on Design-Expert® software (Version 6.0.8, Stat-Ease Inc., Minneapolis, MN, USA). The experiment was studied with a standard RSM design called a central composite design (CCD). One of the challenges that appear in the formulation of drug as an emulgel preparation is the choice of the type of base and concentration used (Sabri, *et al.*, 2009). Therefore, the entry field for ‘Numeric Factors’ was entered ‘3’ for the software, for concentrations of oil, surfactant, and gelling agent with their high and low levels 5 & 15%, 10 & 30%, and 1.5 & 4%, respectively, as the independent variables. The %w/w of surfactant was selected based on the considerations that high concentration of surfactant could cause toxicity and irritation. The WHO has set an estimated acceptable daily intake for Polysorbates at up to 25

mg/kg body-weight (Rowe, *et al.,* 2009), which makes 30% of surfactant solution safe for a 40 kg adult with the dose of 10 mg clotrimazole for five times a day.

During titration of oil and surfactant mixture with aqueous phase (> 40%) at relatively higher oil concentration, w/o emulsions are inverted into o/w emulsions, and/or w/o/w emulsions; a number of liquid crystalline phases are considered to be possible intermediates during this phase inversion process which is indicated by the formation of gel area in the phase diagram (Figure 3.3 a). Systems that remained clear and fluid at higher dilutions with the aqueous phase are expected to be of the o/w type where the oil phase ratio is lower (Abd-Allah, *et al.,* 2010). Therefore, considering the other additives in the formulation the maximum amount of oil that can be incorporated for the possible o/w emulgel system is 15%. And minimum of 5% oil and 10% surfactant mixtures were found to easily solubilize 1% clotrimazole.

Fifteen formulations ($2^n + 2n + n_o$) were prepared using Olive oil and oleic acid as the oil phase at 1:1 ratio and Na CMC as a gelling agent. The concentration of clotrimazole was set to be 1% in the final formulations since it has been shown to be safe in humans. Methylparaben (0.18% w/w) together with propylparaben (0.02% w/w) have been used for the preservation of the formulations due to their additive effects. They exhibit broad spectrum antimicrobial activity over a wide pH range. Propylene glycol (2%) was used to potentiate the antimicrobial activity of the Parabens in the presence of nonionic surfactants, prevents the interaction with Tween 80, and increases their solubility in water. Usually dissolved air found in liquids or possibly incorporated during the preparation of the product may result in oxidative decomposition of certain excipients like oils. Therefore, sodium sulphite (0.1%) was used as an antioxidant to scavenge oxygen from the formulation (Rowe, *et al.,* 2009). Trisodium citrate (0.3% w/w) and citric acid were used as a buffering system to convey the final formulation to a pH of around 6.8, which is considered acceptable to avoid the risk of irritation upon application to the oral mucosa pH ranging between 6.2 and 7.4. And spray dried orange flavor was added to mask the characteristics odor of Tween 80. The compositions of the gels are summarized in Table 3.2.

Table 3.2: Composition of clotrimazole emulgel formulations.

Materials (% w/w)	F0	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14
Clotrimazole	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Oil mixture	10	5	15	5	15	5	15	5	15	1.58	18.41	10	10	10	10
Surfactant solution	20	10	10	30	30	10	10	30	30	20	20	3.18	36.82	20	20
Trisodium citrate	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Citric acid	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
Propyl Paraben	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Methyl Paraben	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18
Propyl glycol	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Sodium Sulphite	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Orange flavor	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Na CMC	2.75	1.5	4	4	1.5	4	1.5	1.5	4	2.75	2.75	2.75	2.75	0.65	4.85
Water qs.	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Three blocks were selected for this design, one for each day, with full CCD type. The design layout (Table 3.3), submitted by the software, contains the completed design in run order showing the actual factor levels for twenty experiments ($2^n + 2n + n_o$).

Table 3.3: Design layout used to build surface response design.

Formulation Identification	Day/Block	Oil %	Surfactant %	SCMC %
F2	Block 1	15.00	10.00	4.00
F1	Block 1	5.00	10.00	1.50
F3	Block 1	5.00	30.00	4.00
F0	Block 1	10.00	20.00	2.75
F4	Block 1	15.00	30.00	1.50
F0	Block 1	10.00	20.00	2.75
F7	Block 2	5.00	30.00	1.50
F0	Block 2	10.00	20.00	2.75
F8	Block 2	15.00	30.00	4.00
F5	Block 2	5.00	10.00	4.00
F0	Block 2	10.00	20.00	2.75
F6	Block 2	15.00	10.00	1.50
F0	Block 3	10.00	20.00	2.75
F14	Block 3	10.00	20.00	4.85
F11	Block 3	10.00	3.18	2.75
F9	Block 3	1.59	20.00	2.75
F13	Block 3	10.00	20.00	0.65
F12	Block 3	10.00	36.82	2.75
F0	Block 3	10.00	20.00	2.75
F10	Block 3	18.41	20.00	2.75

3.2.2 Clotrimazole emulgel characterization

3.2.2.1 Physical appearance, pH, and content

The formulated emulgels were examined for their physical appearance, pH, and content after 24 hr of preparation (Table 3.4). They were transparent to white opaque and from viscous gel preparations to flowable liquids with a smooth homogeneous appearance. The pH values of all prepared formulations ranged from 6.0 to 6.9, which are considered acceptable to avoid the risk of irritation upon application to the oral mucosa because pH of adult oral cavity is between 6.2 and 7.4. The contents of all formulations were between 104.5% and 98.3% of the labeled amount. Therefore, it may be assumed that these formulations were applicable to the oral cavity.

Table 3.4: Physical appearance, consistency, pH and drug content of emulgel formulations.

Formulation Identification	Appearance	Consistency	pH	Content (%)
F0	Transparent	Gel	6.2 ± 0.06	99.48 ± 1.27
F1	Transparent	Flowable	6.5 ± 0.06	98.67 ± 1.84
F2	opaque	Gel	6.2 ± 0.17	99.64 ± 1.49
F3	Transparent	Gel	6.4 ± 0.15	98.37 ± 0.60
F4	opaque	Creamy*	6.2 ± 0.15	100.20 ± 0.95
F5	Transparent	Gel	6.5 ± 0.17	100.35 ± 1.50
F6	opaque	Flowable	6.2 ± 0.35	98.73 ± 1.06
F7	Transparent	Flowable	6.4 ± 0.07	100.52 ± 0.97
F8	opaque	Gel	6.2 ± 0.15	99.39 ± 0.27
F9	Transparent	Gel	6.9 ± 0.35	100.34 ± 0.28
F10	opaque	Gel	6.0 ± 0.10	98.94 ± 1.10
F11	opaque	Gel	6.5 ± 0.17	100.02 ± 0.71
F12	Transparent	Gel	6.5 ± 0.10	98.79 ± 2.01
F13	Transparent	Flowable	6.5 ± 0.12	104.50 ± 1.19
F14	Transparent	Gel	6.5 ± 0.15	99.37 ± 1.27
Mycoheal®	opaque	Gel	6.6 ± 0.20	-
Decozol®	opaque	Gel	6.5 ± 0.17	-

*Creamy: intermediate between gel and flowable consistency.

The commercially available Miconazole oral gels, namely Mycoheal® and Decozol® were also evaluated, for the same parameters except content, for comparison purpose.

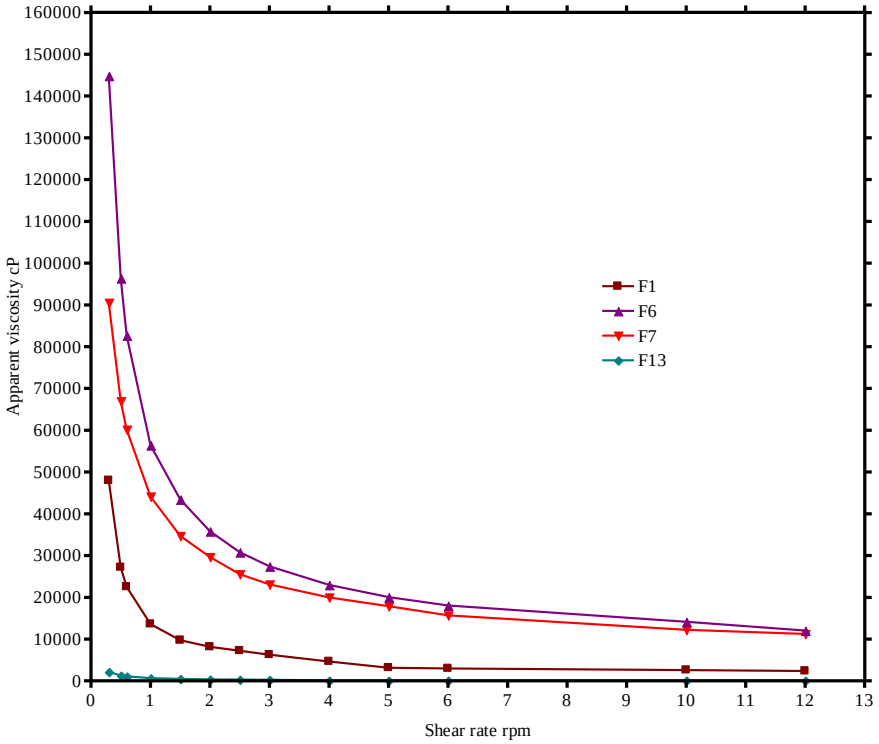
3.2.2.2 Apparent viscosity

An important rheological parameter is the apparent viscosity of a gel. It plays a vital role in the dispensing and formulation of topical formulations. It is related to mechanical and physical properties such as spreadability, consistency and hardness of the preparation which in turn are related to ease of product removal from container, ease of application on the target surface and product feel on the application site. Apparent viscosities of the fifteen emulgel preparations and locally available oral gels of miconazole have been determined using a rotational viscometer with 'T' shaped special spindles exerting minimal disturbance to sample, with shear rate ranging from 0.3 to 12 rpm.

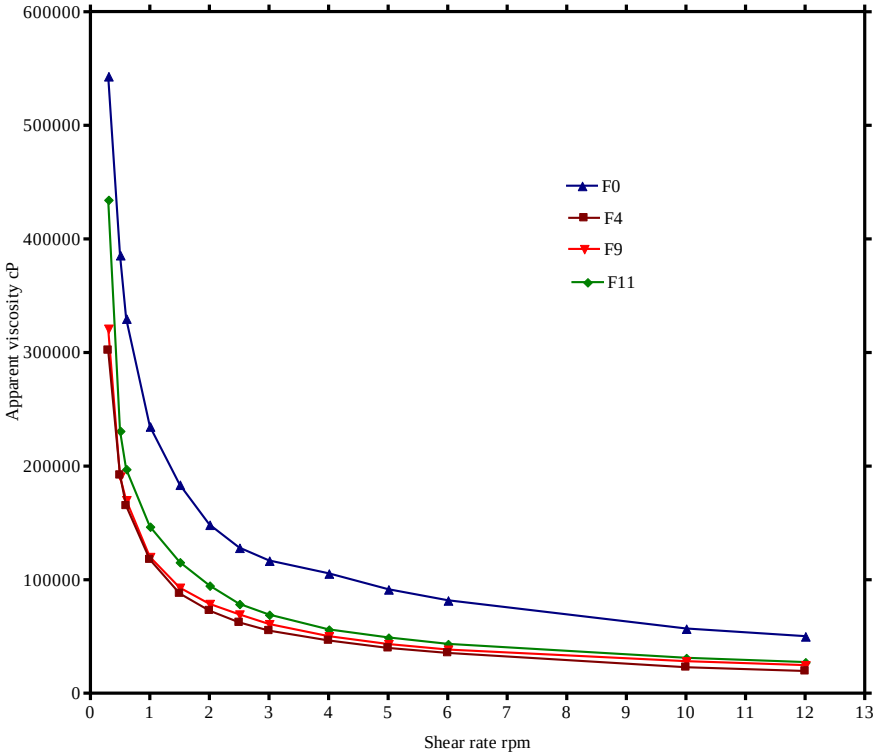
All the design points showed a shear-thinning behavior since the viscosity decreased with increasing shear rate (Figure 3.5 A-D). This is a desirable property in an oromucosal semisolid preparation, since it should thin during application. The cause of pseudoplastic flow revealed by gels may be due to progressive rupture of the internal structure of the formulations and its later reconstruction by means of Brownian movement (El-Houssieny & Hamouda, 2010). This coincides with other studies in which also the degree of such pseudoplastic behavior significantly increased with increasing Na CMC concentration (Mirhosseini & Ping Tan, 2010; Amiri & Radi, 2013).

Semisolid pharmaceutical preparations for topical administration preferably have viscosity of 250,000 – 1,000,000 cps for the ease of application and retention (Hartung, *et al.,* 1994). Formulations F1, F6, F7 and F13 showed less than 200,000 cps of viscosity at minimal shear rate with F13 showing the least resistance to flow (Figure 3.5.A.). Formulations F2, F3 and F14 showed more than 1,500,000 cps of viscosity (Figure 3.5.D). And for the marketed formulations Mycoheal® (1,340,660 cps) is two times as viscous as Decozole® (678,442 cps).

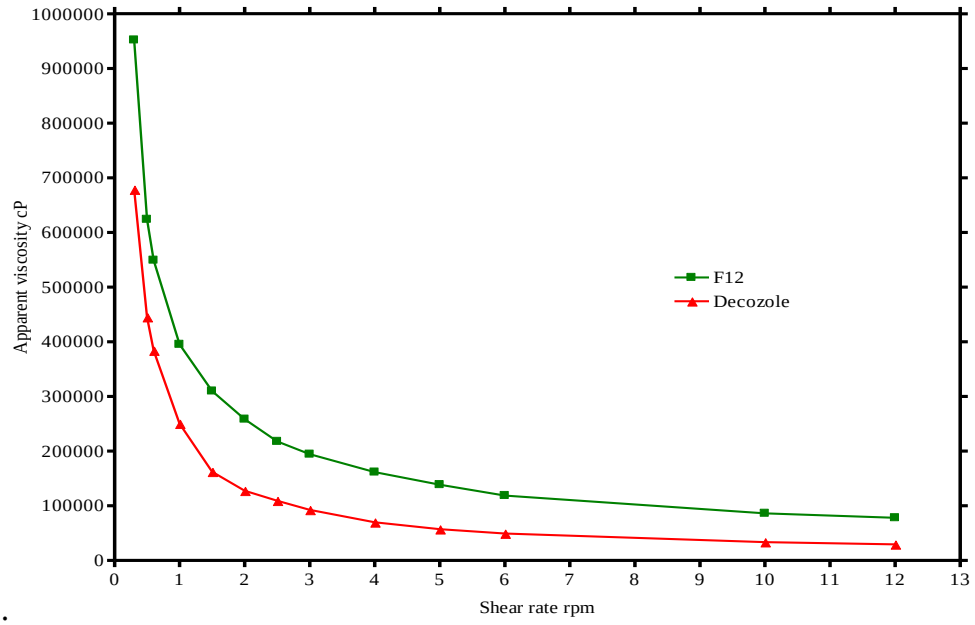
A.


Apparent viscosity cP
Shear rate rpm
F1
F6
F7
F13

B.


Apparent viscosity cP
Shear rate rpm
F0
F4
F9
F11

C.



D.

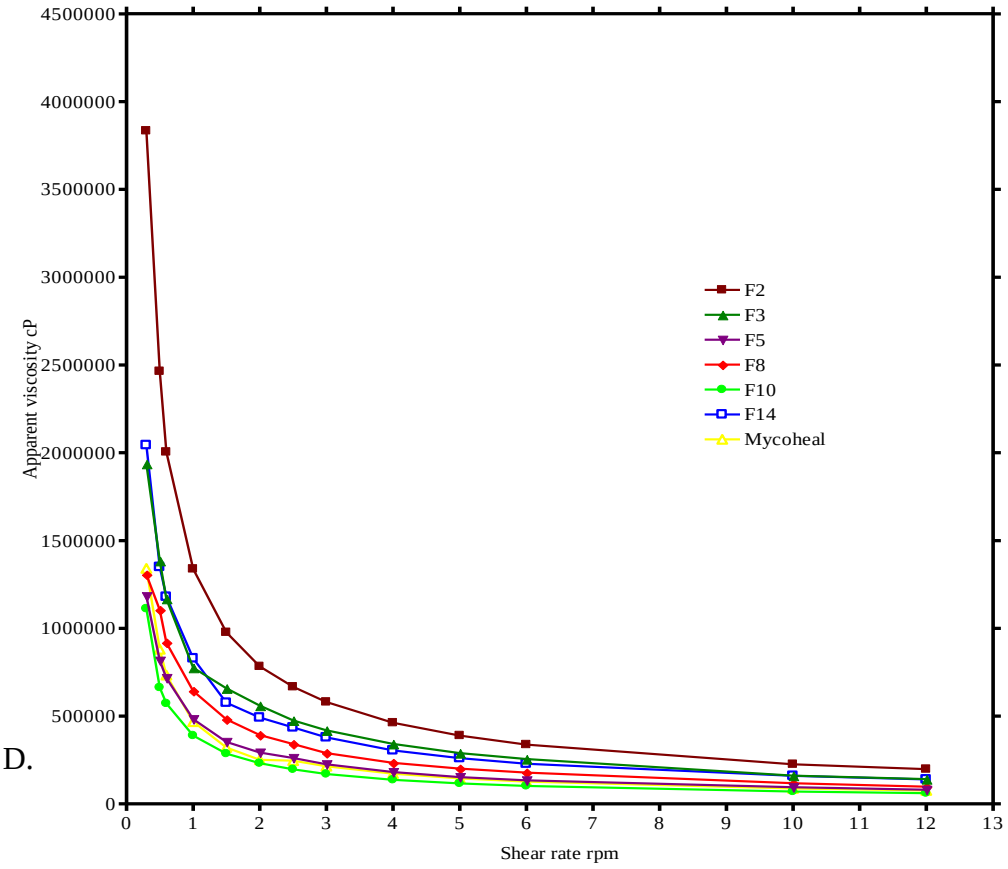


Figure 3.5: Apparent viscosities of the emulgel formulations and marketed oral gels at increasing shear rates (A-D).

3.2.2.3 Spreadability

One of the essential criteria for an emulgel is that it should possess good spreadability. Spreadability is an important factor in therapy and it is shown as index of ease of application. The efficacy of topical therapy depends on the patient spreading the formulation in an even layer to deliver a standard dose. The optimum consistency of such a formulation helps ensure that a suitable dose is applied or delivered to the target site. This is particularly important with formulations of potent drugs. A reduced dose would not deliver the desired effect, and an excessive dose may lead to undesirable side effects. The delivery of the correct dose of the drug depends highly on the spreadability of the formulation (Garg, *et al.,* 2002).

The important factors to consider, during evaluating the spreadability of a formulation, include the rate and time of shear produced upon smearing and the temperature of the target site. The parallel-plate method is the most widely used method for determining and quantifying the spreadability of semisolid preparation. It provides accurate, reproducible, and statistically relevant data. The advantages of the method are simplicity and relative lack of expense. On the other hand, the method is not very precise and sensitive, and data which it generates must be manually interpreted and presented (Garg, *et al.,* 2002; Jelvehgari, *et al.,* 2007).

The spreadability of clotrimazole emulgel formulation following the spreadability test was found to range from 102 g.cm/min to 387 g.cm/min for the formulations F2 and F6, respectively (Table 3.5). Each value is given as mean of the three experiments. Formulations with low Na CMC concentrations, such as F1, F4, F6, and F13, showed the highest spreadability index (Table 3.5). When the concentration of the Na CMC is increased from 1.5% to 4% while keeping other variables constant it showed extremely significant reduction in spreadability except when oil and the surfactant concentration were 5% and 30%, respectively. This may be due to the effect of the concentration and physical characteristics of the polymer used in the emulgels on the contact angle of preparation on the substrate and lubricity, which is directly related to the coefficient of friction (Bonacucina, *et al.,* 2009).

Table 3.5: Spreadability, residence time, and *in vitro* drug release at 1st and 8th hrs of emulgel formulations.

Formulation Identification	Day/Block	Spreadability ($g.cm.min^{-1}$)	Residence time (min)	Drug Release at 1st hr (%)	Drug Release at 8th hr (%)
F2	Block 1	102 ± 2.65	24.15 ± 0.50	0.02 ± 0.02	4.33 ± 0.83
F1	Block 1	375 ± 11.53	0.47 ± 0.03	85.82 ± 1.72	88.39 ± 2.58
F3	Block 1	297.5 ± 9.73	2.22 ± 0.11	15.38 ± 1.28	94.42 ± 5.20
F0	Block 1	314.5 ± 2.52	2.01 ± 0.05	34.78 ± 1.76	92.79 ± 3.47
F4	Block 1	372 ± 4.58	2.49 ± 0.15	62.25 ± 5.23	66.39 ± 2.04
F0	Block 1	335 ± 8.89	9.44 ± 0.26	47.63 ± 1.06	90.81 ± 3.54
F7	Block 2	280.5 ± 7.40	0.47 ± 0.05	90.56 ± 0.89	102.48 ± 1.95
F0	Block 2	303 ± 6.08	6.36 ± 0.49	34.92 ± 0.44	96.99 ± 0.15
F8	Block 2	255 ± 7.94	29.99 ± 0.92	15.74 ± 0.84	78.54 ± 2.43
F5	Block 2	280.5 ± 9.26	7.99 ± 1.052	7.9 ± 1.41	41.53 ± 1.71
F0	Block 2	323 ± 6.08	6.43 ± 0.18	31.53 ± 8.27	82.13 ± 0.94
F6	Block 2	387 ± 10.58	5.16 ± 0.65	0.29 ± 0.04	11.95 ± 0.25
F0	Block 3	324 ± 3.61	1.34 ± 0.08	32.29 ± 4.46	93.96 ± 0.19
F14	Block 3	297.5 ± 4.44	15.18 ± 0.34	3.73 ± 0.39	34.26 ± 1.92
F11	Block 3	348.5 ± 4.44	8.54 ± 0.20	0	12.05 ± 1.15
F9	Block 3	314.5 ± 7.09	5.38 ± 0.20	37.52 ± 0.97	70.46 ± 2.47
F13	Block 3	355 ± 6.24	0.30 ± 0.07	55.18 ± 0.08	104.64 ± 1.98
F12	Block 3	289 ± 1.00	1.41 ± 0.08	22.36 ± 2.78	100.7 ± 1.09
F0	Block 3	297.5 ± 9.12	6.16 ± 0.51	32.01 ± 0.99	89.56 ± 2.49
F10	Block 3	314.5 ± 5.68	19.36 ± 1.26	1.04 ± 0.13	37.64 ± 3.15
Mycoheal®	-	271 ± 3.61	5.43 ± 0.81	-	-
Decozol®	-	285 ± 17.06	2.21 ± 0.37	-	-

The increase in surfactant and co-surfactant mixture from 10% to 30%, at higher polymer (4%) and oil (15%) concentrations while keeping other factors similar, tend to increase the viscosity but has shown extremely significant increase in spreadability. Surface active agents, such as surfactants have been used to improve the lubricity and spreadability of topical–mucosal

formulations, oil–enriched surfactants exhibiting better surface activity (Garg, *et al.,* 2002). And the presence of oil and surfactant may be the reasons for the improvement in the spreadability in the emulgel formulations than the marketed conventional gel formulations.

3.2.2.4 Residence time

A more viscous formulation would have poor spreadability but would have a good residence time at the target site. Increased residence time of the dosage form at the target site tends to prolong drug action. Some polymers, such as Na CMC, also have mucoadhesion property which become adhesive on hydration and hence can be used for targeting a drug to a particular region of the body for extended periods of time.

Formulation F8 showed the highest residence time of 29.99 ± 0.92 min and formulations F2, F10, and F14 showed residence time of more than 15 min on the Agar plate (Table 3.5). Formulations F1, F7, and F13 showed the least residence times which are below one min. Na CMC is anionic polymers which forms strong hydrogen bonding with mucin. As its concentration increased from 1.5% to 4% and 0.65% to 4.85% the residence time also increased very significantly. This can be explained by the increase of polymer chain length available for penetration into the agar layer as the concentration increases. When the concentration of the polymer is too low, the number of penetrating polymer chains per unit volume of the Agar is small and the interaction between polymer and mucus is unstable. Therefore the more concentrated polymer would result in a longer penetrating chain length and better adhesion (Boddupalli, *et al.,* 2010).

In addition to mucoadhesive properties, enhanced viscosity also increases residence time of formulations in the ever diluting and moving environment of the media in the disintegration apparatus simulating the oral cavity state. When the oil concentration increased while keeping other constituents constant, it resulted in increased viscosity and hence residence time very significantly except in formulation F3 with possibly due to the interaction effect of other factors. Usually systems with higher elastic component, which is the rate at which the deformed sample returns to its un-deformed condition after the removal of the deforming force, possess a greater mucoadhesion (Bonacucina, *et al.,* 2009; Senyigit, *et al.,* 2014).

3.2.2.5 In vitro drug release of clotrimazole emulgel

It is expected that drug release might be influenced by gel viscosity. As the viscosity of the gel increased, the release of the drug was expected to be slower. Initial release of the drug is significantly affected ($P<0.02$) by the concentration of gelling agent in all cases. Formulations which contain Na CMC at concentration of 4% and above (F2, F3, F5, F8, and F14) seem to retain the active ingredient at the 1st hr regardless of the oil to surfactant ratio (Table 3.5). Whereas formulations F1, F7, and F13 released more than 80% of the drug in the 1st hr. Dosage forms which are able to sustain the release of the active ingredient in order to maintain a constant drug concentration for a specific period of time are desirable.

Complete drug release (100%) was achieved at the 8th hr for the formulations F7, F12 and F13 and formulations F0, F1, F3, and F13 released more than 85% of the drug (Table 3.5). Percentage of drug released in dissolution medium showed extremely significant difference ($P\leq0.0005$) when the surfactant concentration is varied. This effect may be explained by the ability of these emulsifying agents to lower the interfacial tension between oily and aqueous layer in the dispersion medium, increasing the hydrophilicity of the emulgel which in turn increase penetration of dissolution medium into the emulgel structure and then increasing the amount of drug released (Sabri, *et al.*, 2009).

The effect of oil phase concentration, when increased from 5% w/w to 15% w/w, showed significant ($P<0.05$) decrease in the amount of clotrimazole release from these bases. This result may be explained according to the concept of escaping tendency of drugs; it was supposed that increasing the thermodynamic activity which can be expressed in terms of relative solubility of drug lead to enhance the releasing of drugs from vehicle (Sabri, *et al.*, 2009).

3.3 Mathematical model selection and optimization of responses

3.3.1 Selection of mathematical model

The responses for twenty experiments were used to fit an equation for composite design model which then can predict the properties of all possible formulations. The software fits linear, two-factor interaction (2FI), quadratic and cubic polynomials to the responses and suggests a model based on the least sum of squares.

On the fit summaries for the responses the "Sequential Model Sum of Squares" summary table shows how terms of increasing complexity contribute to the total model. For each source of terms (linear, etc.), the probability ("PROB > F") which falls below 0.05, was selected. For drug release at 1^{st} and 8^{th} h, the quadratic model was suggested by the software with R-square of 0.9483 and 0.9812, respectively (Table 3.6). However, for spreadability, the addition of the quadratic (squared) terms and, for residence time, the addition of the two factor interaction (2FI) and the quadratic (squared) terms were not found to be significant. Therefore, two factor interaction (2FI) and linear models were chosen for spreadability and residence time, respectively. The "Lack of Fit Tests" table compares the residual error to the "Pure Error" from replicated design points. And, in all cases the respective models, identified earlier as the likely models, do not show significant lack of fit. In cases of drug release at 1^{st} and 8^{th} h, the linear and two-factor interaction (2FI) models definitely can be ruled out, because their "PROB > F" falls below 0.05 (Table 3.6). Yet again on "Model Summary Statistics" table lists other statistics useful in comparing models. The software chose models which exhibit low standard deviation (SD), high "R-Square" values and a low "PRESS" (predicted residual sum of squares) for each response. PRESS is a measure of how well the model fits each point in the design. The model is used to estimate each point using all of the design points. The difference between the predicted value and actual value at each point is squared and summed over all of the points. Small values for the same in these models show a good fit of the data points. The quadratic model comes out best for drug release at 1^{st} and 8^{th} h and the linear and the two-factor interaction (2FI) models for residence time and spreadability respectively (Table 3.6).

Table 3.6: Fit summary statistical analysis of the responses by Design-Expert ® software.

Response	Model	Model F-value	Lack of Fit F-value	Std. Dev.	PRESS	R^2	Adj R^2	Pred R^2	Adeq. Precision	Remark
Drug release at the 1st h	Linear	11.57	20.29	1.72	94.61	0.7126	0.6511	0.3414		
	2FI	1.08	21.45	1.70	133.67	0.7781	0.6571	0.0694		
	Quadratic	29.13	5.45	0.88	40.07	0.9408	0.9085	0.7210	16.667	Suggested
	Cubic	8.94	1.09	0.43	159.79	0.9948	0.9779	0.1124		Aliased
Drug release at the 8th h	Linear	9.65	14.51	21.74	13173.75	0.6740	0.6041	0.3509		
	2FI	0.44	17.78	23.18	27333.40	0.7089	0.5501	0.3468		
	Quadratic	46.40	1.28	6.91	3889.60	0.9812	0.9601	0.8084	17.535	Suggested
	Cubic	1.60	0.61	6.06	20000.19	0.9928	0.9693	0.0145		Aliased
Spreadability	Linear	2.91	14.35	53.99	1.033E+005	0.3840	0.2520	0.0004		
	2FI	8.22	1.44	33.13	10232.06	0.7736	0.7214	0.4757	10.310	Suggested
	Quadratic	0.21	8.22	37.41	1.449E+005	0.8310	0.6409	0.0008		
	Cubic	5.09	4.25	21.44	8.573E+005	0.9722	0.8820	0.0020		Aliased
Residence time	Linear	16.63	0.56	0.31	2.65	0.7809	0.7339	0.5709	9.896	Suggested
	2FI	0.054	0.75	0.35	4.70	0.7840	0.6662	0.2379		
	Quadratic	1.60	0.52	0.32	5.71	0.8651	0.7133	0.0757		
	Cubic	0.54	0.64	0.37	76.90	0.9124	0.6278	-11.4590		Aliased

Statistical validation of the polynomial equations generated was established on the basis of ANOVA provision in the software. This confirms the adequacy of the model equations to represent the relationship between the responses and the measured components. ANOVA was performed to identify insignificant factors in the previously selected models. The F-value is the ratio of model mean square to the appropriate error (i.e. residual) mean square. The larger the F-value, the more likely that variance contributed by model are significantly larger than random error. If the F ratio, the ratio of variances lies near the tail of the (F) distribution then the probability of a larger F is small and the variance ratio is judged to be significant. Usually, a probability less than 0.05 is considered significant (Verma, *et al.,* 2013). The ANOVA in this case confirms the adequacy of the selected models, with the model "F-value's of 29.13, 46.4, & 16.63 and where the model "PROB > F" was less than 0.0001 for responses drug release at 1^{st} and 8^{th} hr and residence time, respectively (Table 3.6).

Probability values for each individual term in the model were also seen where "PROB > F" less than 0.05 indicate model terms are significant. If there are many insignificant model terms, model reduction of terms with "PROB > F" values greater than 0.1000, (not counting those required to support hierarchy), may improve the model. Model reduction was required for the drug release at the 1^{st} h, with insignificant terms having probability values greater than 0.1, in-order to improve the quadratic model. However, as there were no significant improvement in the models of drug release at the 8^{th} h, spreadability and residence time, model reduction was not required. The lack of fit for all the models for each response was insignificant as compared to the pure error.

Adequate precision measures the signal to noise ratio which was computed by dividing the difference between the maximum predicted response and the minimum predicted response by the average standard deviation of all predicted responses. A ratio of greater than 4 was achieved for all models (Table 3.6), which is desirable to navigate the design spaces. And in all cases, except spreadability, the "Pred R-Squared" were in reasonable agreement with the "Adj R-Squared" when drug release at 1^{st} h and residence time were transformed to square root and base 10 log, respectively. Final equations in terms of actual factors are depicted below (Eq. 3.1 – Eq. 3.4) which can be used to draw conclusions after considering the magnitude of coefficients and the mathematical signs they carry (i.e., positive or negative). And the response surface prediction

curves (model graphs) for four of the responses in two and three-dimensional displays, for five contour levels, are shown at Annex 2 (Figure 6.3 – 6.14).

Mathematical relationships generated using multiple linear regression analysis for the studied response variables are expressed as follows (Eq. 3.1 – Eq. 3.4):

$Sqrt(Q_1\ (\%)) = +10.08268 - 0.73558\ A + 0.49321\ B - 2.69626\ C - 0.012846\ B^2 + 0.017010\ AB + 0.094365\ AC$ …….....Eq. 3.1

$Q_8\ (\%) = +12.87 + 3.97A + 6.12B - 8.71C - 0.61A^2 - 0.14B^2 - 1.56C^2 + 0.12AB + 0.95AC + 0.30BC$…….....Eq. 3.2

$Log_{10}(R_t) = -0.77114 + 0.063132\ A - 0.015332\ B + 0.38349\ C$…….....Eq. 3.3

$S = +506.07 + 5.35A - 13.36B - 24.75C + 0.54AB - 6.49AC + 2.80BC$……...Eq. 3.4

Where: Q_1 = Drug release at the 1^{st} hr (%)

Q_8 = Drug release at the 8^{th} hr (%)

R_t = Residence time (min)

S = Spreadability ($g.cm.min^{-1}$)

A = Oil mixture concentration (%)

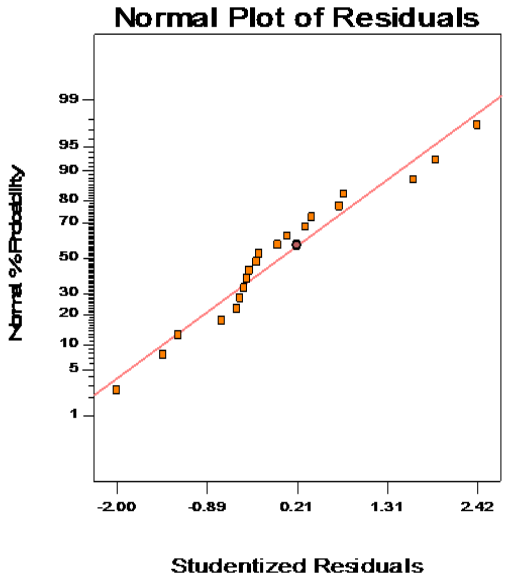
B = Surfactant solution concentration (%)

C = NaCMC concentration (%)

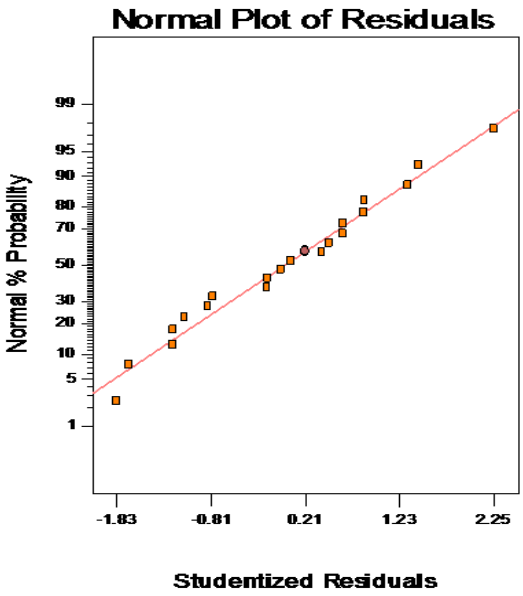
3.3.2 Model adequacy checking

In constructing the regression models, it was assumed that the responses were represented adequately with the independent variables in the β parameters and that the errors were (roughly) normal and (approximately) independently distributed with a mean of 0 and some constant variance. Diagnostic plots of the case statistics were used to check if these assumptions were valid.

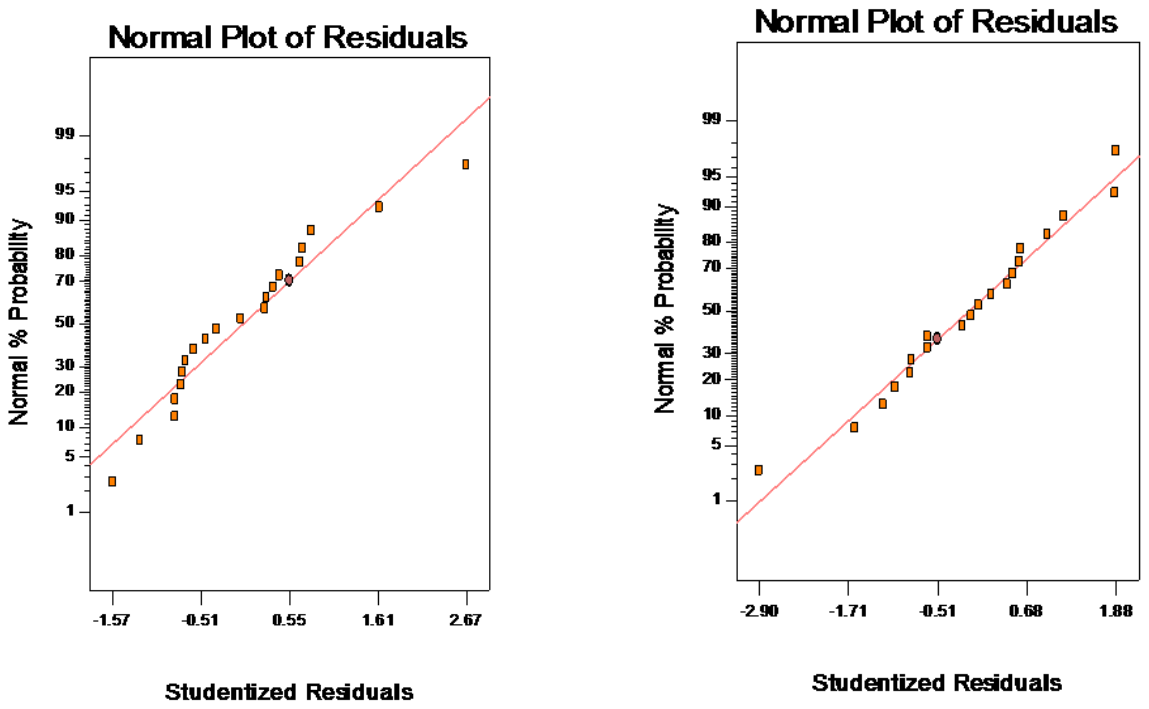
The most important diagnostic tool is the normal probability plot of the residuals. Data points should be approximately linear. A non-linear pattern (such as an S-shaped curve) indicates non-normality in the error term. There are no signs of any problems in all the cases. Numbers of standard deviations of the actual values from their respective predicted values are normally distributed for all the residuals. The size of the studentized residual should also be independent of its predicted value, the run, and the independent variables. In other words the spread of the studentized residuals should be approximately the same for each bowler. In this case all the plots look acceptable (Figure 3.6).



a)



b)



c) d)

Figure 3.6: Normal Probability plots for a) drug release for the 1st hr, b) drug release for the 8th hr, c) relative residence time, and d) spreadability index.

An influential point is one if removed from the data would significantly change the fit. An influential point may either be an outlier or have large leverage, or both, but it will tend to have at least one of those properties. Cook's distance (Figure 3.7) is a commonly used influence measure that combines these two properties. The leverage measures the amount by which the predicted value would change if the observation was shifted one unit. It always takes values between 0 and 1. A point with zero leverage has no effect on the regression model. Center points carry little weight in the fit and thus exhibit low leverage. The leverage plot for all the cases shows less than one value. The Outlier T plot shows if any points stand out. There's nothing out of the ordinary if all the points fall well within the red lines set at ±3.5. During spreadability analysis an outlier was found with spreadability index of 102 $g.cm.min^{-1}$ for the formulation F2 (Figure 3.7d). Ultimately this point was removed to have a better fit.

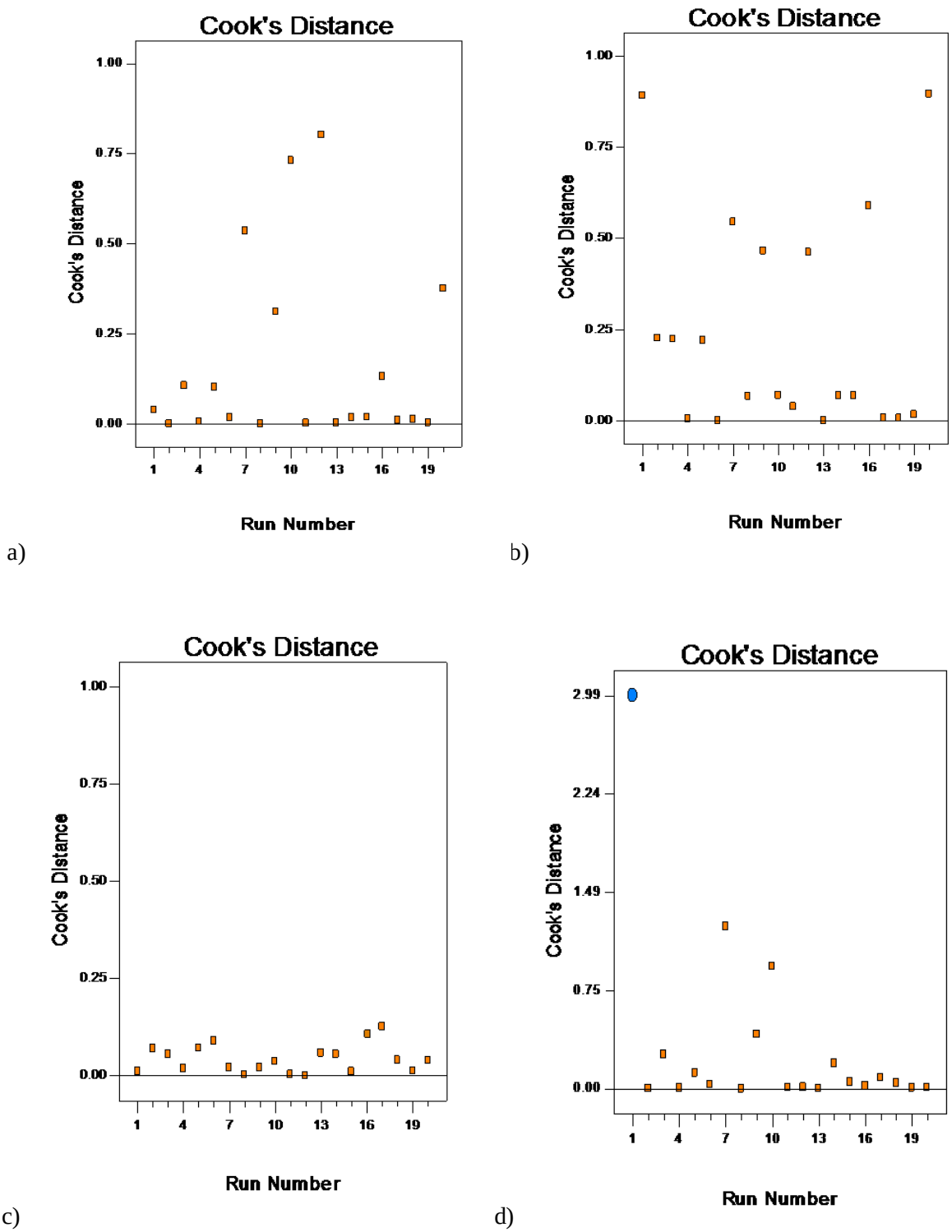
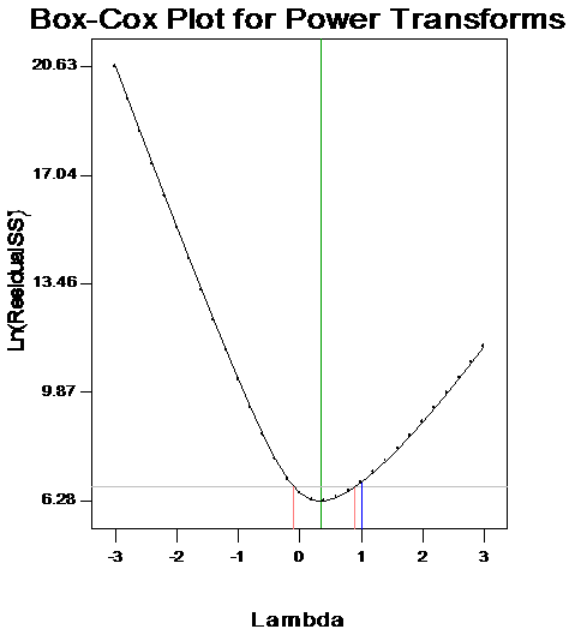


Figure 3.7: Cook's Distance plots for a) drug release for the 1st hr, b) drug release for the 8th hr, c) relative residence time, and d) spreadability index.

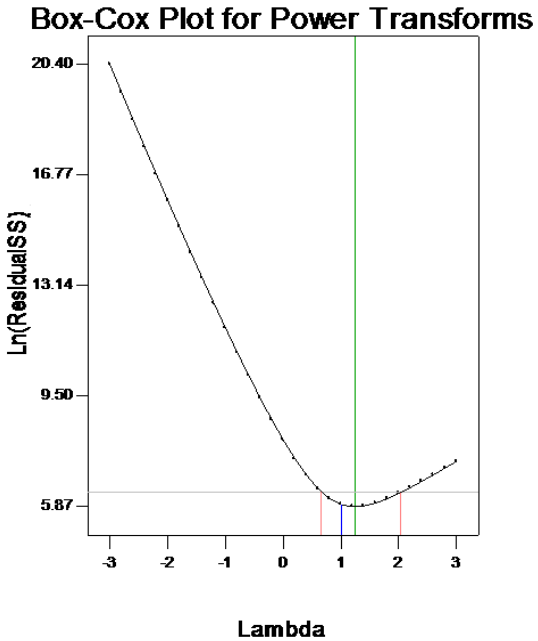
The last diagnostic plot is the Box Cox plot. This was developed to analytically calculate the best power law transformation. On this plot, the blue line shows the current transformation. In the cases of drug release at the 8th hr and spreadability, it points to a value of 1 for "Lambda," which symbolizes the power applied to the response values (Figure 3.8b and 3.8d). A lambda of 1 indicates no transformation. The green line indicates the best lambda value, while the red lines indicate the 95% confidence interval surrounding it. If this 95% confidence interval includes 1, then no transformation is recommended otherwise the software recommends the appropriate transformation like "square root" and "log" in the cases of drug release at the 1st hr and residence time respectively (Figure 3.8a and 3.8c).

3.3.3 Optimization

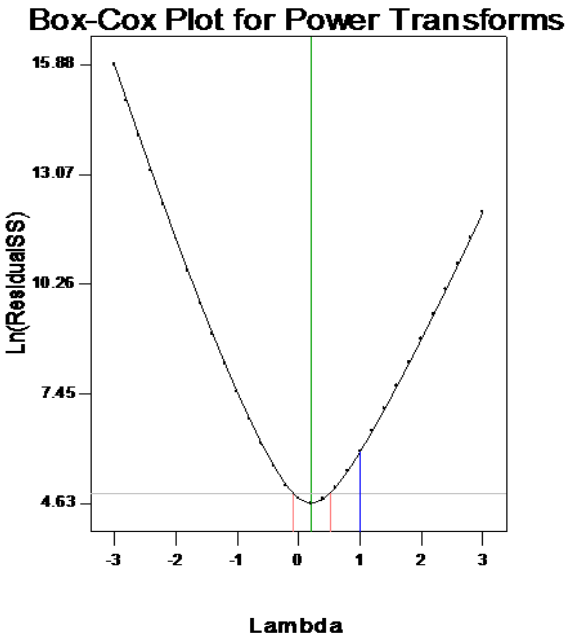
To optimize all the responses with different targets, a multi criteria decision approach like the numerical optimization technique by the desirability function and graphical optimization technique by the overlay plot were used. The optimized formulation was obtained by applying constraints on dependent variable responses and independent variables.



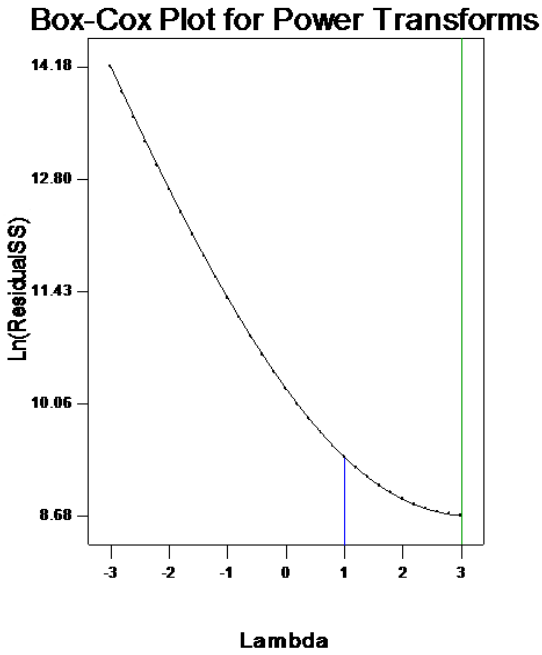
a)



b)



c)



d)

3.8: Box Cox plots for a) drug release for the 1st hr, b) drug release for the 8th hr, c) relative residence time, and d) spreadability index.

Optimized formulation was selected based on the criteria of less than 20% of the drug release at 1st hr and not less than 85% and targeted to 100% of the drug released at the 8th hr. These constrains were common for all the formulations. The optimization of the emulgel was also decided to have maximum spreadability and residence time features not less than 285 g.cm.min^{-1} and 12 min, respectively. The recommended concentrations of the independent variables were calculated by the Design Expert software from the above plots which has the highest desirability near to 1.0. Desirabilities range from zero to one for any given response. The program combines the individual desirabilities into a single number and then searches for the greatest overall desirability.

The optimized concentration was obtained by using design expert software as clears in the 2D and 3D response surface prediction curve (Figure 3.9 a-c). A checkpoint batch was prepared as per Table 3.7. From the full model, it was expected that the Q_8 value should be more than 98% and the value of residence time and spreadability should be more than 15 min and 286 g.cm.min^{-1}, respectively. Table 3.8 indicates that the results were as expected. The average % relative error

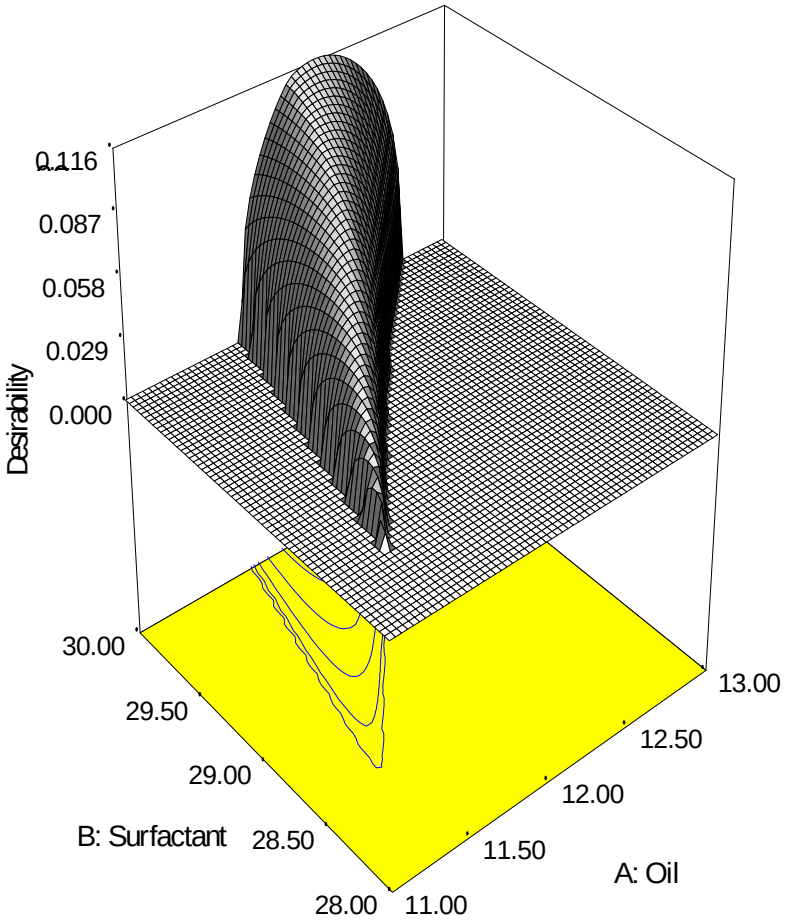
between the predicted values and experimental values of each response was calculated and the values were found to be 5.3%, 1.3%, 0.37%, and 18.5% for Q_1 (drug release at the 1st hr), Q_8 (drug release at the 8th hr), spreadability, and residence time, respectively. Thus, it was concluded that these experimental findings are in close agreement with the model predictions which confirmed the predictability and validity of the model (Verma, *et al.*, 2013).

Table 3.7: Numerical Optimization Report of Solutions and predicted responses.

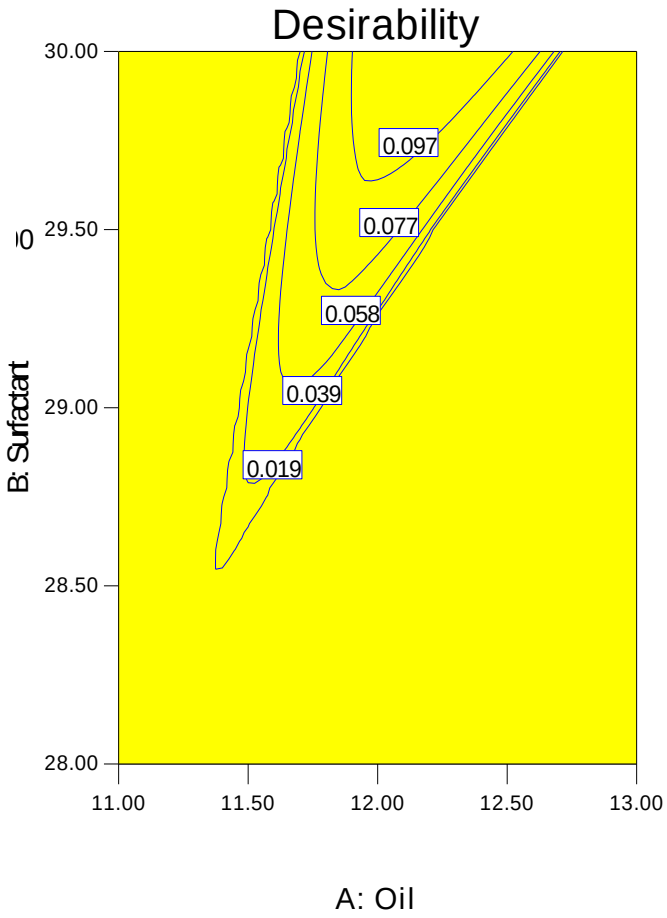
Formulations	Oil	Surfactant	Gelling agent	release at 1st hr (%)	release at 8th hrs (%)	Residence time (min)	Spreadability ($g.cm.min^{-1}$)	Desirability
OP1	12.42	30.00	4.00	13.91	98.60	15.28	286.34	0.116
OP2	12.46	30.00	4.00	14.00	98.44	15.33	286.14	0.115
OP3	12.37	29.89	4.00	13.97	98.70	15.24	286.05	0.105

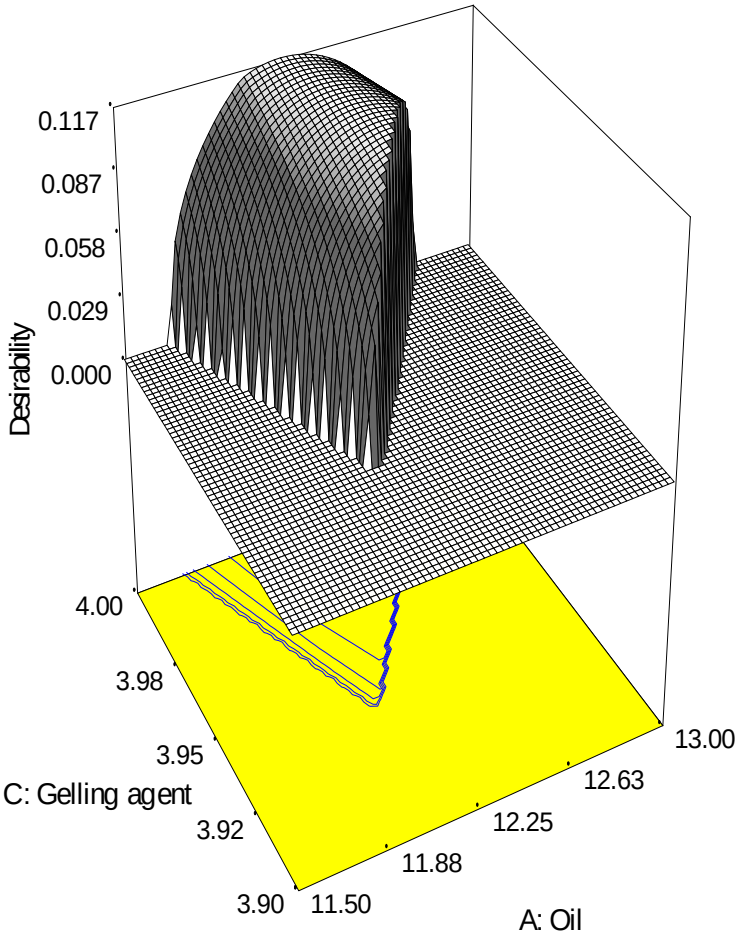
Table 3.8: Experimental response values and residual of prediction at the optimal levels of factors.

Formulations	Actual release at 1st hr (%)	Residues	Actual release at 8th hrs (%)	Residues	Actual retention time (min)	Residues	Actual Spreadability ($g.cm.min^{-1}$)	Residues
OP1	15.30±1.71	1.39	100.26±1.68	1.66	19.00±4.15	3.72	284.9±1.06	-1.44
OP2	14.81±1.10	0.81	99.55±1.59	1.11	20.11±5.89	4.78	285.1±2.25	-1.04
OP3	13.77±0.07	0.20	99.92±1.44	1.22	17.35±1.94	2.11	286.7±0.40	0.65

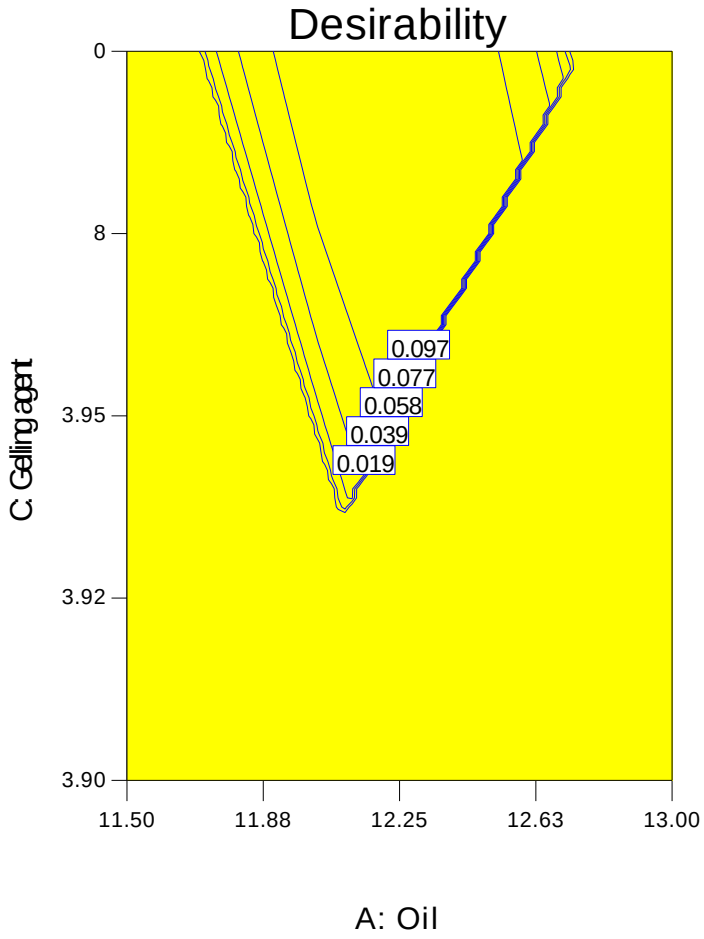

0.116
0.087
0.058
0.029
0.000
Desirability
30.00
29.50
29.00
28.50
28.00
B: Surfactant
11.00
11.50
12.00
12.50
13.00
A: Oil

a.


Desirability
30.00
29.50
29.00
28.50
28.00
B: Surfactant
0.097
0.077
0.058
0.039
0.019
11.00
11.50
12.00
12.50
13.00
A: Oil


Desirability
0.117
0.087
0.058
0.029
0.000
C: Gelling agent
4.00
3.98
3.95
3.92
3.90
A: Oil
11.50
11.88
12.25
12.63
13.00

b.


Desirability
C: Gelling agent
0
8
3.95
3.92
3.90
0.097
0.077
0.058
0.039
0.019
11.50
11.88
12.25
12.63
13.00
A: Oil

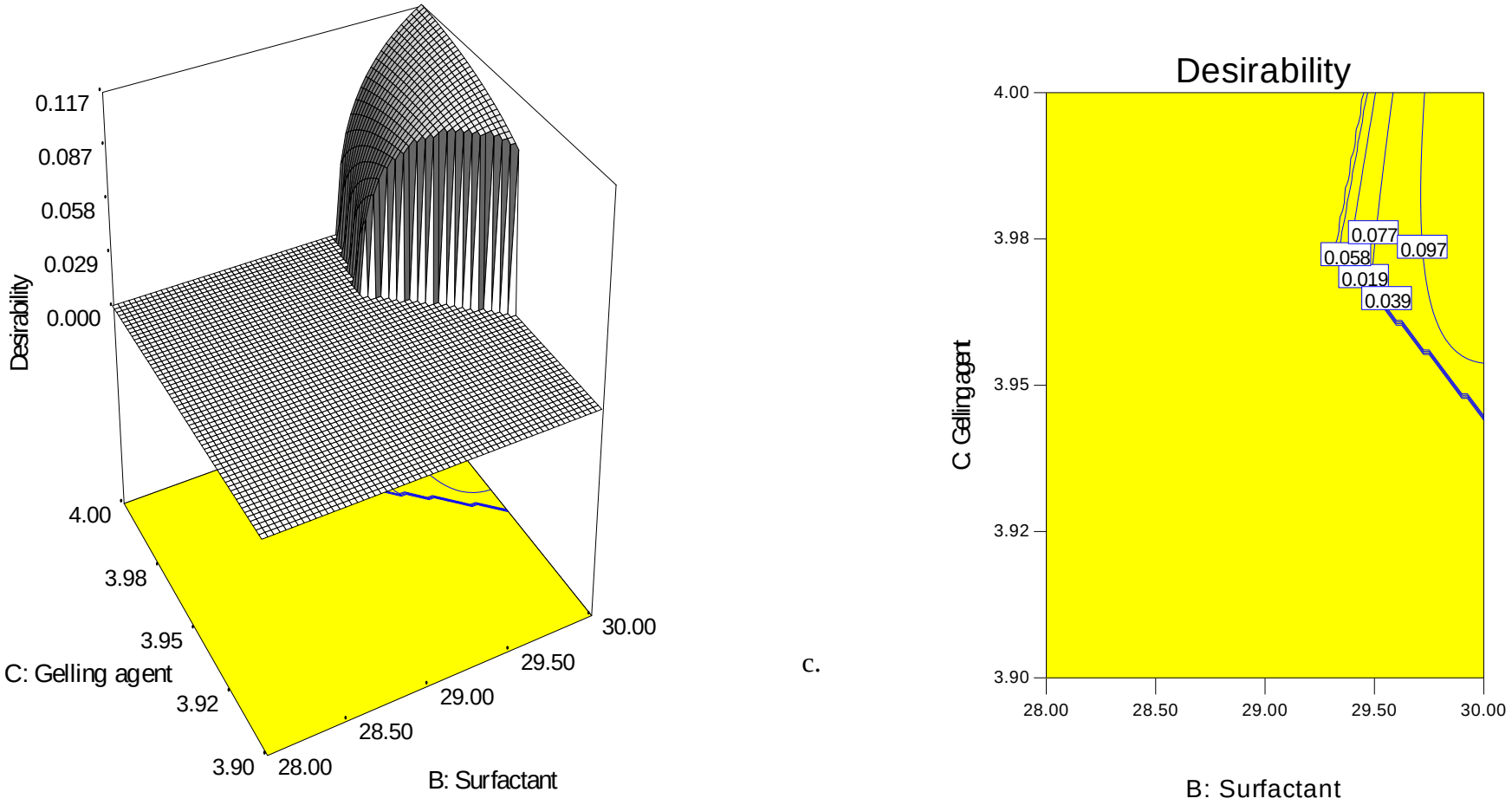


Figure 3.9: percent relative deviation view of most desirable operating conditions a. when gelling agent concentration is 4%, b. When oil concentration is 12%, and c. when surfactant concentration is 30.0%

Design-Expert also overlays response contours to generate graphical optimization for broader operating window. Figure 3.10 shows the overlay plot where the criteria match the numerical optimization. The shaded areas on the graphical optimization plot do not meet the selection criteria. The lines mark the high or low boundaries on the responses. The yellow colored "window" shows where the factors can be set to satisfy the requirements on two responses.

3.4 Evaluation of optimized formulations

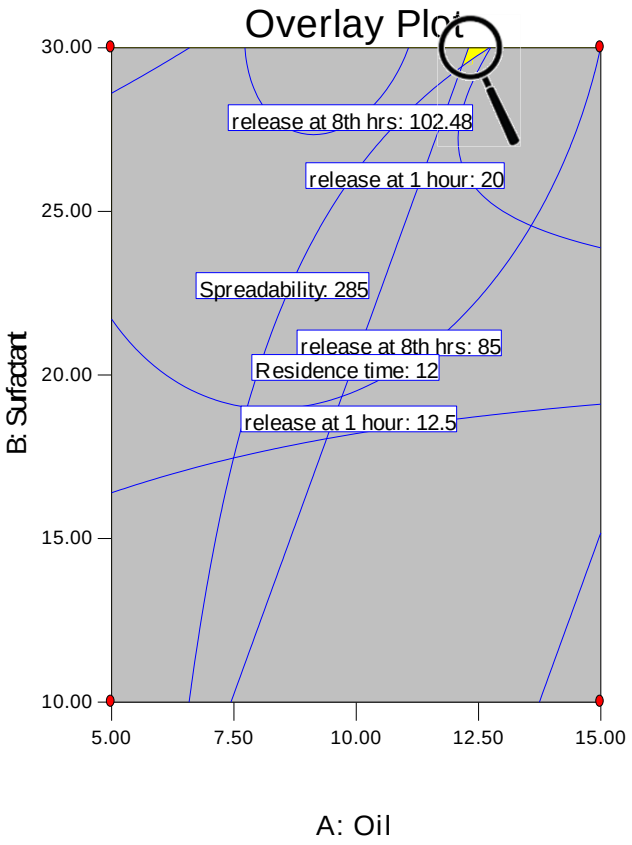
3.4.1 Globule Size Analysis of the emulsion

The average globule size and polydispersity index of 0.2 gm of the optimized formulations were determined in triplicate before and after the addition of the gel system by photon correlation spectroscopy (Brookhaven Instruments Corporation, USA) with inbuilt particle size analyzer software (Brookhaven 90plus nanoparticle size analyzer, USA). The particle size analysis showed that the mean droplet sizes of the formulations are below 200 nm (Table 3.9) before and after the addition of the gel system indicating a microemulsion region (Borhade; *et al.*, 2012). This may enhance penetration of oil globules containing clotrimazole through fungal cell walls to inhibit ergosterol synthesis (Bachhav & Patravale, 2009).

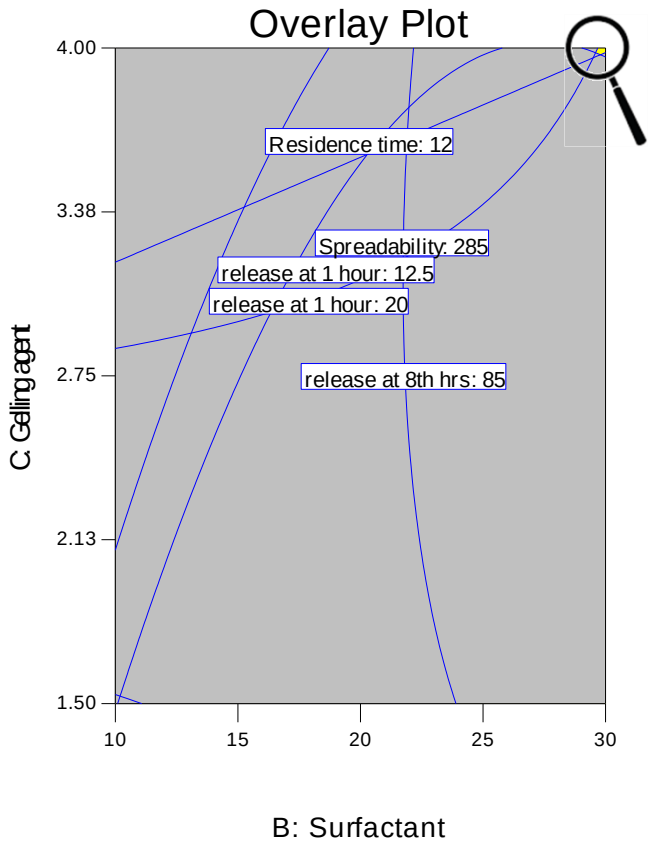
Table 3.9: Effective diameter and polydispersity of the emulsion and emulgel of the optimized formulations.

Optimized formulations	Before addition of gel system		After addition of gel system	
	Effective diameter (nm)	Polydispersity	Effective diameter (nm)	Polydispersity
OP1	146.53 ± 0.75	0.23 ± 0.01	174.13 ± 0.51	0.21 ± 0.02
OP2	139.27 ± 2.48	0.16 ± 0.02	186.13 ± 6.91	0.24 ± 0.02
OP3	140.67 ± 0.32	0.26 ± 0.01	164.13 ± 5.61	0.28 ± 0.02

a)


Overlay Plot
30.00
25.00
20.00
15.00
10.00
B: Surfactant
release at 8th hrs: 102.48
release at 1 hour: 20
Spreadability: 285
release at 8th hrs: 85
Residence time: 12
release at 1 hour: 12.5
5.00
7.50
10.00
12.50
15.00
A: Oil

b)


Overlay Plot
4.00
3.38
2.75
2.13
1.50
C: Gelling agent
Residence time: 12
Spreadability: 285
release at 1 hour: 12.5
release at 1 hour: 20
release at 8th hrs: 85
10
15
20
25
30
B: Surfactant

c)

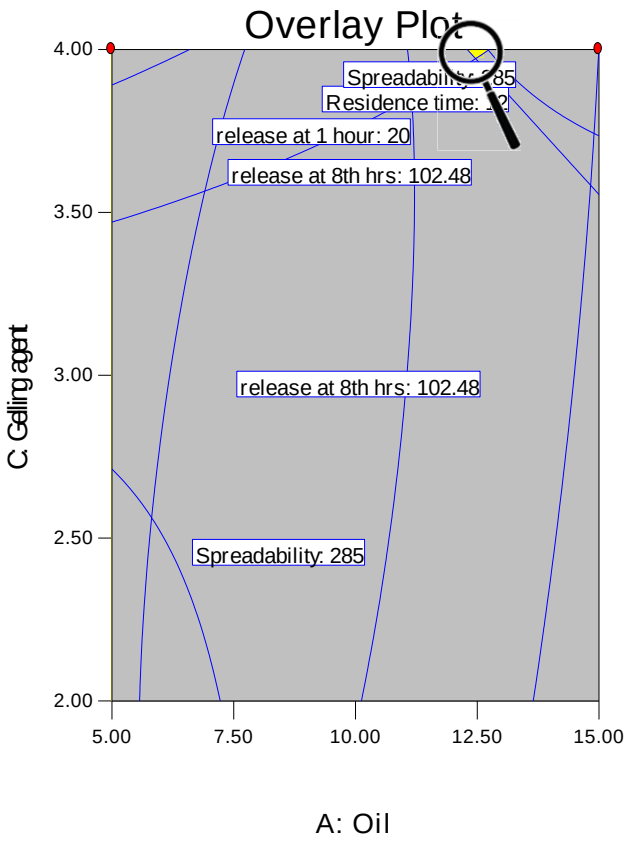


Figure 3.10: Overlay plot of responses a) at gelling agent concentration of 4.00%, b) at oil concentration of 12.4%, and c) at surfactant concentration of 30%.

Polydispersity index is the ratio of standard deviation to mean droplet size; hence, it indicates the uniformity of droplet size within the formulation. All polydispersity index values were around 0.2, which shows a narrow distribution of the particle size except optimized formulation OP3 after the addition of the gel system.

A slight increase in mean droplet size of the internal phase have been seen during addition of gelling agent to all optimized formulations which may be due to altered orientation of clotrimazole at the interface to the change of the dispersion medium pH. As clotrimazole is more soluble in acidic pH, it is likely to migrate more in the external phase leading to reduction in its amount present at interface. This may increase the effective concentrations of surfactants available for emulsion formation, which may be responsible for their low globule size. The

globule size of formed emulgels increased may be as the result of decreased interaction of surfactant with oil at the interface (Borhade, *et al.*, 2012).

3.4.2 *In* vitro release profile comparison

An *in vitro* release profile (Figure 3.11) reveals fundamental information on the structure (e.g., porosity) and behavior of the formulation at molecular level, possible interactions between drug and polymer, and their influence on the rate and mechanism of drug release and model release data.

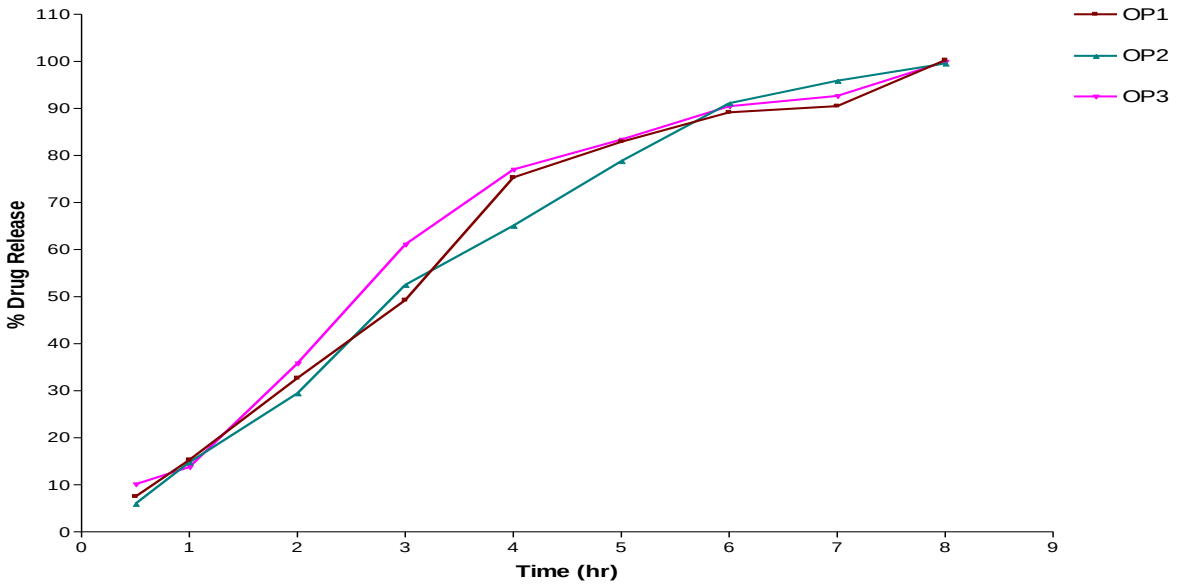


Figure 3.11: *In vitro* release profile of the three optimized formulations.

The release profiles of the optimized formulations were compared with difference factor (f_1) which is an efficient tool for comparison of dissolution profiles. For two formulations to be considered different, f_2 values should be less than 50. Difference factor of 0-15 ensures minor difference between two products and, thus, of their performance.

The three optimized formulations were found to have minor difference in their drug release profile when compared with each other with the f_1 values of 4.4-7.6 and f_2 values between 61.3 and 67.6. In addition, they show minimal difference in their release behavior when packed in aluminum collapsible tube (Figure 3.12 – Figure 3.15) and stored for three months at real and accelerated time conditions (Table 3.10). No visible physical or chemical change was also

observed especially due to the buffering system, degradation of clotrimazole which was inevitable due to acidic nature of free fatty acids in the oil phase, was circumvented since it is stable from pH 6 onwards (Bachhav & Patravale, 2009; Borhade, *et al.*, 2012).

Table 3.10: Similarity and difference factors for drug release profiles of optimized emulgel after storing at real and accelerated time conditions.

Optimized formulation	Real Time (30°C/65%RH)				Accelerated time (40°C/75%RH)			
	2nd month		3rd month		2nd month		3rd month	
	f_1	f_2	f_1	f_2	f_1	f_2	f_1	f_2
OP1	7.49	64.42	6.57	65.52	12.22	51.21	9.97	53.27
OP2	3.28	80.03	2.98	80.69	4.18	74.15	6.10	63.30
OP3	4.79	74.21	7.20	62.28	6.44	62.78	3.83	74.7

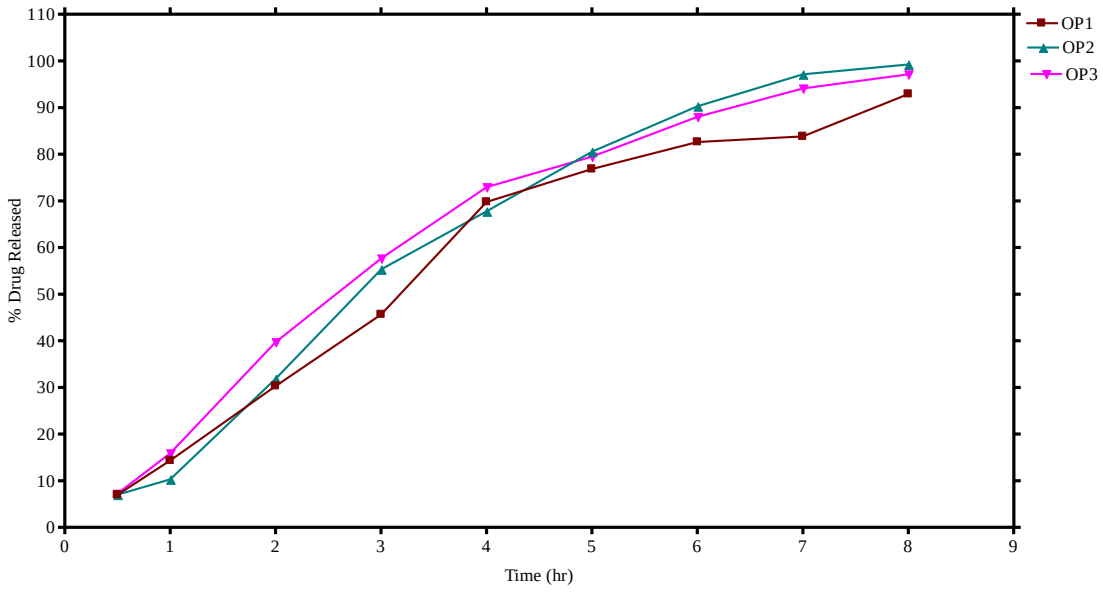


Figure 3.12: In vitro release profile of the three optimized formulations after two months storage at real time (30oC, 65% RH) condition in collapsible aluminum tubes.

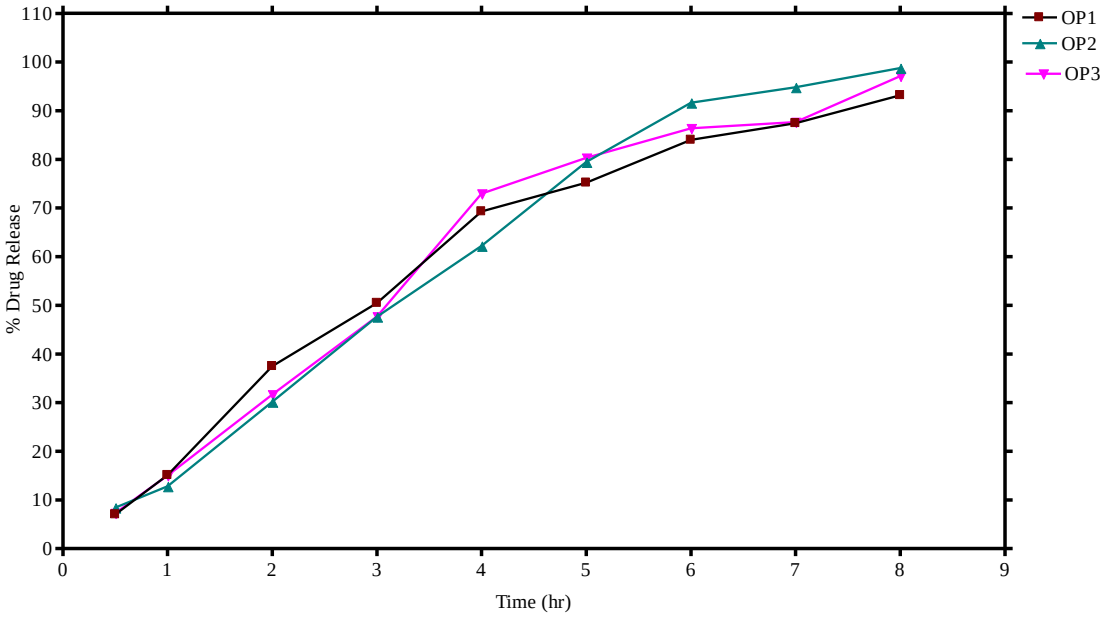


Figure 3.13: *In vitro* release profile of the three optimized formulations after three months storage at real time (30^{o}C, 65% RH) condition in collapsible aluminum tubes.

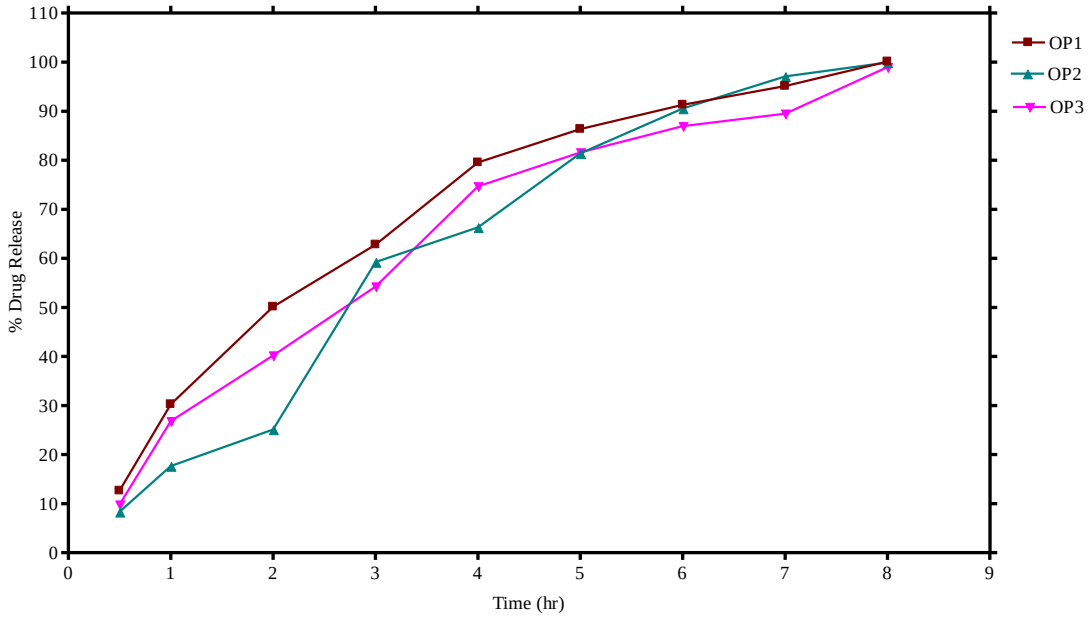


Figure 3.14: *In vitro* release profile of the three optimized formulations after two months storage at accelerated time (40^{o}C, 75% RH) condition in collapsible aluminum tubes.

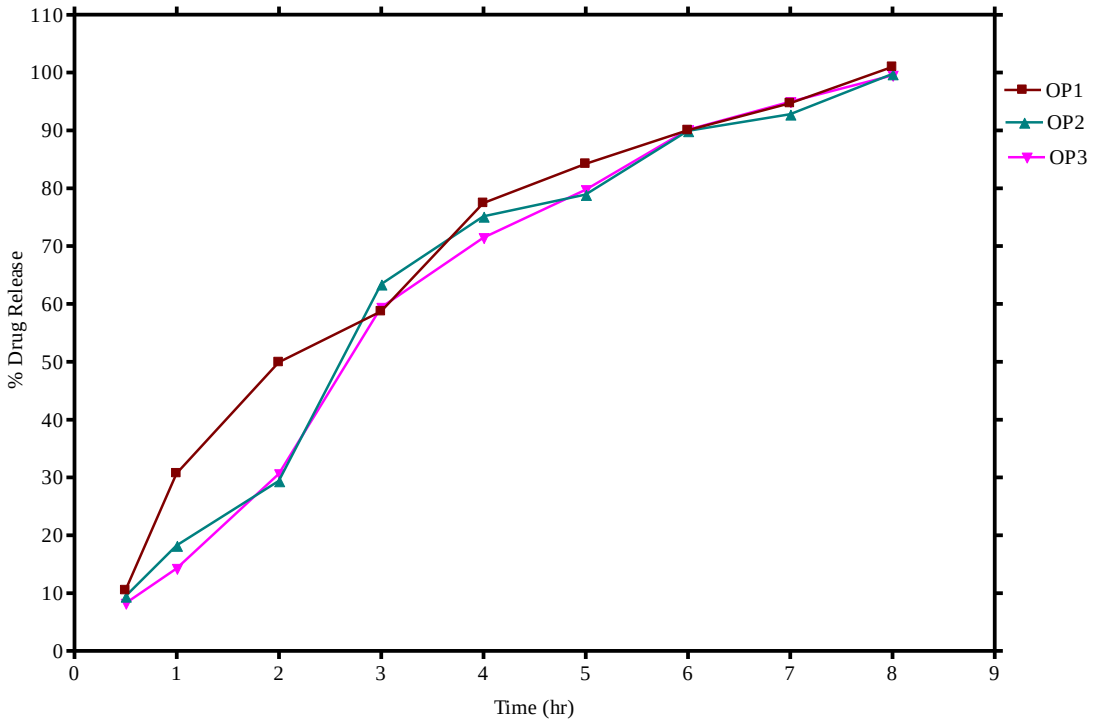


Figure 3.15: *In vitro* release profile of the three optimized formulations after three months storage at accelerated time (40^{o}C, 75% RH) condition in collapsible aluminum tubes.

3.5.2 Study of drug release kinetics and mechanisms

The *in vitro* drug release data were also fitted to five commonly employed release kinetic models, namely; zero order, first order, Hixon-crowell cube root model, and Higuchi model equation to analyze their drug release kinetics, and Korsmeyer-Peppas model up to the first 60% drug release to describe the drug release mechanism from a polymeric system. Table 3.11 presents the linear regression results of the model fitting test of the formulations. Coefficients of determination (R^2) were used to evaluate the goodness of the model fit. Zero order model showed the best fit with high linearity (r^2 ranging: 0.9733 – 0.9885) for all the optimized formulations.

Table 3.11: Kinetic fits of different models for the optimized Clotrimazole emulgels.

Model	OP1		OP2		OP3	
	Slope	r^2	Slope	r^2	Slope	r^2
Zero order	0.0024±0.0001	0.9805	0.0024±0.0001	0.9885	0.002439±0.0001	0.9733
First order	0.0051±0.0010	0.7950	0.0055±0.0010	0.7969	0.005135±0.0010	0.7950
Higuchi model	0.04349±0.0025	0.9738	0.0430±0.0026	0.9712	0.0448±0.0023	0.9692
Hixon-Crowell model	-0.0093±0.00	0.8568	-0.0082±0.00	0.8737	-0.0081±0.00	0.8321

Table 3.12 shows the drug release model obtained by fitting the first 60% drug release to the Korsmeyer-Peppas model. In this model, the value of 'n' illustrates the type of release mechanism. For slab like formulations; $n \leq 0.5$ corresponds to a Fickian diffusion mechanism, $0.5 < n < 1.0$ to non-Fickian transport (which is indicative of drug release mechanisms involving a combination of both diffusion and chain relaxation mechanisms). Therefore, the release of the drug from the prepared formulations is controlled by swelling of the polymer, followed by drug diffusion through the swelled polymer and slow erosion of the polymer (Mohammed & Khedr, 2003), n=1 to Case II (relaxational) transport, and n>1 to super case II transport.

As shown in Table 3.12, the n value for all the optimized formulations were found to be in the range of 0.9051 – 1.031, which is rounded to 1 indicating Case II (relaxational) transport mechanism where the polymer relaxation and erosion are the rate controlling steps.

Table 3.12: Drug release mechanism for optimized clotrimazole emulgels evaluated by Korsmeyer-Peppas model.

Optimized formulations	Slope (n)	y-intercept	k	R^2
OP1	0.9645 ± 0.0491	-2.514 ± 0.1123	0.1751	0.9822
OP2	1.031 ± 0.0479	-2.682 ± 0.1097	0.0021	0.9851
OP3	0.9051 ± 0.0664	-2.351 ± 0.1519	0.0045	0.9637

4. Conclusion

In this study, the *in vitro* results showed that an emulgel formulation can be a potential candidate for the delivery of clotrimazole to the oral cavity, with better *in vitro* physical characteristics than marketed oral gels, using olive oil and oleic acid as lipid drug carriers. Clotrimazole incorporated in oleic acid and olive oil at 1:1 ratio can be emulsified with Tween 80 with oil to surfactant ratio of up to 15:10 (3:2) with the promotional effect of ethanol on the micellization of the surfactant. Furthermore, since both the oil phase and the surfactant system (Tween 80 and ethanol) are good solvents for clotrimazole, drug loading poses no challenge during formulation.

The jellifying of drug incorporated emulsion is the distinctive step of an emulgel formulation. Na CMC can be incorporated in the aqueous phase of the emulsion up to a concentration of 4% resulting in better physical property than formulations containing HPMC and showing non-Newtonian shear thinning pseudo plastic behavior. The pH of the final formulation can be affected by the incorporation of a buffering system such as trisodium citrate and citric acid during this step.

Computer based full CCD can be successfully used to optimize emulgel system setting concentration of oil phase, surfactant, and gelling agent as independent variables. From these variables, gelling agent concentration has more prominent effect on *in* vitro drug release, residence time and spreadability followed by surfactant concentration. When the formulations consist of oil phase around 12.4% and higher surfactant and gelling agent concentrations precisely at 30% and 4%, respectively, it was shown that the drug will be released almost completely at the 8^{th} hr and demonstrate much better spreadability and residence time compared to currently marketed oral gels in the country. And, according to Korsmeyer-Peppas model these emulgels attend a relaxational transport mechanism, and aluminum collapsible tubes packing preserve this property for three months.

5. Suggestion for further work

This study suggests the following for further work:

- Validation of *in vitro* analysis methods,
- *In vivo* and *in vitro* correlation for drug release and residence time studies, and
- Palatability and safety studies for the optimized formulation.

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6. Annexes

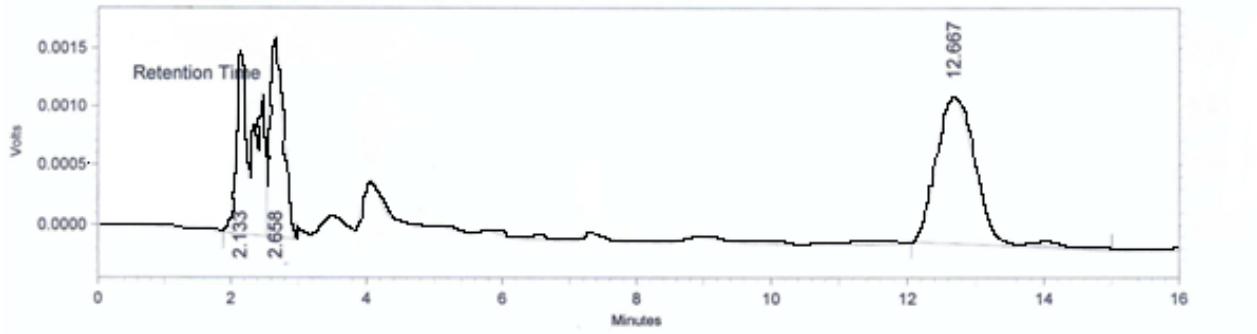
Annex 1: HPLC method validation

An analytical method should be accurate, precise, specific and robust. With this intention, an HPLC method for the quantitative determination of clotrimazole described at section 2.2.3.2.8 was validated according to the guidelines of ICH Q2) (R1) (2005).

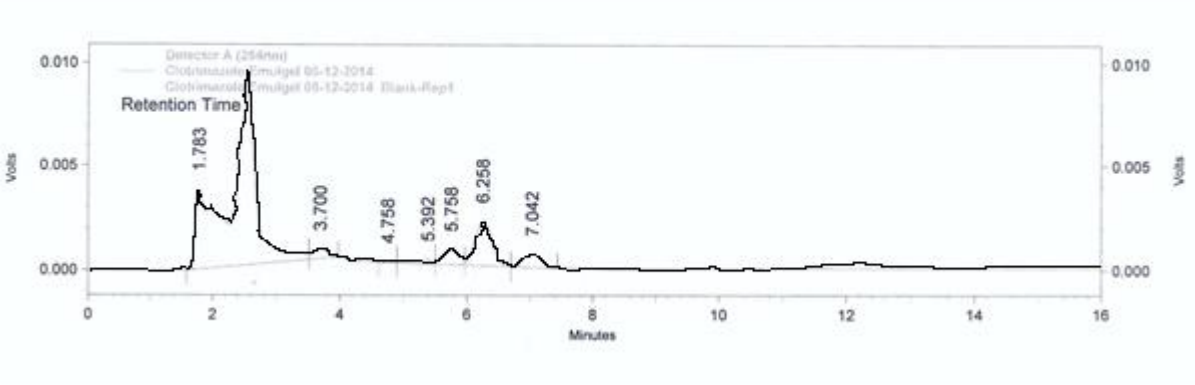
1.1. Specificity

The specificity (selectivity) of the method was studied to demonstrate that this method provides distinguishable response for the clotrimazole from other excipients or solvents. It was verified by comparing the chromatograms of the blank solution (blank emulgel, which contains all the inactive excipients selected for this study at their highest concentration, in dissolution media) and standard solution (20 µg/ml clotrimazole in absolute ethanol).

There were no interfering or overlapping peaks observed with the same retention time (12.7 min) to that of clotrimazole as depicted in Figure 5.1 at the method detection wavelength of 254 nm. Hence, the method is specific enough to detect the product as the dissolution media and other formulation ingredients have negligible interference on the elution of clotrimazole and its detection at the specific retention time.



a)



b)

Figure 6.1: HPLC chromatogram of a standard solution (20 µg/ml) (a) and blank emulgel solution (b).

1.2. Linearity and calibration curve

The linearity of the analytical method was calculated to show its ability to elicit test results that are directly proportional or proportional by means of well-defined mathematical transformations to the concentration of analytes in samples within a given range. A stock solution was prepared by dissolving 50 mg of Clotrimazole USP RS in sufficient volume of absolute ethanol to make 100 ml solution (500 µg/ml of clotrimazole). From this, serial dilutions of solutions having concentrations of 20, 40, 60, 80, 100 and 120 µg/ml were prepared using the same solvent as a diluent. The areas of the resulting peaks were investigated for linearity using GraphPad® Prism statistical software. The HPLC chromatogram peak area versus concentration was plotted with 95% confidence interval as depicted in Figure 5.2. A linear relationship was observed between the response and concentration with a fitted equation: Y = 14410 X, where Y is the chromatogram peak area and X is the concentration in µg/ml. Correlation coefficient (r^2) of 0.9993 was obtained which is very close to one proved good linearity in the concentration range studied.

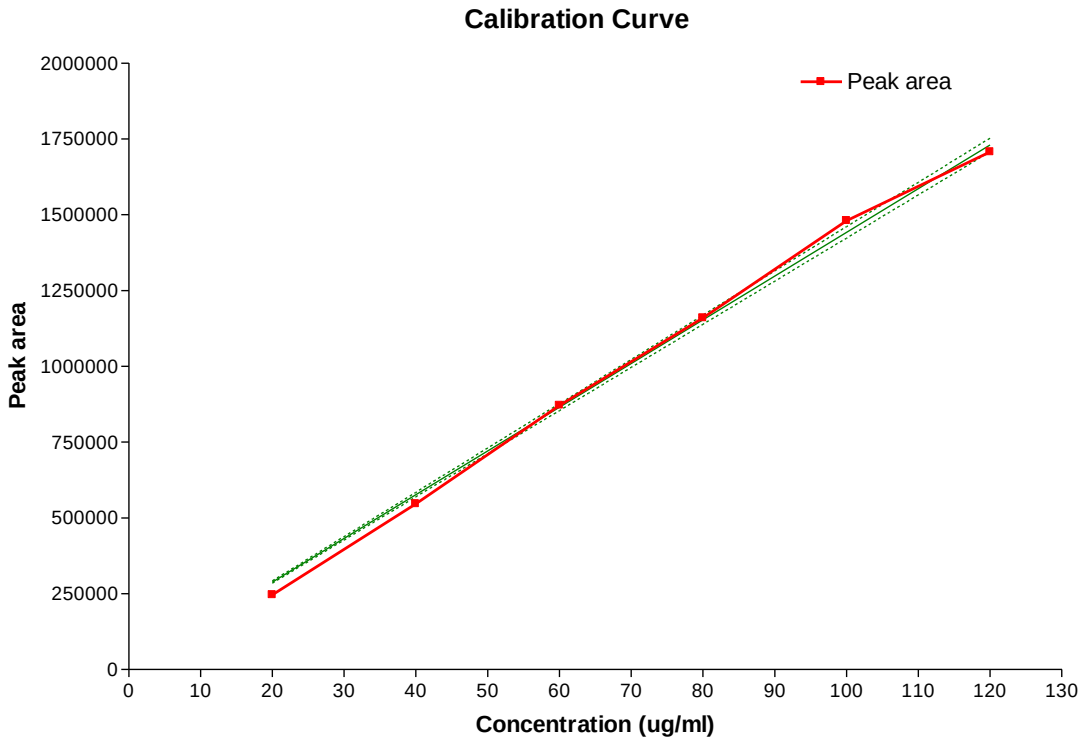


Figure 6.2: Calibration curve for Clotrimazole in the concentration range of 20 – 120 µg/ml.

1.3. Precision (Intermediate precision & Repeatability)

The precision of a method was studied in order to demonstrate the extent to which the individual test results of multiple injections of a series of standards agree. The measured standard deviation can be subdivided into 3 categories: repeatability, intermediate precision and reproducibility. Repeatability was obtained during calibration study on the same equipment (Shimadzu, Tokyo, Japan) with 3 injections at each concentration (20, 40, 60, 80, 100, & 120 µg/ml). And the intermediate precision was determined by comparing the results of three of the concentrations (60, 80, and 100 µg/ml) with the results of same concentrations run over a number of days. Since the method is to be used in the same laboratory with the same equipments it was not necessary to validate its reproducibility.

Measurement precision was achieved for six data points with percent relative standard deviation values of 0.8, 0.4, 0.2, 0.2, 0.3, and 0.9 (n=6) for the 20, 40, 60, 80, 100, & 120 µg/ml concentrations, respectively less than 2 which is the upper limit for precision measurement in

analytical method validations. The variations of the three concentrations run over a week interval were not quite significant. All have P value greater than 0.05 (Table 5.1).

Table 6.1: Data for the inter-day precision study (three aliquots in triplicate on the same days for three consecutive weeks).

Concentration (µg/ml)	Peak area			P value
	Week 1	Week 2	Week 3	
100	1480394±2006.521	1484717±1509.92	1489779±1940.40	0.0532
80	1159894±1740.027	1160391±1206.96	1161304±1077.01	0.8169
60	870929.1±723.62	868501.4±1655.94	877070.3±717.62	0.0697

1.4. Accuracy and Recovery

The accuracy of an analytical method is the extent to which test results generated by the method and the true value agree. It was carried out by standard addition method. A blank sample matrix of the formulation was spiked with a known concentration of clotrimazole by volume. After extraction of the analyte from the matrix with absolute ethanol (as described in section vii) and injection into the analytical instrument, its recovery was determined by comparing the response of the extract with the response of the reference material dissolved in a pure solvent. The concentrations cover the range of concern and include concentrations close to the smallest concentration (20 µg/ml), one in the middle of the range (60 µg/ml) and one at the high end of the calibration curve (100 µg/ml) with three replicates each. Accuracy was reported as percent recovery (Eq. 5.1) by the assay of known added amount of analyte in the sample.

Percentage recovery= 100 (Au/As)…… Eq. 6.1

Where: Au is area of peak for extracted analyte and As is area of peak for standard.

The values obtained for recovery, Table 5.2 (98.00–100.62%), which is in the range of 98-120% showed the accuracy of the method.

Table 6.2: Data for recovery study (using standard addition technique).

Concentration (µg/ml)	Peak area		% Recovery
	Spiked preparation	Standard preparation	
100	1451121±419.9369	1480394±2006.4	98.0226
80	1138376±1075.149	1159894±1740.426	98.14486
60	885646.8±5746.911	870929.1±723.5791	101.6899

1.5. Robustness

The robustness of the analytical procedure was studied to demonstrate its capacity to remain unaffected by small, but deliberate variation in method parameters and provides an indication of its reliability during normal usage. The flow rate and mobile phase composition were varied and the resultant peak areas of three concentrations (100, 80, and 60 µg/ml) were compared with standards which were run according to the normal procedure.

There was no significant change in the result of the method, after the introduction of small deliberate changes (~±5%) from the original method parameters. The standard deviation of peak areas was calculated for each set of conditions and was found to be <2% (Tables 5.3 – 5.4). The low values of %RSD indicate that the method is robust.

Table 6.3: The influence of flow rate variation on average peak areas and their respective %RSD.

Flow rate (ml/min)	Concentration (µg/ml)	Peak area	%RSD
1.05	60	865442.7±299.02	0.63
	80	1165841.3±2024.67	0.53
	100	1479811.3±2311.65	0.13
0.95	60	856322.7±3192.01	1.70
	80	1167365±2170.40	0.66
	100	1490479.3±2853.77	0.70

Table 6.4: The influence of mobile phase concentration variation on average peak areas and their respective percent relative deviation.

Methanol : dibasic potassium phosphate	Concentration (µg/ml)	Peak area	%RSD
79:21	60	869216.7±335.29	0.22
	80	1161281.67±2664.43	0.41
	100	1456062.3±3416.62	1.65
71:29	60	866187.7±575.02	0.55
	80	1138731±2984.19	1.83
	100	1505455±1202.91	1.69

Annex 2: Response surface plots

2.1. Model graphs for drug release at the 1st hr

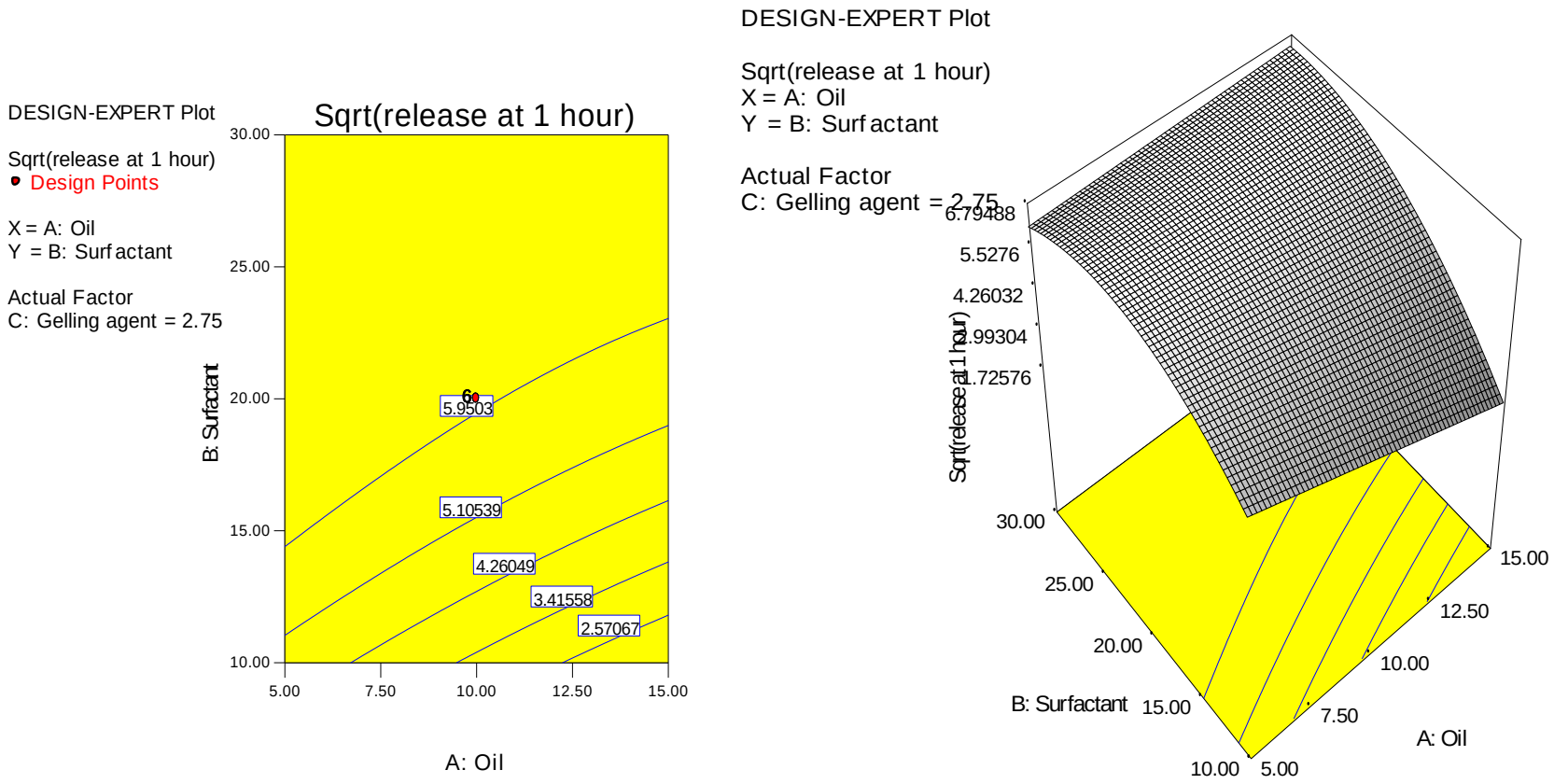


Figure 6.3: Contour and 3D plot of drug release at the 1st hr when surfactant concentration (%) was plotted against oil concentration (%) at a gelling agent concentration of 2.75%.

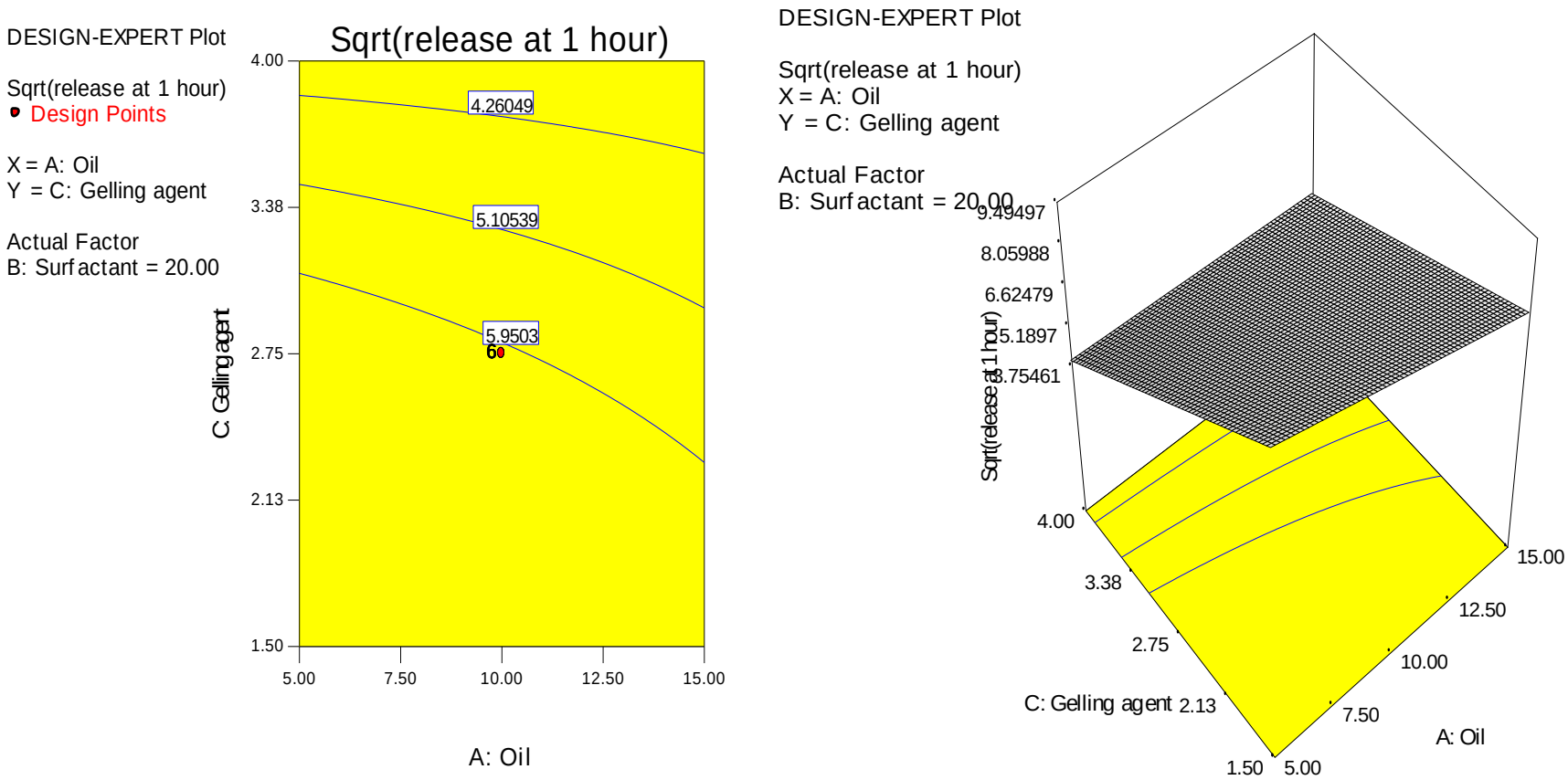


Figure 6.4: Contour and 3D plot of drug release at the 1st hr when gelling agent concentration (%) was plotted against oil concentration (%) at a surfactant concentration of 20.0%.

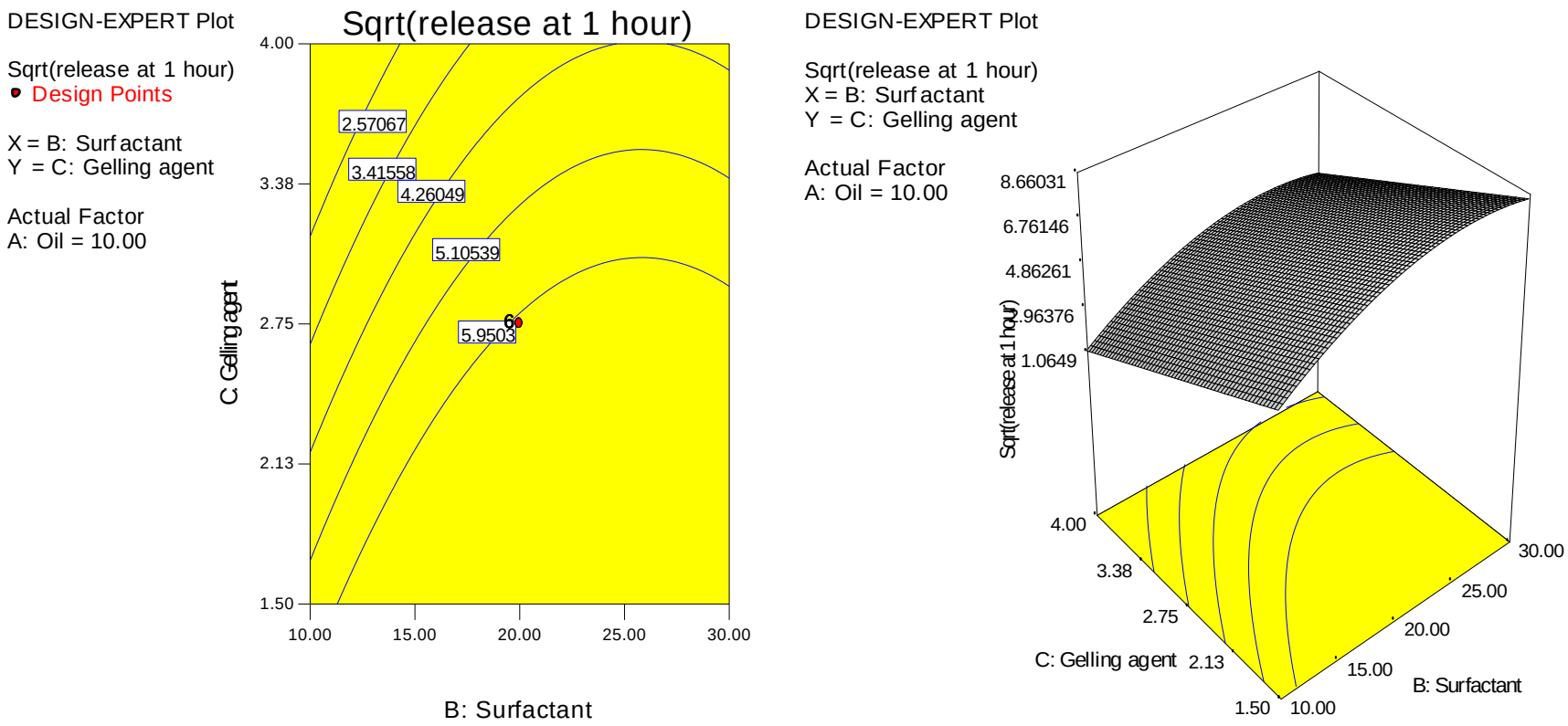


Figure 6.5: Contour and 3D plot of drug release at the 1st hr when gelling agent concentration (%) was plotted against surfactant concentration (%) at an oil concentration of 10.0%.

2.2. Model graphs for drug release at the 8th hr

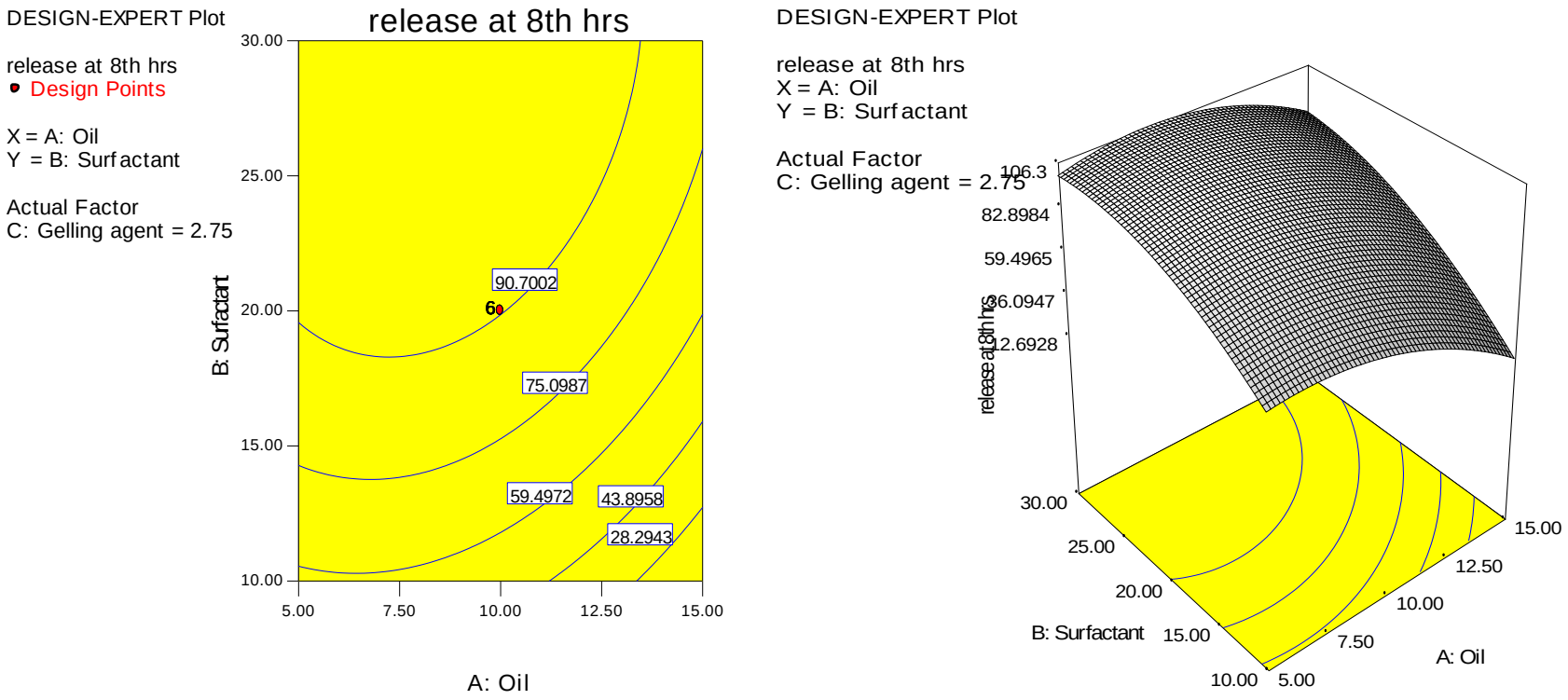


Figure 6.6: Contour and 3D plot of drug release at the 8th hr when surfactant concentration (%) was plotted against oil concentration (%) at a gelling agent concentration of 2.75%.

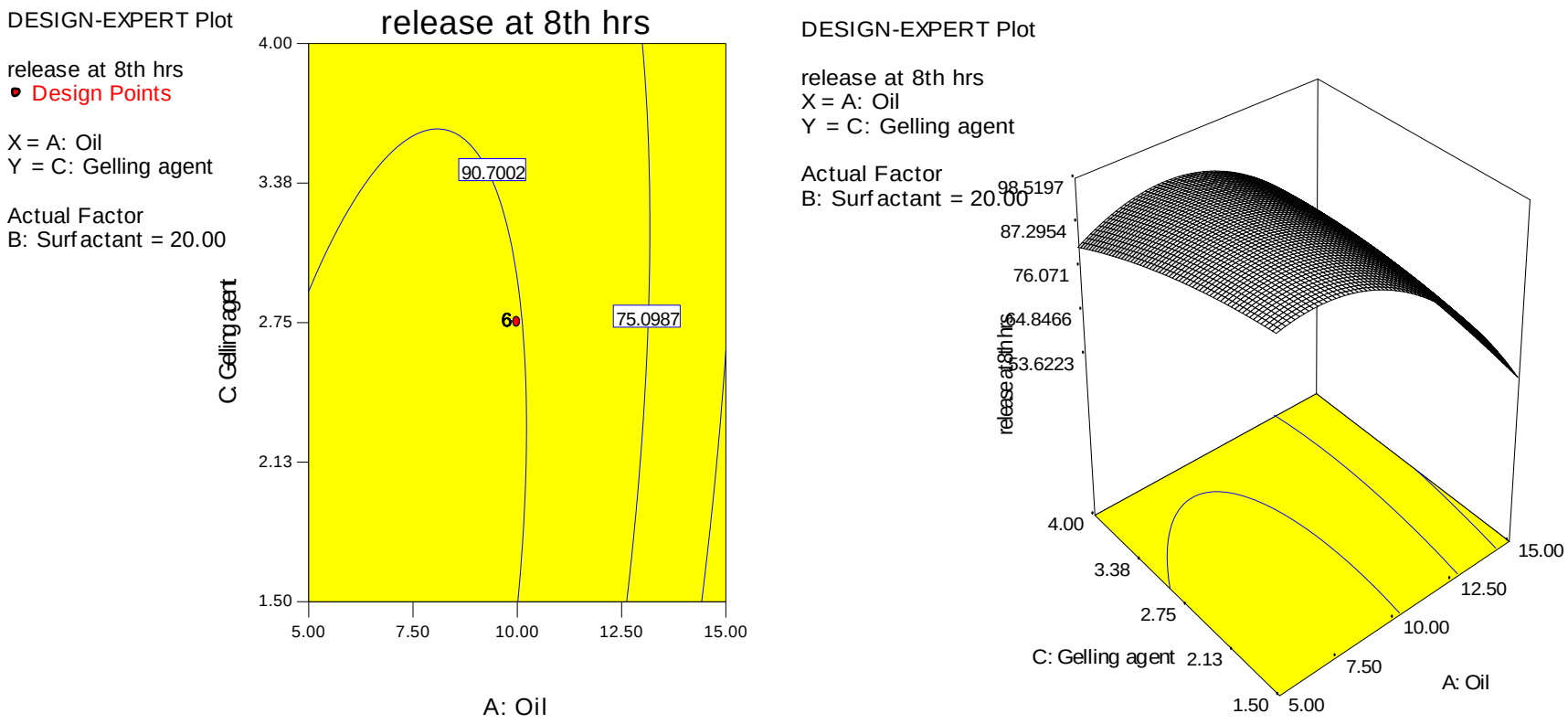


Figure 6.7: Contour and 3D plot of drug release at the 8th hr when gelling agent concentration (%) was plotted against oil concentration (%) at a surfactant concentration of 20.0%.

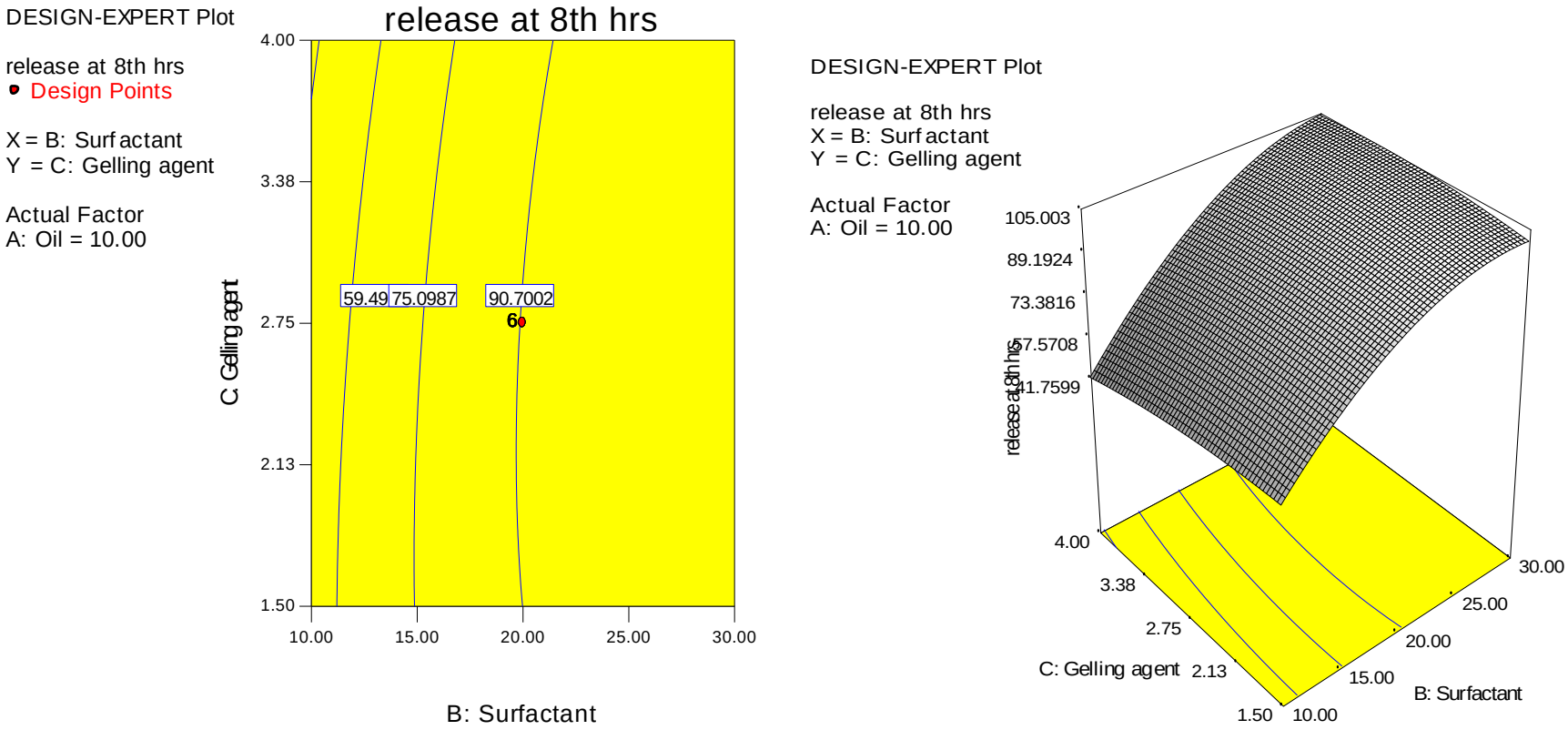


Figure 6.8: Contour and 3D plot of drug release at the 8th hr when gelling agent concentration (%) was plotted against surfactant concentration (%) at an oil concentration of 10.0%.

2.3. Model graphs for Residence time

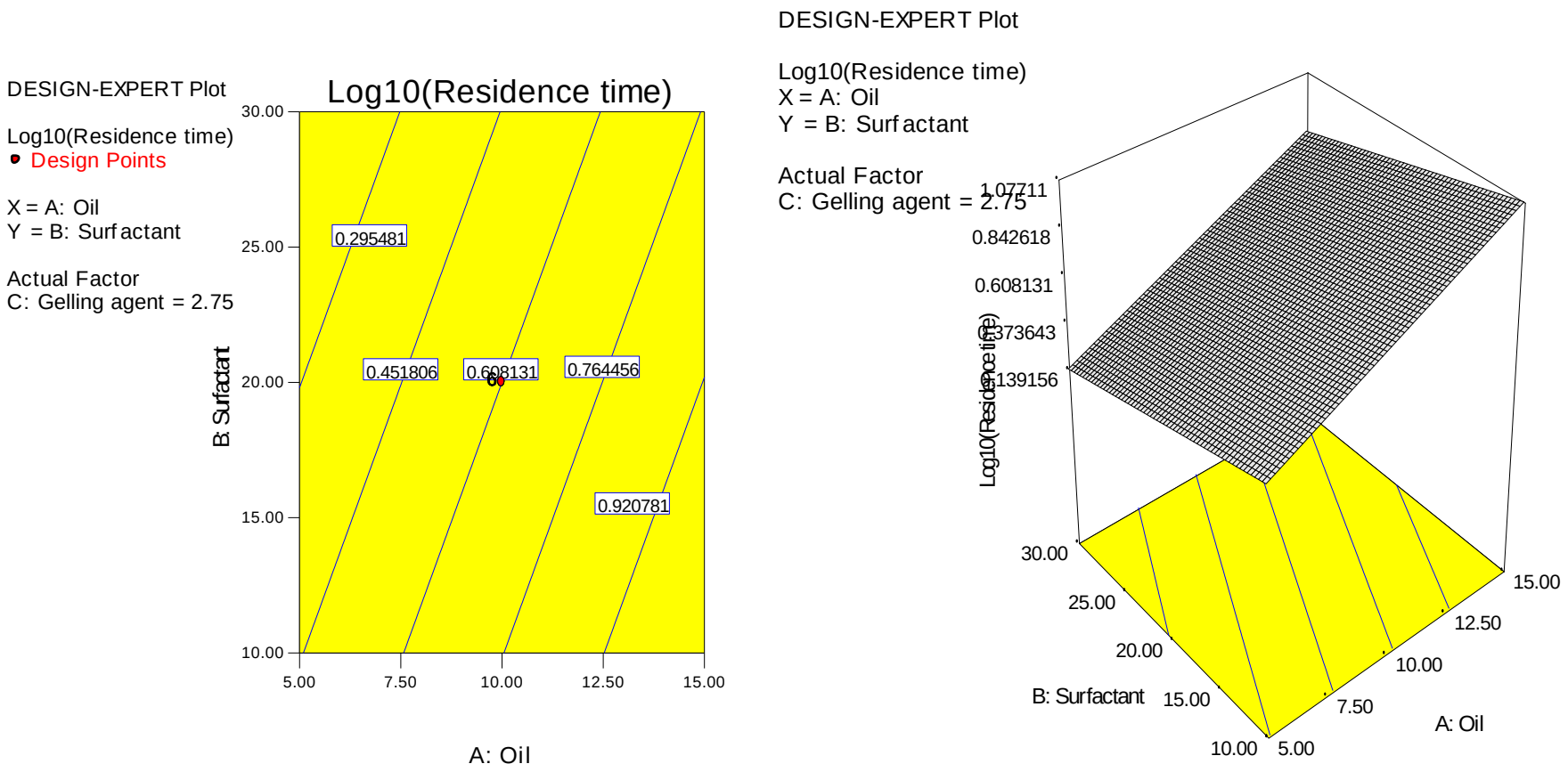


Figure 6.9: Contour and 3D plot of residence time when surfactant concentration (%) was plotted against oil concentration (%) at a gelling agent concentration of 2.75%.

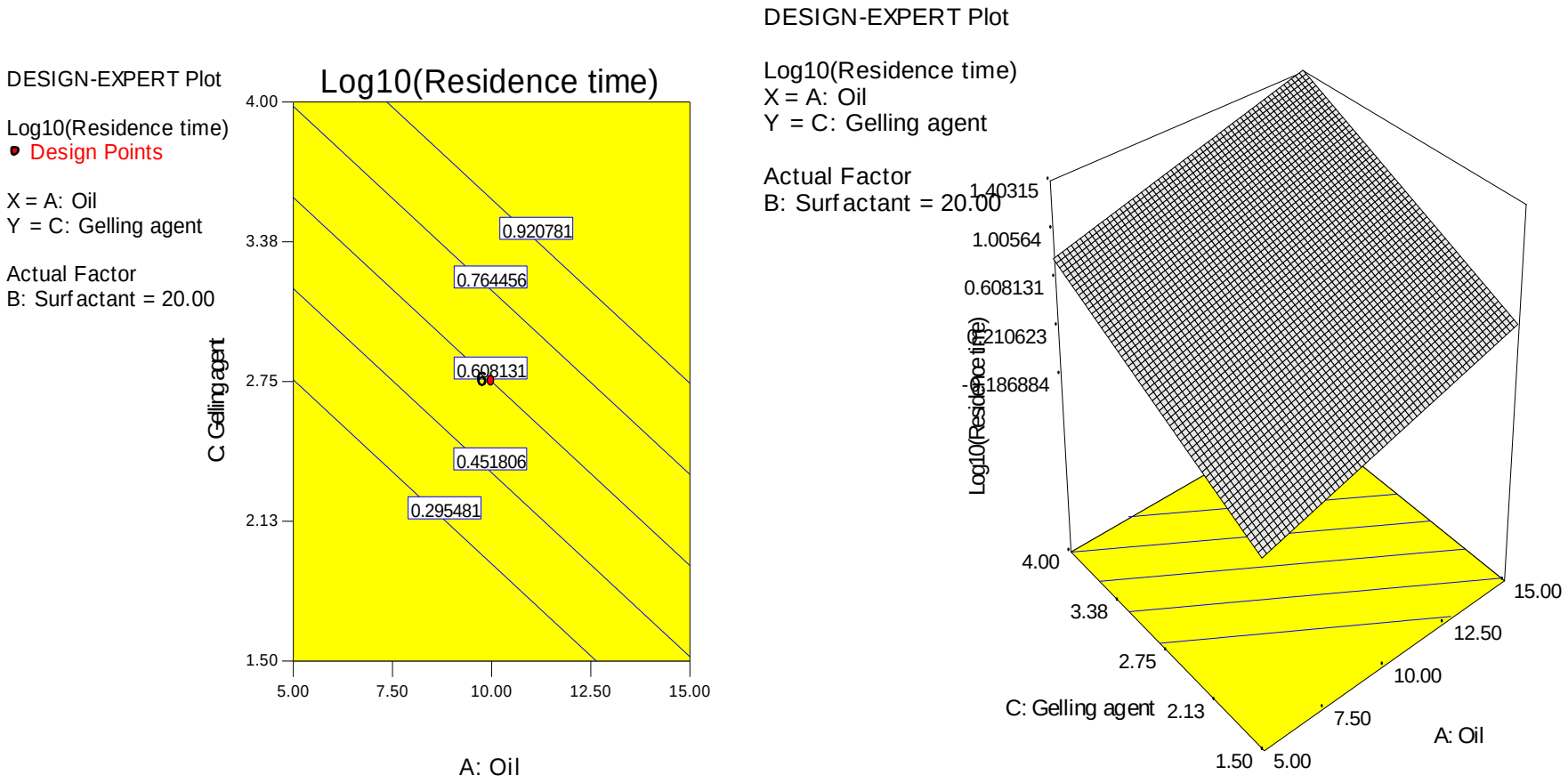


Figure 6.10: Contour and 3D plot of residence time when gelling agent concentration (%) was plotted against oil concentration (%) at a surfactant concentration of 20.0%.

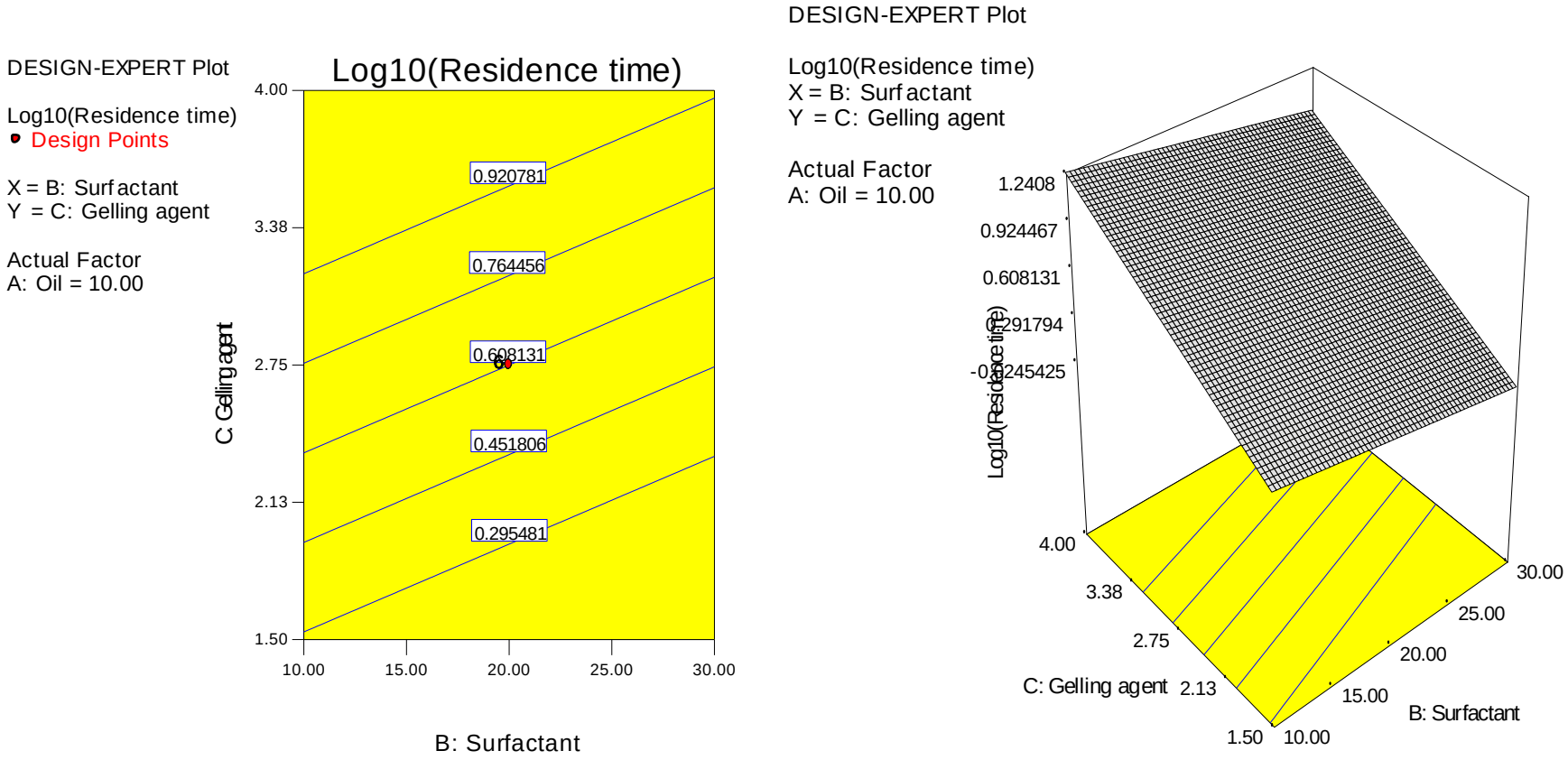


Figure 6.11: Contour and 3D plot of residence time when gelling agent concentration (%) was plotted against surfactant concentration (%) at an oil concentration of 10.0%.

2.4. Model graphs for Spreadability

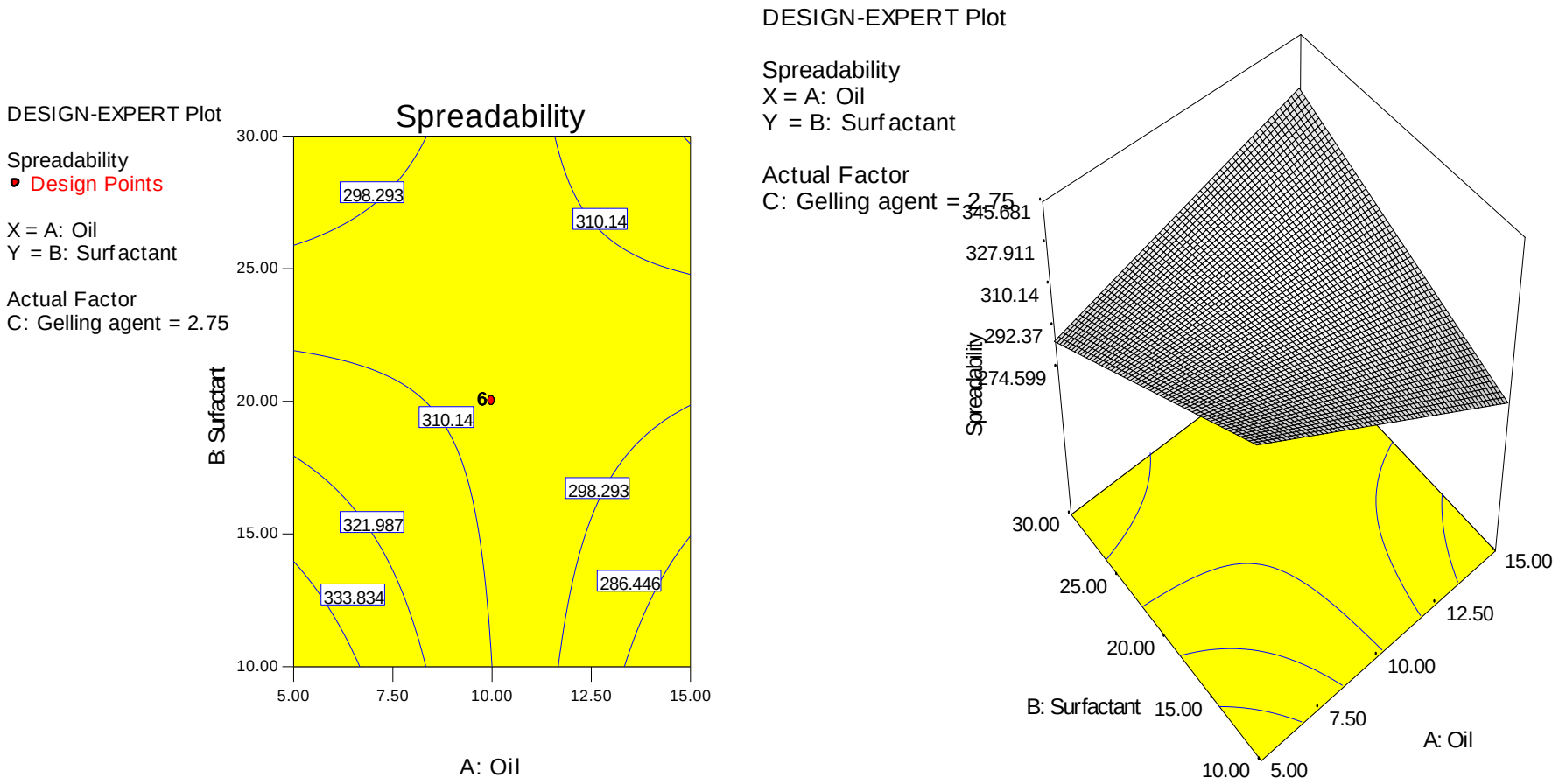


Figure 6.12: Contour and 3D plot of spreadability when surfactant concentration (%) was plotted against oil concentration (%) at a gelling agent concentration of 2.75%.

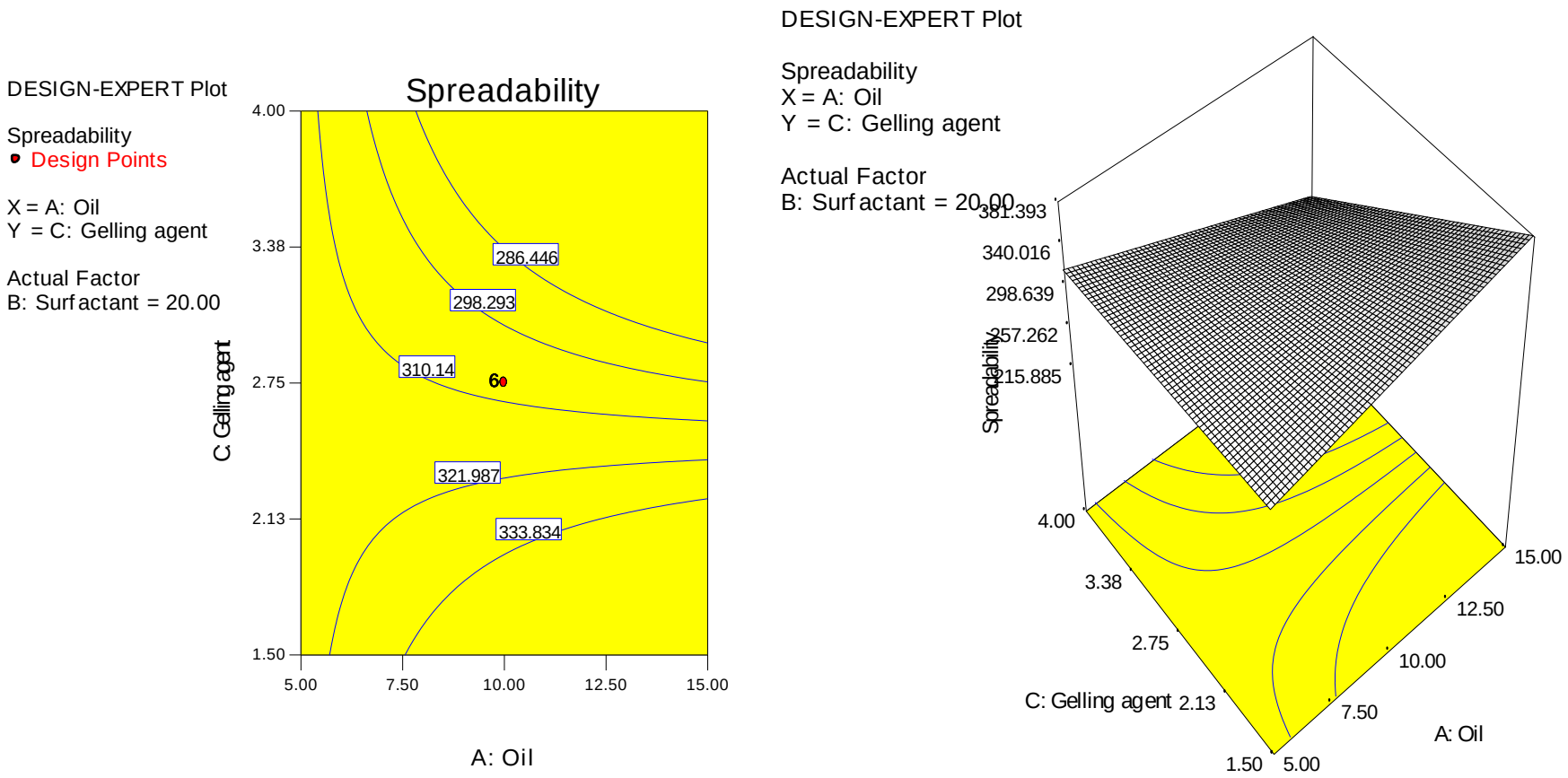


Figure 6.13: Contour and 3D plot of spreadability when Gelling agent concentration (%) was plotted against oil concentration (%) at a surfactant concentration of 20.0%.

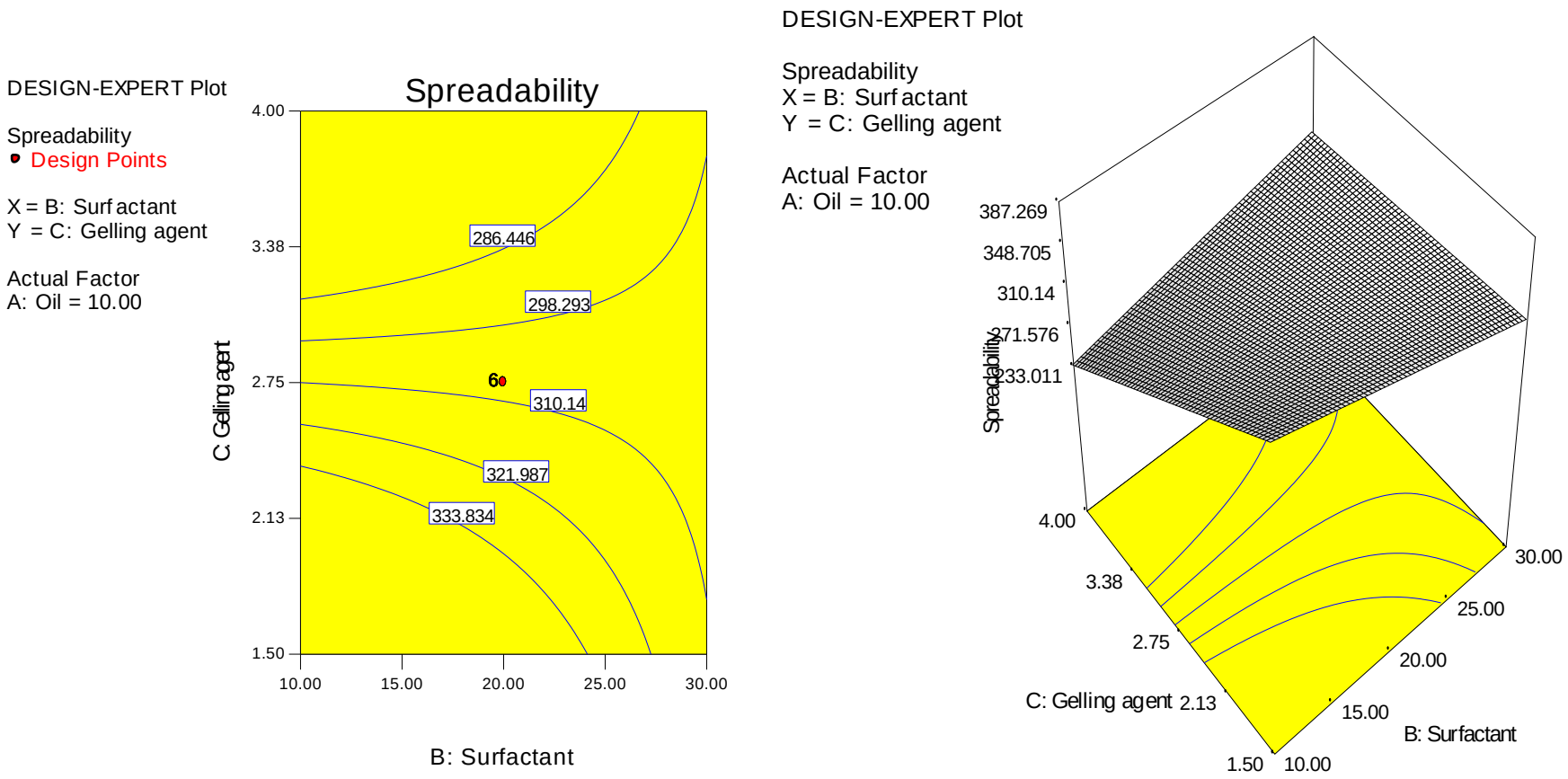


Figure 6.14: Contour and 3D plot of spreadability when Gelling agent concentration (%) was plotted against Surfactant concentration (%) at an oil concentration of 10.0%.