STUDIES ON ISOCONAZOLE NITRATE'S COMPLEXES WITH DIFFERENT CYCLODEXTRIN DERIVATIVES

Master of Science Thesis

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ABSTRACT

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Isoconazole nitrate (ISN) is an anti-fungal active agent within azole family with superior anti-mycotic and antibacterial features. ISN has a wide variety of antimicrobial action against pathogenic yeasts, filamentous spores, dermatophytes, moulds and some Gram-positive bacteria. Many antifungal agents, even ISN, have lower aqueous solubility with variable dissolution kinetics. Literature review recommended the use of inclusion complexes of antifungal drugs-different cyclodextrin derivatives to enhance aqueous solubility and stability of active agents. ISN/β-CD, ISN/HP-β-CD and ISN/M-β-CD inclusion complexes were formulated by using two different techniques such as spray-drying and lyophilization method. The characterization of the prepared complexes were studied and according to the results of the characterization tests, a decision was taken to use spray-dried complexes in the following studies. *In situ* gel formulations containing spray-dried complexes were prepared by using Pluronic® F127 and HPMC based on their improved solubility, gelling features, stability, antimicrobial efficiency, rheological behavior and *in vitro* release characteristics. As a result, ISN/M-β-CD and ISN/HP-β-CD inclusion complexes included *in situ* gel formulations were considered as effective drug delivery systems to the vaginal therapy, owing to their prolonged-release behavior, stability and *in vitro* efficacy.

Keywords: Isoconazole nitrate, Pluronic® F127, HPMC, *In situ* gel, Inclusion complex.

ÖZET

İZOKONAZOL NİTRATIN FARKLI SİKLODESKTRİN TÜREVLERİ İLE GERÇEKLEŞTİRİLEN KOMPLEKSLERİ ÜZERİNE ÇALIŞMALAR

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İzokonazol nitratın (ISN), azol ailesinden mükemmel antimikotik ve antibakteriyel (gram-pozitif) özelliklere sahip antifungal nitelikli bir ilaçtır. ISN, dermatofitlere, patojenik mayalara, filamentli mantarlara, küflere, bazı gram-pozitif bakterilere karşı geniş bir antimikrobiyal aktivite spektrumuna sahiptir. ISN, suda düşük çözünürlüğe bir ilaç ve sulu çözeltilerde çökebilir. Literatürde, suda çözünürlüğü ve ilaç kararlılığını arttırmak için antifungal ilaçlar ile farklı siklodekstrin tiplerinin inklüzyon komplekslerinin kullanılmasını önermiştir. ISN/β-CD, ISN/HP-β-CD ve ISN/M-β-CD inklüzyon kompleksleri püskürterek kurutma ve dondurularak kurutma yöntemi gibi iki farklı yöntem kullanılarak hazırlanmıştır. Hazırlanan komplekslerin karakterizasyonu incelenmiş ve karakterizasyon testlerinin sonuçlarına göre püskürterek kurutma yöntemi ile hazırlanan komplekslerin ileriki çalışmalarda bunların kullanılmasına karar verilmiştir. Pluronic® F127 ve HPMC kullanılarak geliştirilmiş çözünürlükleri, jelleşme özellikleri, kararlılıkları, antimikrobiyal aktivite özellikleri, reolojik davranışları ve *in vitro* salım özellikleri esas alınarak püskürterek kurutma yöntemi ile hazırlanan kompleksler içeren *in situ* jel formülasyonları hazırlanmıştır. Sonuç olarak, ISN/M-β-CD ve ISN/HP-β-CD inklüzyon komplekslerini içeren *in situ* jel formülasyonları, uzatılmış salım profilleri, *in vitro* etkinlik ve kararlılıkları nedeni ile vajinal terapide etkili formülasyonlar olarak kabul edilmiştir.

Anahtar Sözcükler: İzokonazol nitrat, Pluronic® F127, HPMC, *İn situ* jel, İnklüzyon kompleksi

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I dedicate this thesis to the memory of my father and my mother for sacrificing and giving me the care, education and love for my whole life that have made me who I am now and will stay with me forever.

14/01/2021

STATEMENT OF COMPLIANCE WITH ETHICAL PRINCIPLES AND RULES

I hereby truthfully declare that this thesis is an original work prepared by me; that I have behaved in accordance with the scientific ethical principles and rules throughout the stages of preparation, data collection, analysis and presentation of my work; that I have cited the sources of all the data and information that could be obtained within the scope of this study, and included these sources in the references section; and that this study has been scanned for plagiarism with "scientific plagiarism detection program" used by Anadolu University, and that "it does not have any plagiarism" whatsoever. I also declare that, if a case contrary to my declaration is detected in my work at any time, I hereby express my consent to all the ethical and legal consequences that are involved.

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LIST OF SYMBOLS AND ABBREVIATIONS

α-CD	**:** Alpha Cyclodextrin
AIC	**:** Akaike Information Criteria
ATCC	**:** American Type Culture Collection
AUC	**:** Area Under Curve
β-CD	**:** Beta Cyclodextrin
CDs	**:** Cyclodextrins
CS	**:** Chitosan
DC	**:** Drug Content
DMSO	**:** Dimethyl Sulfoxide
DS	**:** Distilled Water
DSC	**:** Differential Scanning Calorimetry
DTA	**:** Differential Thermal Analysis
EMA	**:** European Medicines Agency
EP	**:** European Pharmacopoeia
ESI-MSMS	**:** Electrospray Ionization Mass Spectrometry
F	: The Fraction % of Released Drug
FD	**:** Freeze-Drying
FT-IR	**:** Fourier Transform Infrared Spectroscopy
F127	: Pluronic® F127
γ-CD	**:** Gamma Cyclodextrin
GC	**:** Gas Chromatography
GRAS	**:** Generally Recognized as Safe
h	**:** Hour

HEC	**:** Hydroxyethyl Cellulose
HP-β-CD	**:** 2- Hydroxypropyl Cyclodextrins
HPLC	**:** High-Pressure Liquid Chromatography
HPMC	**:** Hydroxypropyl-Methylcellulose
ICH	**:** International Committee on Harmonization
ISN	**:** Isoconazole Nitrate
K_0	: Release Constant of Zero Order
K_1	: Release Constant of first-order
K_{BL}	: Release Constant of Baker-Lonsdale
K_H	: Release Constant of Higuchi
K_{HB}	**:** Release Constant of Hopfenberg
K_{HC}	**:** Release Constant of Hixson-Crowell
K_{PS}	**:** Release Constant of Peppas-Sahlin
K_s	**:** The Apparent Stability Constant
LCST	**:** Lower Critical Temperature
LOD	**:** The Limit of Detection
LOQ	**:** The Limit of Quantitation
M-β-CD	**:** Methyl Beta Cyclodextrin
MSC	: Model Selection Criteria
n	**:** The Diffusional Exponent
NMP	: N-Methyl-Pyrrolidone
PAA	**:** Polyacrylic Acid
PEG	**:** Polyethylene Glycol
PEO	**:** Polyethylene Oxide

PMA	**:** Polymethacrylic Acid
PPO	**:** Polypropylene Oxide
r^2	**:** Square Regression
RM-β-CD	**:** Randomly Methylated Beta Cyclodextrin
RSD	**:** Relative Standard Deviation
Rpm	**:** Revolutions per Minute
S_0	**:** Slope at Zero Time
SBE-β-CD	: Sulfobutyl Ether Beta Cyclodextrin
SD	**:** Spray Drying
SDV	**:** Standard Deviation
SE	**:** Standard Error
SEM	**:** Scanning Electron Microscope
SVF	**:** Simulated Vaginal Fluid
TAS	: Thermodynamic Affinity Structure
UCST	**:** Upper Critical Temperature
UV	**:** Ultraviolet
v/v	**:** Volume/volume
w/w	**:** Weight/weight

1. INTRODUCTION AND PURPOSE

Vaginal candidiasis, also namely as vulvovaginal candidiasis, is a widespread vaginal mucosal infection, primarily caused by the Candida species and stated to be the second most common mucosal infectious disease after bacterial vaginosis. The most favored traditional topical formulations for vaginal therapy involve semi-solid formulations such as creams, ointments, and gels, but these formulations still suffer from drawbacks of messiness, weakness in spreading, and vaginal cavity leakage based on self-cleansing function of vaginal cavity.

In recent years, many studies have been carried out to increase the bioavailability of vaginal preparations. Cyclodextrins (CDs) are highly preferred because they successfully increase the solubility. CDs are naturally occurring hydrophilic, cyclic oligosaccharides called α, β, γ, composed of six, seven or eight glucose monomers, respectively. While inclusion complexes of active substances with low solubility and unstable effect are formed with CDs, their solubility and stability are increased.

Vaginal *in situ* gelling systems are viscous fluids that pass into the gel phase when exposed to the physiological condition, causing an increase in residence time with their increased viscosity and mucoadhesive properties. These systems increase the availability of vaginal active agents by prolonging the contact time between the drug and the site of action.

Isoconazole nitrate (ISN) is belong to the azole category with superior antifungal and antibacterial (gram-positive) features. ISN is an active substance with low water solubility and has light-sensitive properties. For these reasons, the scope of this master's thesis was aimed to preparing vaginal *in situ* gelling formulations containing ISN/CDs inclusion complexes and characterize these formulations, evaluate the stability and *in vitro* release properties.

Taking into consideration the drawbacks of traditional topical semi-solid formulations, in the current study inclusion complexes with different CD derivatives incorporated into *in situ* gel systems (developed by Pluronic® F127 and HPMC) to provide prolonged release, increase vaginal retention time and increase availability and compatibility.

2. LITERATURE REVIEW

2.1. Isoconazole Nitrate

2.1.1. The chemical structure and description of isoconazole nitrate (ISN)

Isoconazole nitrate (ISN) is one of the antifungal drugs that belong to the imidazole group and similar in structure to miconazole and econazole. ISN has been released in many countries under many brand names (Kessler, 1979). The molecular weight of ISN is 476.8 g/mol and its chemical structure is presented in Figure 2.1. ISN has the corresponding empirical formula $C_{18}H_{15}Cl_4N_3O_4$ and the following molecular formula 1-[2-(2,4-dichlorophenyl)-2-[(2,6-dichlorophenyl)methoxy]ethyl]-1H-imidazolemononitrate (http-1).

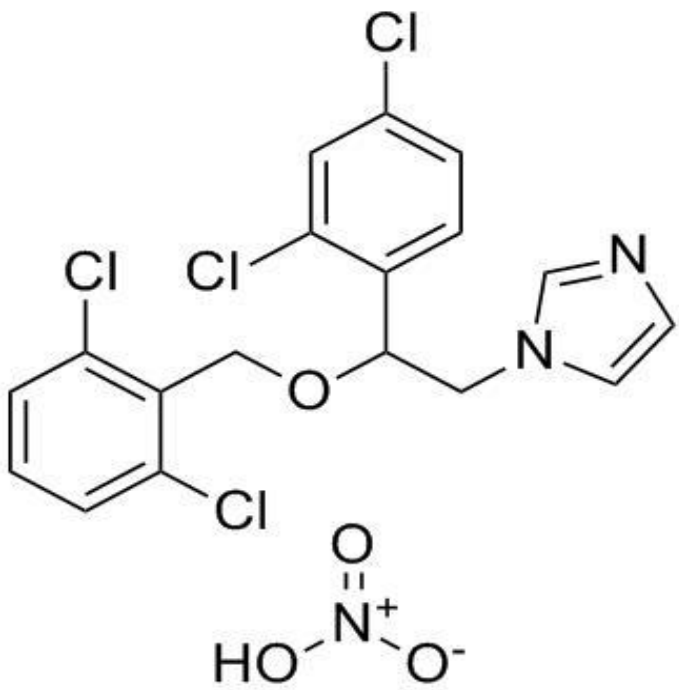


Figure 2.1. *Chemical structure for ISN (http-1).*

ISN is a white or nearly white substance that is very slightly water-soluble, soluble in methanol and slightly soluble in ethanol (EP, 2008).

2.1.2. Pharmacological properties

ISN is a wide-spectrum imidazole-derived antifungal substance (Table 2.1). ISN is used to treat superficial fungi that infect the skin as it treats vaginal candidiasis (against *Candida spp.*). Moreover, it has a broad spectrum of clinical efficacy against Gram-

positive bacteria (Czaika et al., 2012). ISN is one of the N-substituted (mono) imidazole and this group highly effective against dermatophytes, yeast and moulds (Havlickova and Friedrich, 2008). It is also effective in mixed fungal infections with gram-positive bacteria that cause secondary infectious lesions such as staphylococci, streptococci and micrococci (Rx Media Pharma®, 2020).

ISN has a fungistatic and fungicidal effect on microorganisms that cause fungal infections in the skin and mucous membranes (Rx Media Pharma®, 2020). ISN's fungistatic effect is exerted by interfering with the biosynthesis of some compounds in the membranes of fungal cells (not present in humans) (Veraldi, 2013).

The fundamental fungistatic mechanism of ISN is the inhibition of 14 α-demethylation of lanosterol, which leads to inhibit the ergosterol biosynthesis in the fungal membrane (Ivancic et al., 2016). Fungistatic mechanism of ISN is presented in Figure 2.2. (Veraldi, 2013). At the same time, a fungicidal impact, which is not attributed to inhibition of ergosterol synthesis but includes rapid membrane disruption, can be observed in the case of extended use of ISN (Ivancic et al., 2016). Furthermore, it is effective against bacterial or fungal cells and conidiospores that are in the growth phase or not (Rx Media Pharma®, 2020).

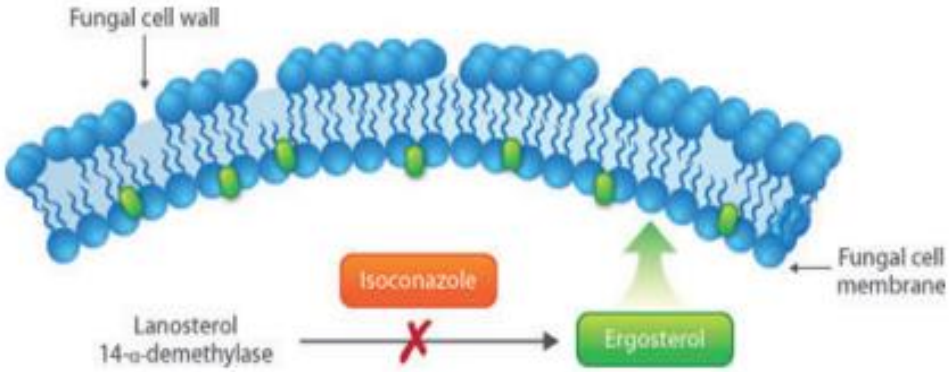


Figure 2.2. *Antimycotic mechanism of action of ISN (Veraldi, 2013).*

Table 2.1. *Indications and susceptible microorganisms of ISN (Rx Media Pharma, 2020)*

Indications	Erythrasma, Candidiasis, Candidiasis prophylaxis, Malassezia fungal prophylaxis, mucocutaneous candidiasis, tinea caitis, tinea corporis, tinea cruris, tinea manuum, tinea pedis, tinea versicolor Vulvovaginal candidiasis
Susceptible microorganism	*Candida albicans* *Candida krusei* *Candida sp.*

2.1.3. Pharmacokinetics

ISN can quickly penetrate human skin and reach to high levels in the horny layer and in the living layer of the skin after one hour from application. After topical application to rabbits, high levels of antimycotic activity were obtained as compared with preparations containing free corticosteroids (http-2). In the injured skin, the topical concentration of ISN cream is five times higher that of uninjured skin. In addition, ISN penetrates the skin rapidly compared to other azole compounds (Tauber,1979).

It is said that there is a neglected systemic absorption of isoconazole after administering the intravaginal pessaries in two doses of 300 mg. The fungicidal concentrations of isoconazole on the vagina continue for three days after a single dose of 600 mg inserted intravaginally (Grayson, 2018).

Although its systemic absorption is very low, the absorbed active substance is completely metabolized in the organism and rapidly eliminated, 1/3 through the kidney and 2/3 with bile (Rx Media Pharma®, 2020).

2.1.4. Isoconazole nitrate preparation, dosage, side effects and proprietary names

2.1.4.1. *Dosage and dosage form*

ISN is given to vaginal infections especially due to Candida spp. in the form of pessaries in a single dose of 600 mg or 300 mg or as a 1% vaginal cream daily for 7 days. ISN is also given to skin infections in the form of 1 or 2 percent cream or topically given in other formulation (EP, 2008).

2.1.4.2. *Side effects*

Local reactions, including burning or itching, may appear following the application of ISN. Intravaginal formulations of azole antifungals may damage latex contraceptives.

Therefore, contraceptive measurements are necessary when topical use of ISN (EP, 2008).

2.1.4.3. *Proprietary names*

There are creams, ovules and spray forms of ISN used against fungal infections in the skin and vagina in the market. The proprietary names of ISN in the market were presented in Table 2.2.

2.1.5. Storage conditions

ISN is damaged by light, so it must be protected from light in a dark place (EP, 2008).

2.1.6. Quantitative determination

The spectrophotometric methods in comparison with the chromatographic method is more economical (Ozen et al, 2014). Solutions of ISN (50 mg mL^{-1}) with different pH. (in the scale of 1.0 to 12.0) were detected by UV-vis examination in the region of 200–500 nm (Von Ahn and Dos Santos, 2012).

In high-performance liquid chromatography (HPLC), in amount of ISN cream formulations have been efficiently processed on a phenyl-based column. The sample was isolated at a flow rate of 1 mL min^{-1} at 45 °C, with a mobile phase (pH 2.9) consisting of 1 percent triethylamine (v/v), methanol, acetonitrile and tetrahydrofuran (4:3:2:2 v/v/v/v). The volume of injection was 10 μL and the sample was analyzed at 225 nm (Von Ahn and Dos Santos, 2012). High performance liquid chromatography (HPLC)-electrospray ionization mass spectrometry (ESI-MSMS) was developed to determine quantitative and qualitative values for isoconazole in human stratum corneum by MS characteristics (Lademann et al., 2012).

Table 2.2. *Brand names of ISN in some country (Rx Media Pharma®, 2020).*

Brand Names	Firm name	Country
ALBACORT® cream [+ Diflucortolone]	KENTFARMA	TURKEY
FAZOL G® cream FAZOL G® lotion FAZOL G® skin powder FAZOL G® ovule	SINCLAIR	France
FUGGY® cream [+ Diflucortolone]	TRIPHARMA	TURKEY
FUGUSIT® cream [+ Diflucortolone]	İSTANBUL PHARMA	TURKEY
FUNGOİD® cream [+ Diflucortolone]	TERRA PHARMA	TURKEY
FUNOCORD® cream [+ Diflucortolone]	KOÇAK PHARMA	TURKEY
GYNO_TRAVOGEN® ovule	BAYER	TURKEY
IZOSOL S® cream [+ Diflucortolone]	ECZACIBAŞI PHARMA	TURKEY
IZOVEF® cream [+ Diflucortolone]	VEFA PHARMA	TURKEY
MANTAZOL® cream [+ Diflucortolone]	PHARMACTIVE	TURKEY
MAXADERM® cream [+ Diflucortolone]	SIPLA MEDPRO	THE REPUBLIC OF SOUTH AFRICA
OLY® cream OLY ® spray	SANTA PHARMA	TURKEY
OLY PLUS® cream [+ Diflucortolone]	SANTA PHARMA	TURKEY
TRAVACOL ® cream [+ Diflucortolone]	DEVA PHARMA	TURKEY
TRAVAZOL® cream [+ Diflucortolone]	BİLİM PHARMA	TURKEY
TRAVOCORT® cream	BAYER INTENDIS	SWITZERLAND POLAND
TRAVOCORT® cream [+ Diflucortolone]	JENAPHARM	GERMANY
TRAVOGEN® cream	BAYER INTENDIS	AUSTRIA, ITALY POLAND
TRAVOGEN® ovule	BAYER	GREECE
TRODERM® cream [+ Diflucortolone]	BİOPHARMA	TURKEY
TROKAR® cream [+ Diflucortolone]	BİKAR PHARMA	TURKEY

For determination of isoconazole nitrate in creams Zorbax C_{18} column and 2% ammonium acetate-methanol-acetonitrile (270: 375: 355) mixture used as mobile phase, the UV detector used was set to 235 nm (http-3).

2.2. Cyclodextrins

2.2.1. Structure and properties

Cyclodextrins (CDs) are cyclic oligosaccharide utilized to promote the solubility in water and the bioavailability of medicines. For instance, they have been used in tablets, nasal sprays, and eye drop solutions, aqueous parenteral solutions (EMA, 2013). CDs comprised of (α-1,4-)-linked α-D-glucopyranose components with central lipophilic cavity (Figure 2.3) (Loftsson et al., 2005). Alpha cyclodextrin (α-CD) contains six units, beta cyclodextrin (β-CD) (Figure 2.3) contains seven units and gamma cyclodextrin (γ-CD) contains 8 units of glucose (Pio di Cagno, 2017). Various properties of natural CDs were presented in Table 2.4. The CD molecules have a special shape since they have a hydrophobic hole and the surface is hydrophilic such that guest molecules can be encapsulated. CDs may also generate complex compounds with a wide range forms of substances like solid, liquid and gaseous substances (Cheirsilp and Rakmai, 2016). CDs are popular as food additives and are considerably used in pharmaceutical, cosmetic and in many other industrial products (Jansook et al., 2018).

Table 2.3. *Usage of cyclodextrins for different administration routes (EMA, 2013)*

	α-CD	**β-CD**	**γ-CD**	**HP-β-CD**	**SBE-β-CD**	**RM-β-CD**
Oral		+	+	+	+	
Nasal						+
Rectal		+		+		
Dermal		+	+	+		
Ocular		+		+		+
Parenteral	+			+	+	

(SBE: Sulfobutyl Ether Beta Cyclodextrin, RM-β-CD: Randomly Methylated beta cyclodextrin)

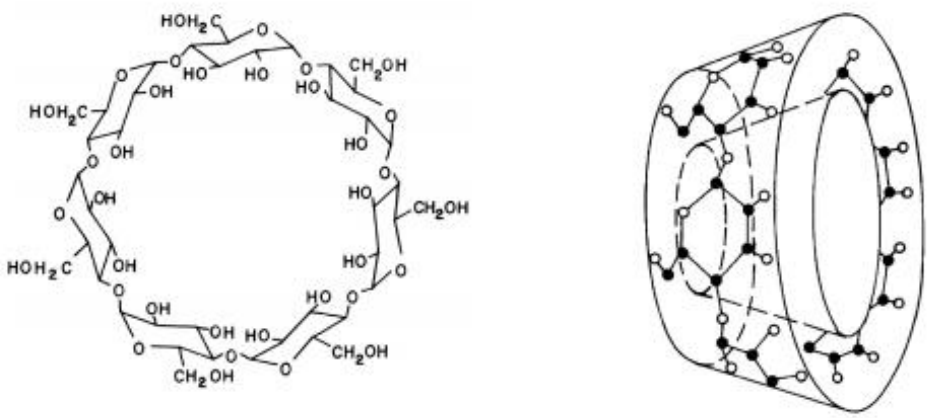


Figure 2.3. *The chemical composition and the unique conical shape of the β-CD (Loftsson et al., 2005)*

Table 2.4. *Characteristics of parent cyclodextrins (Miranda et al., 2011)*

	α-CD	**β-CD**	**γ-CD**
Units of glucose	6	7	8
Molecular weight (g/mol)	972	1135	1297
Outer diameter (Å)	14.6	15.4	7.5-8.3
Inner diameter (Å)	4.7-5.3	6.0-6.5	7.5-8.3
Height (Å)	7.9	7.9	7.9
Volume of core (Å)	174	262	427
The shape of the crystals	Hexagonal lattice	Monocyclic parallelograms	Quadratic prism
pKa (25 °C)	12.333	12.202	12.081
Diffusion constant (40 °C) (m/s)	3.443	3.232	3.000
Hydrolysis by α-amylase	Unremarkable	Slow	Rapid

CDs have fairly aqueous solubility. However, β-CD shows a significantly lower aqueous solubility than α-CD and γ-CD, but at higher temperatures, all CDs solubility in water will be increased (Das et al., 2013). CDs are hydrolyzed in the presence of strong acids, the hydrolysis process is related with the temperature and the acid concentration. CDs are stable in the presence of bases (Hedges, 2009). According to differential scanning calorimetry (DSC) thermograms for α -, β - and γ-CDs are identical. Two heat absorption peaks exist: the first take place at 100 °C as water is evaporated from the crystals; the second, take place at 250 °C, resulting from crystal melting and pyrolysis (Yang et al., 2008).

CDs including 2-Hydroxypropyl-beta-cyclodextrin (HP-β-CD) and sulfobutylether-β-Cyclodextrin (SBE-β-CD) are considered safe for parenteral products and are used frequently with antitumor and immunomodulatory drugs (Carneiro et al., 2019).

2.2.2. Cyclodextrin derivatives

To enhance the solubility of natural CD compounds, CD derivatives were prepared by reacting the first or second hydroxyl group of CD molecules with different substitutes (Saokham et al., 2018). CD derivatives that have acquired wide medicinal attention involve hydroxy propane variants of β, γ and, methylated β-CDs (M-β-CD), SBE-β-CD, etc. (Table 2.5) (Arun et al., 2008).

Table 2.5. *Some of cyclodextrin derivatives (Stella and He,2008)*

Cyclodextrin	R	n
α-cyclodextrin (α-CD)	-H	4
β-cyclodextrin (β-CD)	-H	5
γ-cyclodextrin (γ-CD)	-H	6
Carboxymethyl-β-cyclodextrin (CM-β-CD)	$-CH_2CO_2H$/ -H	5
Carboxymethyl-ethyl-β-cyclodextrin (CME-β-CD)	$-CH_2CO_2H$/ CH_2CH_3/ -H	5
Diethyl-β-cyclodextrin (DE-β-CD)	$-CH_2CH_3$ / -H	5
Dimethyl-β-cyclodextrin (DM-β-CD)	$-CH_3$/ -H	5
Glucosyl-β-cyclodextrin (G1-β-CD)	-Glucosyl / -H	5
Hydroxyethyl-β-cyclodextrin (HE-β-CD)	$-CH_2CH_2OH$ / -H	5
Hydroxybutenyl-β-cyclodextrin (HBU-β-CD)	$-CH_2CH(CHCH_2)OH$ / -H	5
Hydroxypropyl-β-cyclodextrin (HP-β-CD)	$-CH_2CHOHCH_3$ / -H	5

2.2.3. Cyclodextrin phase solubility

Analysis of the phase solubility of the impact of complex factors on the solubilized substance is a conventional method not only to determine the value of constant solubility but also gives a clear idea about the stoichiometry equilibrium (Brewster and Loftsson, 2007).

A-type refers to the inclusion complexes are soluble (Nicolescu et al., 2010), A_L-type refers to a linear increment in drug solubility against concentration of CD, A_P profiles refers to a positive deviation from the linear pattern (i.e. solubilization is more efficient at higher concentrations) and A_N-type refers to a negative deviation from the linear curve

(i.e., the CD is directly proportional less efficient at higher concentrations) (Loftsson et al., 2002). B-type refers to the generation of inclusion complexes have poor solubility, B_S-type refers to the inclusion complexes have limited solubility and B_I-type refers to the inclusion complexes are insoluble (Abhiman and Anantrao, 2017) (Figure 2.4).

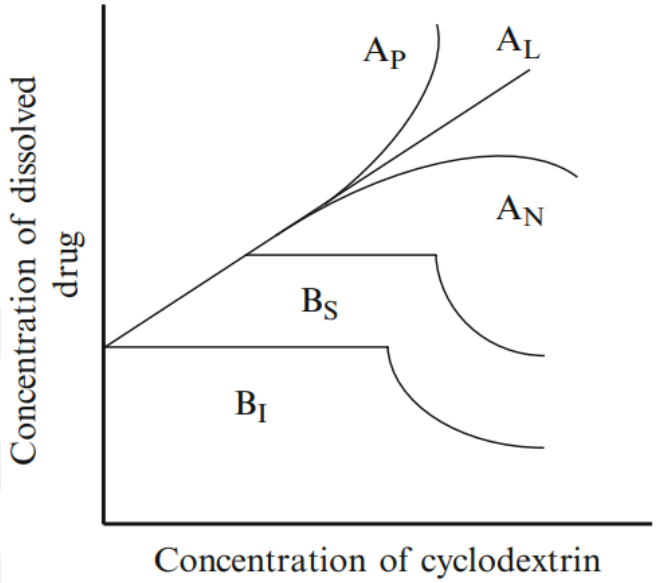


Figure 2.4. *Cyclodextrin phase solubility diagram (Higuchi and Connors, 1965)*

2.2.4. Approaches for preparation of inclusion complexes

2.2.4.1. *Milling or co-grinding technique*

With the aid of a mechanical device, a solid inclusion complexes could be formulated using grinding and milling the active agent and CDs. Drugs and CDs are well blended and the physical blend is put into the oscillating mill and grinded for the appropriate period (Hiremath et al., 2015).

2.2.4.2. *Kneading method*

In this method, a small volume of pure water, ethanol or a combination of water: ethanol (50:50, v/v) is added onto the physical mixture of CDs and guest molecule to obtain a paste form. In order to obtain a homogeneous slurry, the resulting mixture is kneaded within the specified time depending on the guest molecule or until the solvent is fully eliminated. The resulting mixture is dried under vacuum at higher temperatures or optionally at room temperature. The hard mass that results from drying the pastes

completely can be obtained in the form of powder by grinding (Al-Marzouqi et al., 2009; Demirel et al., 2011; Yurtdaş et al., 2011).

2.2.4.3. *Co-precipitation technique*

It is the most used method in laboratories where a solution of the drug that is dissolved in the organic solvent is slowly added to the aqueous solution of CD. The precipitate can be separated from the medium by transferring the cycle, centrifugation and filtration (Del Valle, 2004).

2.2.4.4. *Solution-solvent evaporation method*

In this process, the drug is dissolved in an alcohol solvent, and CD is dissolved in water, and then an aqueous CD solution is transferred to the alcoholic drug solution for chemical dispersion of the drug and the complex agent. Then the solvent is evaporated by using a vacuum at 45 °C to obtain the complex solid powder form (Yurtdaş et al., 2011; Octavia et al., 2015).

2.2.4.5. *Neutralization precipitation method*

The basic drugs are dissolved in an acidic solution and the acidic drugs are dissolved in a basic solution. Once all is dissolved, CD is applied to the solution through constant stirring. The stirring continuous until a clear solution is formed. The water solubility of the substance is decreased by adjusting the pH of the resulting solution. Consequently, the complexation process is formed (Özkan et al., 2000).

2.2.4.6. *Spray-drying or atomization method*

The CD and the drug molecule are dissolved in a combination of water/organic solvent and the mixture is sprayed through a nozzle into a heated closet with spray-drying device. The complex is formed by the rapid removal of the solvent from the medium (Mura et al., 1999; Demirel et al., 2011).

The spray drying method has the advantage of being able to control the internal and external temperature, flow rate and air pressure. Spray drying conditions can be made suitable for volatile substances that have stability problems against temperature (Yurtdaş, 2010).

2.2.4.7. *Lyophilization (freeze-drying) technique*

Lyophilization is a suitable for heat sensitive compounds such as volatile compounds, which have low stability in some drying methods in which the pressure temperature and the flow velocity are high (Güleç and Demirel, 2016). This method has many advantages, including that it is less harmful to the drug than it is compared to other methods that are use higher temperatures. However, it is a time-consuming method and freeze-drying faces difficult challenges, as the sensitivity, complexity and price of processed products increase steadily (Shukla, 2011).

The CD and the drug molecule are dissolved in a blend of water/organic solvent. The resulting solution is frozen at -18-86 ºC then dried by lyophillization for the solid inclusion complex to be acquired (Yurtdaş et al., 2011).

2.2.4.8. *Microwave irradiation method*

In this way, the microwave radiation is used to cause interference between the drug molecule and CDs. In the given molar CDs and the active ingredient are dissolved in a solution of water and organic solvents. The solution is left for two minutes in the microwave at a temperature of 60 ºC until the reaction between the drug and CDs is carried out (Badr-Eldin et al., 2013).

2.2.4.9. *Supercritical CO_2 technology*

In recent years, supercritical fluid technology has great importance in research and application areas. Supercritical CO_2 is frequently preferred in pharmaceutical processes as it is not flammable, toxic and expensive, chemically stable, environmentally acceptable, and easily separated from the mixture. In the supercritical CO_2 method, which is also utilized in the formulation of the inclusion complex, the physical mixture of CD and guest molecule is placed in the cell in the instrument. Pressure is applied to the system up to the desired pressure and temperature and the system is heated. Generally, the system is operated in stationary mode at the desired pressure and temperature for three hours. After the pressure is reduced, the complex inside the cell is taken and homogenized (Al-Marzouqi et al., 2009; Yurtdaş, 2010).

2.2.5. Characterization methods of CD inclusion complexes

Analysis of the resulting complexes can be performed quickly and directly by using physiochemical and solid-state methods to confirm the determination of inclusion complexes and host-guest interactions of drug and CD molecules.

2.2.5.1. *X-ray diffraction method*

This method is also used to assess the development of the inclusion complex. Each material has specific rolled curves that can be perceived by the diffraction end detectors in the crystal structure. If the inclusion complex is formed, there are significant differences in the X-ray analysis results of the complex (Mura, 2015).

2.2.5.2. *Thermal methods*

Thermal methods include many techniques such as thermodynamic affinity structure (TAS), thermodynamic gradient structure, differential scanning calorimetry (DSC) and differential thermal analysis (DTA) methods (Mura, 2015). DSC is useful for quantitatively detects the conversion temperatures, in addition DSC has the ability to assess the degree of purity of the substance (Gill et al., 2010).

2.2.5.3. *Nuclear magnetic resonance method (^{1}H-NMR)*

^{1}H-NMR is an important method in qualitative analysis of the CDs inclusion complex. If the CDs inclusion complex was formed, the ^{1}H-NMR spectra of the complex varies from the spectra of the guest molecule and CD (Mura, 2014).

2.2.5.4. *Scanning electron microscopy (SEM)*

SEM is used to identify the solid state of the resulting complex and it allows in-depth examination of the morphological properties of pure materials and related physical mixtures, in addition to examining the resulting complex which was obtained through various methods of complex preparation (Yurtdaş-Kırımlıoğlu, 2020).

2.2.5.5. *Fourier transform infrared spectroscopy (FT-IR)*

FT-IR spectroscopy is widely used to examine the inclusion of CD and drug. This method makes it possible to determine which bands of stretching vibrations of pure compounds and CDs alter the complexing dynamics (Yurtdaş-Kırımlıoğlu, 2020).

2.2.5.6. *Chromatographic method*

Gas chromatography (GC) allows the quantitative analysis and separation of volatile compounds. The stability of the CD infusion complexes can be studied by using HPLC method (Kfoury et al., 2018).

2.2.6. Cyclodextrins advantages

The applications and benefits of the CD complexation in pharmaceutical industries have been well recognized by several reviews in recent years (Table 2.6). These benefits are improving active stabilization, masking unpleasant odors or taste, reducing irritation and material handling benefits (Das et al., 2013). In addition, enhanced solubility and dissolution properties of CDs thus increases the oral bioavailability of substances such as haloperidol and HP-β-CD. CDs also enhance the chemical, physical stability and half-life of active agents, such as sulfamethoxazole/trimethoprim and HP-β-CD. CDs also mask the smell and unwanted taste of some medicines, like dextromethorphan HBr and β-CD (Hanumegowda, 2014).

Table 2.6. *Various pharmaceutical advantages of cyclodextrin complexes (Vikas et al., 2018)*

Drugs	Cyclodextrins	Pharmaceutical Application
Voriconazole	HP-β-CD and 2-O-methyl-β-CD	Enhance dissolution rate, solubility and chemical stability
Glimepiride	HP-β-CD, β-CD	Improvement in the dissolution rate extended time of activity and improved therapeutic efficacy of the medication
Latanoprost	propylamino-β-CD	Higher stability, solubility, and ocular tolerance
Nateglinide	SBE- β-CD	Higher aqueous solubility
Sulfamethoxazole	β-CD	Enhance bioavailability and dissolution rate
Eslicarbazepine acetate	β-CD	Faster onset of action and higher bioavailability
Polylactic acid	β-CD	Improve the thermal expansion stability
Prostaglandin E1	α-CD, β-CD, CME-β-CD	Enhance topical drug delivery in the presence of water
Cetirizine dihydrochloride	β-CD	Enhance bitter taste and organoleptic properties

2.2.7. Commercial products containing cyclodextrin

Commercial products containing CD derivatives were demonstrated in Table 2.7.

2.2.8. Cyclodextrins toxicity

Although CDs are classified as a GRAS (Generally Recognized as Safe) substances, its safety and toxicity depend on the route of administration and on the type of CD used (Carneiro et al., 2019). When CDs are administered orally it will be absorbed negligently through the gastrointestinal tract, therefore there are essentially non-toxic due to the large size of the molecules and hydrophilic properties (Gidwani and Vyas, 2015).

Table 2.7. *Examples of branded preparations including cyclodextrin (Loftsson et al.,2005).*

Drug	Formulation	Trade name	Company
α- CD			
Cefotiam hexetil HCl	IV solution	Prostavasin®	Ono (Japan)
Cefotiam hexetil HCl	Oral tablet	Pansporin T®	Takeda (Japan)
β-CD			
Benexate HCl	Oral capsule	Ulgut®	Teikoku Kagaku
Dexamethasone	Dermal pomatum	Glymesason®	Sangyou (Japan)
Nicotine	Sublingual tablet	Nicorette®	Fujinaga (Japan)
Nitroglycerin	Sublingual tablet	Nitropen®	Pharmacia (Sweden)
Piroxicam	Oral tablet	Brexin®	Nihon Kayaku (Japan)
Tiaprofenic acid	Oral tablet	Surgamyl®	Roussel-Maestrelli (Italy)
2-HP-β-CD			
Cisapride	Suppository	Propulsid®	Janssen (Belgium)
Indomethacin	Eye drop	Indocid®	Chauvin (France)
Itraconazole	Oral and IV	Sporanox®	Janssen (Belgium)
Mitomycin	solutions	Mitozytrex®	SuperGen (USA)
	IV solution	MitoExtra®	Novartis (Switzerland)
RM-β-CD			
17β-Oestradiol	Nasal spray	Aerodiol®	Servier (France)
Chloramphenicol	Eye drop	Clorocil®	Oftalder (Portugal)
SBE-β-CD			
Voriconazole	IV solution	Vfend®	Pfizer (USA)
Ziprasidone maleate	IM solution	Geodon®, Zeldox®	Pfizer (USA)
2-Hydroxypropyl-γ-CD			
Diclofenac sodium	Eye drop solution	Voltaren®	Novartis (Switzerland)

The effect of β-CD is demonstrated by comparatively lower oral doses where reactions appear in the main target organ and in the kidneys. The osmotic nephrosis are stimulated and globulin fraction proteins released as an indicator of glomeruli damage. In contrast, the greatest oral doses applicable to 20 per cent of the daily regular diet of dogs and rats, were impaired by α- and ɣ-CD (Antlsperger and Schmi,1996).

It has been reported that synthetic alterations to the α-CD glucopyranose rings can improve or minimize its cytotoxic impacts. Methyl and succinyl replacement can raise toxicity based on the amount of substitutes, however hydroxypropyl-CDs such as β-CDs

are ideal for parenteral delivery systems and even acetylation significantly decrease the toxic impact (Róka et al., 2015).

2.3. *In Situ* Gelling Systems for Drug Delivery

2.3.1. Definition

"In situ" is a latin term meaning 'in its original location' or 'in position'. The *in situ* gelling drug carrier system is able to release the active agent in a sustainable pattern while ensuring reasonably stable plasma curves (Devasani et al., 2016). In recent years a growing number of *in situ* gel systems and numerous patents have been researched and reported for utilize in different biomedical applications, especially drug delivery (Madan et al., 2009). *In situ* gelling systems are polymeric based formulations in solution state before introducing the body, but under physiological environment they are converted into gel states (Kouchak, 2014).

The *in situ* gel system demonstrated anticipated viscosity, drug quality and sustainable drug release, along with ease of administration and decreased frequency of dosage regime, which improves patient compliance and convenience. *In situ* gel form took place owing to one or a combination of various factors, such as pH alteration, temperature variation and ion bonding. *In situ* gel systems are also applicated through oral, ocular, injectable and intraperitoneal, rectal and vaginal paths (Nikode et al., 2016).

2.3.2. Importance of *in situ* gelling system

In situ gelling systems play important role in the controlled and prolonged release of the drug, reduce the number of doses needed, decreases wastage of the drug, increasing the drug bioavailability, increasing in residence time of the drug due to the formation of the gel and it keeps the drug free from the liquid pharmaceutical form, so the drug stays in contact with the site of action at the ideal time (Mohanty et al., 2018). In addition, incorporation of novel drug delivery approaches like liposomes, nanoparticles, microsphere, pegylation, nanoemulsion, microemulsion etc. again make this delivery system more promising (Ajazuddin et al., 2012).

2.3.3. Approaches of *in situ* gel drug delivery

There are four commonly described pathways used to trigger gel formation at the site of action: Physiological factors (like temperature and pH), physical alterations in biomaterials (like exchanging of solvents and swelling), chemical reactions (like enzymatic, chemical and photo-induced polymerization) (Nirmal et al., 2010).

2.3.3.1. *Physiological stimuli*

2.3.3.1.1. *Thermally trigged system*

The use of biomaterials that have the potential to transfer from the solution to the gel triggered by a rise in temperature is a desirable way to form *in situ* gel systems. The optimum critical temperature range for this carrier system is ambient and physiological temperature, and without external source other than body temperature is required to trigger the gelation process.

The thermosensitive hydrogels are classified into three classes: Negatively thermosensible, positively thermosensible and thermally reversible. The negatively thermosensible hydrogels get lower critical temperature (LCST) and the temperature should be raised above the LCST to formation the gel. Positively thermosensitive hydrogels have upper critical temperature (UCST) and the temperature should be less than the UCST to formation the gel. Thermally reversible hydrogels are polymer solutions and have a free-flowing liquid at ambient temperature and gel at body temperature. When administrated into the body it creates a firm, stable gel in minutes (Patil et al., 2014).

2.3.3.1.2. *pH triggered systems*

Another way to form gel on site depends on the change in pH. Some polymers including polyacrylic acid (PAA) (Carbopol®) or its derivatives, a mixture of polymethacrylic acid (PMA) and poly(ethylene glycol) (PEG) demonstrate a transition from sol to gel with pH alteration. Hydrogel swelling rises with an increase in outer pH in presence of weak anionic acid groups but reduces if the polymer includes weak base fractions (Kumbhar et al., 2013).

2.3.3.2. *Chemical stimuli*

2.3.3.2.1. *Ionic cross-linking*

Some ions sensitive natural polysaccharides, including carrageenan, gellan gum, sodium alginate go through a transition phase in the presence of different ions like K^+, Ca^{2+}, Na^+. Alginic acid transforms gel in the case of divalent/polyvalent cations such as Ca^{2+} owing to interaction with glucuronic acid in alginate linkages (Majeed et al., 2019).

2.3.3.2.2. *Enzymatic cross-linking*

Enzyme-induced *in situ* gel formation has not been commonly studied but tends to have more benefits than chemical and photochemical techniques. It offers a useful pathway for regulating the rate of gel forming and is used in pharmaceutical forms containing insulin (Kumar et al., 2011).

2.3.3.3. *Physical stimuli*

2.3.3.3.1. *Swelling*

In situ formation can also exist as the material takes water molecules from the external atmosphere and expands. One of these substances is Myverol® 18-99 (mono-glycerol), a polar fat that enlarges in water to generate liquid crystal phase. It has certain bioadhesive features and can be degraded *in vivo* by an enzyme activity (Kumar et al., 2011).

2.3.3.3.2. Diffusion

This process includes spreading the solvent from the polymer solution to the surrounding tissues, resulting in accumulation or hardening of the polymer network. N-methyl-pyrrolidone (NMP) was considered to be a good solvent for this process (Kumar et al., 2011).

2.3.4. *In situ* gel preparation methods

There are two methods for preparing *in situ* gel formulation: hot method and cold method. In cold method, the drug is mixed with an adequate amount of distilled water and kept overnight at 4 °C in the refrigerator. The polymer is slowly added with constant stirring and the dispersion is stored in the refrigerator until a clear solution is formed.

This method is appropriate when Poloxamer®, Chitosan (CS) or Carbopol® is used as a gelling polymer (Ban et al., 2018). The hot method is suitable when gellan gum pectin are used as a gel forming agent. In this way, the polymer is dispersed in deionized water and the temperature is raised between 60 to 90 °C with constant stirring. After that the solution is cooled below 40 °C and finally the drug is added into the polymeric solution (Swain et al., 2019).

2.3.5. Physicochemical evaluation of *in situ* gel formulation

The analyses that are used to characterize *in situ* gels are: Rheological behavior, determination of pH, infrared profile, measurement of gelation temperature, measurement of gelation capacity, *in vitro* release studies and stability tests (Sharma et al., 2014).

2.3.6. Polymers used in *in situ* gel systems

2.3.6.1. *Pectin*

Pectins are polysaccharide compounds in which the polymer network usually consists of (1-4)-d-galacturonic acid traces. Low methoxy pectins (the esterification degree <50%) easily generate gels in case of free calcium ions in aqueous solution (Shastri et al, 2016). The gelation process of pectin occurs in the case of H^+ ions and calcium ions are essential to make the gels that are appropriate as drug carrier systems. The pectin used mainly in these preparations is soluble in water, so organic solvents not used in these formulations (Mohanty et al., 2018).

2.3.6.2. *Xanthan gum*

Xanthan gum is a high cellular polysaccharide with high molecular weight. Xanthan gum is provided by fermentation process of gram-negative bacteria such as Xanthomonas campestris. It is dissolved in cold and hot water, in aditon in alkaline and acidic solutions. It show good stability under the alkaline conditions (Ahmed and Goli, 2018).

2.3.6.3. *Guar gum*

Guar gum is a natural compound and extracted from the endosperm of the seed. Guar gum compounds are dispersible in both cold and hot water. Guar gum derivatives

are used widely in the delivered systems to form nano-microparticles and hydrogels (Wang et al., 2018).

2.3.6.4. *Chitosan*

CS is naturally extracted from shrimp peel and crab by alkaline deacetylation of chitin. The gel is formed by using CS through a rapid change in temperature or pH. It has many unique properties that made it one of the most important polymers to use owing to its biocompatibility, biodegradability and low immunogenicity features (Chen et al., 2011).

2.3.6.5. *Poloxamers®*

Poloxamers® are synthetic water-soluble tri-block copolymer. It contains two molecules of polyethylene oxide (PEO) and one molecule of polypropylene oxide (PPO) core in an ABA arrangement (PEO-PPO-PEO). Poloxamers® have many properties that make it used for many purposes such as gelling, emulsifying and solubilizing agent. Pluronic®, Kolliphor® and Lutrols® are the other commercially names of Poloxamers®. Concentrated aqueous solutions of Poloxamers® generate thermo-reversible gels and increased residence time of the drug (Soliman et al., 2019). Different studies of Pluronics® include use of Pluronic® F127 (Poloxamer® 407) for delivery of human growth hormone, through the use of Pluronic® F127 and enantiomeric D- or L-lactide oligomer spacer a stereocomplexed hydrogels was obtained and lead to increased mechanical strength with rapid dissolution in aqueous media (Chung et al., 2008). Mixture of CS with Pluronic® F127 used as an injectable cell delivery carrier for cartilage renewal, the hydrogel showed improved mechanical properties, hydrogel stability and aqueous dissolution ability (Park et al., 2009). Pluronic® F 68 and HPMC K4M as independent variables used in combination with Pluronic® F 127 for vaginal delivery of antifungal drug and play an important role at the level of the following characteristics: gelation temperature, viscosity, mucoadhesive strength and gel strength drug release (Jolly and Chetna, 2014). Combination of Pluronic® F127 (20% w/v) with Pluronic® F68 (1% w/v) used for vaginal drug delivery for HIV prophylaxis and this mixture of polymers lead to the gel did not result in nanoparticle aggregation and the gel showed thermo-gelation at 32.5 °C. (Date et al., 2012).

2.3.6.6. *Hydroxy propyl methyl cellulose (HPMC)*

HPMC is consists of repeating β-(1, 4)-D-glucopyranose unit and as thickening agent, suspending agent and as stabilizing agent. HPMC is a mucoadhesive polymer and it is useful in gel formulations as viscosity enhancer. The viscosity of cellulose derivatives such as HPMC and methyl cellulose (MC) increases with increasing temperature. Some cellulose derivatives remain liquid at low temperatures and turn into gel at high temperatures such as HPMC become gel between 75-90 °C. HPMC is soluble in organic and aqueous solvent system (Jain et al., 2016). When different studies on HPMC were investigated, it was reported that HPMC was used as a viscosity enhancer in *in situ* gelling ophthalmic systems containing puerarin using Carbopol® and HPMC (Wu et al., 2007). Another study showed the effect of combination of HPMC with alginate in preparing gatifloxacin loaded *in situ* gel ophthalmic delivery system. Rheological studies indicate the significant influence of HPMC on the formulation viscosity (Liu et al., 2006). Moreover, the combination of HPMC with Carbopol® 940 in preparation of sinomenine hydrochloride loaded *in situ* gel for uveitis therapy reflect the positive role for HPMC on the viscosity and gel strength in the physiological condition (Song et al., 2013).

2.3.7. Ideal characteristics of polymers

One of the most important components in the manufacture of *in situ* gel is the polymer. The formulation of *in situ* gel may contain different types of polymers such as hydroxyethyl cellulose (HEC), Carbopol®, sodium alginate, and gums such as guar hydroxypropyl, xanthan gum (Kurniawansyah et al., 2018). It should be biocompatible, able to adherence to mucus, have pseudoplastic flow pattern, has well tolerance and optical activity and able to lower viscosity with an increased shear rate (Kalia et al., 2014).

2.3.8. Commercial products of *in situ* gel systems

Commercial products containing *in situ* gel systems were summarized in Table 2.8.

Table 2.8. *Some examples of marketed formulations (Patil et al., 2014)*

Type of Drug Delivery Formulation	Name of Drug	Marketed
Oral	Betamethasone	Celestone®, Celestone Soluspan®
Nasal	Fluconazole Zinc gluconate, Zinc acetate	Diflucan® Zicam®
Ocular	Ganciclovir Lidocaine Loteprednol etabonate Pilocarpine Timolol	Zirgan® Akten® Lotemax Gel® Pilostat® Carpine® Timoptic®
Rectal and Vaginal	Diazepam Dinoprostin Metronidazole Nonoxynol-9	Diastat® Prostin E® Metrogel Vaginal® Advantage S® Conceptrol® Gynol II®
Parenteral	Oxyquinoline sulphate, Ricinolic acid Progesterone Ganciclovir Doxycycline	Acid jelly® Crinone® Vitrasert® Atridox®

2.3.9. Vagina

2.3.9.1. *Anatomy and physiology of the vagina*

The vagina is a 7 to 10 cm long elastic muscular tube that reaches from the vulva to the cervix of the uterus which finishes at the anterior and posterior fornix (Krantz, 1959). The vaginal opening is located behind the urethral opening, behind the vulvar vestibule. It is surrounded medially by the labia minora and laterally by the labia majora on both sides. A thin, perforated hymen comes as part of the entry to the vaginal opening (Wells, 1959).

Histologically, the vaginal wall composed of three segments: the superficial non-secretory stratified squamous epithelial membrane, the middle muscularis of smooth

muscle fibers flowing in both radial and longitudinal areas, and the tunica adventitia composed of the areolar connective tissue (Platzner et al., 1978).

The surface of the vagina is protected with a film layer. This fluid is secreted from Bartholin and Skene glands. Vaginal fluid consists of electrolytes (calcium, potassium, sodium and chloride), enzymes, various enzyme inhibitors, carbohydrates, proteins, amino acids, alcohols, hydroxyketones, aromatic compounds, lactic acid, acetic acid, glycerin, urea, glycogen and glucose. The composition of this fluid varies according to age, disease state and menstrual cycle (Owen and Katz, 1999; Acartürk, 2007).

Normally, the pH of vaginal fluid is between 4-5. It is known that this acidity is due to lactic acid, which is the degradation product of glycogen (Çalış et al., 1994). After menopause, the pH increases and rises to 7 due to the increase in glycogen content in the cells. During pregnancy, the glycogen content increases. The volume and pH of the vaginal fluid is important as it affects the bioavailability of intravaginally administered local and systemic drugs (Acartürk, 2007).

2.3.9.2. *Vaginitis*

Candida is the most common microorganism in vaginitis, which is an infection of the vaginal mucosa. Microorganisms such as Trichomonas and Hemophilus are also factors of vaginitis (Aşıkoğlu, 1992). These microorganisms, which can be found in normal conditions in this region, can cause infection due to the effects of certain factors such as emotional sensitivity, tissue change, bacterial flora, pH, nutrition and hygienic conditions (Çalış et al., 1994).

Vaginal infection is common and over 80 % of females infected during their lifetime (Deshkar and Palve, 2019). Vaginal candidiasis is a common infection that 75% of women encounter at least one time in their lives. Vaginal candidiasis can be acute, as well as chronic infections. Candida albicans is the most important cause of vaginal candidiasis. It has been reported that many patients with candidal vaginitis respond to the topical treatment of antifungal agents in the form of nystatin or imidazole (Aşıkoğlu, 1992; Wang and Tang, 2008; Demirel et al., 2011).

2.3.9.3. *Applications for vaginal drug delivery*

The vagina is a suitable site for many drugs to exercise its local and systemic effects in females because of the greater surface area, rich blood flow, avoiding the first pass

effect, significantly higher permeability and self-penetration of several medicines. For many years, tablets, creams, injections ointments, and suppositories have been used vaginally (Taksande et al., 2013).

The gel formulations of many drugs are widely used vaginally to exert various effects such as moisturizing and lubricating effects, physiological pH regeneration and contraceptive effect and antimicrobial activity (Tuğcu-Demiröz, 2017). The direct application of the gel at the infected vaginal site may be difficult and uncomfortable. In addition, many doses will be used because the traditional gel does not stay for a long time at the application site (Chopra et al., 2007). In recent years gels for vaginal application with mucoadhesive properties and consisting of a mixture of polycarbophil, Poloxamer® and cellulose derivatives have gained great importance.

A new method has been devised that combines the features of liquid and gel form and thus a precise dose can easily be given vaginally like *in situ* gel system. These formulations stay in their liquid state prior to administration but transform into a gel following administration to the vaginal cavity. The applied liquid in the topical areas becomes gel as a consequence of physical or chemical alterations such as pH for cellulose acetate phthalate, the concentration of Ca^{+2} ions for gellan gum, temperature for poloxamers® (Gupta and Sharma, 2009).

3. MATERIALS

3.1. Chemicals

Substance	Company
Acetic acid	: Sigma-Aldrich, Germany
Agar agar	: Doğa Pharma, Turkey
Benzalkonium chloride	: Fluka, Denmark
Beta-cyclodextrin	: Sigma-Aldrich, Germany
Bovine serum albumin	: Merck, Germany
Calcium hydroxide	: Merck, Germany
Ethanol	: Sigma-Aldrich, Germany
Glucose	: Sigma-Aldrich, Germany
Glycerin	: Doğa Pharma, Turkey
Hydroxy propyl-beta-cyclodextrin	: Sigma-Aldrich, Germany
Hydroxy propyl methylcellulose	: Sigma-Aldrich, Germany
Isoconazole Nitrate (ISN)	: Deva Pharma, Turkey
Lactic Acid	: Doğa Pharma, Turkey
Methanol	: Sigma-Aldrich, Germany
Methyl-beta-cyclodextrin	: Sigma-Aldrich, Germany
Mueller Hinton Broth	: Labm, United Kingdom
Potassium Dihydrogen Phosphate	: J.T.Baker, Netherlands
Potassium hydroxide	: Merck, Germany
Pluronic F127	: Sigma-Aldrich, Germany
Urea	: Merck, Germany
Sodium chloride	: Merck, Germany

3.2. Used Devices

Device	Company
Column	: VDSpher, C18, Germany
Differential Scanning Calorimetry	: Shimadzu DSC-60, Japan
Fourier Transform Infrared Spectroscopy	: Schimadzu, Japan
Horizontal Shaker	: WiseShake SHR-1D, Korea
Lyophilizer	: Leybold-Heraeus Lyovac GT-2, Germany
Magnetic stirrer	: IKA, Germany
Micropipette	: Eppendorf, Germany
Micropipette tip	: Eppendorf, Germany
Nuclear Magnetic Resonance Spectrophotometer (^{1}H-NMR)	: Brucker, USA
pH meter	: Mettler Toledo, USA
Pure Water Device	: Millipore, France
Refrigerator	: Arçelik 5274 NMS No Frost, Turkey
Rheometer	: Brookfield, USA
Scanning Electron Microscope (SEM)	: Zeiss, Supratm 50 VP, Germany
Sensitive balances	: Mettler Toledo, USA
Spray-Drying Device	: Büchi, Nano Spray-Dryer B-90, Switzerland
Thermometer	: Ebro TFX 410, Germany
Ultrasonic bath	: Wisd Laboratory Instruments, South Korea
Vortex	: Jeiotech, South Korea
Water bath	: GFL, Germany

4. METHODS

4.1. The Characterization Analyses of ISN

4.1.1. Morphological studies

The surface properties of ISN were examined by using SEM, which apply electrons transmitted from the surface of the particle.

4.1.2. Thermal analysis

Approximately 4 mg of ISN was placed in an aluminum cell and closed with the help of pressure. The thermal analysis was done by using DSC and the thermograms of the ISN was obtained.

4.1.3. Infrared (FT-IR) analysis

FT-IR is used to study and define the active substance and its functional groups. Spectra was taken between 500-4000 cm^{-1} for FT-IR analysis of ISN. This range (500-4000 cm^{-1}) is called far infrared and can be used for rotational spectroscopy and low frequency vibrations.

4.1.4. Proton nuclear magnetic resonance (^{1}H-NMR) analysis

^{1}H-NMR is a strong instrument to analyze the dynamic phenomenon and properties of the substances. Because of the different chemical changes, it is possible to give ^{1}H-NMR signals to specific molecules or their portions (Al-Heibshy et al., 2019). ^{1}H-NMR spectra of the active ingredient (ISN) was obtained using deuterated dimethyl sulfoxide (dDMSO).

4.1.5. ISN solubility in distilled water

The solubility of ISN in water was determined by adding excess amounts of ISN (~5 mg) to 1 ml of distilled water to form saturated solution. The dispersions were shaken at 25 °C for 24h at horizontal shaker after that the solution was filtered by 0.45 µm nylon filter and then analyzed by HPLC. This analysis was replicated three times (Yurtdaş-Kırımlıoğlu, 2020).

4.1.6. Analytical validation studies

Validation studies in high pressure liquid chromatography (HPLC) have a great importance for subsequent analysis. Validation provides accurate, reliable and reproducible data from analytical studies. Analytical parameters such as linearity, precision, accuracy, selectivity, sensitivity were tested and statistically assessed according to the analytical process validation guidelines of the International Harmonization Committee (ICH) (ICH, 1995).

Table 4.1. *HPLC working conditions*

Device	**Shimadzu, LC 20-AT, Japan**
Column	VDSpher 100 C18-E (250 × 4.6 mm, 5 µm)
Oven Temperature	40 °C
Mobile Phase	Methanol : Potassium dihydrogen phosphate buffer (0.05 M) 50:50 (v/v)
Detector	Diode array detector
Wavelength	210 nm
Flow rate	0.5 $mL.min^{-1}$
Injection Volume	20 µL

4.1.6.1. Linearity

Linearity is the test of whether analytical studies have a linear relationship throughout the study. In the linearity study, if the material to be analyzed in the specified range contains samples of different concentrations, it is expected to be directly proportional to the peak area measured on the chromatogram. Through the peak areas obtained in different concentrations, the standard curve (straight-line equation) can be determined. Thus, it is possible to determine the concentration of any peak area in later studies by using (straight-line equation) equation (ICH, 1995).

For linearity study, dilutions were made from ISN stock solution prepared at 500 $\mu g.mL^{-1}$ concentration and solutions at concentrations within the level of 20-500 $\mu g.mL^{-1}$ in mobile phase were obtained. The solution in each concentration was analyzed by HPLC and the studies were repeated three times.

4.1.6.2. Range

It is the range between the highest and lowest concentration of the material to be analyzed in the sample to show that the analytical method used has linear, accuracy and

precision properties. For the active substance, the concentration range should be between 70% and 130%, but for the final pharmaceutical dosage form the range should be between 80% to 120% of the test concentration.

4.1.6.3. Accuracy

The accuracy of the analytical procedure is the proximity between the actual value or the approved reference value and the value obtained at the end of the analysis. When examined this parameter, the calculation should be repeated at three times for three different concentrations. Samples were prepared in 4 different concentrations (20-50-80-200 µg.mL^{-1}) and analyzed by HPLC and repeated 3 times for each concentration. The outcomes are presented as (recovery %), standard error (SE), standard deviation (SDV) and relative standard deviation (RSD %).

4.1.6.4. Precision

Precision can be verified at three parts: repeatability (intra-day precision), intermediate precision (inter-day precision) and reproducibility (between laboratories precision). Repeatability (intra-day precision) is the accuracy of at least 6 repetitions of the same stock solution or 3 repetitions of 3 different concentrations under the same processing conditions and short time interval. Samples were prepared in 4 different concentrations (20-50-80-200 µg.mL^{-1}), analyzed by HPLC and repeated 3 times for each concentration.

Intermediate precision (inter-day precision) expresses the accuracy of independent analyzes, even on different days, with variables such as other analysts, different materials and tools used. Analyzes were made on different days under the same conditions for repeatability between days.

Reproducibility (between laboratories precision) is not a mandatory parameter for validation. It is an indication of the precision of the two analyzes as a result of repeating the analysis in different laboratories.

4.1.6.5. Selectivity

Selectivity is an important analytical parameter showing that a single substance in the mixture or in the existence of other substances can be accurately determined in the formulation. The determination of any component should not be influenced by

interference from other ingredients (biological compounds, metabolites, impurities, decomposition products) that may be present in the environment (ICH, 1995).

In order to determine the selectivity of the method; ISN mobile phase solution, distilled water, ISN-free β-CD aqueous solution, ISN-free M-β-CD aqueous solution, ISN-free HP-β-CD aqueous solution and methanol 50 % were analyzed by HPLC.

4.1.6.6. Sensitivity

The capability of the analytical method to detect low concentrations is called sensitivity. There are two types of sensitivity, detection and quantification limits. The limit of detection (LOD) is the lowest detectable concentration of analyte in the sample. This value may not express the quantitative value. The limit of quantitation (LOQ) is the minimum quantity of the analyte to be analyzed, which can be detected accurately and quantitatively.

LOD and LOQ are determined by the equations shown below (ICH, 1995).

$$\text{LOD} = \frac{3.3\ x\ SDV}{Slope} \qquad (4.1)$$

$$\text{LOQ} = \frac{10 \text{ x SDV}}{\text{Slope}} \qquad (4.2)$$

4.1.7. Stability in working conditions

4.1.7.1. *Stability under in vitro release environment conditions*

The stability of the active ingredient has been studied in simulated vaginal fluid (SVF), which is the *in vitro* release medium (n=3).

4.2. Formulation of Inclusion Complexes

4.2.1. Phase solubility studies

Phase solubility studies were performed to examine the ratios of both materials (ISN β-CD), (ISN/HP-B-CD) or (ISN/M-β-CD) in order to form their inclusion compounds.

4.2.1.1. *Determination of equilibrium time for solubility phase diagram of ISN/β-CD, ISN/M-β-CD and ISN/HP-β-CD*

Aqueous solutions of β-CD, M-β-CD and HP-β-CD were prepared with concentration of $20x10^{-3}$ M. Excessive quantity of ISN was added to a mixture of the prepared CDs solution and ethanol in a ratio (3:1) respectively. In order to determine the balance time, the samples taken on the 1st, 2nd and 3rd days then the samples were filtered by using a nylon (0.45 µm) filter and analyzed by HPLC. Then ISN quantities were calculated by using the calibration equation determined for ISN. The experiment was repeated 3 times.

4.2.1.2. *Determine the phase solubility diagram of ISN/β-CD, ISN/M-β-CD and ISN/HP-β-CD*

Aqueous solutions of β-CD, M-β-CD and HP-β-CD were prepared with concentration range of $2\text{-}20x10^{-3}$ M ($2x10^{-3}$ M, $4x10^{-3}$ M, $6x10^{-3}$ M, $8x10^{-3}$ M, $10x10^{-3}$ M and $20x10^{-3}$ M). Excessive quantity of ISN (20 mg) was added to a mixture of the prepared CD solution and ethanol in a ratio (3:1) respectively. It was shaken to generate supersaturated solutions using horizontal shaker at 25 °C for a predetermined equilibrium period (1 day). The samples taken on the 1st day then the samples were filtered using nylon (0.45 µm) filter and analyzed by HPLC. The test was replicated three times and the resulting data was used to establish the phase solubility diagram.

A chart was drawn according to the obtained values and a diagram type was determined as regards to the phase solubility diagram of Higuchi (Higuchi and Connors, 1965).

4.2.2. Preparation of ISN/β-CD, ISN/M-β-CD and ISN/HP-β-CD inclusion complexes

Spray-drying method and lyophilization methods were used to formulate the inclusion complexes. The ISN/CD (1:1) molar ratio that obtained from the ISN/β-CD, ISN/M-β-CD and ISN/HP-β-CD study of the phase solubility, was used in the preparation of ISN /CDs inclusion complexes.

4.2.2.1. *Spray-drying method*

An accurate amount of ISN and CD derivatives for 1:1 molar ratio were dissolved in methanol and pure water, respectively. After mixing the two prepared clear solutions by using magnetic stirring, they were dried in a spray-dryer with a 0.7 mm diameter spray head using a feed temperature of 100 °C and an output temperature of 60 °C and a feed rate of 7 $mL.min^{-1}$. At the end of drying, fine powders of complexes were achieved in the collection container (Yurtdaş et al., 2011).

4.2.2.2. *Lyophilization method*

The solutions of ISN and CDs for 1:1 molar ratio were dissolved in methanol and pure water, respectively. After mixing the two prepared clear solutions by using magnetic stirring, the final solutions of ISN/CDs complex were freezed at -86 °C and then lyophilized. The final powders were collected and stored at room temperature.

4.3. Characterization of the inclusion complexes

4.3.1. Morphological studies of inclusion complexes

The surface properties of ISN/CDs complexes prepared by different methods were examined by SEM.

4.3.2. Thermal analysis of inclusion complexes

Approximately 4 mg of ISN/CDs inclusion complexes were placed in an aluminum cell and closed with the help of pressure. The thermal analysis was done by using DSC at a temperature range of 50-400 ºC with a temperature increase of 10º$C.min^{-1}$. The thermograms of the ISN/CDs inclusion complexes were obtained.

4.3.3. FT-IR analysis of inclusion complexes

Spectra was taken between 500-4000 cm^{-1} for FT-IR analysis of ISN/CDs inclusion complexes.

4.3.4. ^{1}H-NMR analysis of inclusion complexes

^{1}H-NMR spectra of CDs inclusion complexes were acquired by dissolving in DMSO.

4.3.5. Determination of aqueous solubility in distilled water of inclusion complexes

The solubility of ISN/CDs inclusion complexes in distilled water were calculated by adding excess quantities of ISN/CDs inclusion complexes to 1 ml of distilled water to form saturated solution. The dispersions were mixed at 25 °C for 24h at horizontal shaker after that the solution was filtered by 0.45 µm nylon filter and then analyzed by HPLC. This test was replicated three times.

4.3.6. Determination of drug content (DC) %

The amount of drug ISN (DC %) loaded into the prepared inclusion CDs complexes was determined by taking 1 mg of the inclusion complexes and dissolved it in 1 mL of methanol 50% and the final solution was diluted to the appropriate concentration with the methanol 50% and analyzed under HPLC.

4.4. Formulation of *In Situ* Gels

In this study *in situ* gel formulations were formulated with Pluronic® F127 as temperature sensitive *in situ* gelling polymer, HPMC as mucoadhesive agent, benzalkonium chloride as a preservative agent and distilled water as a solvent.

4.4.1. Formulation development studies

4.4.1.1. *Preparation of placebo (active substance-free) formulations*

The development study for the formulations was started by creating polymer solutions with different ratios within the lower and upper limits determined by the information obtained from the relevant sources. Placebo formulations were prepared without addiction of active agent. Benzalkonium chloride was selected as preservative agent and was used at a concentration of 0.01% (w/w) (Ibrahim et al., 2012). HPMC was used at a concentration of 0.5% (w/w) as a mucoadhesive agent (Rençber et al., 2017).

There are two different methods in the literature for preparing *in situ* gel formulation: hot and cold method. In temperature sensitive gel systems long-term mixing to dissolve the polymers in water is required. The cold method is one of the preferred techniques of preparing a polymer solution because a clear solution is obtained in this method and it is more appropriate to use because in the hot method, lumpy solution will be resulted (Borole et al., 2013).

The formulations were prepared by using Pluronic® F127 and HPMC was added to some formulations to study its impact on the physicochemical properties of formulations. Pluronic® F127, HPMC and benzalkonium chloride were weighed in the specified proportions and placed in a beaker and sufficient amount of water added to beaker. Magnetic stirring was continued at ~ 4 ° C for 24 hours at 500 rpm with magnetic stirring bar of 2 mm diameter. Ice molds were renewed every 4 hours in order to keep the temperature constant. The prepared formulations rates and codes are shown in Table 4.2.

Table 4.2. *Placebo formulations components and codes*

Formulation Code	Pluronic® F127	HPMC	Benzalkonium chloride	Distilled water
P12.75	12.75	-	0.01	To 100 g
P12.75H05	12.75	0.5	0.01	To 100 g
P13	13	-	0.01	To 100 g
P13H05	13	0.5	0.01	To 100 g
P13.25	13.25	-	0.01	To 100 g
P13.25H05	13.25	0.5	0.01	To 100 g
P13.5	13.5	-	0.01	To 100 g
P13.5H05	13.5	0.5	0.01	To 100 g
P13.75	13.75	-	0.01	To 100 g
P13.75H05	13.75	0.5	0.01	To 100 g
P14	14	-	0.01	To 100 g
P14H05	14	0.5	0.01	To 100 g
P15	15	-	0.01	To 100 g
P15H05	15	0.5	0.01	To 100 g
P16	16	-	0.01	To 100 g
P16H05	16	0.5	0.01	To 100 g
P17	17	-	0.01	To 100 g
P17H05	17	0.5	0.01	To 100 g
P18	18	-	0.01	To 100 g
P18H05	18	0.5	0.01	To 100 g

4.4.1.2. *Gelation temperature of placebo formulations*

The ideal temperature for temperature sensitive *in situ* gel systems to be applied vaginally is 37 °C. The first parameter to be considered in the selection of the formulation to be prepared is the gelation temperature. For this reason, all formulations prepared were

tested with the "Test Tube Tilting Method" from room temperature 25 °C to 45 °C. When measuring the temperature of formulations, the thermometer probe was held in the middle of the solution, in the region where the temperature reached the ambient temperature at the latest. The studies were repeated three times.

4.4.1.3. *pH measurements of placebo formulations*

The pH measurements of placebo formulations prepared were done by using pH meter at 25 °C.

4.4.2. Preparation of the formulations containing ISN or ISN/CDs complexes

Placebo formulations which have appropriate gelation temperature, pH value and the lowest polymer concentration were selected. In comparison with branding products which containing isoconazole nitrate, 100 mg of the active ingredient or its equivalent from CD complexes was added to the selected formulations. The formulations containing the active ingredient were prepared in the same way as preparing the placebo formulations and the active substance was added to the formulation after getting a clear solution (after the complete dissolution of the polymer).

4.4.2.1. *Gelation temperature of the formulations containing ISN or ISN/CDs* complexes

"Test Tube Tilting Method" was repeated and the formulation containing active ingredient were tested for determination the gelation temperature. The gelation temperature of the formulations with the active substance prepared with the optimum ratio found was determined by repeating 3 times.

4.4.2.2. *pH measurement of the formulations containing ISN or ISN/CDs* complexes

The pH measurements of the formulations containing the active substance prepared were performed by pH meter.

4.4.3. Selection of the optimum *in situ* gel formulations

Following the formulation development study, seven formulations that would be most suitable for vaginal use were selected by evaluating the gelation temperature and

pH values. The optimum formulation became a gel at ~ 37 ° C, has the lowest polymer concentration and has the pH value closest to the range between 3.8 to 4.5, which is the pH of the vagina.

4.5. Characterization of the *in situ* gel formulations

As a result of the formulation development study, gelation temperature and pH measurement of formulations were performed. In addition these characterization analyses, gelling capacity, swelling, rheological behavior and drug content (%) were carried out on the selected *in situ* gel formulations.

4.5.1. Gelling capacity

The gelling capacity was detected by taking a drop from the formulation into vial containing 2 mL of the SVF and kept at 37 °C, visually considering the gelation time and the gel dissolution time. Gelation capacity was evaluated by using the codes shown in Table 4.3 (Makwana et al., 2016).

Table 4.3. *Gelling capacity codes*

Observation	Coding
No gelation	-
Gelation occurred in few minutes and lasted for few hour	+
Gelation immediate and lasted for few hour	++
Gelation quickly and for longer time	+++
Very strong gel	++++

4.5.2. Swelling test

For swelling test, 1 mL of formulation was put into dialysis membrane and closed with dialysis membrane sealer. The gel was weighed before being placed in SVF medium and kept at 37 °C. The swelling ratio was calculated using equation 4.3.

$$\text{Swelling percentage } \% = \frac{Gel\ weighting\ t}{Gel\ weighting\ t0} \times 100 \qquad (4.3)$$

Which is t_0 zero time and t is the taken sample time

4.5.3. Rheological studies

The rheological behavior of the formulations was examined at two different temperatures 25 °C and 37 °C. The shear stress and viscosity change against the shear rate were recorded using rheometer at two temperatures.

4.5.4. Determination of drug content (DC) %

The amount of drug ISN (DC%) in the prepared formulations was calculated by taking 100 mg of the formula and dissolved in 1 mL of methanol 50% and the final solution was diluted to the appropriate concentration with the methanol 50% and analyzed with validated HPLC method.

4.6. *In Vitro* Release Studies of *In Situ* Gel Formulations

An accurate quantity of the *in situ* gel formulations were put into dialysis bag included magnet. The dialysis bag was put in a beaker contained 50 mL SVF. SVF was prepared using the materials mentioned in Table 4.4. The experiment was performed at 37 ± 0.5 °C with a magnetic stirrer at a stirring speed of 100 rpm. 1 mL of samples were collected at fixed time intervals (0.25, 0.5, 1, 2, 3, 4, 6, 9, 12, 24, 48, 72, 96 hours). In order to preserve sink conditions, required volume of SVF was applied to the receptor medium. The samples were analyzed using the validated HPLC process.

Table 4.4. Simulated vaginal fluid (SVF) components

Component	Amount
Sodium chloride	3.510 g
Potassium chloride	1.400 g
Calcium hydroxide	0.222 g
Bovine serum albumin	0.018 g
Lactic acid	2.000 g
Acetic acid	1.000 g
Glucose	5.000 g
Urea	0.400 g
Glycerin	0.160 g
Distilled water	To 1000 mL

4.6.1. Determination of release kinetics

Release kinetics were examined using the software DD Solver program to assess the drug release mechanism from *in situ* gel systems.

4.7. Stability Studies

For the stability study, the *in situ* gel formulations including ISN and ISN/CDs complexes were tested for 90 days at 4 °C and 25 °C. The physical properties were determined at certain intervals (30th, 60th and 90th days).

4.7.1. Gelation temperature

The gelation temperature of the formulations stored at 4 °C and 25°C was determined at 30th, 60th and 90th days.

4.7.2. Gelling capacity

For the formulations stored at 4 °C and 25 °C, the gelling capacity study was repeated on 90th day.

4.7.3. pH measurement

The pH measurements of the formulations kept at 4 °C and 25 °C were performed at 30th, 60th, and 90th days.

4.7.4. Rheological studies

The rheological pattern of the formulations kept at 4 °C and 25 °C was measured at 30, 60, and 90 days at two distinct temperatures 25 °C and 37 °C.

4.7.5. Determination of drug content (DC) %

Determination of the drug content of the formulations kept at 4 °C and 25 °C was carried out at 30, 60, and 90 days by the validated HPLC method.

4.8. Antimicrobial Efficacy Testing of *In Situ* Gel Formulations

4.8.1. Microorganisms

In the current work, the following organisms were used: *Candida albicans* (*C. albicans*) (ATCC 90028), *Candida glabrata (C. glabrata)* (ATCC 90030) and *Candida krusei* (*C. krusei)* (ATCC 6258) were grown in Saboraud Dextrose Broth (Merck).

4.8.2. Inoculum

The standardization of the yeast cell number used for susceptibility testing has vital significance to obtain reliable and reproducible performance. The suggested final inoculum size for agar diffusion test is $0.5x10^5$ cells/ml. For this cause, all inoculums have been set to 0.5 McFarland norm with McFarland Tube Densitometer for reliable and reproducible performance.

4.8.3. Agar diffusion test

The agar diffusion analysis is a significant tool for evaluating microbial susceptibility to antibiotics or molecules whose potential results are curious, and has been introduced globally over the last 60 years. In order to perform the tests, 20/25 mL of Saboraud Dextrose Agar medium was added to each of the 90mm diameter petri dishes at aseptic conditions and allowed to solidify. Then, the agar surfaces were inoculated by 100 µL yeast suspension (regulated spectrophotometrically at 530 nm to the turbidity of a 0.5 McFarland norm) and dispensed using a sterile, disposable and nontoxic dispenser. After excess humidity was absorbed into the agar and the surface was totally dry (10 minutes at 25 °C), 6 mm holes were punched with a Cork Borer. Each hole was filled with 35 µL substance to each inoculated plate. Samples were applied at an ISN concentration of 3 µg/mL. All plates have been incubated at 37 °C for 24 hours.

5. RESULTS

5.1. The Characterization Tests of ISN

5.1.1. Morphology

The morphological structure of ISN were evaluated by SEM and SEM microphotograph was illustrated in the Figure 5.1.

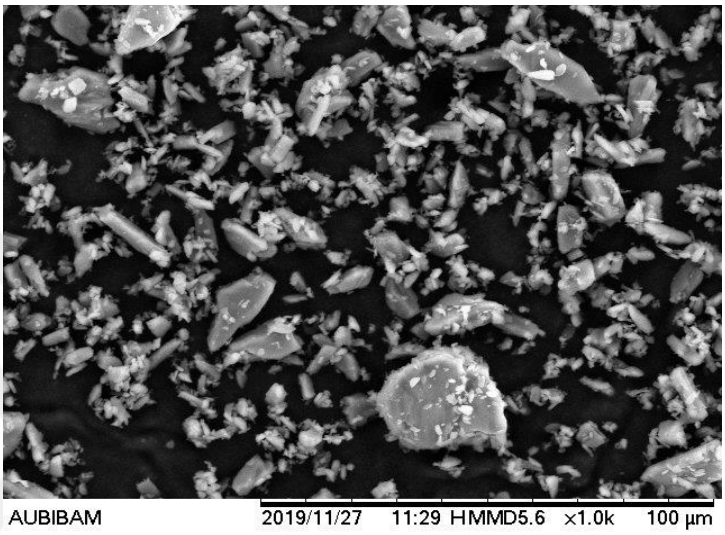


Figure 5.1. *SEM image of ISN*

5.1.2. Thermal analysis

ISN was analyzed by using DSC between 50-300 °C and melting point was found as 187.50 °C. The thermogram of ISN was presented in Figure 5.2.

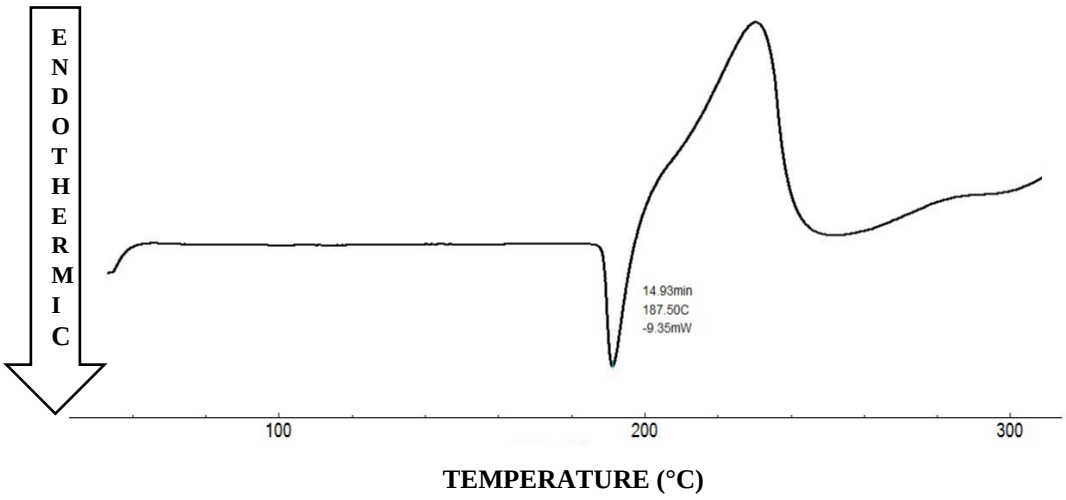


Figure 5.2. *DSC thermogram of ISN*

5.1.3. Infrared (FT-IR) analysis

FT-IR analysis for ISN was carried out by FT-IR between 500-4000 cm^{-1}. FT-IR spectrum was presented in Figure 5.3.

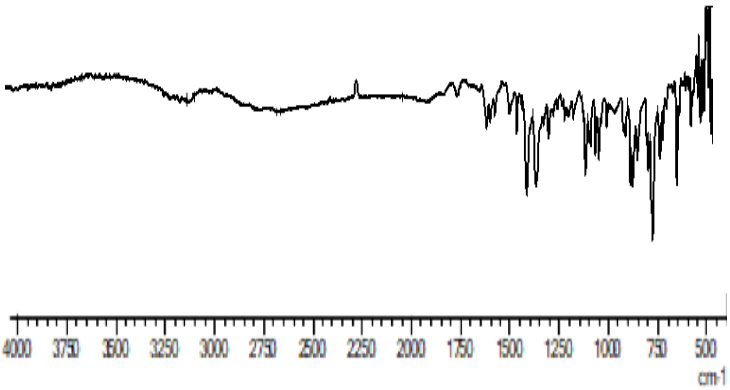


Figure 5.3. *FT-IR spectrum of ISN*

5.1.4. ^{1}H-NMR analysis of ISN

The ^{1}H-NMR spectrum of ISN was demonstrated in Figure 5.4.

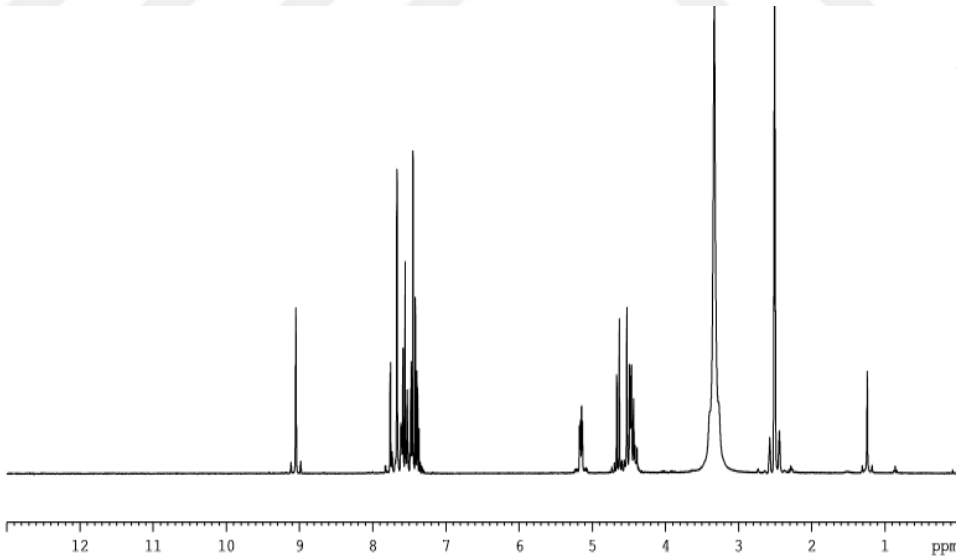


Figure 5.4. *^{1}H-NMR spectrum of ISN*

5.1.5. Aqueous solubility of ISN

The aqueous solubility of pure ISN was calculated as 0.5088 ± 0.0062 $\mu g.mL^{-1}$ at 25 °C.

5.1.6. Analytical validation studies

5.1.6.1. *Linearity*

The ISN solutions were prepared in the mobile phase and analyzed by HPLC. ISN concentrations within the level of 20-500 µg.mL^{-1} were used in the study. The line graph was drawn using the Area/R_t against the concentrations (Figure 5.5). Linearity was examined using the least squared regression equation. The AUC values obtained by HPLC analysis for standard ISN concentrations were presented in Table 5.1. The curve and linearity equation were illustrated in Figure 5.5.

Table 5.1. *AUC values obtained by standard ISN HPLC analysis*

			Curve (AUC)	
Concentrations µg. mL^{-1}	**1.Set**	**2. Set**	**3. Set**	**Mean ± SE**
20	205380.265	219117.641	213934.949	212810.954 ± 4005.265
30	317030.714	330989.257	322306.797	323442.256 ± 4069.283
40	407466.807	413940.436	435388.409	418931.884 ± 8437.810
50	528645.752	520180.897	527636.699	525487.783 ± 2669.384
60	631536.079	617056.169	628925.332	625839.194 ± 4455.713
70	727477.437	753526.865	751039.246	744014.516 ± 8299.665
80	842521.452	858832.256	857010.201	852787.970 ± 5160.136
90	938723.040	970264.842	980321.672	963103.185 ± 12531.009
100	1059635.629	1150381.672	1163990.985	1124669.429 ± 32753.370
200	2224295.758	2294645.081	2312256.604	2277065.814 ± 26870.371
300	3368158.234	3441876.768	3513875.595	3441303.532 ± 42065.956
400	4709068.4974	4694695.914	4720129.713	4707964.708 ± 7362.819
500	5710705.9372	5853118.379	5918280.515	5827368.277 ± 61289.211

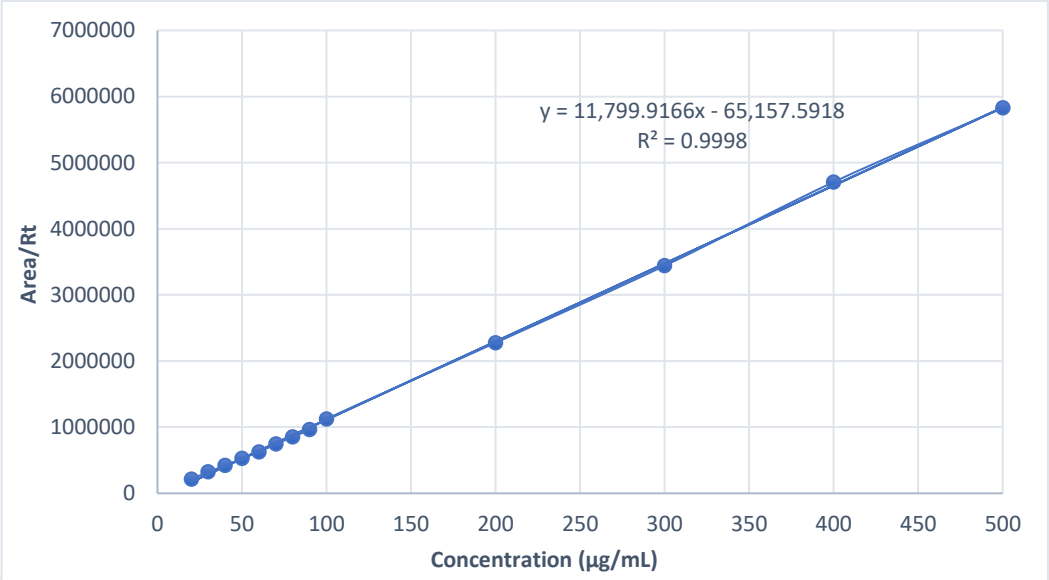


Figure 5.5. *Calibration curve and linearity equation of standard ISN*

5.1.6.2. *Range*

The extent of the analytical method was studied in this limitation of concentration (5-500 µg.mL^{-1}. The calibration curve was selected at the concentration range (20-500 µg.mL^{-1}).

5.1.6.3. *Accuracy*

Samples were prepared in 4 different concentrations (20-50-80-200 µg.mL^{-1}) and analyzed by HPLC and repeated 3 times for each concentration. Acceptance level of recovery percent for accuracy is ± 2%. The achieved outcomes refer to that the analytical approach is appropriate for recovery and accuracy. The findings were shown in Table 5.2.

Table 5.2. *Recovery % of standard ISN analysis by HPLC*

Concentration µg. mL^{-1}	**20**	**50**	**80**	**200**
Difference %	1.1908	1.1339	0.3518	0.7585
Recovery %	101.1908	101.1339	99.6482	99.2415
SDV	1.3828	0.6557	0.9740	1.5682
SE	0.7984	0.3786	0.5623	0.9054
RSD %	1.3665	0.6484	0.9775	1.5801
Confidence Intervals (95%)	3.4351	1.6289	2.4196	3.8955

SDV: Standard Deviation; SE: Standard error

5.1.6.4. *Precision*

The repeatability measurement (intra-day) and intermediate precision (interday) were performed to confirm the precision of the analysis method. Standard ISN solutions at concentrations of 20, 50 and 80 μg.mL^{-1} were tested on three days, three times in a day. The determined RSD value is less than 2%. This denote that the analytical procedure is highly precise and analytically agreeable. The statistical estimate was demonstrated in Table 5.3-5.5.

Table 5.3. *Precision study results for standard ISN 20 µg.mL^{-1}*

	Intra-day (n = 3)			**Inter-day**
	1.DAY	**2.DAY**	**3.DAY**	
Average	21.2021	20.7319	20.7938	20.9093
SDV	0.3352	0.2399	0.2751	0.3322
SE	1.5811	1.1571	1.3230	1.5887
RSD	0.4162	0.2979	0.3416	0.1652

SDV: Standard Deviation; SE: Standard error, RSD: Relative standard deviation

Table 5.4. *Precision study results for standard ISN 50 µg.mL^{-1}*

	Intra-day (n = 3)			**Inter-day**
	1.DAY	**2.DAY**	**3.DAY**	
Average	49.9089	51.5447	50.9321	50.7952
SDV	0.2381	0.4397	0.6807	0.3408
SE	0.4772	0.8530	1.3364	0.6710
RSD	0.2957	0.5460	0.8452	0.1695

SDV: Standard Deviation; SE: Standard error, RSD: Relative standard deviation

Table 5.5. *Precision study results for standard ISN 80 µg.mL^{-1}*

	Intra-day (n = 3)			**Inter-day**
	1.DAY	**2.DAY**	**3.DAY**	
Average	80.1316	79.7385	80.3602	80.0768
SDV	0.3516	0.8198	0.5508	0.5907
SE	0.4388	1.0281	0.6855	0.7377
RSD	0.4366	1.0179	0.6840	0.2938

SDV: Standard Deviation; SE: Standard error, RSD: Relative standard deviation

5.1.6.5. *Sensitivity*

To determine the ability of the analytical method used to detect small changes in concentration and low concentrations, LOD and LOQ values were determined using the equations (4.1 and 4.2) described in section (4.1.7). LOD and LOQ was found as 1.3047 $\mu g.mL^{-1}$ and 3.9536 $\mu g.mL^{-1}$, respectively. These results indicate that the method is sensitive.

5.1.6.6. *Selectivity*

Selectivity studies were conducted to show that any peak originating from its components does not interfere with the active substance peak. In these studies, the chromatograms of the mobile phase (MP), distilled water (DS), dilution solution (mixture of DS with methanol 50:50), SVF, CD solutions, ISN/CD inclusion complexes and placebo *in situ* gel formulations (P12.75H05, P13.5H05, P14, P14H05 and P15) were analyzed by HPLC. Chromatograms showing the selectivity of the analytical method are given in Figure 5.6. – Figure 5.13.

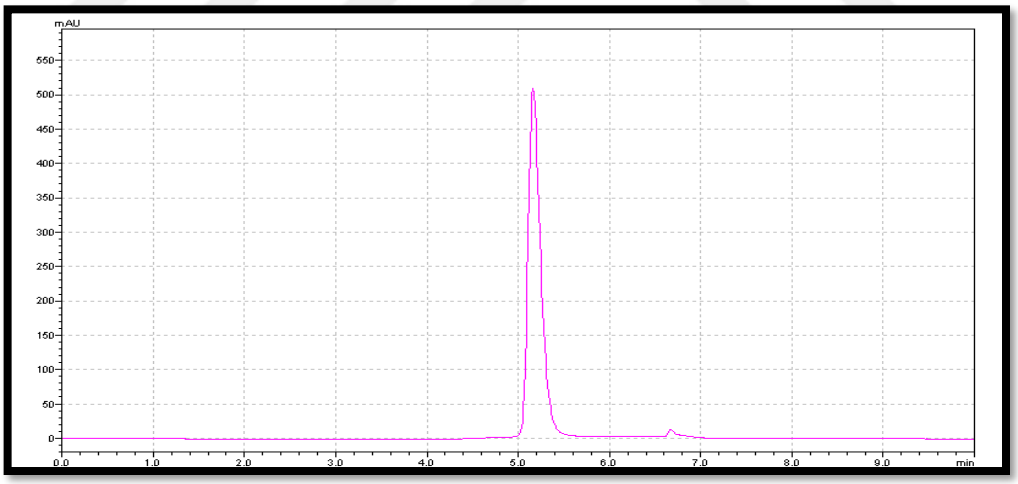
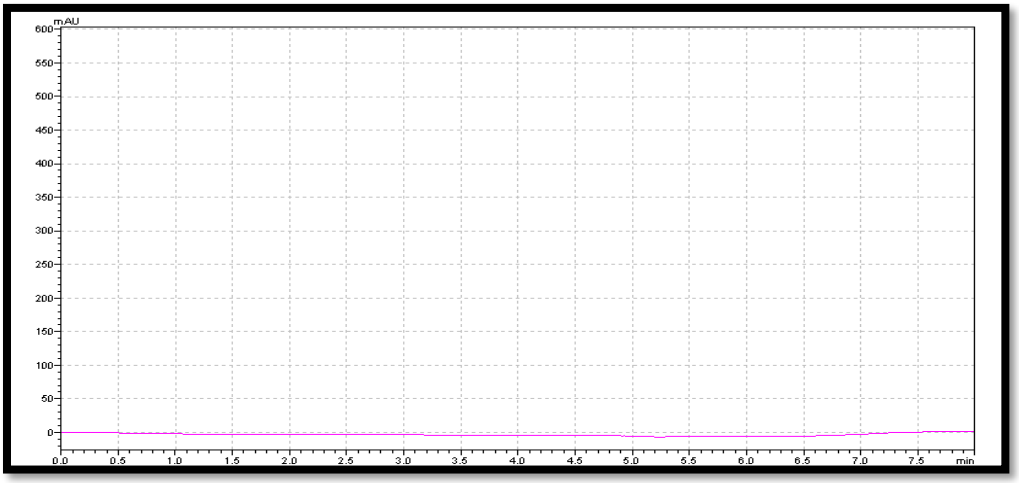
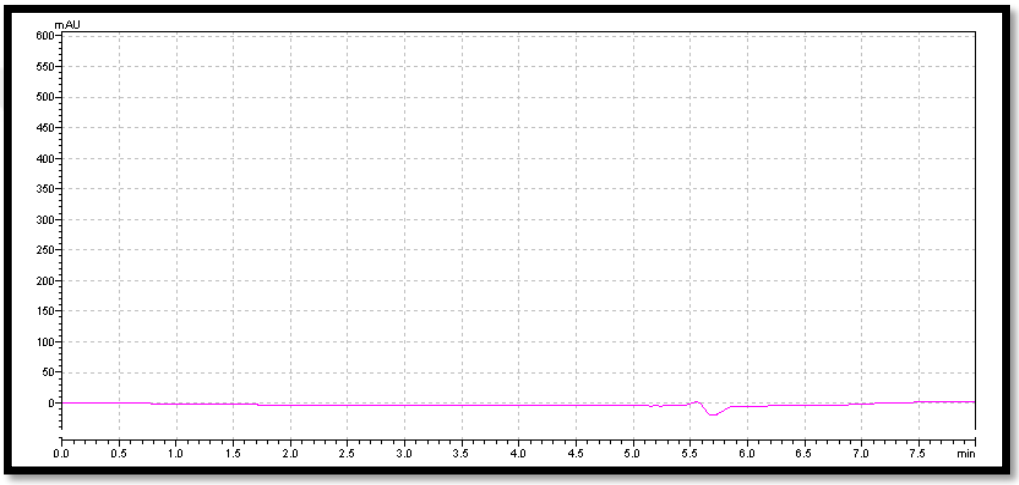


Figure 5.6. *Chromatogram of ISN*

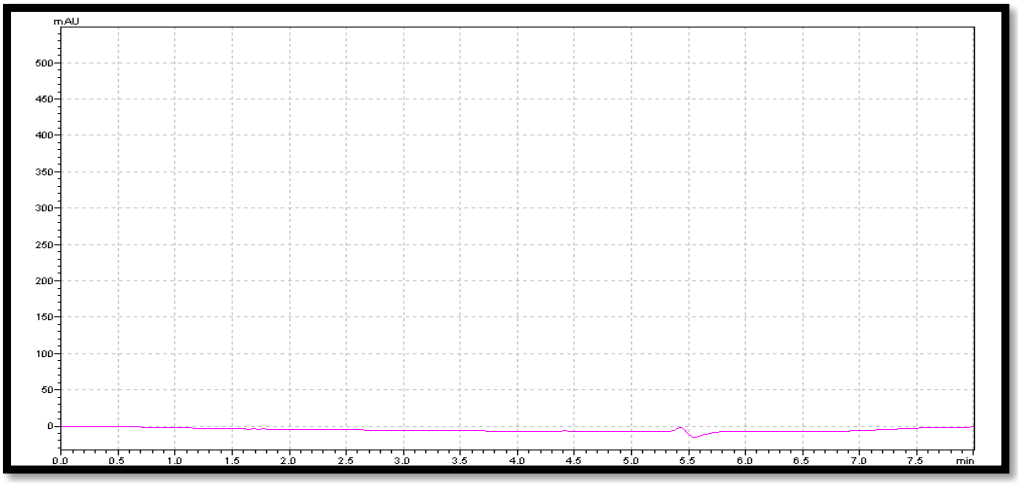


A

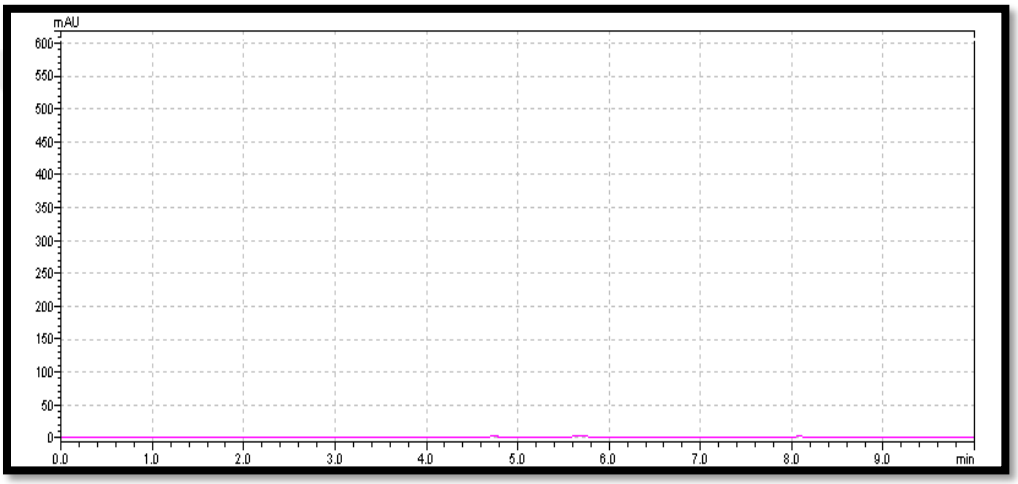


B

Figure 5.7. *Chromatogram of A: Mobil phase (MP) and B: Distilled water (DS)*

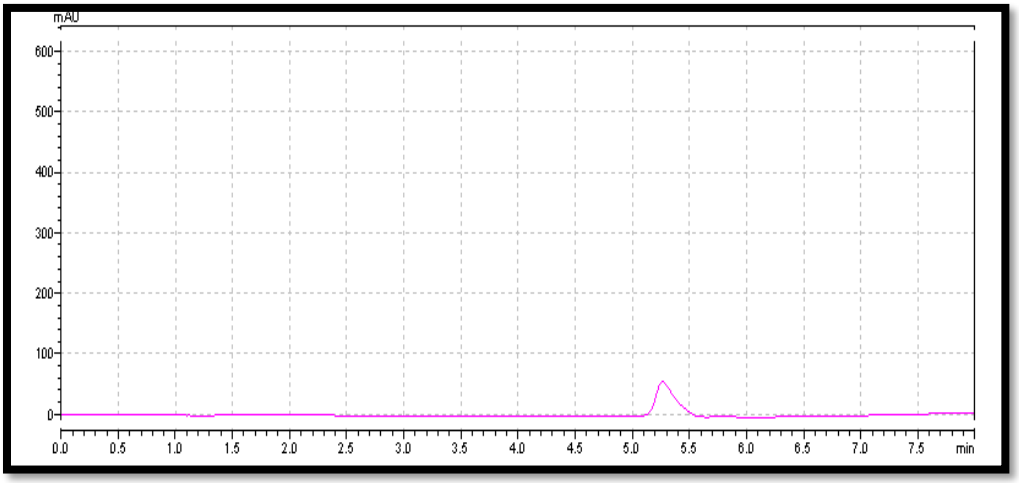


C

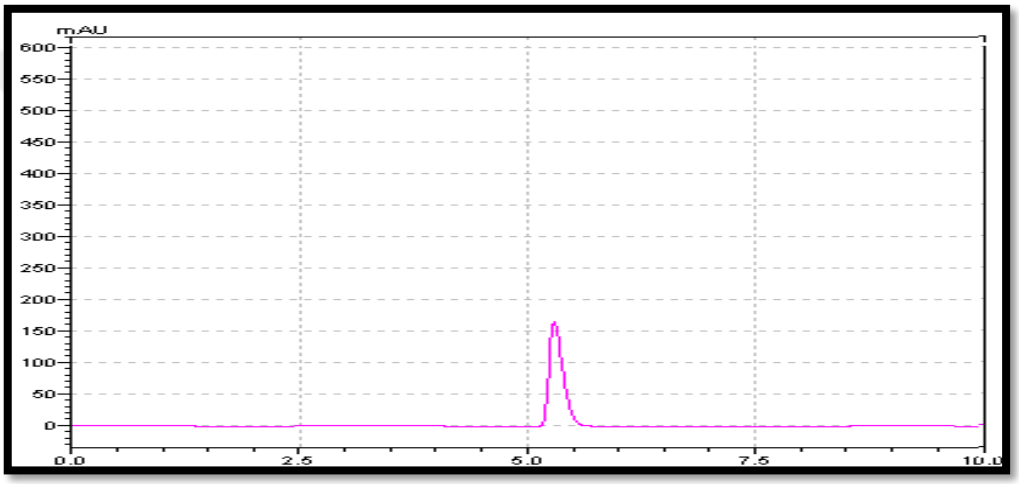


D

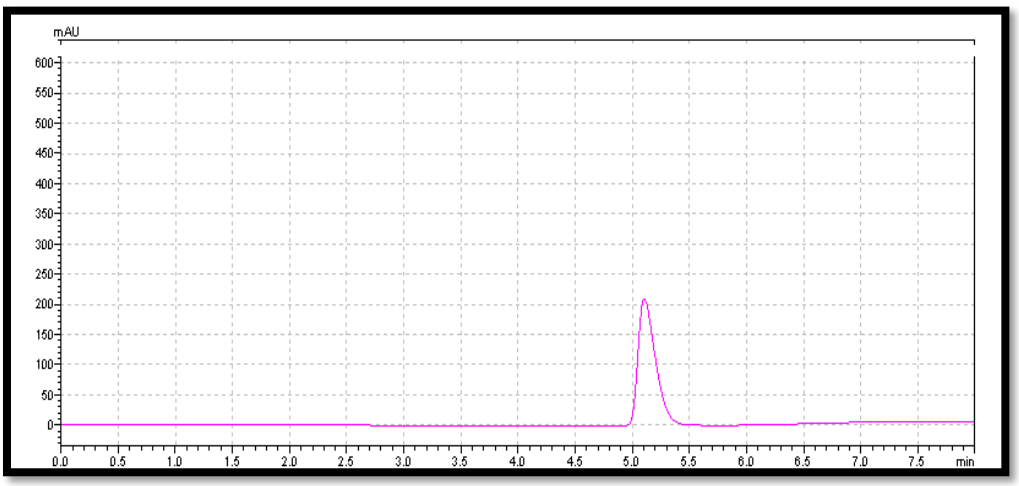
Figure 5.8. *Chromatogram of C: Dilution solution (50% methanol), D: SVF*



A

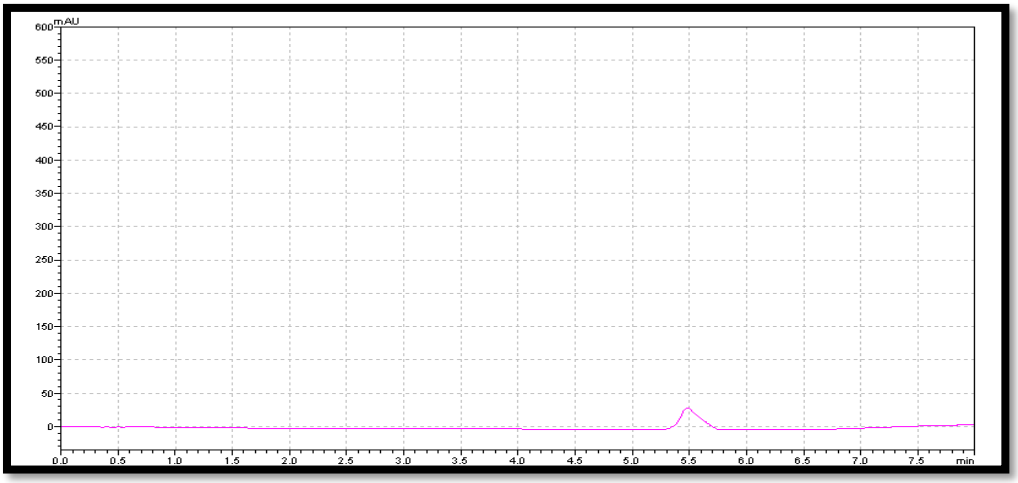


B

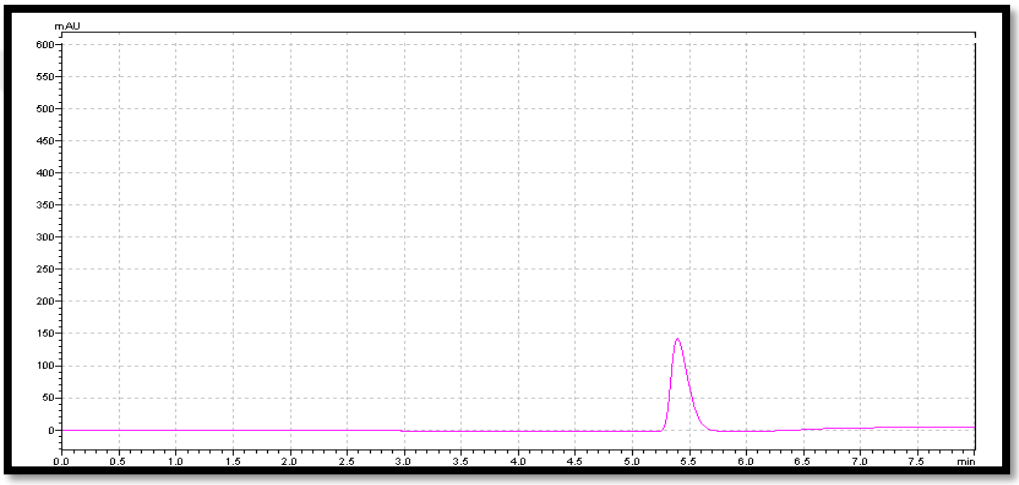


C

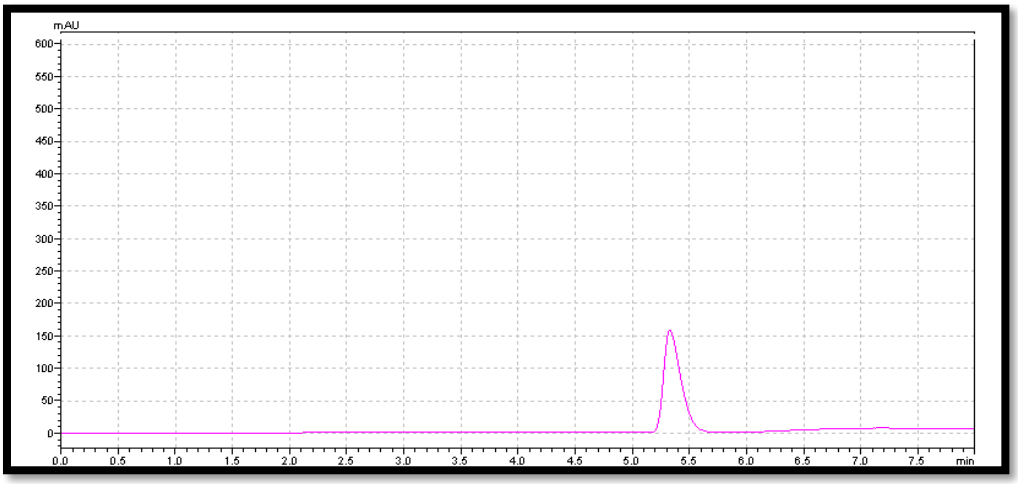
Figure 5.9. *Chromatograms of A: β-CD Solution, B: ISN/ β-CD complex prepared by Freeze-Drying (FD) and C: ISN/ β-CD complex prepared by Spray-Drying (SD)*



A

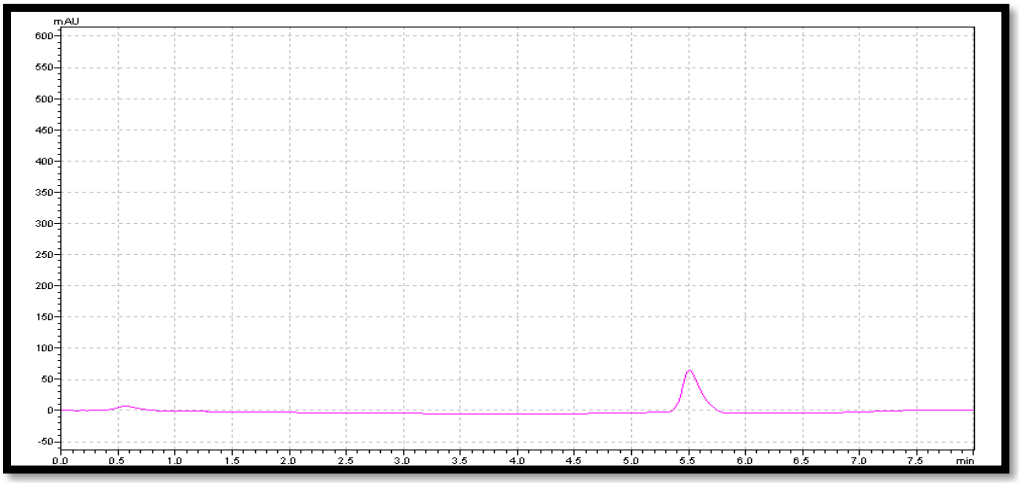


B

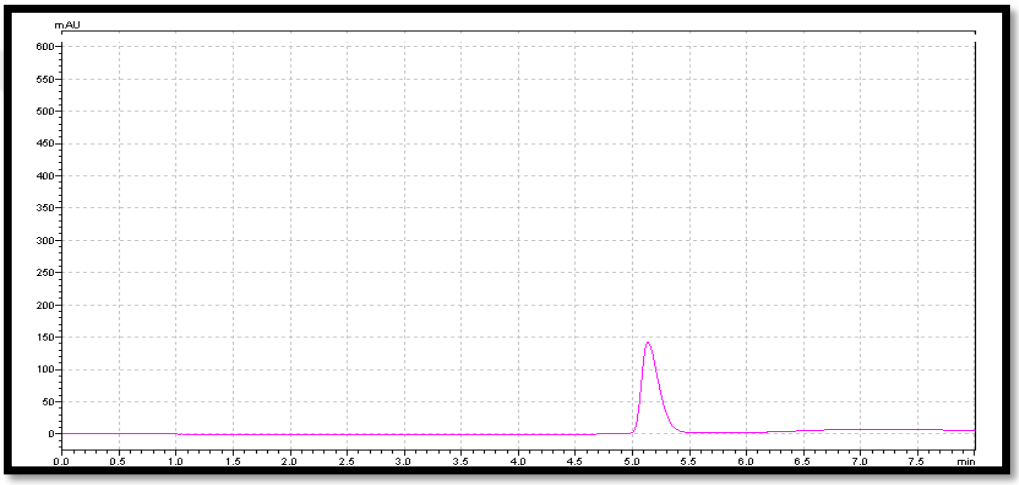


C

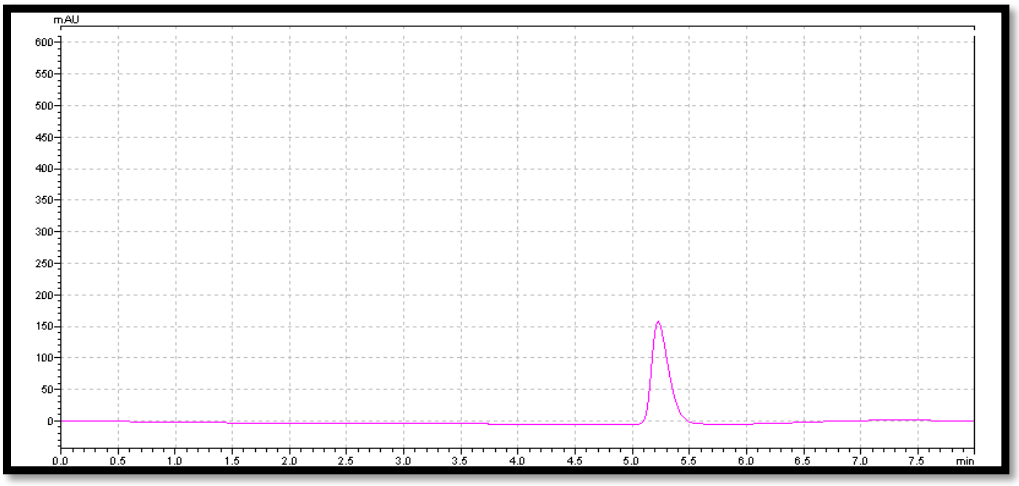
Figure 5.10. *Chromatograms of A:M-β-CD Solution, B: ISN/M-β-CD complex prepared by freeze-drying (FD) and C: ISN/M-β-CD complex prepared by spray-drying (SD).*



A

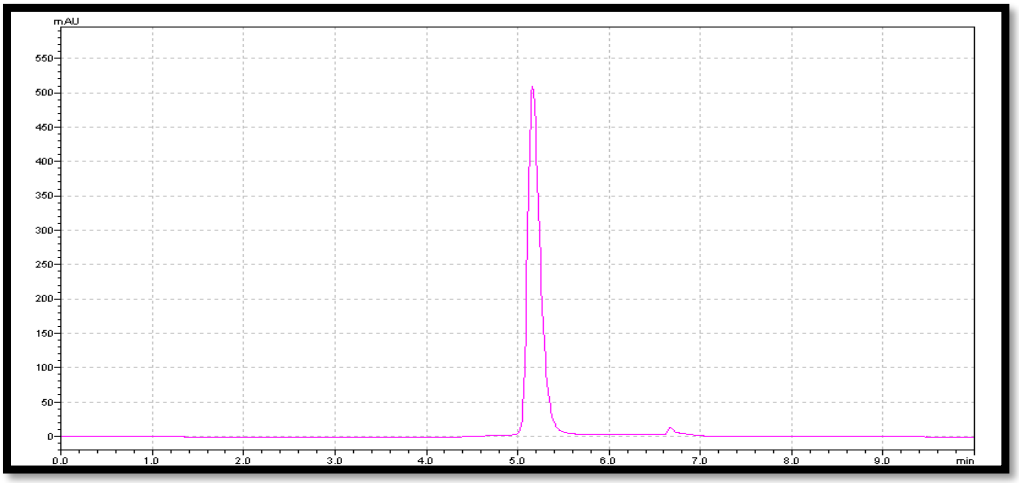


B

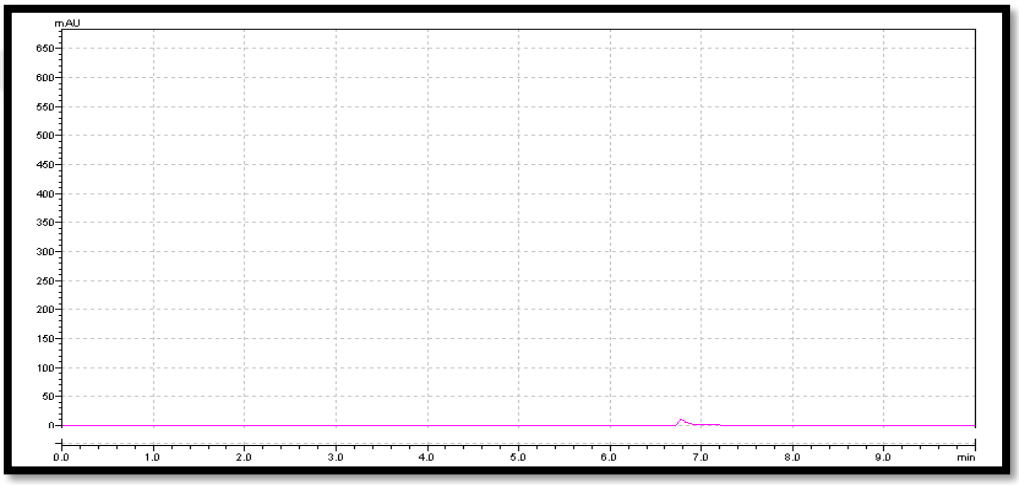


C

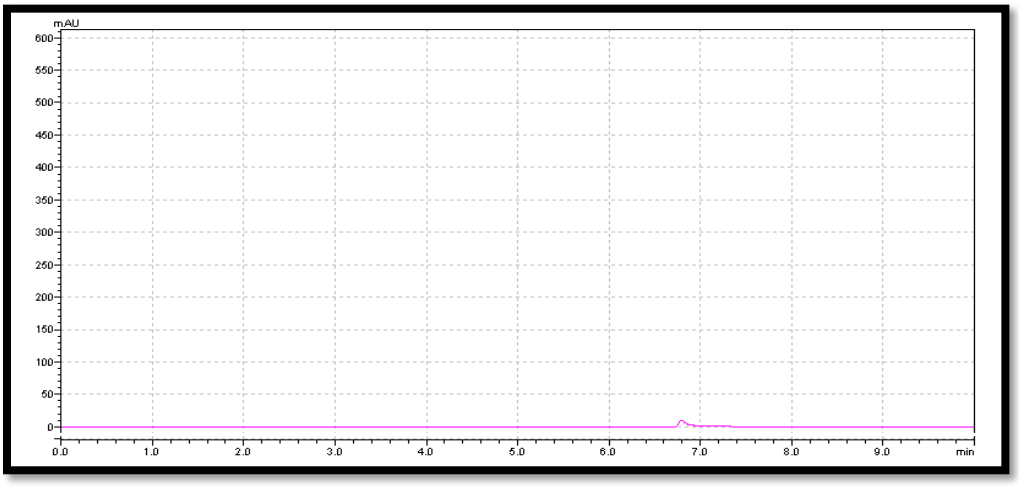
Figure 5.11. *Chromatograms of A: HP-β-CD Solution, B: ISN/HP-β-CD complex prepared by freeze-drying (FD) and C: ISN/HP-β-CD complex prepared by spray-drying (SD)*



A

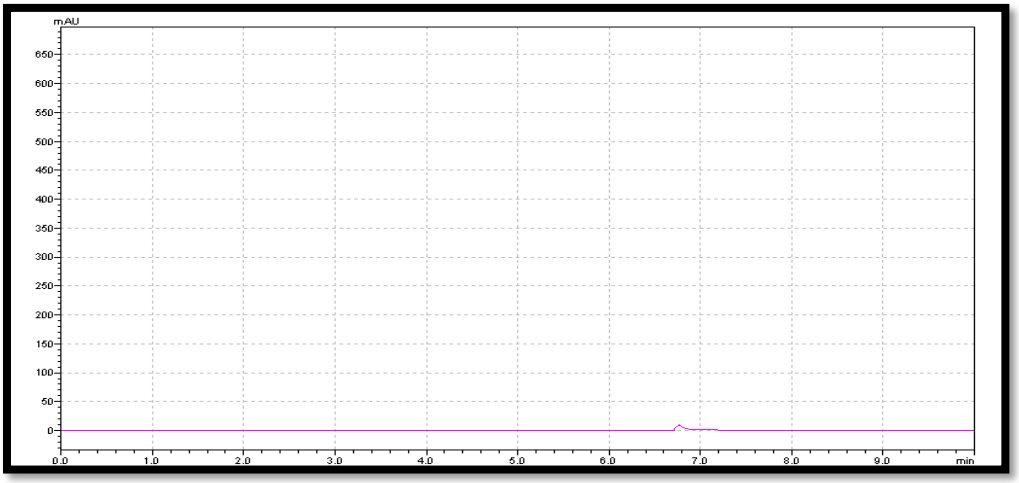


B

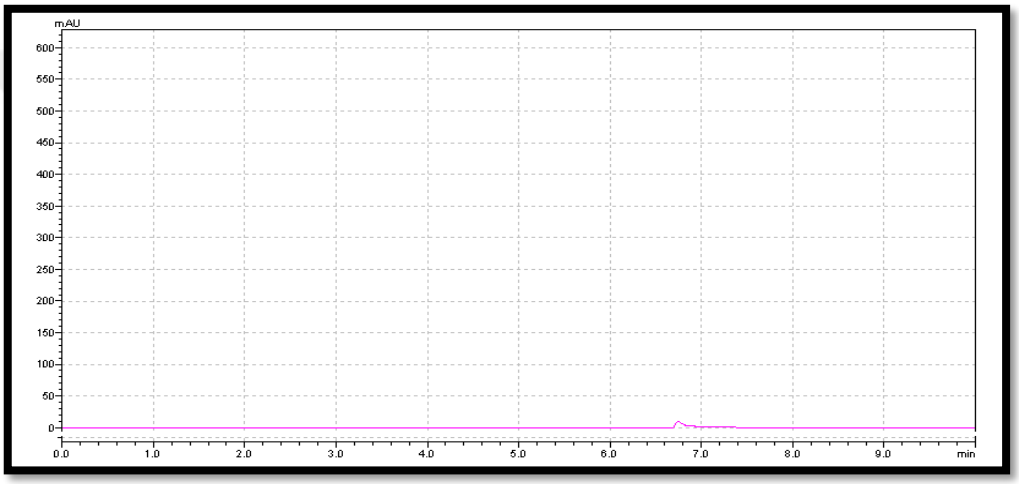


C

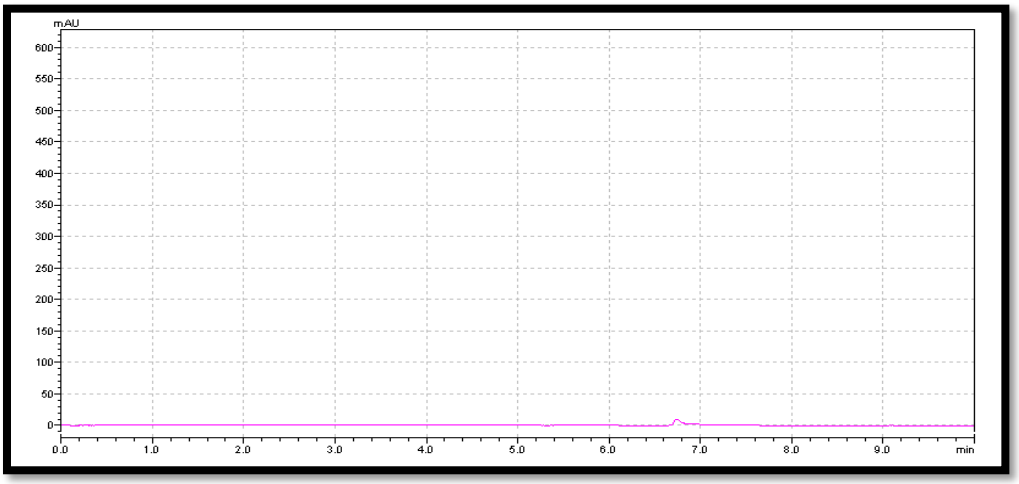
Figure 5.12. *Chromatograms of A: ISN, B: P12.75H05 and C: P13.5H05*



A



B



C

Figure 5.13. *Chromatograms of A: P14, B: P14H05 and C: P15.*

5.1.7. Stability in working conditions

5.1.7.1. *Stability under in vitro release environment conditions*

It was found that the ISN remains stable in the release medium throughout the release study.

5.2. Formulation of Inclusion Complexes

5.2.1. Phase solubility studies

5.2.1.1. *Determination of equilibrium time for solubility phase diagram of ISN/β-CD, ISN/M-β-CD and ISN/HP-β-CD*

The experiments were performed in the manner described in the 4.2.1 Section. Since the ISN resolution increased in relation to the shaking time but reached to the highest solubility value in first day and did not increase after this time, it was decided that the 1st day period was sufficient for ISN to reach the solubility balance.

5.2.1.2. *Determination of the phase solubility diagram of ISN/β-CD, ISN/M-β-CD and ISN/HP-β-CD*

Following equilibration, the quantity of dissolved ISN (mM) against the quantty of added β-CD, M-β-CD and HP-β-CD (mM) was used tp construct the phase solubility diagrams. It was determined that the A_L type diagram is in accordance with the diagrams in Figure 5.14-5.16. Regarding the A_L type diagram, the preparation of ISN/β-CD, ISN/M-β-CD and ISN/HP-β-CD complexes were carried out at a 1:1 molar ratio (Higuchi and Connors, 1965). The Ks value of complexation can be determined according to the following equation from the phase solubility curves:

$$Ks = Slope/S_o \times (1-Slope) \qquad (5.1)$$

Where slope is the value measured from linear adjustment of the data where So is the solubility of ISN without CD derivatives (Yurtdaş, 2010).

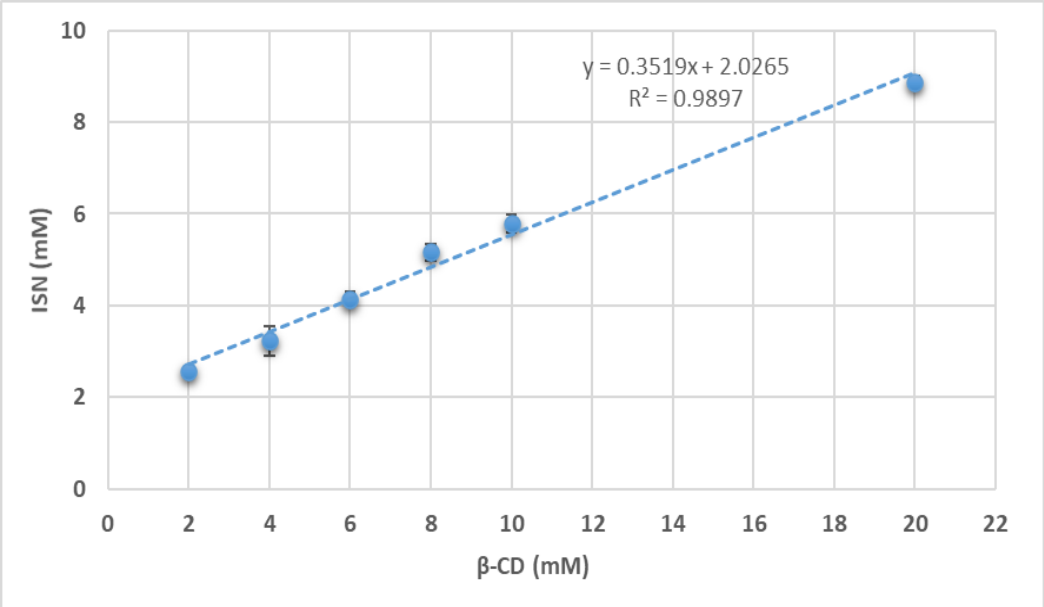


Figure 5.14. *Phase solubility diagram of ISN/β-CD*

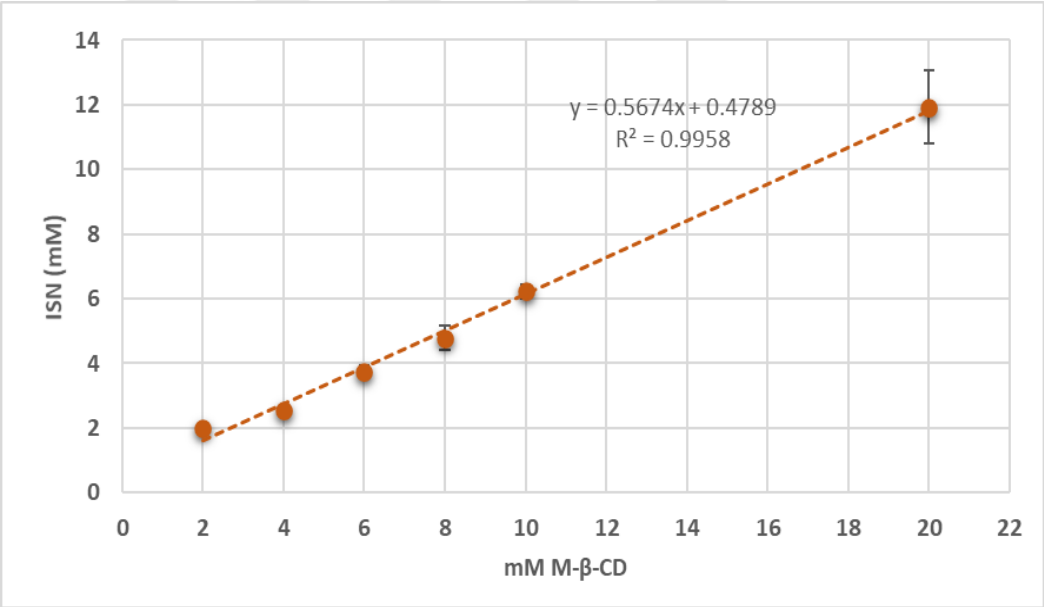


Figure 5.15. *Phase solubility diagram of ISN/M-β-CD*

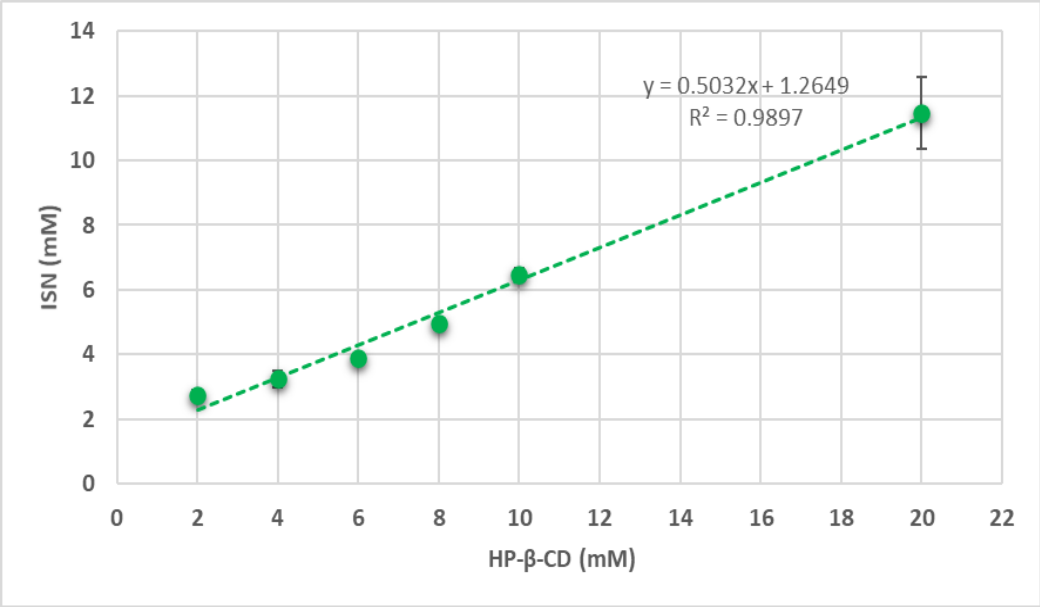


Figure 5.16. *Phase solubility diagram of ISN/HP-β-CD*

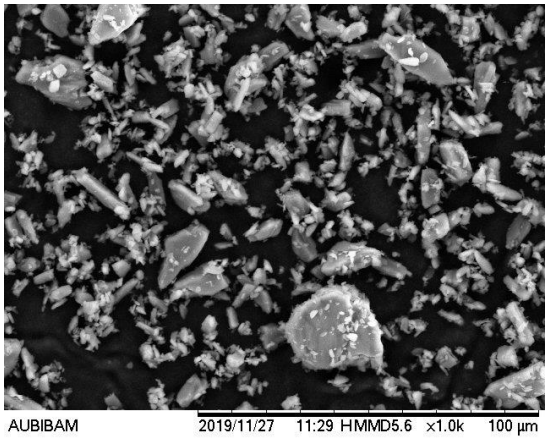
5.2.2. Preparation of ISN/β-CD, ISN/M-β-CD and ISN/HP-β-CD inclusion complexes

Inclusion complexes have been successfully produced by SD and FD methods, and the necessary characterization analyzes have been performed on the complex.

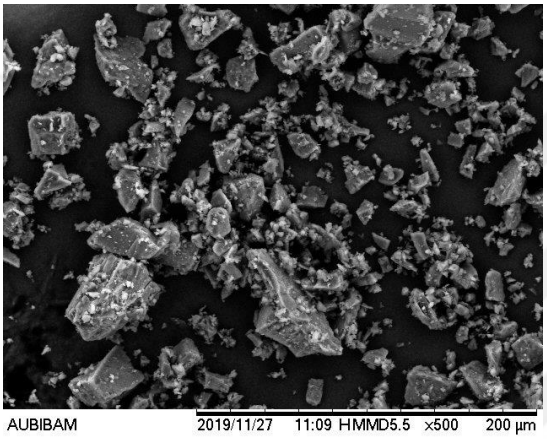
5.3. Physicochemical characterization tests of cyclodextrin inclusion complexes

5.3.1. Morphological studies of inclusion complexes

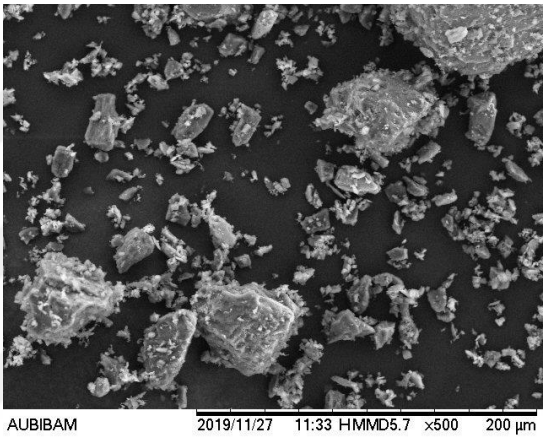
The morphological structures of CDs, physical mixtures of ISN with CDs and inclusion complexes were investigated by SEM and microphotographs were illustrated in the Figure 5.17-5.19.



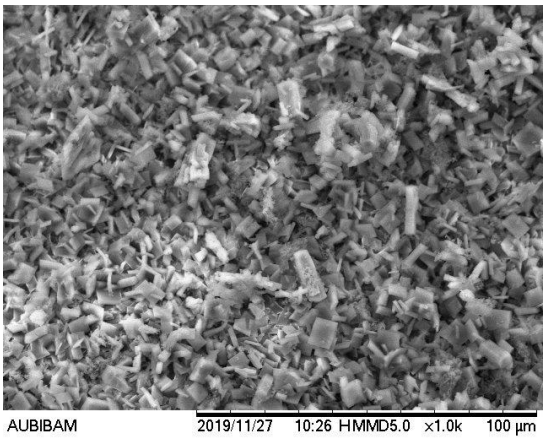
A



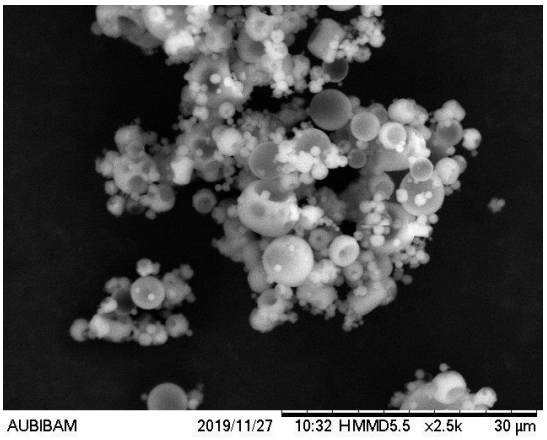
B



C

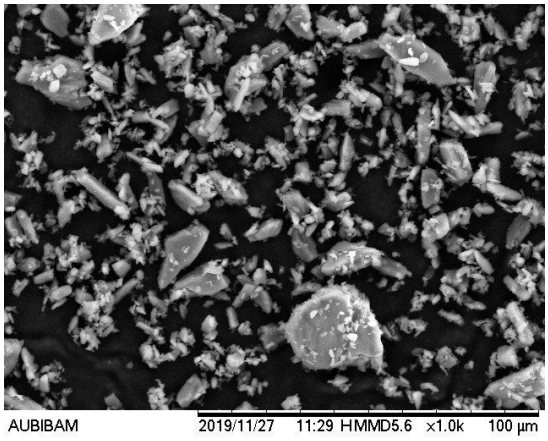


D

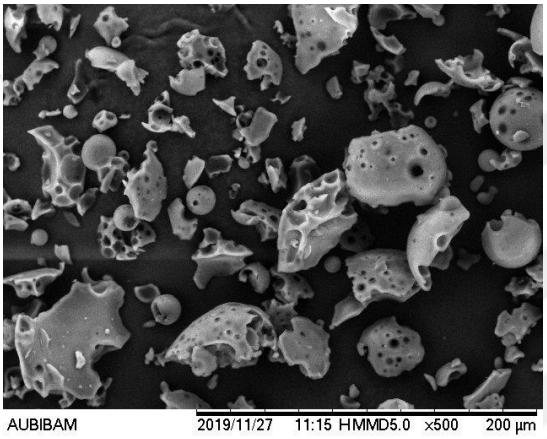


E

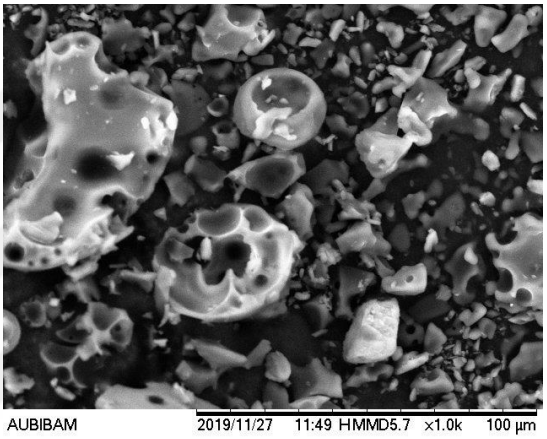
Figure 5.17. *SEM images of pure ISN, pure β-CD, physical mixture and ISN/β-CD inclusion complexes (A: ISN, B: β-CD, C: ISN-β-CD PM, D: ISN-β-CD FD and E: ISN-β-CD SD)*



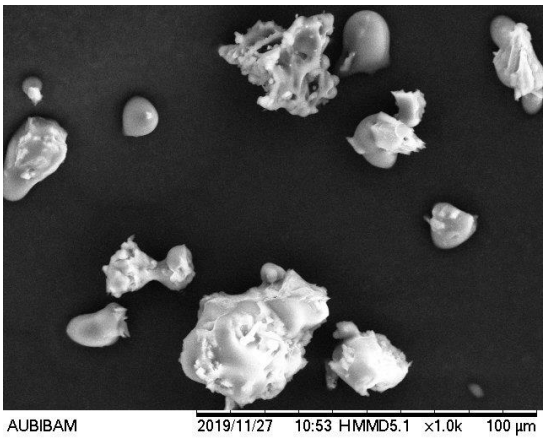
A



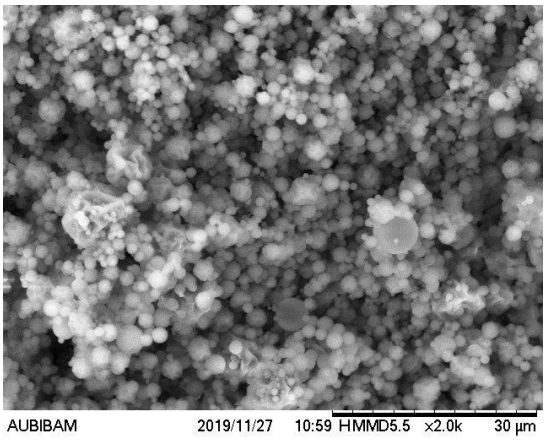
B



C



D



E

Figure 5.18. *SEM images of pure ISN, pure M-β-CD, physical mixture and ISN/M-β-CD inclusion complexes (A: ISN, B: M-β-CD, C: ISN-M-β-CD PM, D: ISN-M-β-CD FD and E: ISN-β-CD SD)*

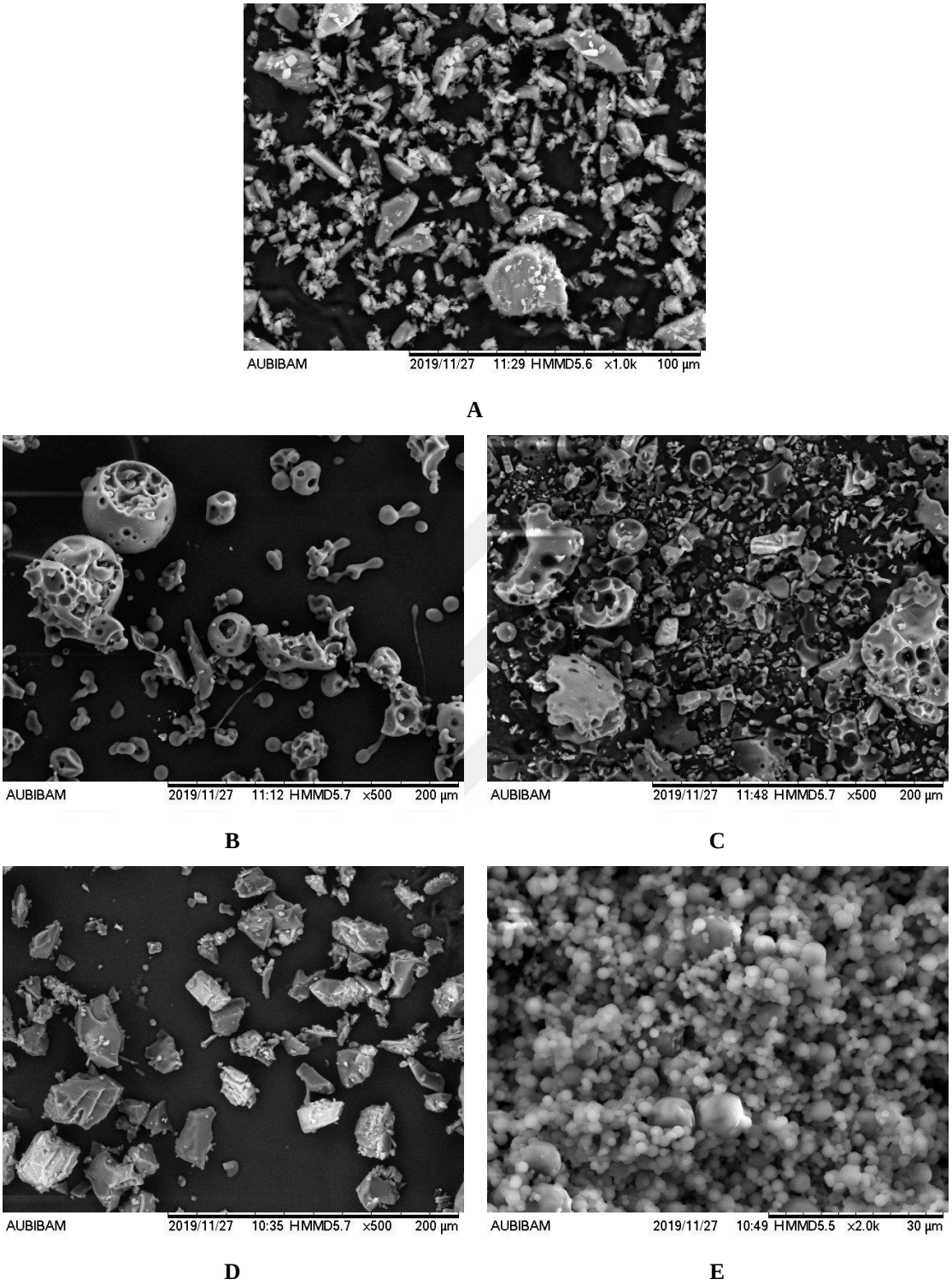


Figure 5.19. *SEM images of pure ISN, pure HP-β-CD, physical mixture and ISN/HP-β-CD inclusion complexes (A: ISN, B: HP-β-CD, C: ISN-HP-β-CD PM, D: ISN-HP-β-CD FD and E: ISN-β-CD SD)*

5.3.2. Thermal analysis of CDs inclusion complexes

Pure ISN, pure CD derivatives, physical mixtures of ISN and CDs and the inclusion complexes were analyzed by DSC between 50-300 °C. DSC thermograms were presented in Figure 5.20 - 5.22.

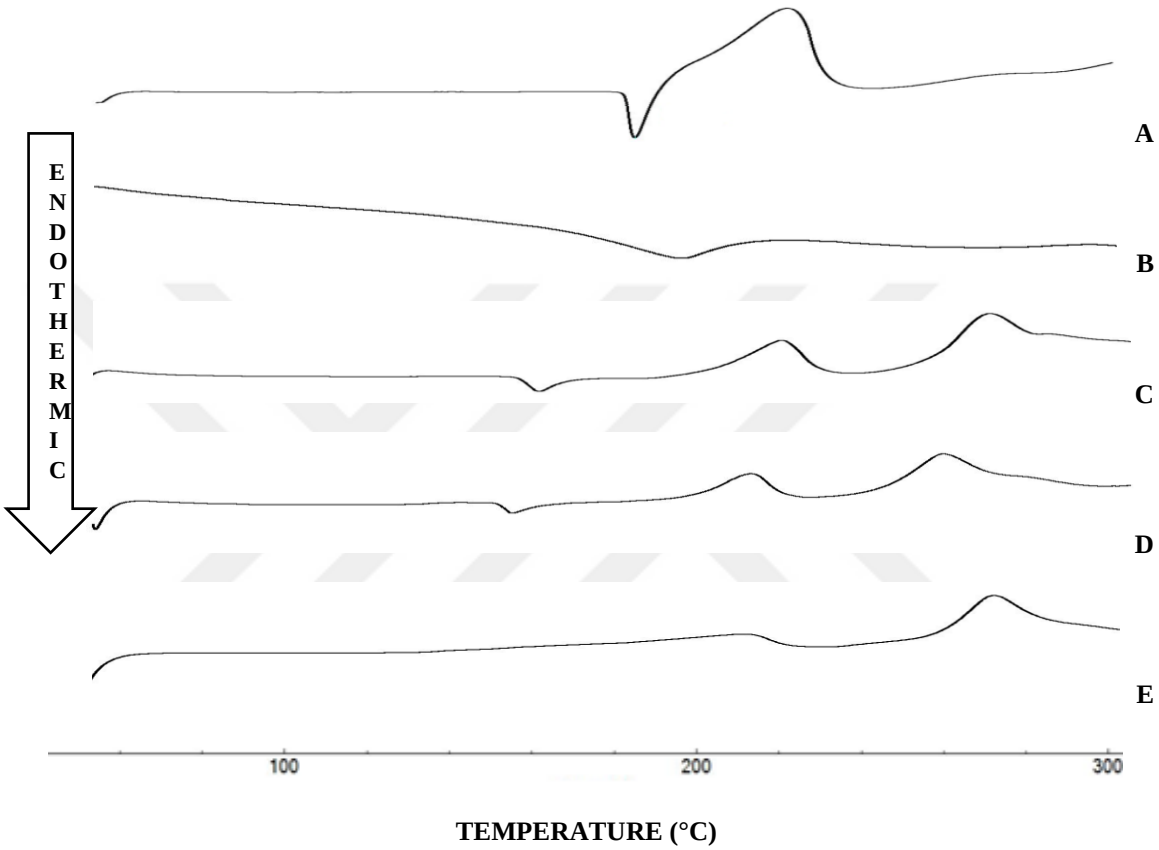


Figure 5.20. *DSC thermograms of pure ISN, pure β-CD, physical mixture and ISN/β-CD inclusion complexes (A: ISN, B: β-CD, C: ISN-β-CD PM, D: ISN-β-CD FD and E: ISN-β-CD SD)*

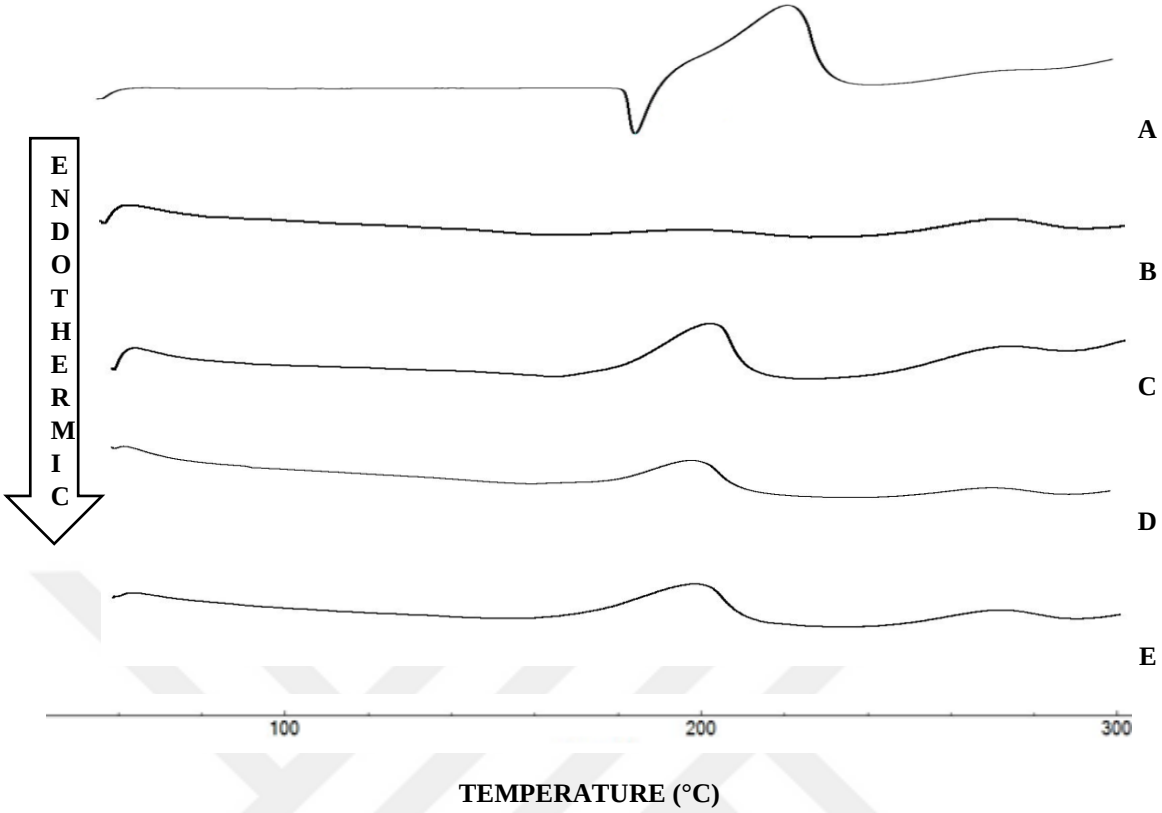


Figure 5.21. *DSC thermograms of pure ISN, pure M-β-CD, physical mixture and ISN/M-β-CD inclusion complexes (A: ISN, B: M-β-CD, C: ISN-M-β-CD PM, D: ISN-M-β-CD FD and E: ISN-M-β-CD SD)*

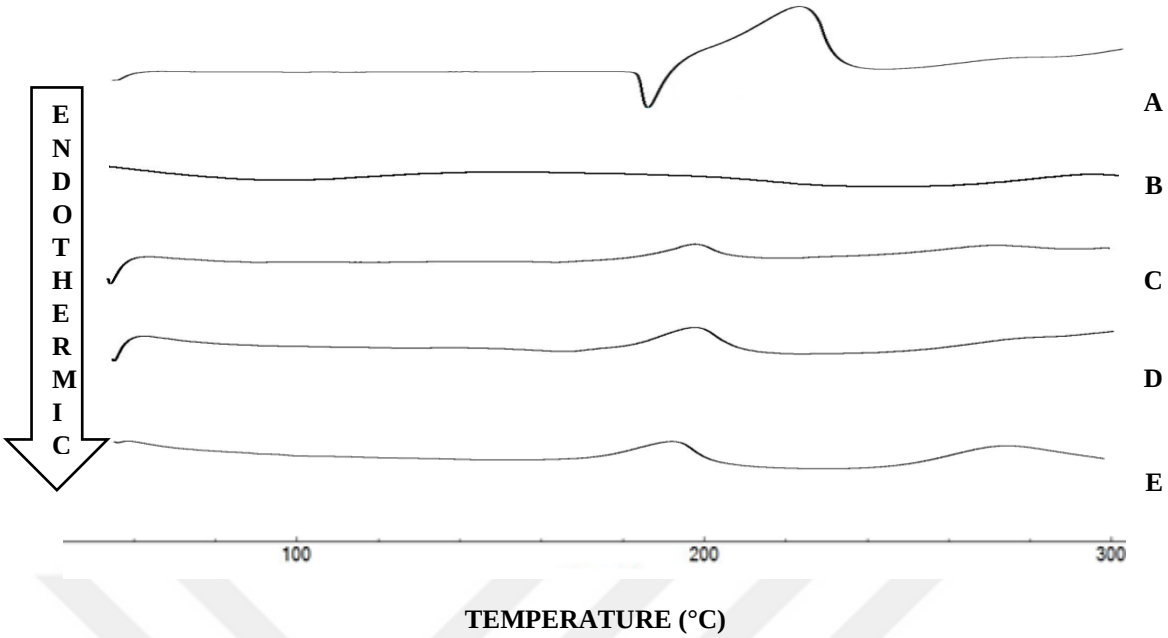


Figure 5.22. *DSC thermograms of pure ISN, pure HP-β-CD, physical mixture and ISN/HP-β-CD inclusion complexes (A: ISN, B: HP-β-CD, C: ISN-HP-β-CD PM, D: ISN-HP-β-CD FD and E: ISN-HP-β-CD SD)*

5.3.3. FT-IR analysis of inclusion complexes

Pure ISN, pure CD derivatives, physical mixtures of ISN and CDs and the inclusion complexes were analyzed by FT-IR between 500-4000 cm^{-1} to investigate the interactions between substances. FT-IR spectra were presented in Figure 5.23 - 5.25.

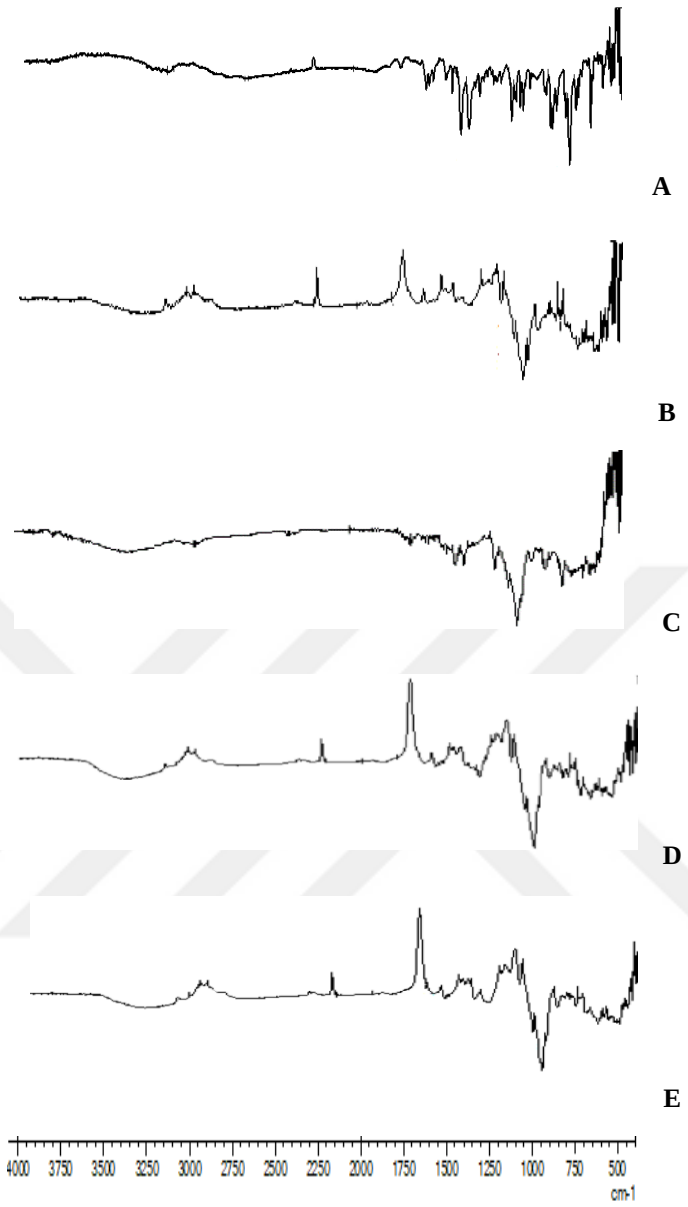


Figure 5.23. *FT-IR spectra of pure ISN, pure β-CD, physical mixture and ISN/β-CD inclusion complexes (A: ISN, B: β-CD, C: ISN-β-CD PM, D: ISN-β-CD FD and E: ISN-β-CD SD)*

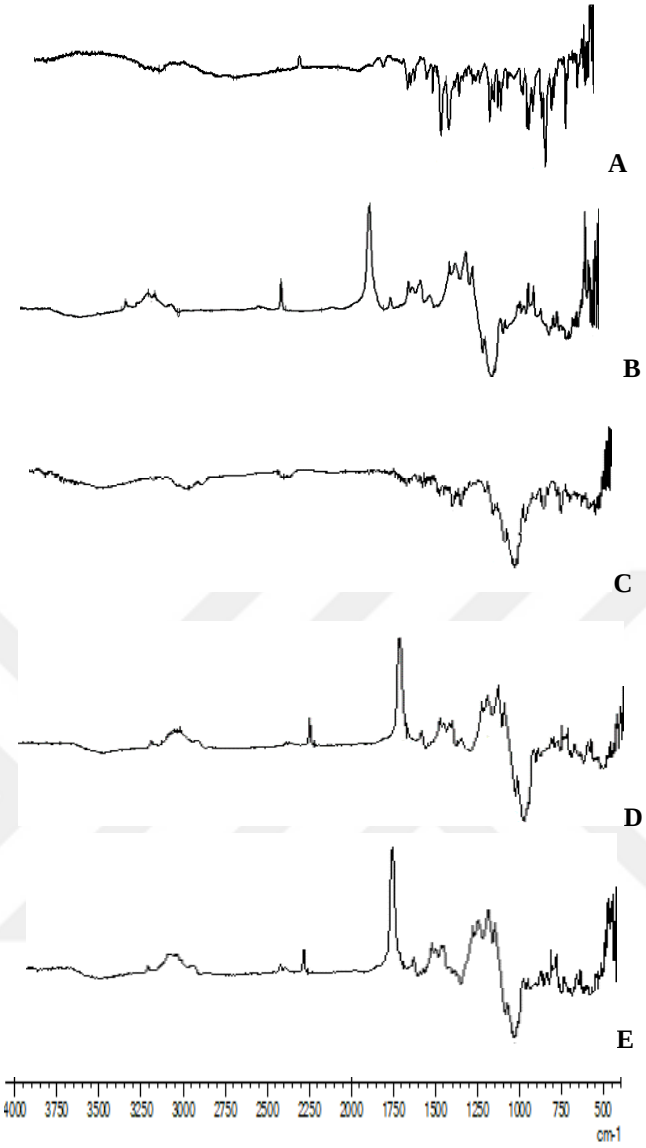


Figure 5.24. *FT-IR spectra of pure ISN, pure M-β-CD, physical mixture and ISN/M-β-CD inclusion complexes (A: ISN, B: M-β-CD, C: ISN-M-β-CD PM, D: ISN-M-β-CD FD and E: ISN-M-β-CD SD)*

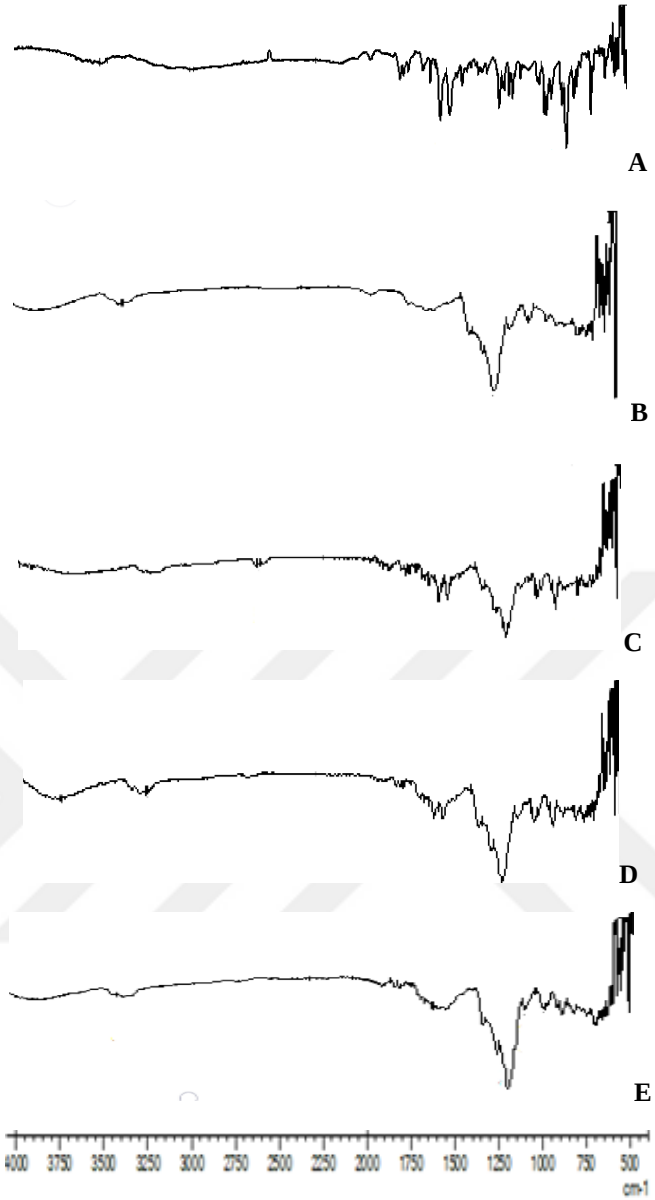


Figure 5.25. *FT-IR spectra of pure ISN, pure HP-β-CD, physical mixture and ISN/HP-β-CD inclusion complexes (A: ISN, B: HP-β-CD, C: ISN-HP-β-CD PM, D: ISN-HP-β-CD FD and E: ISN-HP-β-CD SD)*

5.3.4. ^{1}H-NMR analysis of inclusion complexes

The ^{1}H-NMR spectra of pure ISN, pure CD derivatives, physical mixtures of ISN and CDs and the inclusion complexes were presented in Figure 5.26 - 5.28. The chemical shifts of H-3 and H-5 protons of CD derivatives were shown in Table 5.6-5.8.

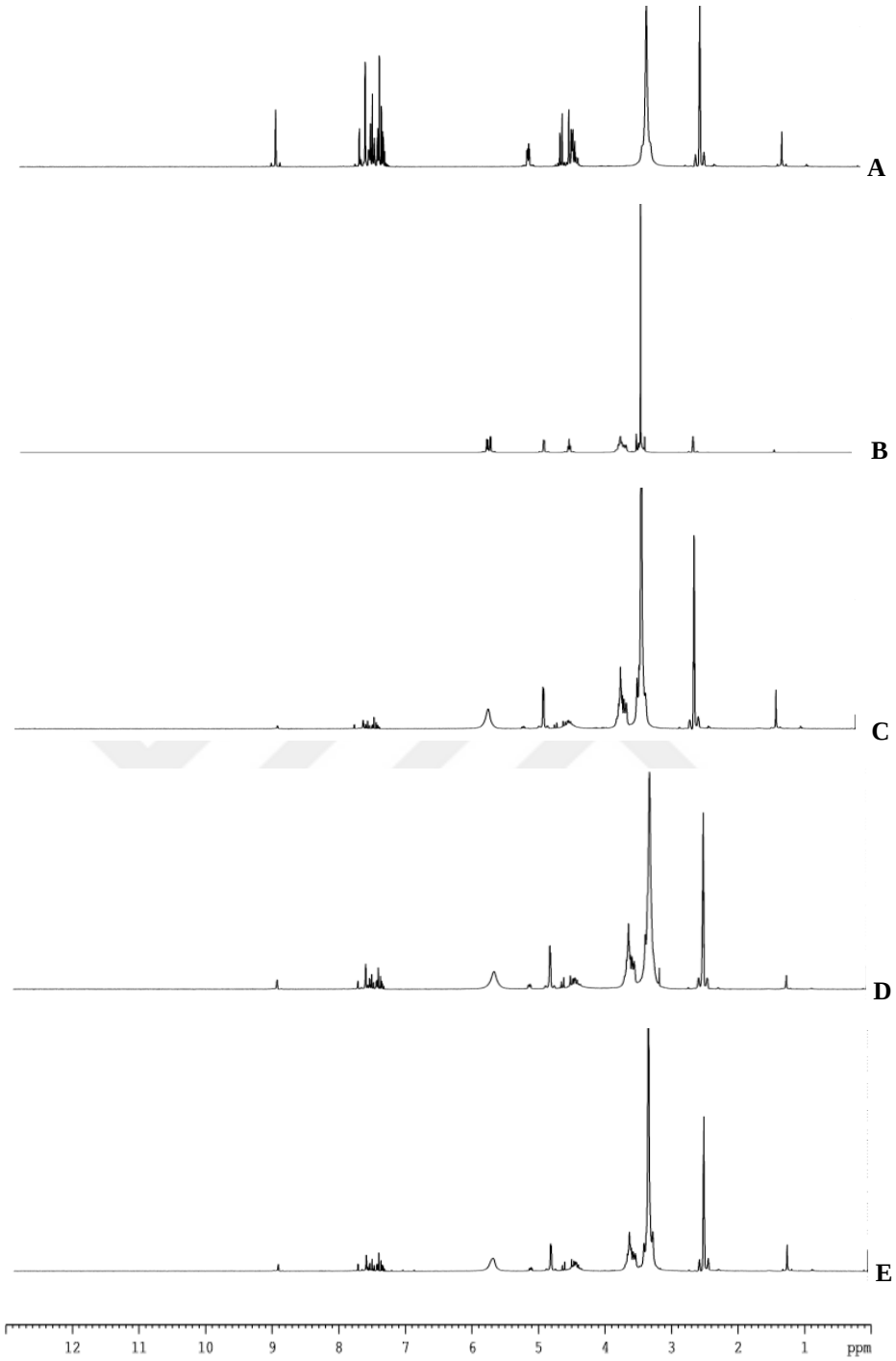


Figure 5.26. *^{1}H-NMR spectra of pure ISN, pure β-CD, physical mixture and ISN/β-CD inclusion complexes (A: ISN, B: β-CD, C: ISN-β-CD PM, D: ISN-β-CD FD and E: ISN-β-CD SD)*

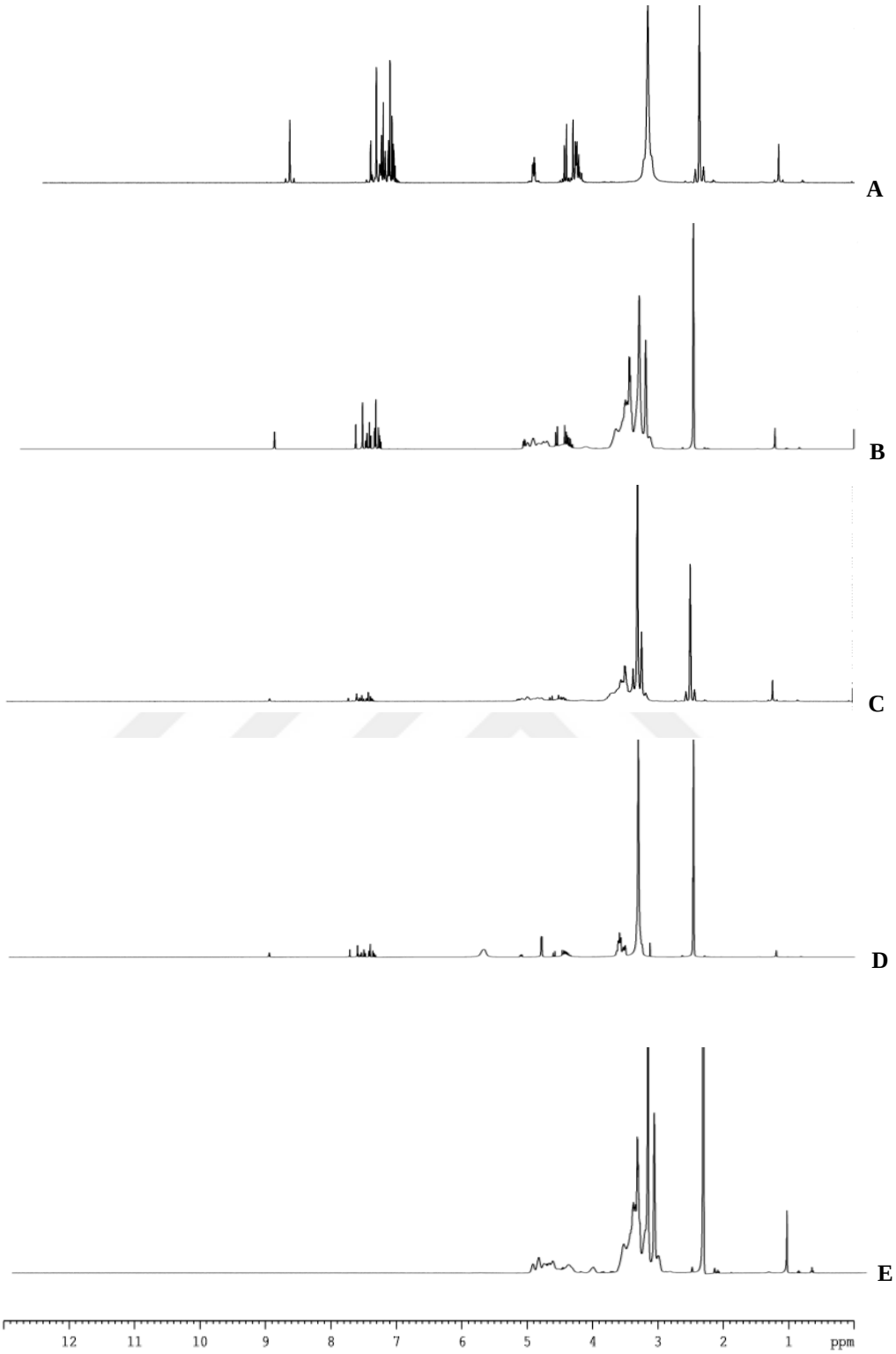


Figure 5.27. *^{1}H-NMR spectra of pure ISN, pure M-β-CD, physical mixture and ISN/M-β-CD inclusion complexes (A: ISN, B: M-β-CD, C: ISN-M-β-CD PM, D: ISN-M-β-CD FD and E: ISN-M-β-CD SD)*

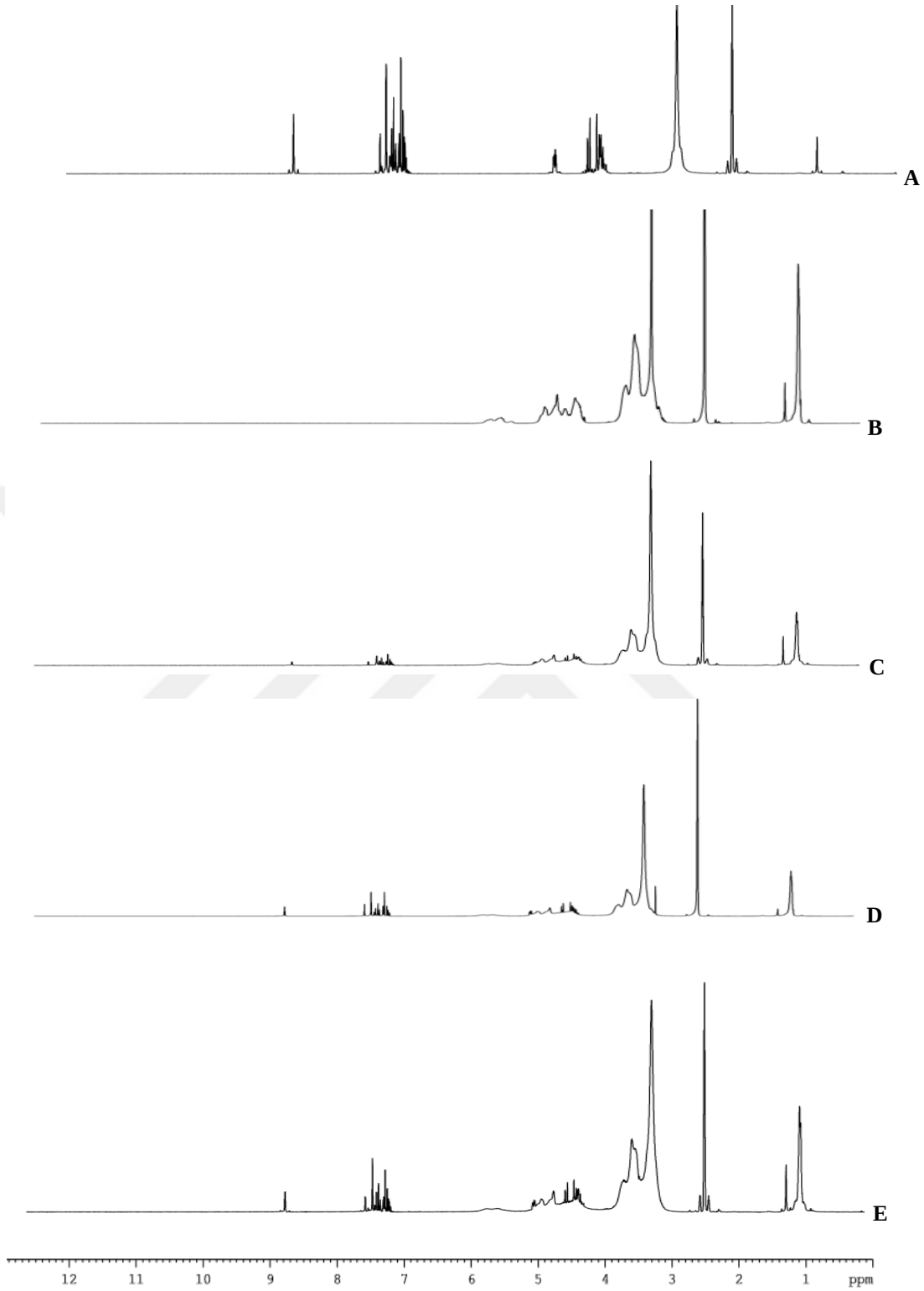


Figure 5.28. *^{1}H-NMR spectra of pure ISN, pure HP-β-CD, physical mixture and ISN/HP-β-CD inclusion complexes (A: ISN, B: HP-β-CD, C: ISN-HP-β-CD PM, D: ISN-HP-β-CD FD and E: ISN-HP-β-CD SD)*

Table 5.6. *Chemical shifts variation of H-3 and H-5 protons of β-CD in free and complex status*

Protons	β-CD (free) (δ)	FD (δ)	SD (δ)
H-3	3.6320	3.6385	3.6392
H-3 (Δ δ)	-	+ 0.0065	+ 0.0072
H-5	3.5628	3.5765	3.5802
H-5 (Δ δ)	-	+ 0.0137	+ 0.0174

Table 5.7. *Chemical shifts variation of H-3 and H-5 protons of M-β-CD in free and complex status*

Protons	M-β-CD (free) (δ)	FD (δ)	SD (δ)
H-3	3.6089	3.6342	3.5625
H-3 (Δ δ)	-	+ 0.0253	- 0.0464
H-5	3.4612	3.4034	3.4012
H-5 (Δ δ)	-	- 0.0578	- 0.0600

Table 5.8. *Chemical shifts variation of H-3 and H-5 protons of HP-β-CD in free and complex status*

Protons	HP-β-CD (free) (δ)	FD (δ)	SD (δ)
H-3	3.437	3.7623	3.7675
H-3 (Δ δ)	-	+ 0.0186	-0.0278
H-5	3.6073	3.5795	3.6378
H-5 (Δ δ)	-	+ 0.0238	+ 0.0305

5.3.5. Determination of aqueous solubility in distilled water of inclusion complexes

The solubility studies of ISN/CDs inclusion complexes were performed at room temperature 25ºC in distilled water and the outcomes were presented in Table 5.9.

Table 5.9. *Saturated solubility values of ISN/CDs inclusion complexes in distilled water at 25 °C.*

Code	Saturated Solubility ($mg.mL^{-1}$)	± SDV	± SE
IS	0.5088	0.0087	0.0062
ISN-β-CD FD	1.4363	0.3448	0.1542
ISN-β-CD SD	1.5613	0.1622	0.0725
ISN-M-β-CD FD	2.5684	0.3658	0.1636
ISN-M-β-CD SD	3.8869	1.1368	0.5084
ISN-HP-β-CD FD	2.0900	0.1311	0.0757
ISN-HP-β-CD SD	3.6550	0.4461	0.1995

5.3.6. Determination of drug content (DC)

The ISN amount of inclusion complexes were shown in Table 5.10.

Table 5.10. *Drug content of ISN/CDs inclusion complexes*

Code	Drug content % ±SE (n=3)
ISN-β-CD FD	30.5399 ± 0.6826
ISN-β-CD SD	32.4722 ± 0.4559
ISN-M-β-CD FD	23.2348 ± 1.3774
ISN-M-β-CD SD	23.9850 ± 0.1871
ISN-HP-β-CD FD	24.9778 ± 0.3972
ISN-HP-β-CD SD	25.9132 ± 0.4880

5.4. Formulation of *In Situ* Gels

5.4.1. Formulation development studies

5.4.1.1. *Preparation of placebo (active substance-free) formulations*

In formulation development studies, twenty *in situ* gel formulations comprised of Pluronic® F127 and/or HPMC at different concentrations were formulated.

5.4.1.2. *Gelation temperature of placebo formulations*

Gelation temperature of placebo formulations was measured from 25 °C to 45 °C and the findings were given in Table 5.11.

Table 5.11. *Gelation temperature of placebo formulations*

Formulation code	Gelation Temperature (°C)
P12.75	43
P12.75H05	35
P13	42
P13H05	38
P13.25	37
P13.25H05	30
P13.5	32
P13.5H05	28
P13.75	31
P13.75H05	25
P14	28
P14H05	26
P15	26
P15H05	25
P16	25
P16H05	25
P17	25
P17H05	25
P18	25
P18H05	25

5.4.1.3. *pH measurements of placebo formulations*

The pH measurements of placebo formulations were performed using pH meter at 25 °C and results are presented in Table 5.12.

Five formulations with suitable gelation temperature and lower polymer content (P12.75H05, P13.5H05, P14, P14H05 and P15) were selected among these formulations. ISN and ISN/CDs complexes were added to these formulations in the amount calculated for the addition of 1% (w/w). According to the results of drug content and saturated solubility analyses of the inclusion complexes, inclusion complexes formulated by SD process were used in the formulation of *in situ* gels.

Table 5.12. *pH measurements of placebo formulations*

Formulation code	pH
P12.75	7.09
P12.75H05	7.05
P13	7.03
P13H05	6.99
P13.25	6.97
P13.25H05	7.03
P13.5	7.02
P13.5H05	6.99
P13.75	6.97
P13.75H05	7.07
P14	6.99
P14H05	7.02
P15	7.06
P15H05	7.13
P16	7.13
P16H05	7.07
P17	7.13
P17H05	7.02
P18	7.07
P18H05	7.08

5.4.2. Preparation of the formulations containing ISN and ISN/CDs complexes

5.4.2.1. *Gelation temperature of the formulations containing ISN or ISN/CDs* complexes

According to the gelation temperature of placebo formulations, P12.75H05, P13.5H05, P14, P14H05 and P15 formulations were selected to prepare the formulations containing ISN, ISN/β-CD SD, ISN/M-β-CD SD and ISN/HP-β-CD SD complexes. Gelation temperatures of these formulations were given in Table 5.13 (Formulation

containing only pure ISN starts with the “F” code, and formulations containing inclusion complexes start with the “C” code).

Table 5.13. *Gelation temperatures of formulations containing ISN and ISN/CDs complexes*

Content	Code	Gelation Temp.	Content	Code	Gelation Temp.
ISN	**F12.75H05**	33	**ISN/ M-β-CD**	**C12.75H05-MβCD**	No gelling
	F13.5H05	26		**C13.5H05-MβCD**	34
	F14	26		**C14-MβCD**	38
	F14H05	25		**C14H05-MβCD**	32
	F15	25		**C15-MβCD**	34
β-CD	**C12.75H05-βCD**	<25	***ISN/ HP*-β-CD**	**C12.75H05-HPβCD**	No gelling
	C13.5H05-βCD	<25		**C13.5H05-HPβCD**	30
	C14-βCD	<25		**C14-HPβCD**	30
	C14H05-βCD	<25		**C14H05-HPβCD**	27
	C15-βCD	<25		**C15-HPβCD**	28

In situ gel formulations containing ISN/β-CD complexes were excluded due to its rapid gelatinization at lower temperatures below 25 °C.

5.4.2.2. *pH measurement of the formulations containing ISN or ISN/CDs* complexes

pH measurements of formulations containing ISN, ISN/β-CD SD, ISN/M-β-CD SD and ISN/HP-β-CD SD complexes were shown at Table 5.14.

Table 5.14. *pH measurements of formulations containing ISN and ISN / CDs complexes*

Content	Code	pH
ISN	**F12.75H05**	4.04
	F13.5H05	4.21
	F14	4.50
	F14H05	4.33
	F15	4.52
ISN/M-β-CD	**C12.75H05-MβCD**	4.09
	C13.5H05-MβCD	3.92
	C14-MβCD	4.06
	C14H05-MβCD	4.18
	C15-MβCD	4.22
***ISN/ HP*-β-CD**	**C12.75H05-HPβCD**	3.92
	C13.5H05-HPβCD	3.86
	C14-HPβCD	4.37
	C14H05-HPβCD	4.10
	C15-HPβCD	4.24

5.4.3. Selection of the optimum *in situ* gel formulations

F12.75H05, F14, C13.5H05-MβCD, C14H05-MβCD, C15-MβCD, C13.5H05-HPβCD and C14-HPβCD formulations, which have the lowest polymer concentration, suitable pH values and gelation temperature close to ~ 37 °C were selected as optimum formulations for further studies. Formulation components and ratios of the optimum formulations were presented in Table 5.15.

Table 5.15. *Components of optimum formulations (w/w %)*

Formulation code	ISN (%)	Pluronic® F127 (%)	HPMC (%)	Benzalkonium chloride (%)	Distilled water (%)
F12.75H05	1	12.75	0.5	0.01	To 100 g
F14	1	14	-	0.01	To 100 g
C13.5H05MβCD	1	13.5	0.5	0.01	To 100 g
C14H05MβCD	1	14	0.5	0.01	To 100 g
C15MβCD	1	15	-	0.01	To 100 g
C13.5H05HPβCD	1	13.5	0.5	0.01	To 100 g
C14HPβCD	1	14	-	0.01	To 100 g

5.5. Characterization of The *In Situ* Gel Formulations

5.5.1. *Gelling capacity*

The gelling capacity of the *in situ* gels prepared were shown in Table 5.16.

Table 5.16. Gelling capacity of *in situ gel formulations*

Formulation code	Gelling capacity
F12.75H05	+++
F14	+++
C13.5H05MβCD	+++
C14H05MβCD	++
C15MβCD	++
C13.5H05HPβCD	+++
C14HPβCD	+++

5.5.2. *Swelling test*

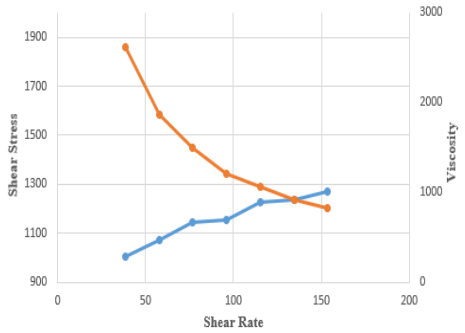
This test was performed according to the method described in “4.5.2. Swelling test” section. The ratio of swelling (%) was calculated after several time periods were shown in the table 5.17.

Table 5.17. *Swelling ratio of in situ gel formulations*

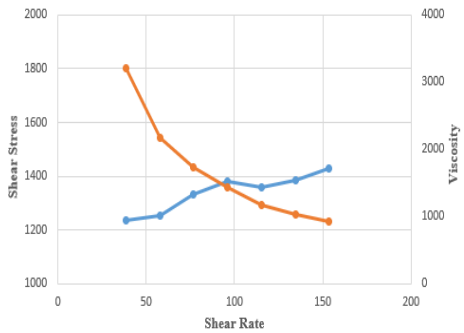
Time (h)	Swelling Ratio %						
	F12.75H05	**F14**	**C13.5H05 MβCD**	**C14H05 MβCD**	**C15 MβCD**	**C13.5H05 HPβCD**	**C14 HPβCD**
0.5	11.289	11.046	17.365	16.034	16.741	20.665	19.982
1	20.468	11.732	27.463	22.472	20.221	28.108	25.723
2	20.927	19.855	35.073	30.726	32.173	40.761	37.249
3	25.929	26.281	42.439	40.165	42.693	50.087	45.527
4	31.344	30.577	49.756	47.906	50.716	61.12	52.558
6	41.303	42.454	63.390	60.347	65.779	67.556	63.061
24	83.249	95.306	106.487	112.796	111.092	121.278	82.777
48	105.277	150.18	165.2019	148.42	145.599	167.075	121.183
72	118.219	184.837	201.365	169.905	166.88	195.096	142.456

5.5.3. Rheological studies

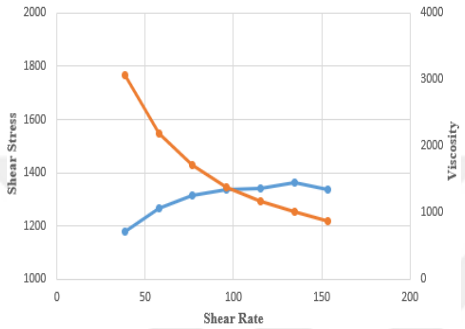
The rheological behavior of the formulations were examined at two seperate temperatures as 25 °C and 37 °C. The shear stress and viscosity change against the shear rate were recorded using rheometer at two temperatures. The results shown in Figure 5.29- 5.30.



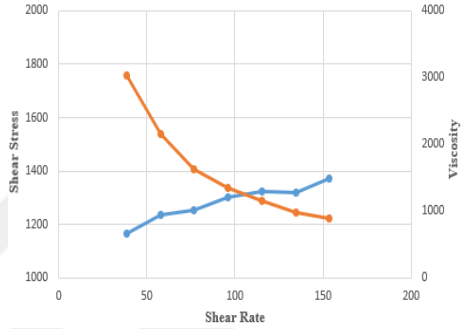
F12.75H05



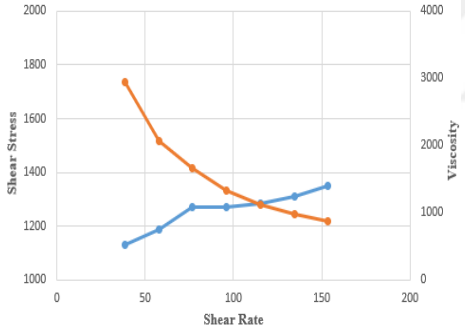
F14



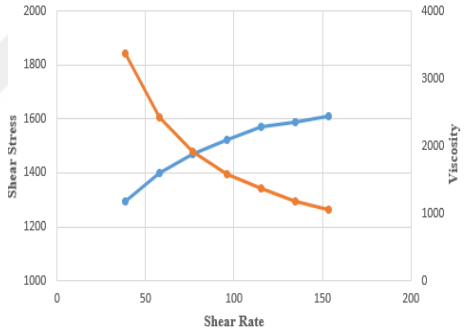
C13.5H05 MβCD



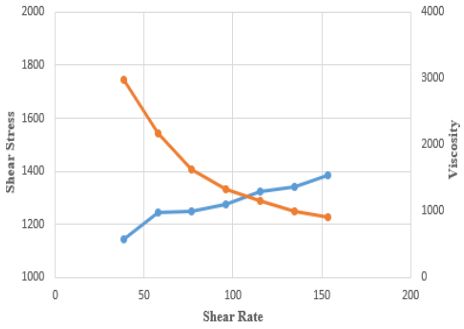
C14H05 MβCD



C15 MβCD

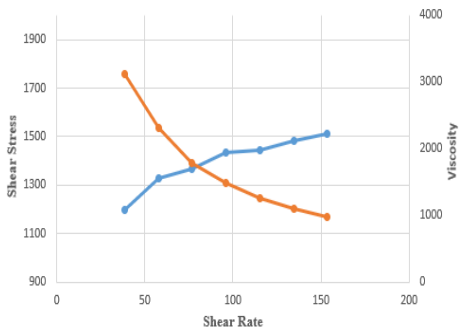


C13.5H05 HPβCD

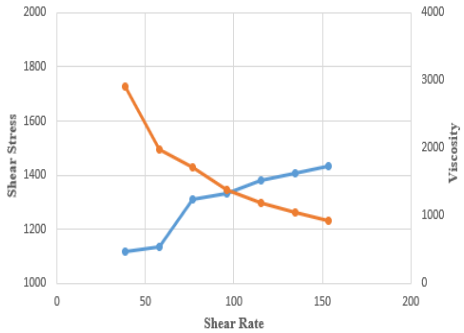


C14 HPβCD

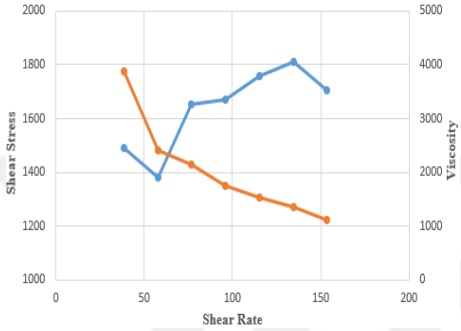
Figure 5.29. *Rheograms of the in situ gel formulations at 25 °C (0th day) (blue line: shear stress, orange line: viscosity)*



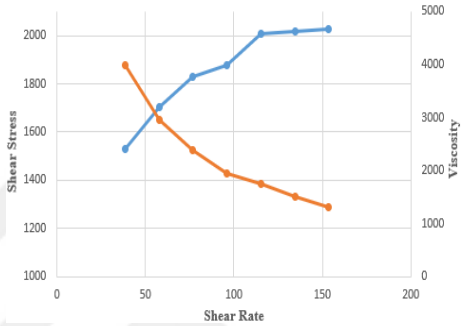
F12.75H05



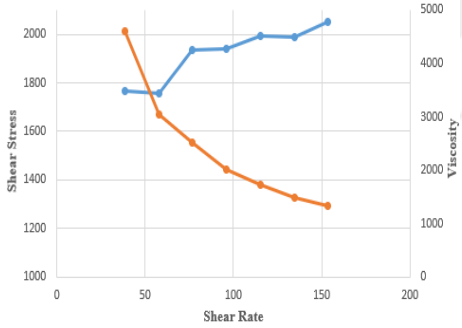
F14



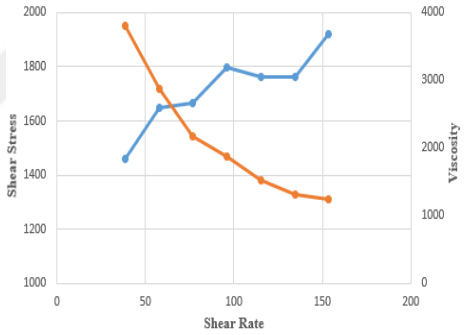
C13.5H05 MβCD



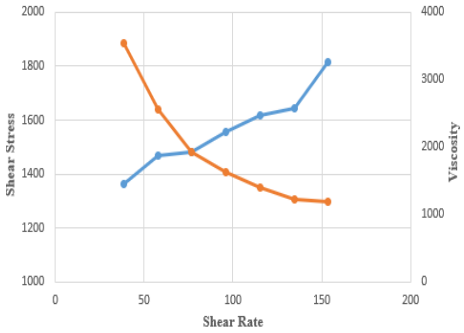
C14H05 MβCD



C15 MβCD



C13.5H05 HPβCD



C14 HPβCD

Figure 5.30. *Rheograms of the in situ gel formulations at 37 °C (0th day) (blue line: shear stress, orange line: viscosity)*

5.5.4. Determination of drug content (DC) %

Determination of the drug contents of the formulations were carried out by the validated HPLC method mentioned in 4.5.4 section. The results shown in Table 5.18.

Table 5.18. *Drug content of in situ gel formulations*

Formulation Code	Drug Content % ±SE (n=3)
F12.75H05	0.615 ± 0.044
F14	0.749 ± 0.096
C13.5H05MβCD	0.862 ± 0.150
C14H05MβCD	0.738 ± 0.148
C15MβCD	0.716 ± 0.075
C13.5H05HPβCD	0.655 ± 0.142
C14HPβCD	0.652 ± 0.253

SE: Standard error

5.6. *In vitro* Release Studies

In vitro release data for *in situ* gel formulations containing the ISN and ISN/CDs inclusion complexes were presented in Table 5.19-5.20. The release profile of formulations were illustrated in Figure 5.31-5.32.

Table 5.19. *In vitro release data of in situ gel formulations containing ISN (n=3)*

Time (h)	Mean cumulative amount % ± SE	
	F12.75H05	**F14**
0.25	1.250 ± 0.145	1.952 ± 0.207
0.5	3.150 ± 0.782	4.309 ± 0.885
1	5.250 ± 1.115	7.002 ± 0.954
2	10.240 ± 0.215	12.232 ± 1.715
3	16.160 ± 0.335	18.215 ± 1.006
4	19.789 ± 0.748	22.125 ± 1.251
6	25.415 ± 1.008	28.116 ± 2.735
9	29.812 ± 1.471	32.115 ± 1.925
12	32.148 ± 1.275	34.125 ± 1.436
24	34.178 ± 2.005	37.115 ± 1.132
48	35.287 ± 1.008	38.234 ± 1.228
72	37.113 ± 1.781	40.113 ± 0.571

Table 5.20. *In vitro release data of in situ gel formulations containing ISN/M-β-CD or ISN/HP-β-CD inclusion complexes (n=3)*

Time (h)	Mean cumulative amount % ± SE				
	C13.5H05-MβCD	**C14H05-MβCD**	**C15-MβCD**	**C13.5H05-HPβCD**	**C14-HPβCD**
0.25	6.221 ± 0.415	6.002 ± 0.507	7.010 ± 0.496	5.032 ± 0.249	5.542 ± 0.112
0.5	9.890 ± 0.669	9.524 ± 0.375	12.710 ± 0.157	9.001 ± 0.914	9.115 ± 0.338
1	14.871 ± 0.491	14.415 ± 0.532	20.185 ± 0.583	13.118 ± 0.778	14.112 ± 0.899
2	22.452 ± 1.212	21.118 ± 0.231	29.478 ± 0.258	20.270 ± 0.564	23.378 ± 1.025
3	28.478 ± 1.602	27.145 ± 0.975	36.123 ± 0.100	28.145 ± 0.803	30.564 ± 1.516
4	36.145 ± 0.119	35.115 ± 0.889	41.276 ± 1.009	35.127 ± 1.094	37.118 ± 1.263
6	47.147 ± 0.340	45.214 ± 1.559	50.478 ± 1.516	44.452 ± 1.077	44.519 ± 2.008
9	54.478 ± 0.993	52.665 ± 0.812	59.008 ± 1.464	51.145 ± 1.741	52.116 ± 1.489
12	58.145 ± 0.897	56.118 ± 1.237	63.873 ± 2.008	54.074 ± 1.408	55.445 ± 1.874
24	63.142 ± 0.328	62.115 ± 1.885	70.459 ± 0.852	60.332 ± 1.017	61.513 ± 1.453
48	66.451 ± 1.378	64.112 ± 1.578	73.103 ± 0.041	63.778 ± 0.942	65.172 ± 1.991
72	69.145 ± 0.906	66.274 ± 0.221	76.118 ± 1.078	66.615 ± 0.824	67.115 ± 1.100

SE: Standard error

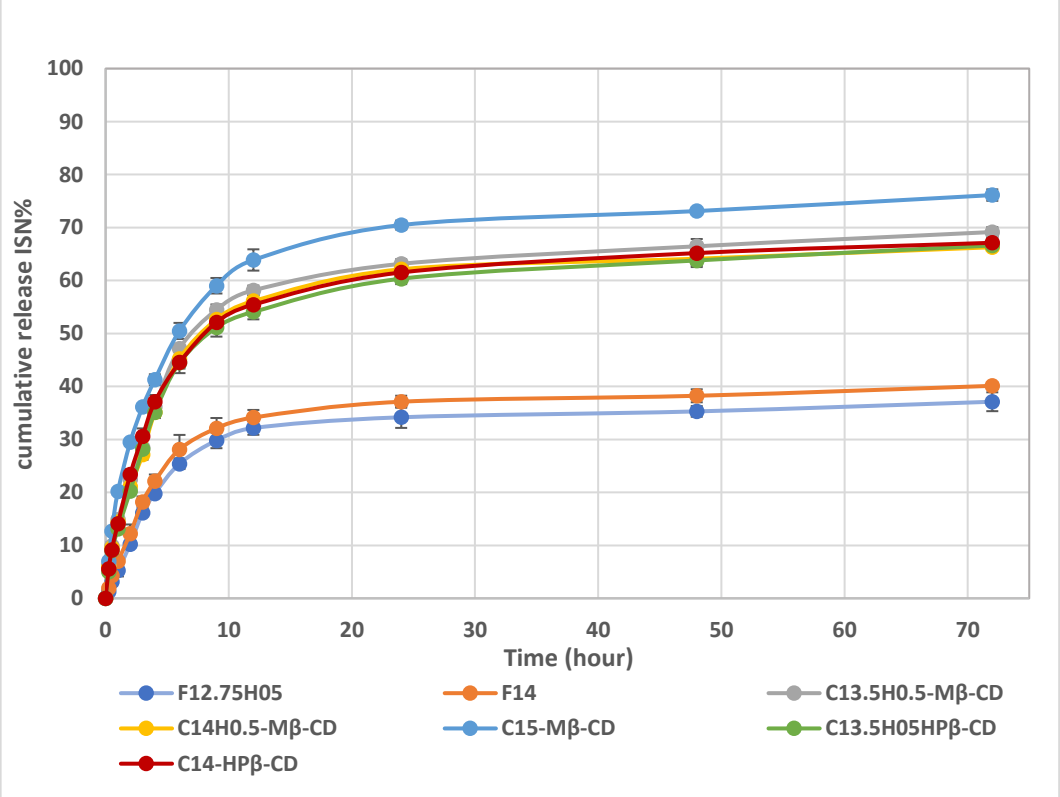


Figure 5.31. *In vitro release profiles of in situ gel formulations containing ISN and ISN/M-β-CD or ISN/HP-β-CD inclusion complexes (72h)*

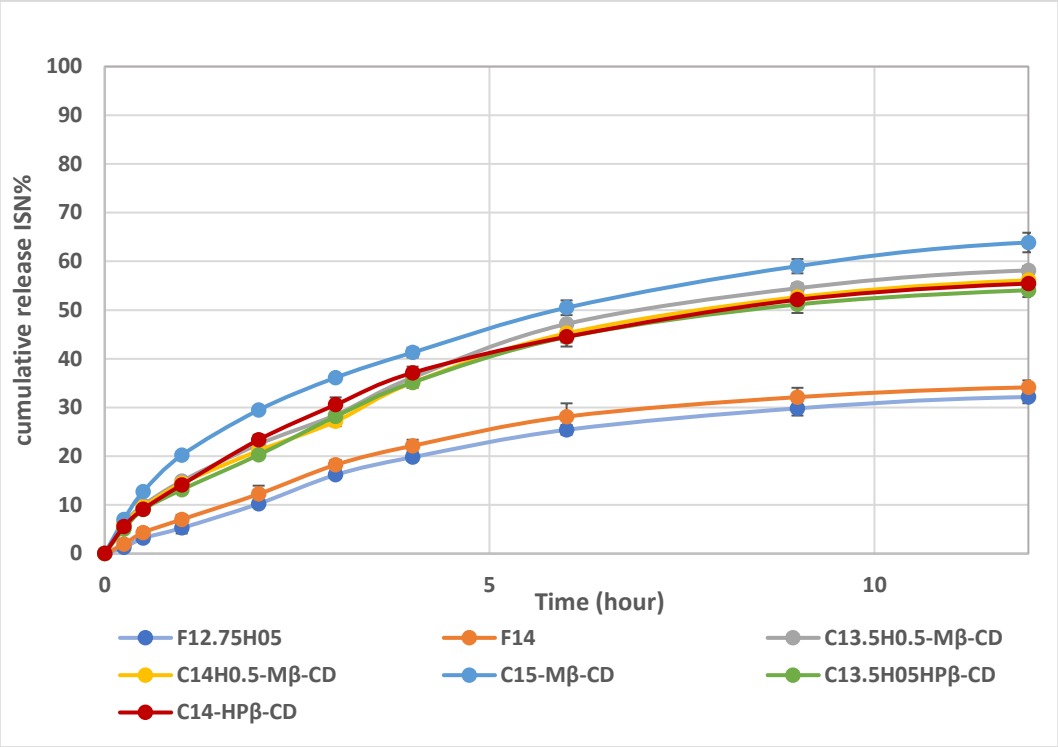


Figure 5.32. *In vitro release profiles of in situ gel formulations containing ISN and ISN/M-β-CD or ISN/HP-β-CD inclusion complexes (12h)*

5.6.1. Determination of release kinetics

In order to understand the mechanism of drug release, the cumulative release findings of *in situ* gel systems were fitted with various kinetic models with the DD solver program that can be used to support the modelling of cumulative release findings using non-linear optimization approaches based on distinct kinetic models (Yurtdaş-Kırımlıoğlu et al., 2020).

The findings were given in Table 5.21-5.22.

Table 5.21. *Release kinetic modelling of in situ gel systems containing ISN*

Code	Model and Equation	Evaluation Criteria				
		k	***R^2***	**AIC**	**MSC**	**n**
F12.75H05	**Zero-order model***	0.736	0.117	104.380	0.485	-
F14	$F=k_0*t$	0.797	0.205	106.802	0.527	-
F12.75H05	**First-order model***	0.014	0.061	102.126	0.262	-
F14	$F=100*[1-Exp(-k_1*t)]$	0.015	0.031	103.979	0.310	-
F12.75H05	**Higuchi model***	5.942	0.687	87.843	0.837	-
F14	$F=k_H*t^{0.5}$	6.463	0.664	90.221	0.748	-
F12.75H05	**Hixson-Crowell model***	0.003	0.002	102.961	0.326	-
F14	$F=100*[1-(1-k_{HC}*t)^3]$	0.003	0.071	105.273	0.410	-
F12.75H05	**Korsmeyer-Peppas Tlag***	5.720	0.552	96.489	0.172	0.417
F14	*F=kKP*(t-Tlag)^n*	10.665	0.612	96.085	0.297	0.249
F12.75H05	**Hopfenberg model***	0.003	0.002	104.961	0.480	3.000
F14	$F=100*[1-(1-k_{HB}*t)^n]$	0.003	0.050	107.020	0.544	4.500
F12.75H05	**Baker-Lonsdale***	0.001	0.711	86.808	0.917	-
F14	*3/2*[1-(1-F/100)^(2/3)]-F/100=*k_{BL}*t*	0.001	0.691	89.125	0.832	-
F12.75H05	**Peppas-Sahlin ***	**12.214**	**0.933**	**71.880**	**2.065**	**-**
F14	*F=k1*t^m+k2*t^(2*m)*	**13.551**	**0.947**	**70.317**	**2.279**	**-**

*In all kinetic models, ***F*** is the fraction % of drug released in time **t**, k_0: release constant of zero-order, k_1: release constant of first-order, k_H: release constant of Higuchi, k_{HC} : release constant of Hixson-crowell, k_{KP}: release constant of Korsmeyer-Peppas Tlag, k_{HB}**:** release constant of Hopfenberg, k_{BL}: release constant of Baker-Lonsdale, k_{PS}: release constant of Peppas-Sahlin, ***n***: is the diffusional exponent indicating the drug-release mechanism, ***AIC:*** Akaike information criteria, ***MSC:*** Model selection criteria, R^2: coefficient of determination

Table 5.22. *Release kinetic modelling of in situ gel systems containing ISN/M-β-CD or ISN/HP-β-CD inclusion complexes*

Code	Model and Equation	Evaluation Criteria				
		k	***R^2***	**AIC**	**MSC**	**n**
C13.5H05-MβCD	**Zero-order model*** $F=k_0*t$	1.370	0.245	120.425	0.577	-
C14H05-MβCD		1.322	0.245	115.594	0.576	-
C15-MβCD		1.513	0.397	123.643	0.721	-
C13.5H05HPβCD		1.312	0.187	118.808	0.523	-
C14-HPβCD		1.334	0.267	119.792	0.600	-
C13.5H05-MβCD	**First-order model*** $F=100*[1-Exp(-k_1*t)]$	0.033	0.393	111.096	0.140	-
C14H05-MβCD		0.031	0.365	110.847	0.097	-
C15-MβCD		0.040	0.388	112.911	0.104	-
C13.5H05HPβCD		0.031	0.395	110.032	0.152	-
C14-HPβCD		0.032	0.355	111.022	0.075	-
C13.5H05-MβCD	**Higuchi model*** $F=k_H*t^{0.5}$	11.087	0.671	103.140	0.752	-
C14H05-MβCD		10.703	0.669	102.394	0.748	-
C15-MβCD		12.319	0.623	106.620	0.588	-
C13.5H05HPβCD		10.575	0.695	101.143	0.836	-
C14-HPβCD		10.803	0.666	102.453	0.734	-
C13.5H05-MβCD	**Hixson-Crowell model*** $F=100*[1-(1-k_{HC}*t)^3]$	0.009	0.248	113.867	0.073	-
C14H05-MβCD		0.009	0.219	113.530	0.109	-
C15-MβCD		0.011	0.233	115.853	0.122	-
C13.5H05HPβCD		0.009	0.253	112.774	0.059	-
C14-HPβCD		0.009	0.208	113.686	0.130	-
C13.5H05-MβCD	**Korsmeyer-Peppas*** $F=k_{KP}*t^n$	16.872	0.760	103.012	0.762	0.416
C14H05-MβCD		16.229	0.757	102.377	0.749	0.417
C15-MβCD		20.483	0.778	103.711	0.812	0.390
C13.5H05HPβCD		15.166	0.732	103.461	0.658	0.438
C14-HPβCD		16.193	0.732	103.571	0.648	0.425
C13.5H05-MβCD	**Hopfenberg model*** $F=100*[1-(1-k_{HB}*t)^n]$	0.006	0.273	115.427	0.193	4.500
C14H05-MβCD		0.006	0.249	115.018	0.224	4.500
C15-MβCD		0.007	0.243	117.681	0.262	4.500
C13.5H05HPβCD		0.006	0.285	114.220	0.170	4.500

Table 5.22. (Continue) *Release kinetic modelling of in situ gel systems containing ISN/M-β-CD or ISN/HP-β-CD inclusion complexes*

C14-HPβCD		0.006	0.237	115.194	0.246	4.500
C13.5H05-MβCD	**Baker-Lonsdale*** *3/2*[1-(1-F/100)^(2/3)]-F/100=k_{BL}*t*	0.003	0.779	97.962	1.151	-
C14H05-MβCD		0.002	0.767	97.794	1.101	-
C15-MβCD		0.003	0.765	100.462	1.062	-
C13.5H05HPβCD		0.002	0.795	95.958	1.235	-
C14-HPβCD		0.002	0.770	97.593	1.108	-
C13.5H05-MβCD	**Peppas-Sahlin *** *F=k1*t^m+k2*t^(2*m)*	**23.067**	**0.964**	**78.212**	**2.670**	**-**
C14H05-MβCD		**22.335**	**0.963**	**77.716**	**2.646**	**-**
C15-MβCD		**26.394**	**0.981**	**71.634**	**3.280**	**-**
C13.5H05HPβCD		**21.625**	**0.964**	**77.331**	**2.668**	**-**
C14-HPβCD		**22.591**	**0.971**	**74.824**	**2.859**	**-**

*In all kinetic models, ***F*** is the fraction % of drug released in time **t**, k_0: release constant of zero-order, k_1: release constant of first-order, k_H: release constant of Higuchi, k_{HC} : release constant of Hixson-crowell, k_{KP}: release constant of Korsmeyer-Peppas Tlag, k_{HB}: release constant of Hopfenberg, k_{BL}: release constant of Baker-Lonsdale, k_{PS}: release constant of Peppas-Sahlin, ***n***: is the diffusional exponent indicating the drug-release mechanism, ***AIC:*** Akaike information criteria, ***MSC:*** Model selection criteria, R^2: coefficient of determination

5.7. Stability studies

5.7.1. Gelation temperature

The gelation temperature of the *in situ* gels kept at 4 °C and 25 °C was repeated on the 30th, 60th and 90th days. The results were shown at Table 5.23-5.24.

Table 5.23. *Gelation temperature of the in situ gel formulations stored at 4 °C*

	Gelation temperature °C (Mean ± SE)			
Code	**0 Day**	**30th Day**	**60th Day**	**90th Day**
F12.75H05	33 ± 0.00	No gelling	No gelling	No gelling
F14	26 ± 0.00	No gelling	No gelling	No gelling
C13.5H05MβCD	34 ± 0.00	36 ±0.00	35 ±0.00	35 ±0.00
C14H05MβCD	32 ± 0.00	32 ±0.00	33 ±0.00	33 ±0.00
C15MβCD	34 ± 0.00	33 ±0.00	33 ±0.00	33 ±0.00
C13.5H05HPβCD	30 ± 0.00	No gelling	No gelling	No gelling
C14HPβCD	30 ± 0.00	31 ±0.00	32 ±0.00	32 ±0.00

Table 5.24. *Gelation temperature of the in situ gel formulations stored at 25 °C* (Mean ± SE)

	Gelation temperature °C			
Code	**0 Day**	**30th Day**	**60th Day**	**90th Day**
F12.75H05	33 ±0.00	No gelling	No gelling	No gelling
F14	26 ±0.00	No gelling	No gelling	No gelling
C13.5H05MβCD	34 ±0.00	No gelling	No gelling	No gelling
C14H05MβCD	32 ±0.00	33 ±0.00	33 ±0.00	33 ±0.00
C15MβCD	34 ±0.00	33 ±0.00	34 ±0.00	34 ±0.00
C13.5H05HPβCD	30 ±0.00	34 ±0.00	34 ±0.00	34 ±0.00
C14HPβCD	30 ±0.00	31 ±0.00	31 ±0.00	31 ±0.00

5.7.2. Gelling capacity

For the *in situ* gels stored at 4°C and 25°C, the gelling capacity study was repeated on 90th day. The results shown in Table 5.25- 5.26.

Table 5.25. *Gelling capacity of the in situ gel formulations stored at 4°C (90th day)*

Code	Gelling capacity
F12.75H05	-
F14	-
C13.5H05MβCD	++
C14H05MβCD	++
C15MβCD	++
C13.5H05HPβCD	-
C14HPβCD	+++

Table 5.26. *Gelling capacity of the in situ gel formulations stored at 25 °C (90th day)*

Code	Gelling capacity
F12.75H05	-
F14	-
C13.5H05MβCD	-
C14H05MβCD	++
C15MβCD	++
C13.5H05HPβCD	+++
C14HPβCD	+++

5.7.3. pH measurements

The pH measurements of the formulations kept at 4 °C and 25 °C were done at 30th, 60th, and 90th days. The results shown in Table 5.27-5.28.

Table 5.27. *pH measurements of the in situ gel formulations stored at 4 °C*

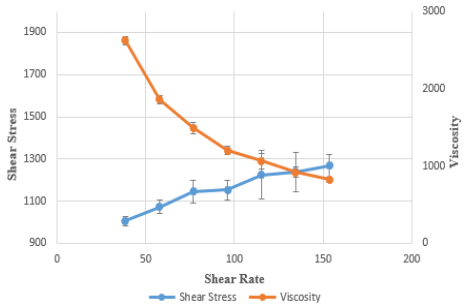
	pH value			
Code	**0 Day**	**30th Day**	**60th Day**	**90th Day**
F12.75H05	4.04 ± 0.00	4.53 ± 0.00	4.03 ± 0.00	3.96 ± 0.00
F14	4.50 ± 0.00	4.53 ± 0.00	4.06 ± 0.02	4.01 ± 0.00
C13.5H05MβCD	3.92 ± 0.00	4.91 ± 0.02	4.23 ± 0.04	4.25 ± 0.01
C14H05MβCD	4.18 ± 0.00	4.89 ± 0.01	4.30 ± 0.00	4.28 ± 0.01
C15MβCD	4.22 ± 0.00	3.96 ± 0.01	3.89 ± 0.00	3.86 ± 0.00
C13.5H05HPβCD	3.86 ± 0.00	4.01 ± 0.01	3.82 ± 0.00	3.82 ± 0.01
C14HPβCD	4.37 ± 0.00	4.01 ±0.00	3.91 ± 0.00	3.96 ± 0.01

Table 5.28. *pH measurements of the in situ gel formulations stored at 25 °C*

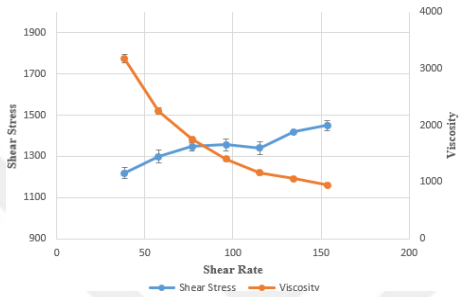
	pH value			
Code	**0 Day**	**30th Day**	**60th Day**	**90th Day**
F12.75H05	4.04 ± 0.00	4.22 ± 0.01	4.32 ± 0.03	4.30 ± 0.01
F14	4.50 ± 0.00	4.15 ± 0.00	4.09 ± 0.00	4.12 ± 0.01
C13.5H05MβCD	3.92 ± 0.00	4.23 ± 0.00	4.14 ± 0.00	4.12 ± 0.01
C14H05MβCD	4.18 ± 0.00	4.24 ± 0.01	4.36 ± 0.00	4.30 ± 0.01
C15MβCD	4.22 ± 0,00	3.88 ± 0.01	3.77 ± 0.00	3.73 ± 0.00
C13.5H05HPβCD	3.86 ± 0,00	3.82 ± 0.01	3.74 ± 0.00	3.72 ± 0.01
C14HPβCD	4.37 ± 0.00	3.83 ± 0.00	3.80 ± 0 .00	3.75 ± 0.00

5.7.4. *Rheological studies*

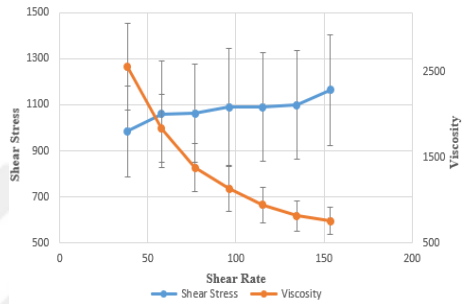
The rheological pattern of the *in situ* gel systems stored at 4 °C and 25 °C were examined at 30th, 60th, and 90th days at two distinct temperatures, 25 °C and 37 °C. The rheological curves were shown in Figure 5.33 - Figure 5.46.



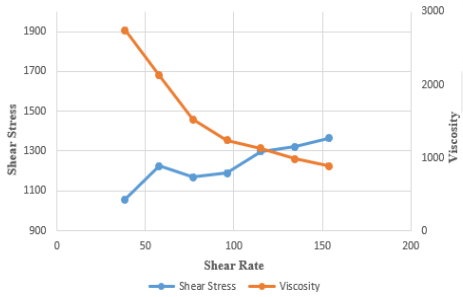
F12.75H05, 0th day



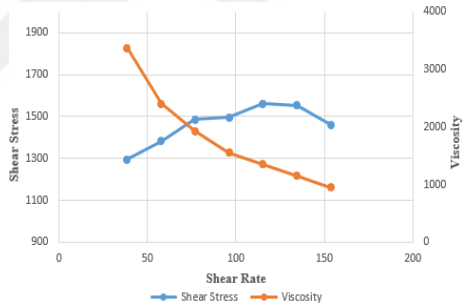
Stored at 4 °C, 30th day



Stored at 25 °C, 30th day



Stored at 4 °C, 60th day



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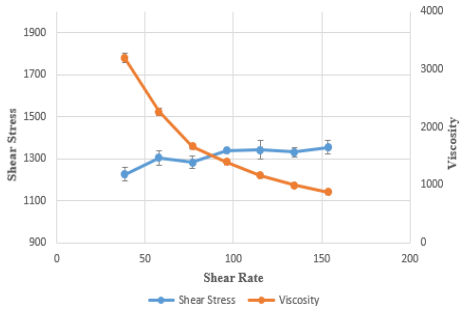
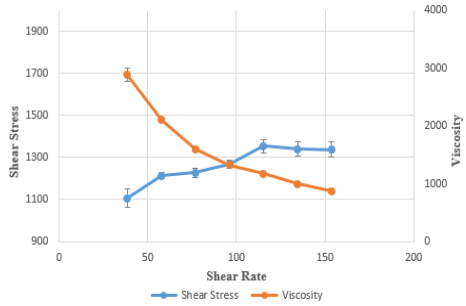
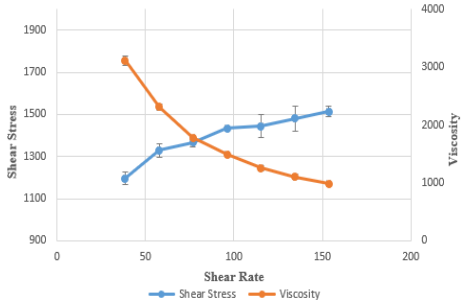


Stored at 4 °C, 90th day

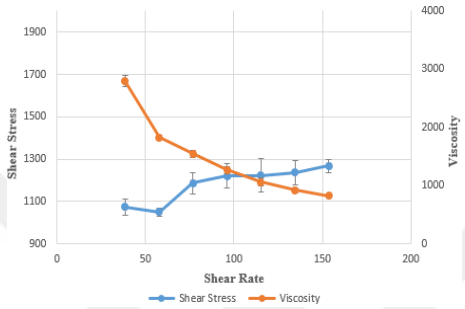


Stored at 25 °C, 90th day

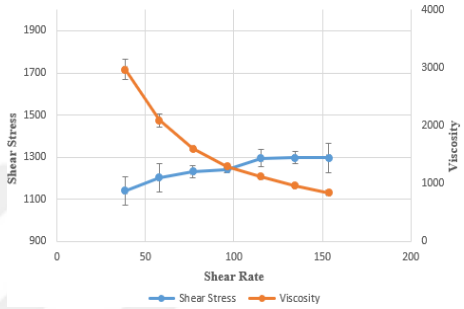
Figure 5.33. *Rheograms at 25 °C of the F12.75H05 formulation stored at 4°C and 25°C (blue line: shear stress, orange line: viscosity)*



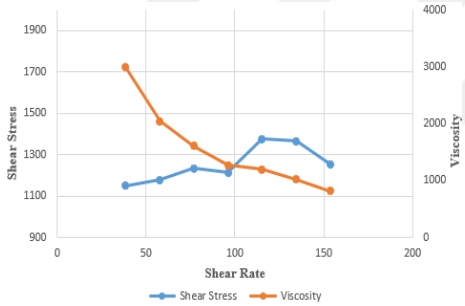
F12.75H05, 0th day



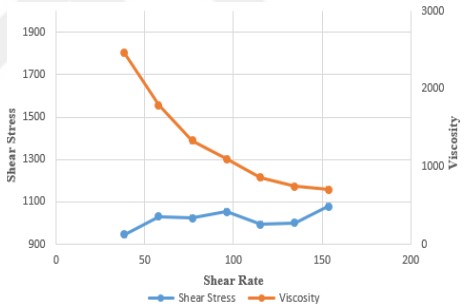
Stored at 4 °C, 30th day



Stored at 25 °C, 30th day



Stored at 4 °C, 60th day



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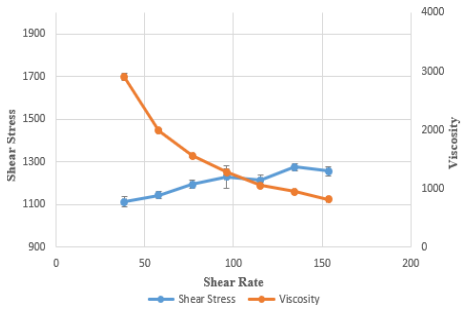

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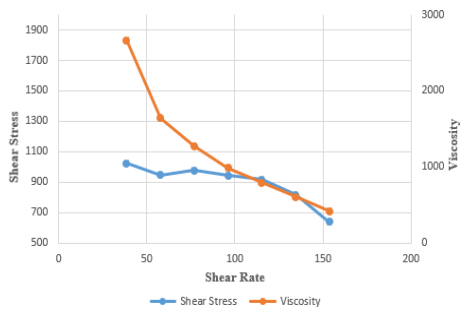
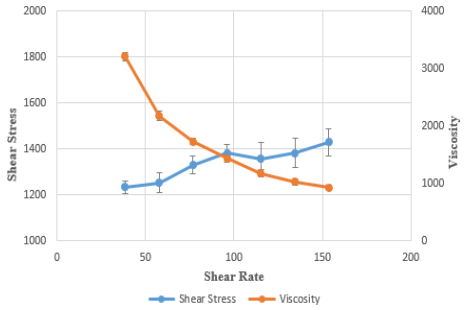
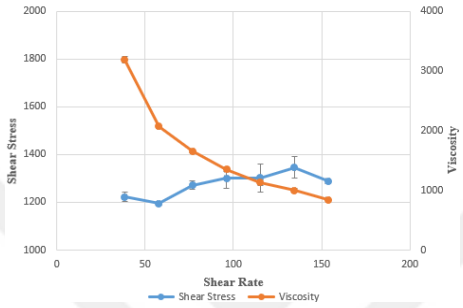


Stored at 25 °C, 90th day

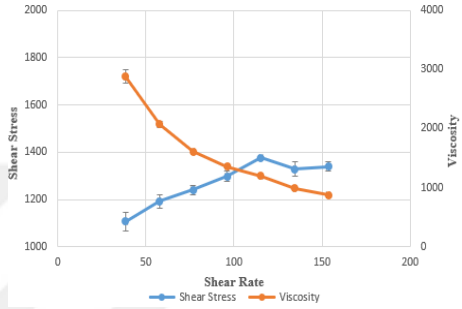
Figure 5.34. *Rheograms at 37 °C of the F12.75H05 formulation stored at 4°C and 25°C (blue line: shear stress, orange line: viscosity)*



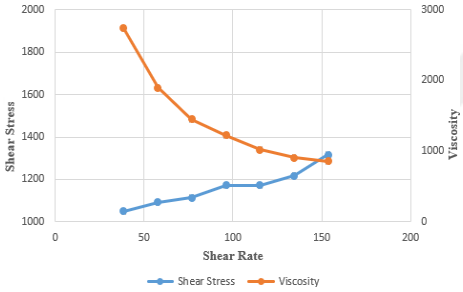
F14, 0th day



Stored at 4 °C, 30th day



Stored at 25 °C, 30th day



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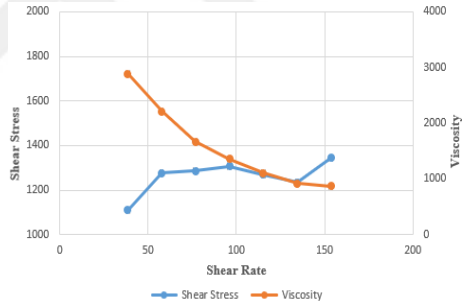

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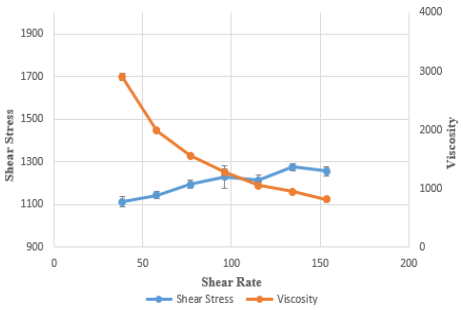

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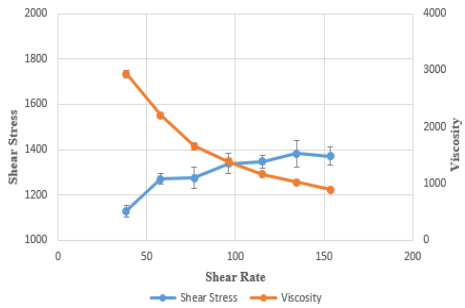
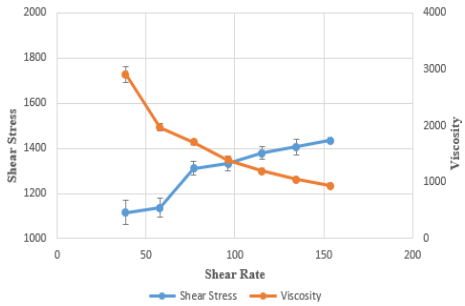
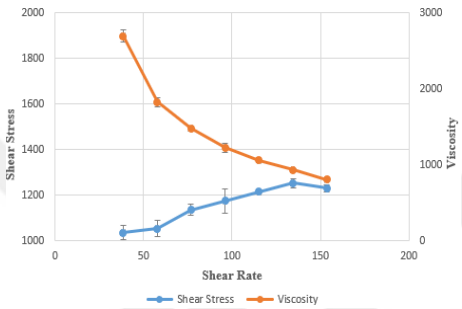


Stored at 25 °C, 90th day

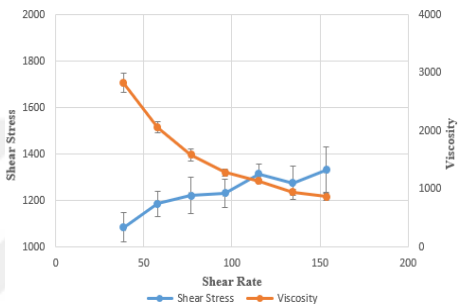
Figure 5.35. *Rheograms at 25 °C of the F14 formulation stored at 4 °C and 25 °C (blue line: shear stress, orange line: viscosity)*



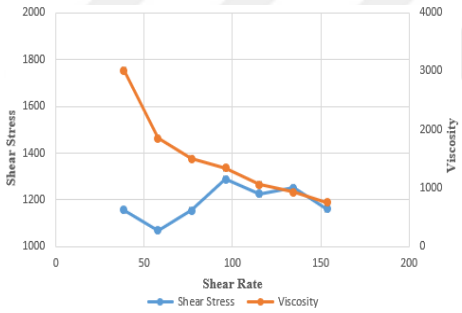
F14, 0th day



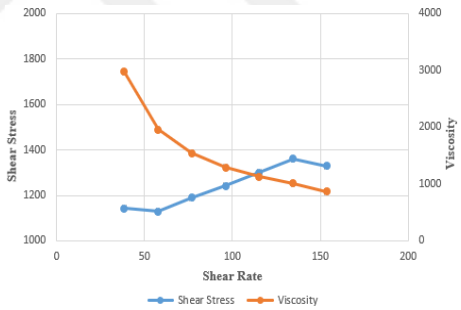
Stored at 4 °C, 30th day



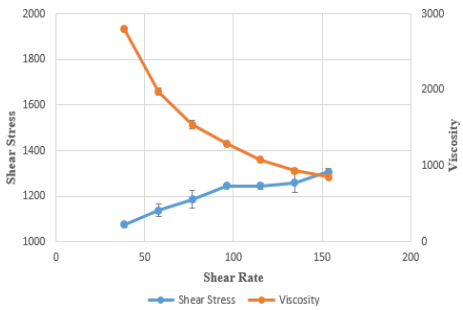
Stored at 25 °C, 30th day



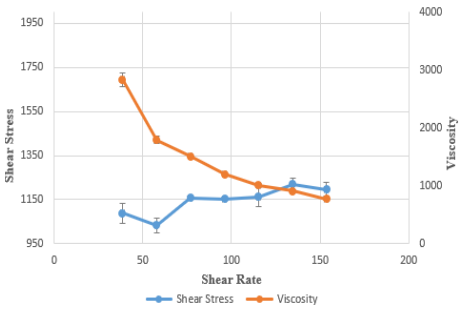
Stored at 4 °C, 60th day



Stored at 25 °C, 60th day

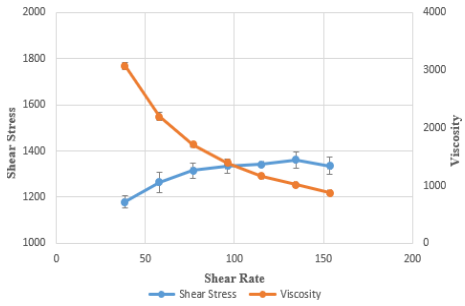


Stored at 4 °C, 90th day

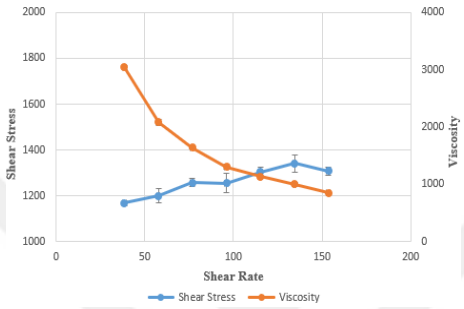


Stored at 25 °C, 90th day

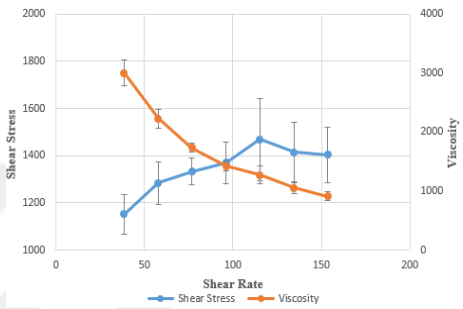
Figure 5.36. *Rheograms at 37°C of the F14 formulation stored at 4°C and 25°C (blue line: shear stress, orange line: viscosity)*



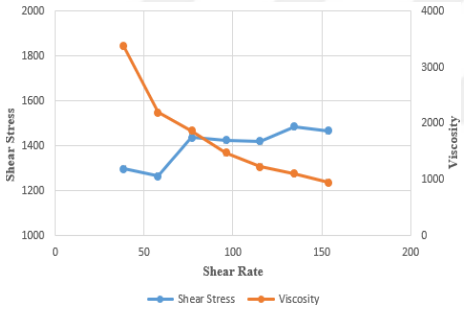
C13.5H05MβCD, 0th day



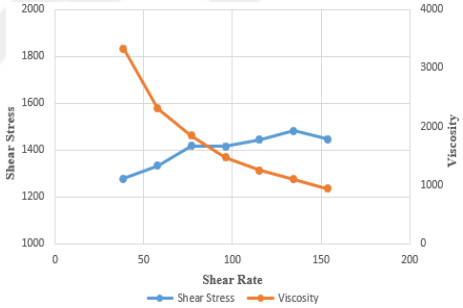
Stored at 4 °C, 30th day



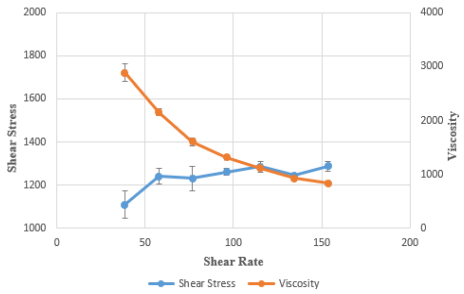
Stored at 25 °C, 30th day



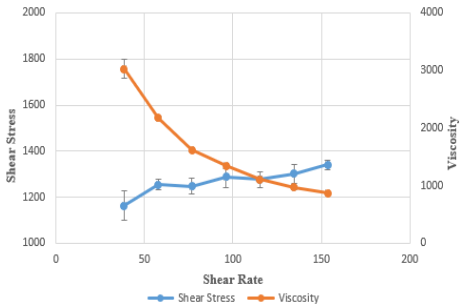
Stored at 4 °C, 60th day



Stored at 25 °C, 60th day

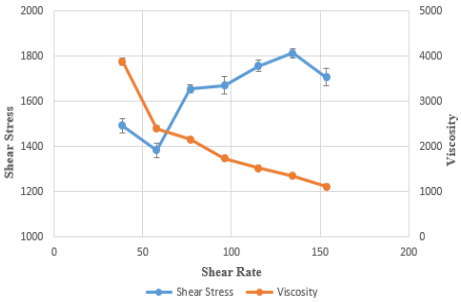


Stored at 4 °C, 90th day

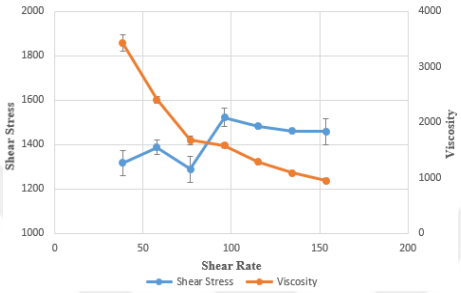


Stored at 25 °C, 90th day

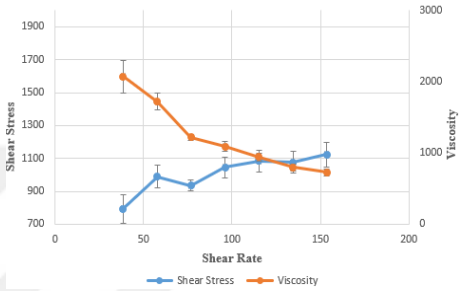
Figure 5.37. *Rheograms at 25°C of the C13.5H05MβCD formulation stored at 4°C and 25°C(blue line: shear stress, orange line: viscosity)*



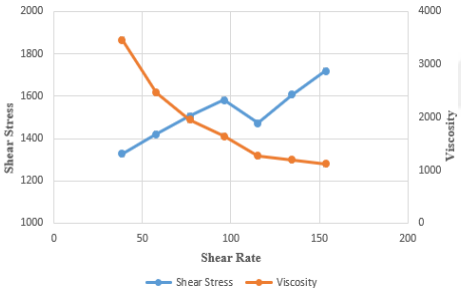
C13.5H05MβCD, 0th day



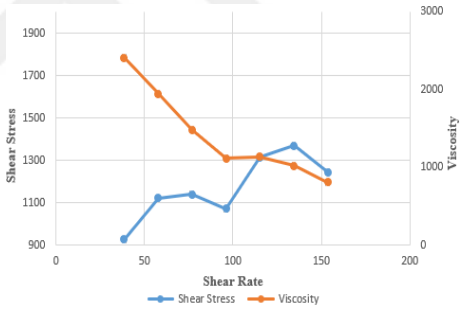
Stored at 4 °C, 30th day



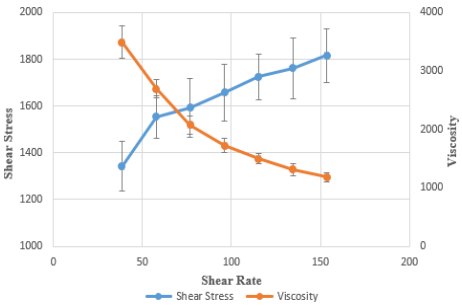
Stored at 25 °C, 30th day



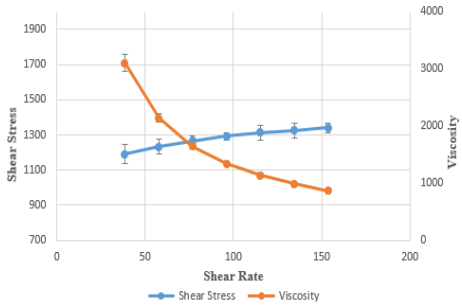
Stored at 4 °C, 60th day



Stored at 25 °C, 60th day

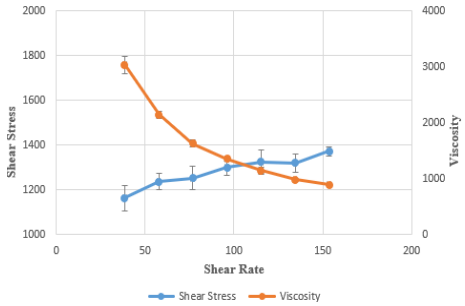


Stored at 4 °C, 90th day

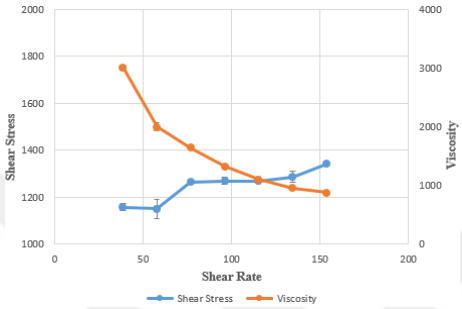


Stored at 25 °C, 90th day

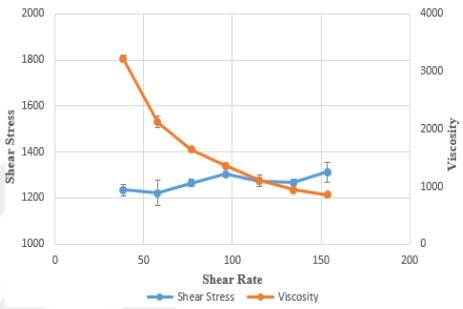
Figure 5.38. *Rheograms at 37°C of the C13.5H05 MβCD formulation stored at 4°C and 25°C (blue line: shear stress, orange line: viscosity)*



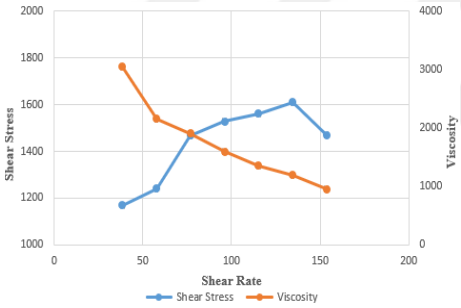
C14H05 MβCD, 0th day



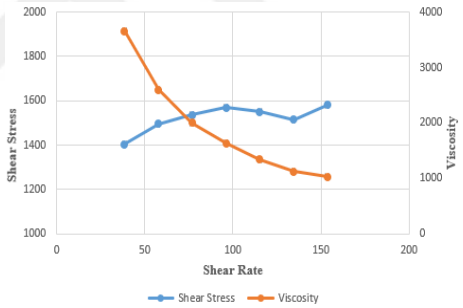
Stored at 4 °C, 30th day



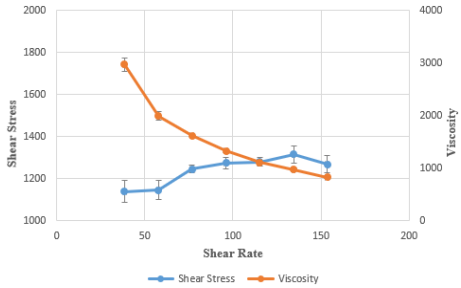
Stored at 25 °C, 30th day



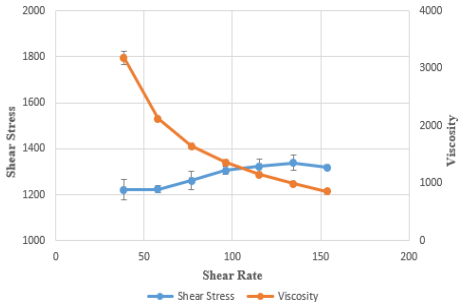
Stored at 4 °C, 60th day



Stored at 25 °C, 60th day

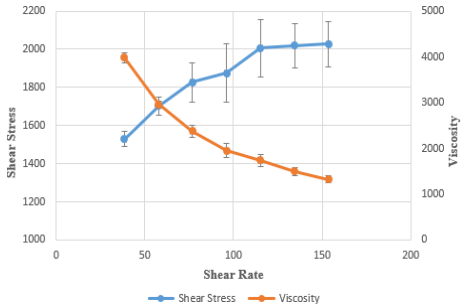


Stored at 4 °C, 90th day

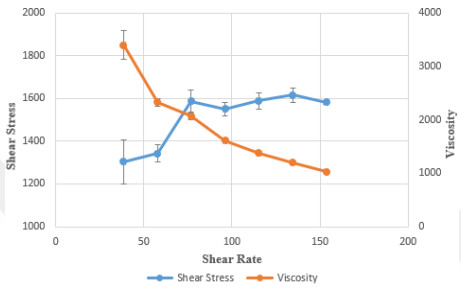


Stored at 25 °C, 90th day

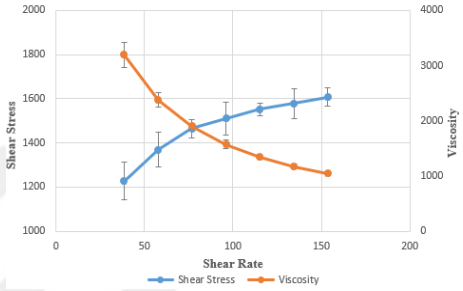
Figure 5.39. *Rheograms at 25°C of the C14H05 MβCD formulation stored at 4°C and 25°C (blue line: shear stress, orange line: viscosity)*



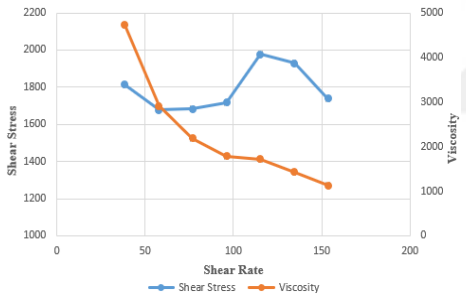
C14H05MβCD, 0th day



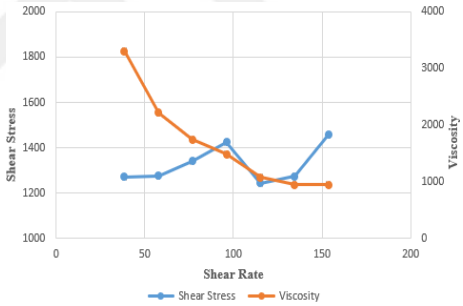
Stored at 4 °C, 30th day



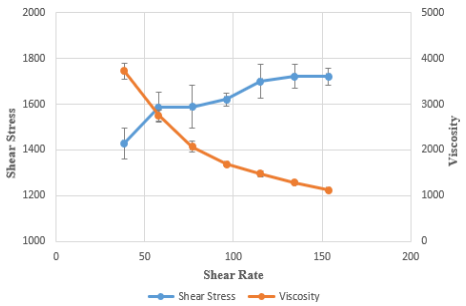
Stored at 25 °C, 30th day



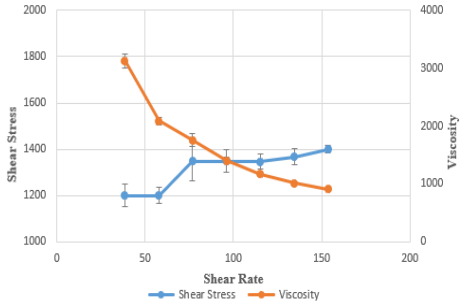
Stored at 4 °C, 60th day



Stored at 25 °C, 60th day

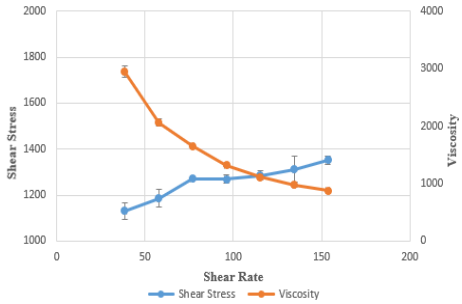


Stored at 4 °C, 90th day

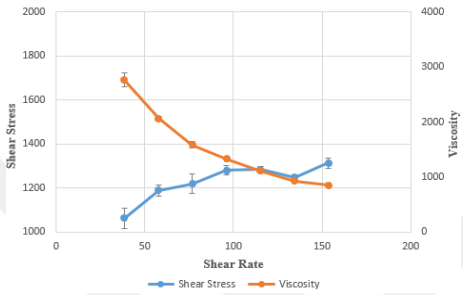


Stored at 25 °C, 90th day

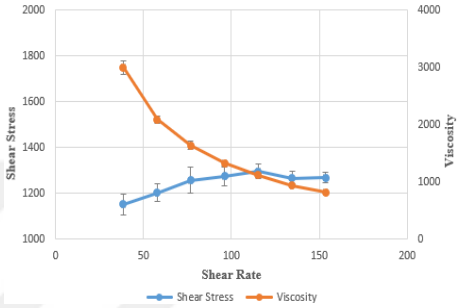
Figure 5.40. *Rheograms at 37°C of the C14H05 MβCD formulation stored at 4°C and 25°C (blue line: shear stress, orange line: viscosity)*



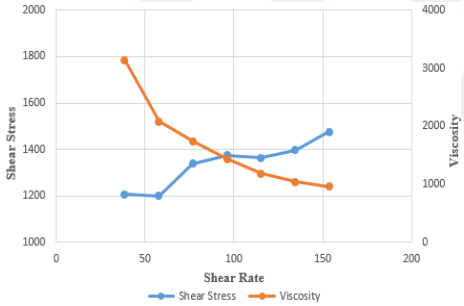
C15MβCD, 0th day



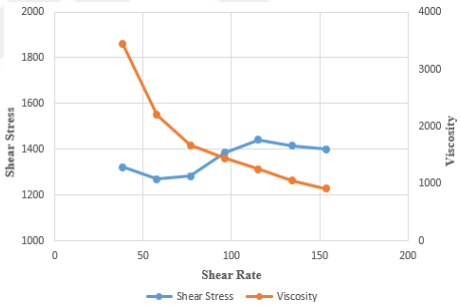
Stored at 4 °C, 30th day



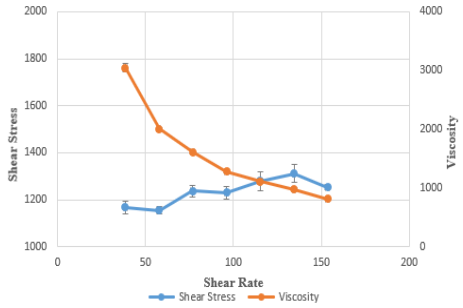
Stored at 25 °C, 30th day



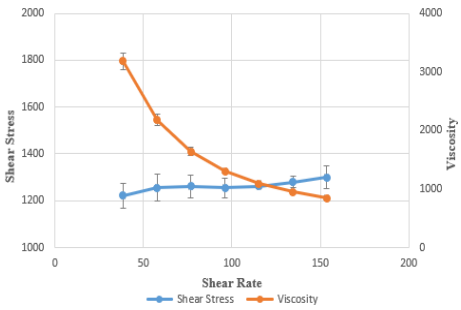
Stored at 4 °C, 60th day



Stored at 25 °C, 60th day

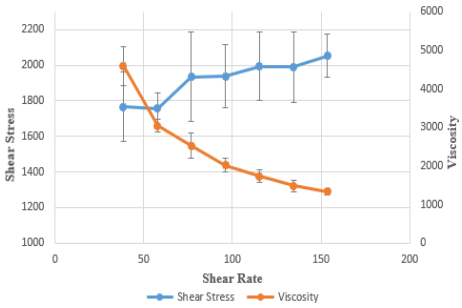


Stored at 4 °C, 90th day

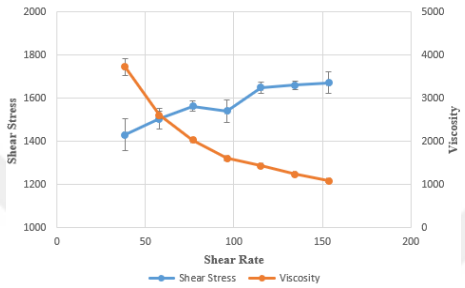


Stored at 25 °C, 90th day

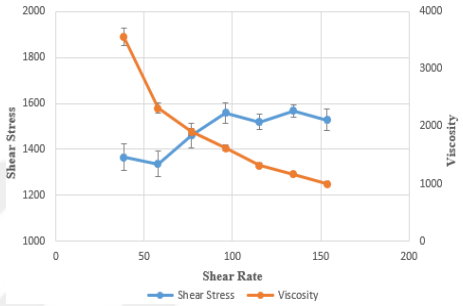
Figure 5.41. *Rheograms at 25°C of the C15MβCD formulation stored at 4°C and 25°C (blue line: shear stress, orange line: viscosity)*



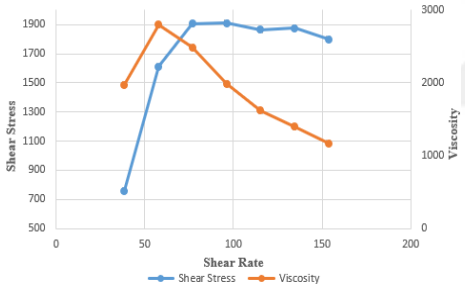
C15MβCD, 0th day



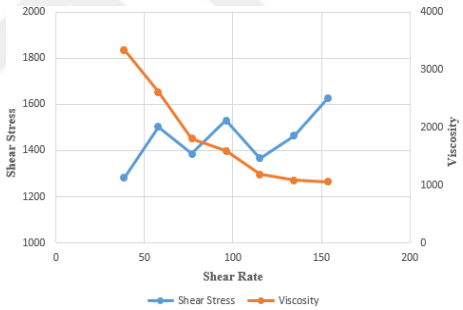
Stored at 4 °C, 30th day



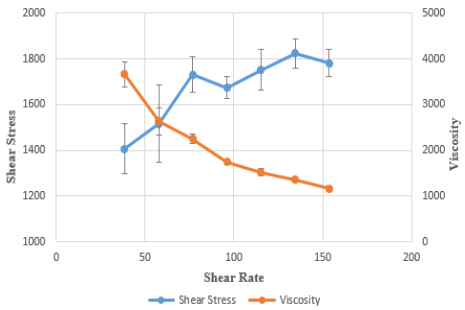
Stored at 25 °C, 30th day



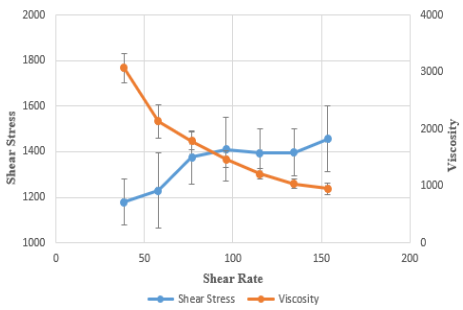
Stored at 4 °C, 60th day



Stored at 25 °C, 60th day

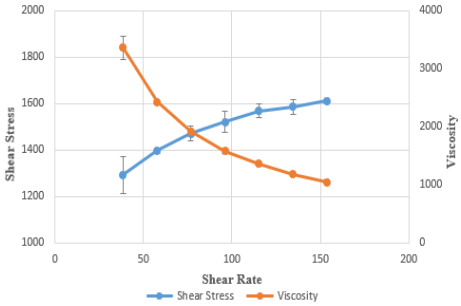


Stored at 4 °C, 90th day

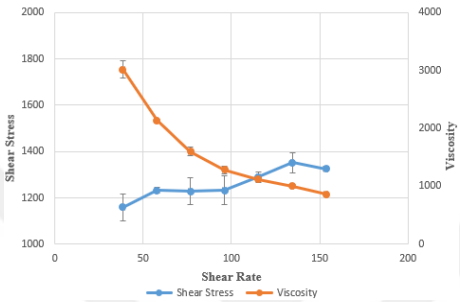


Stored at 25 °C, 90th day

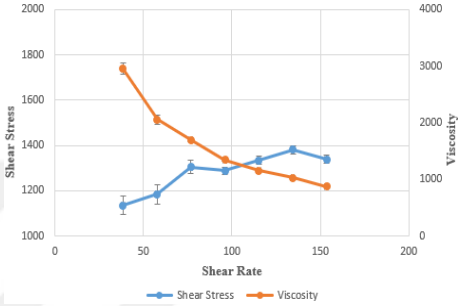
Figure 5.42. *Rheograms at 37°C of the C15MβCD formulation stored at 4°C and 25°C (blue line: shear stress, orange line: viscosity)*



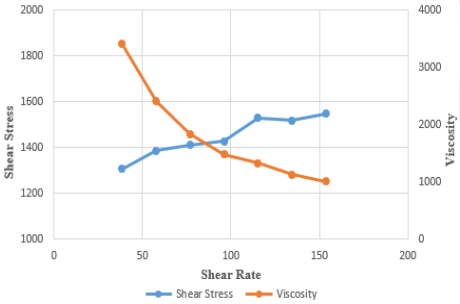
C13.5H05 HPβCD, 0th day



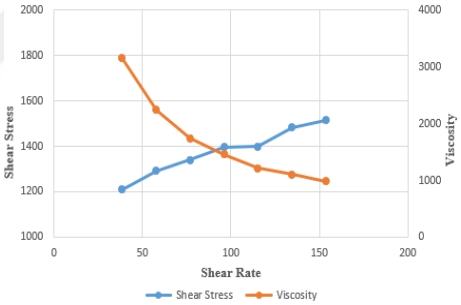
Stored at 4 °C, 30th day



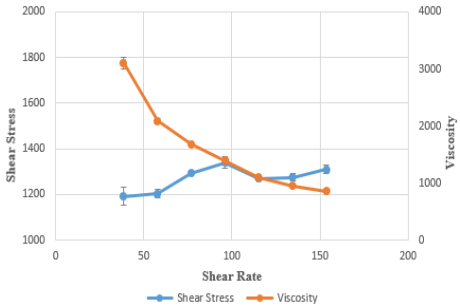
Stored at 25 °C, 30th day



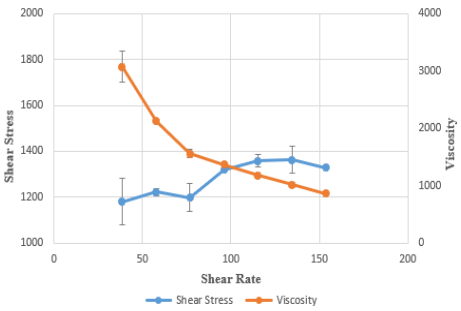
Stored at 4 °C, 60th day



Stored at 25 °C, 60th day



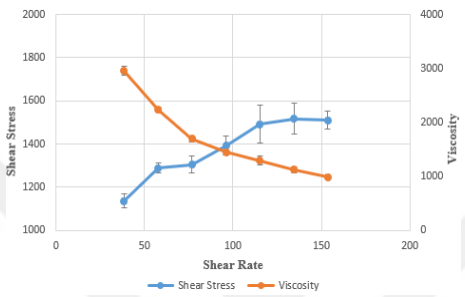
Stored at 4 °C, 90th day



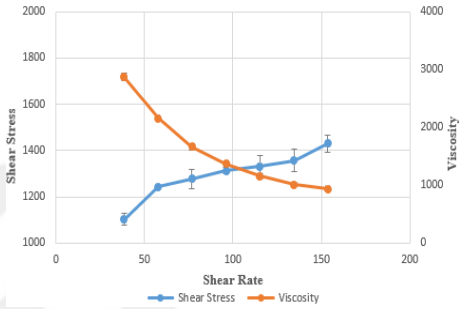
Stored at 25 °C, 90th day

Figure 5.43. *Rheograms at 25°C of the C13.5H05HPβCD formulation stored at 4°C and 25°C (blue line: shear stress, orange line: viscosity)*

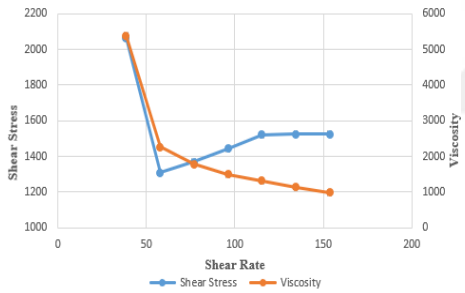
C13.5H05HPβCD, 0th day



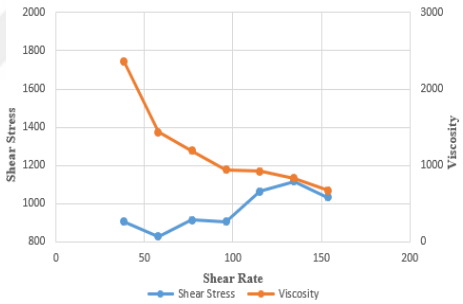
Stored at 4 °C, 30th day



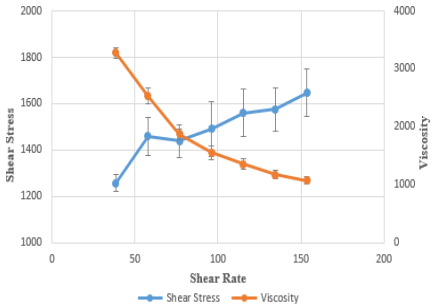
Stored at 25 °C, 30th day



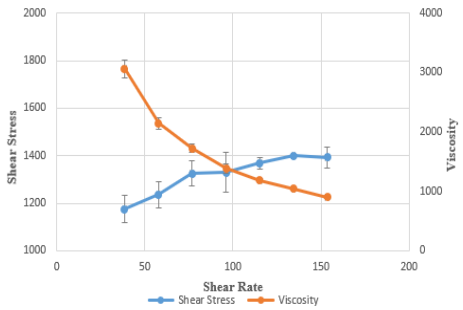
Stored at 4 °C, 60th day



Stored at 25 °C, 60th day

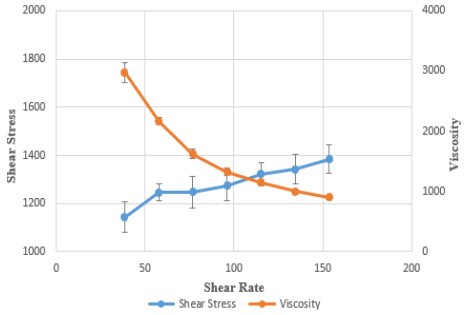


Stored at 4 °C, 90th day

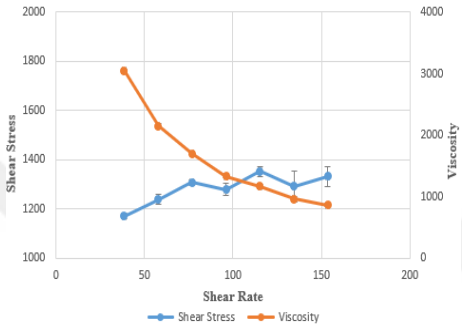


Stored at 25 °C, 90th day

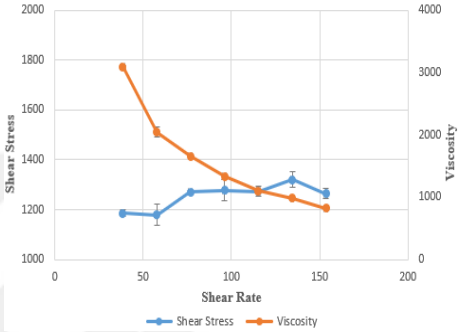
Figure 5.44. *Rheograms at 37°C of the C13.5H05HPβCD formulation stored at 4°C and 25°C (blue line: shear stress, orange line: viscosity)*



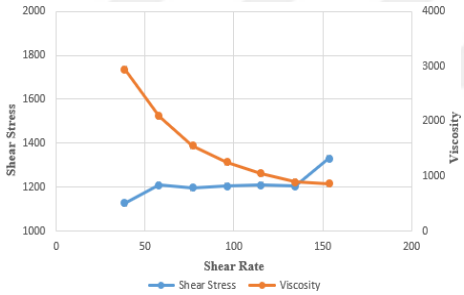
C14HPβCD, 0th day



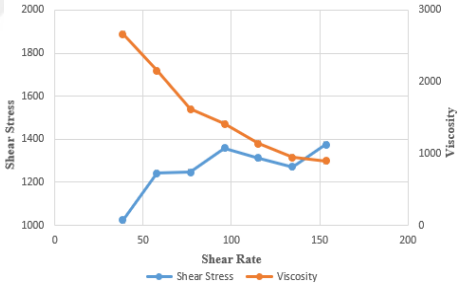
Stored at 4 °C, 30th day



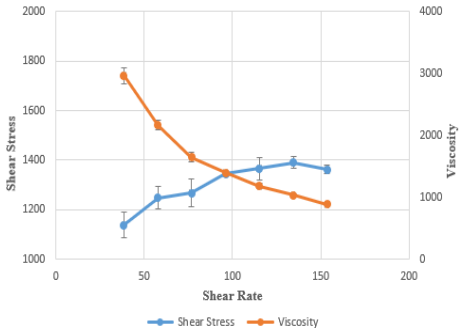
Stored at 25 °C, 30th day



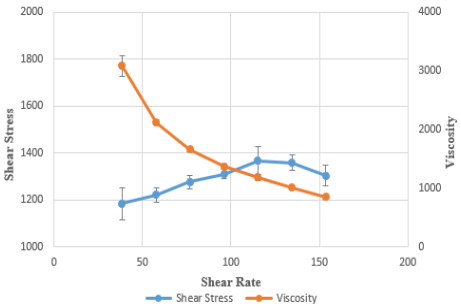
Stored at 4 °C, 60th day



Stored at 25 °C, 60th day

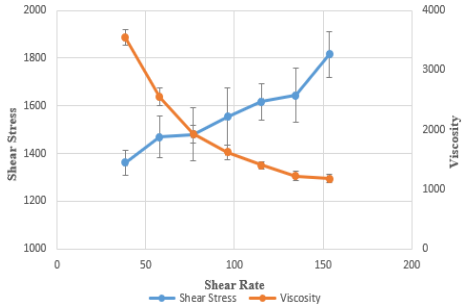


Stored at 4 °C, 90th day

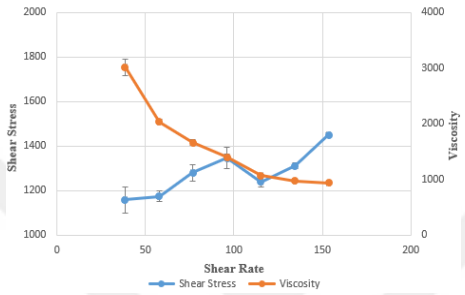


Stored at 25 °C, 90th day

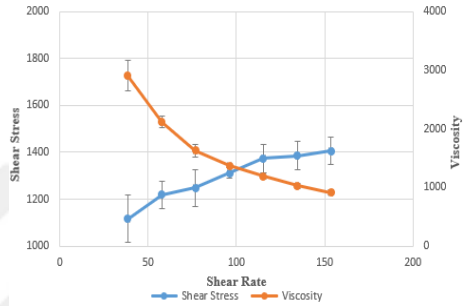
Figure 5.45. *Rheograms at 25°C of the C14 HPβCD formulation stored at 4°C and 25°C (blue line: shear stress, orange line: viscosity)*



C14HPβCD, 0th day

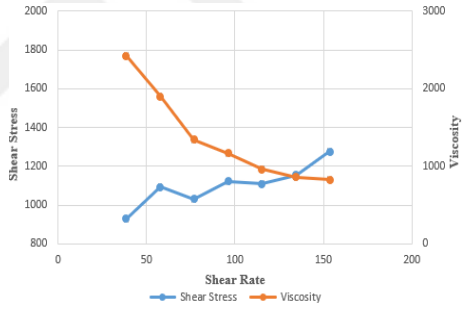


Stored at 4 °C, 30th day

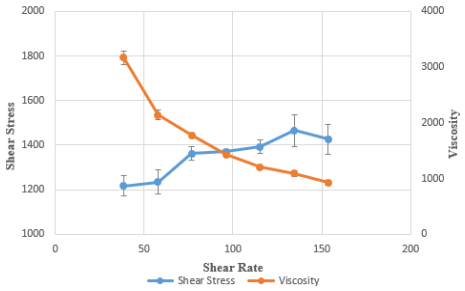


Stored at 25 °C, 30th day

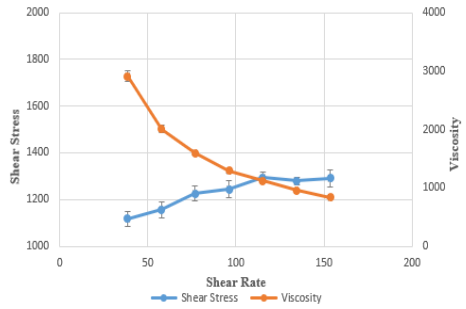
Stored at 4 °C, 60th day



Stored at 25 °C, 60th day



Stored at 4 °C, 90th day



Stored at 25 °C, 90th day

Figure 5.46. *Rheograms at 37 °C of the C14 HPβCD formulation stored at 4 °C and 25 °C(blue line: shear stress, orange line: viscosity)*

5.7.5. Determination of drug content (DC) %

Determination of the drug content of the *in situ* gel formulations kept at 4°C and 25°C were carried out at 30th, 60th, and 90th days by the validated HPLC method. The results shown in Table 5.29-5.30.

Table 5.29. *Drug content % of in situ gel formulations kept at 4 °C (n=3)*

	Drug Content % (mean ± SE)			
Code	**0 day**	**30th Day**	**60th Day**	**90th Day**
F12.75H05	0.615 ± 0.044	0.673 ± 0.153	0.681 ± 0.125	0.618 ± 0.014
F14	0.749 ± 0.096	0.793 ± 0.038	0.653 ± 0.018	0.660 ± 0.035
C13.5H05MβCD	0.862 ± 0.150	0.835 ± 0.164	0.840 ± 0.060	0.818 ± 0.058
C14H05MβCD	0.738 ± 0.148	0.780 ± 0.097	0.759 ± 0.038	0.744 ± 0.067
C15MβCD	0.716 ± 0.075	0.761 ± 0.128	0.776 ± 0.142	0.759 ± 0.014
C13.5H05HPβCD	0.655 ± 0.142	0.685 ± 0.115	0.636 ± 0.160	0.611 ± 0.231
C14HPβCD	0.652 ± 0.253	0.678 ± 0.107	0.657 ± 0.102	0.659 ± 0.118

Table 5.30. *Drug content % of in situ gel formulations kept at 25 °C (n=3)*

	Drug Content % (mean ± SE)			
Code	**0 day**	**30th Day**	**60th Day**	**90th Day**
F12.75H05	0.615 ± 0.044	0.602± 0.050	0.670 ± 0.020	0.652 ± 0.145
F14	0.749 ± 0.096	0.670 ± 0.009	0.736 ± 0.039	0.653 ± 0.019
C13.5H05MβCD	0.862 ± 0.150	0.848 ± 0.185	0.853 ± 0.143	0.799 ± 0.265
C14H05MβCD	0.738 ± 0.148	0.728 ± 0.046	0.736 ± 0.107	0.803 ± 0.258
C15MβCD	0.716 ± 0.075	0.745 ± 0.097	0.732 ± 0.037	0.753 ± 0.027
C13.5H05HPβCD	0.655 ± 0.142	0.630 ± 0.082	0.716 ± 0.171	0.708 ± 0.262
C14HPβCD	0.652 ± 0.253	0.688 ± 0.094	0.634 ± 0.077	0.660 ± 0.014

5.8. Antimicrobial Efficacy Testing of In Situ Gel Formulations

Antimicrobial efficacy test was performed in the procedue described in 4.5.5. Section and results were given in Table 5.31 and Figure 5.47 – 5.52.

Table 5.31. *Inhibition zone diameters of the in situ gel formulations against C. albicans, C. glabrata and C.krusei.*

	Zone diameter mm ± SDV		
	Microrganisms		
Code	***C. albicans***	***C. glabrata***	***C. krusei***
F12.75H05	39.66 ± 0.47	39.00 ± 2.94	37.66 ± 0.94
F14	33.66 ± 1.25	33.66 ± 2.16	35.66 ± 2.87
C13.5H05 MβCD	40.66 ± 0.00	40.00 ± 2.05	39.66 ± 0.94
C14H05 MβCD	38.33 ± 2.87	38.33 ± 4.50	36.33 ± 0.47
C15 MβCD	40.00 ± 1.25	39.33 ± 1.70	40.33 ± 0.94
C13.5H05 HPβCD	40.00 ± 1.70	42.33 ± 2.36	38.33 ± 0.47
C14 HPβCD	37.33 ± 0.82	38.33 ± 2.05	40.66 ± 0.47

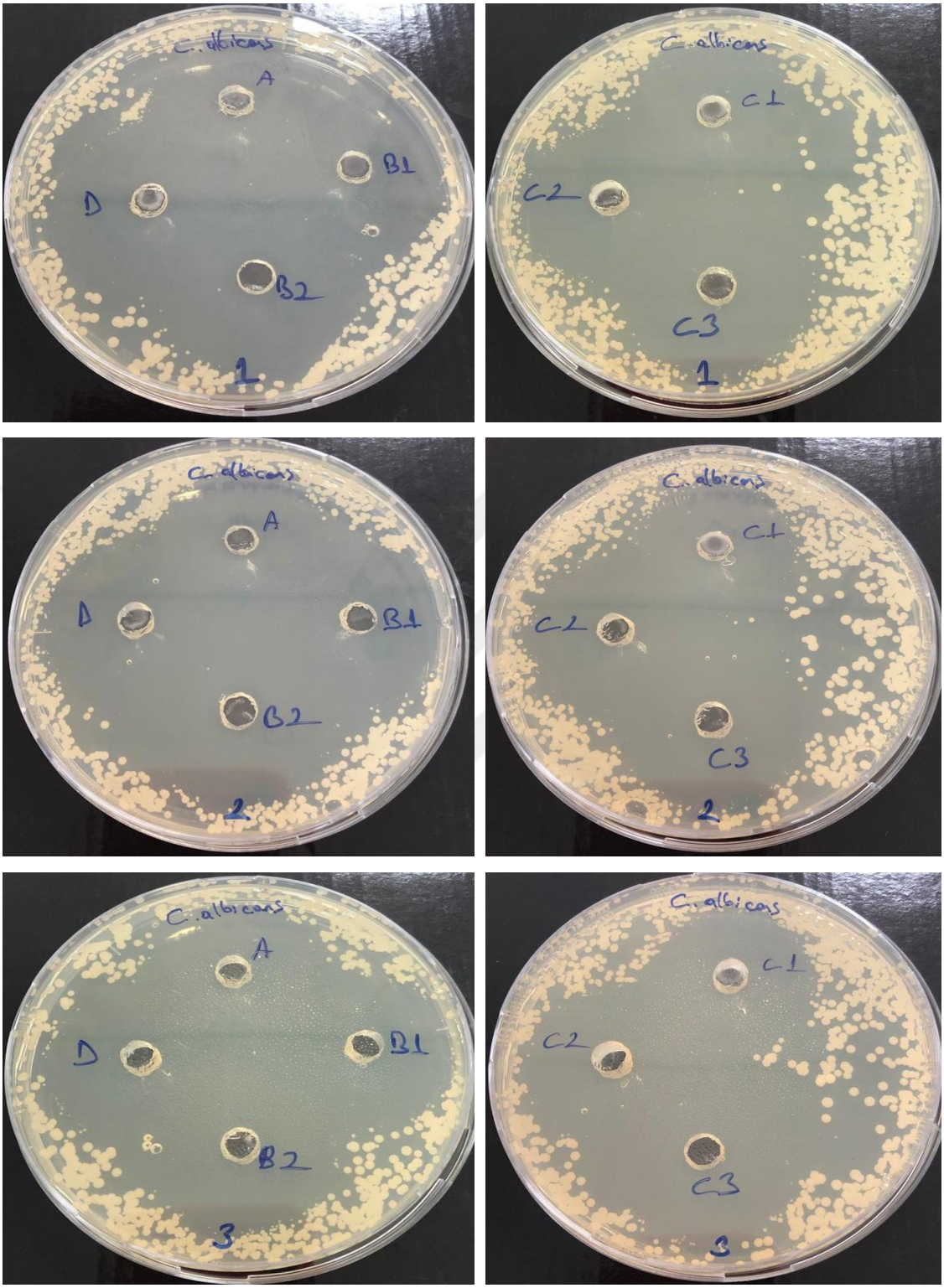


Figure 5.47. *Zone views of in situ gel formulations against C. albicans (A: F12.75H05, B_1: C13.5H05MβCD, B_2: C13.5H05HPβCD, C_1: F14, C_2: C14 HPβCD, $C_{3.}$: C14H05MβCD, D: C15MβCD)*

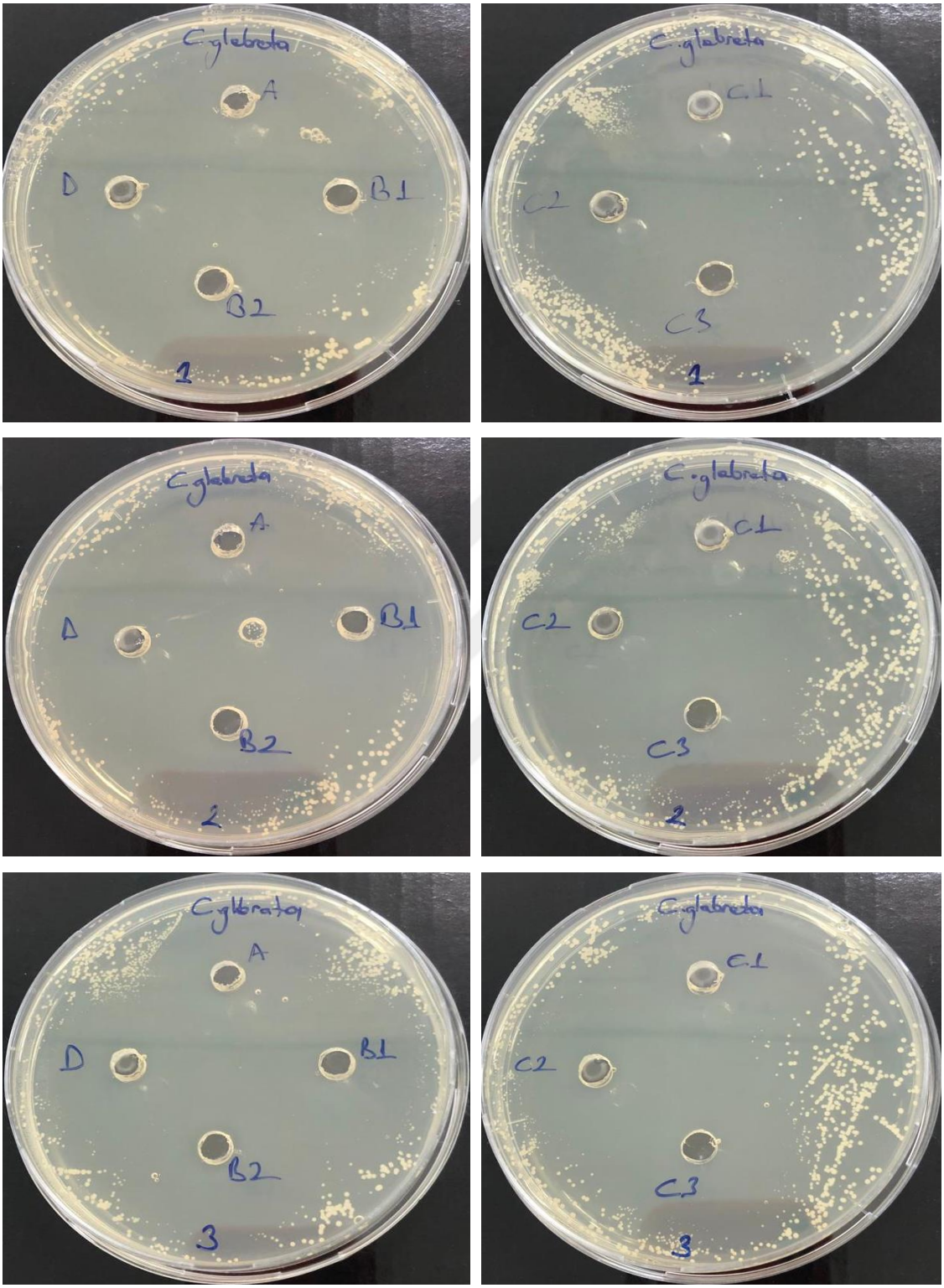


Figure 5.48. *Zone views of in situ gel formulations against C. glabrata (A: F12.75H05, B_1: C13.5H05MβCD, B_2: C13.5H05HPβCD, C_1: F14, C_2: C14 HPβCD, $C_{3.}$: C14H05MβCD, D: C15MβCD)*

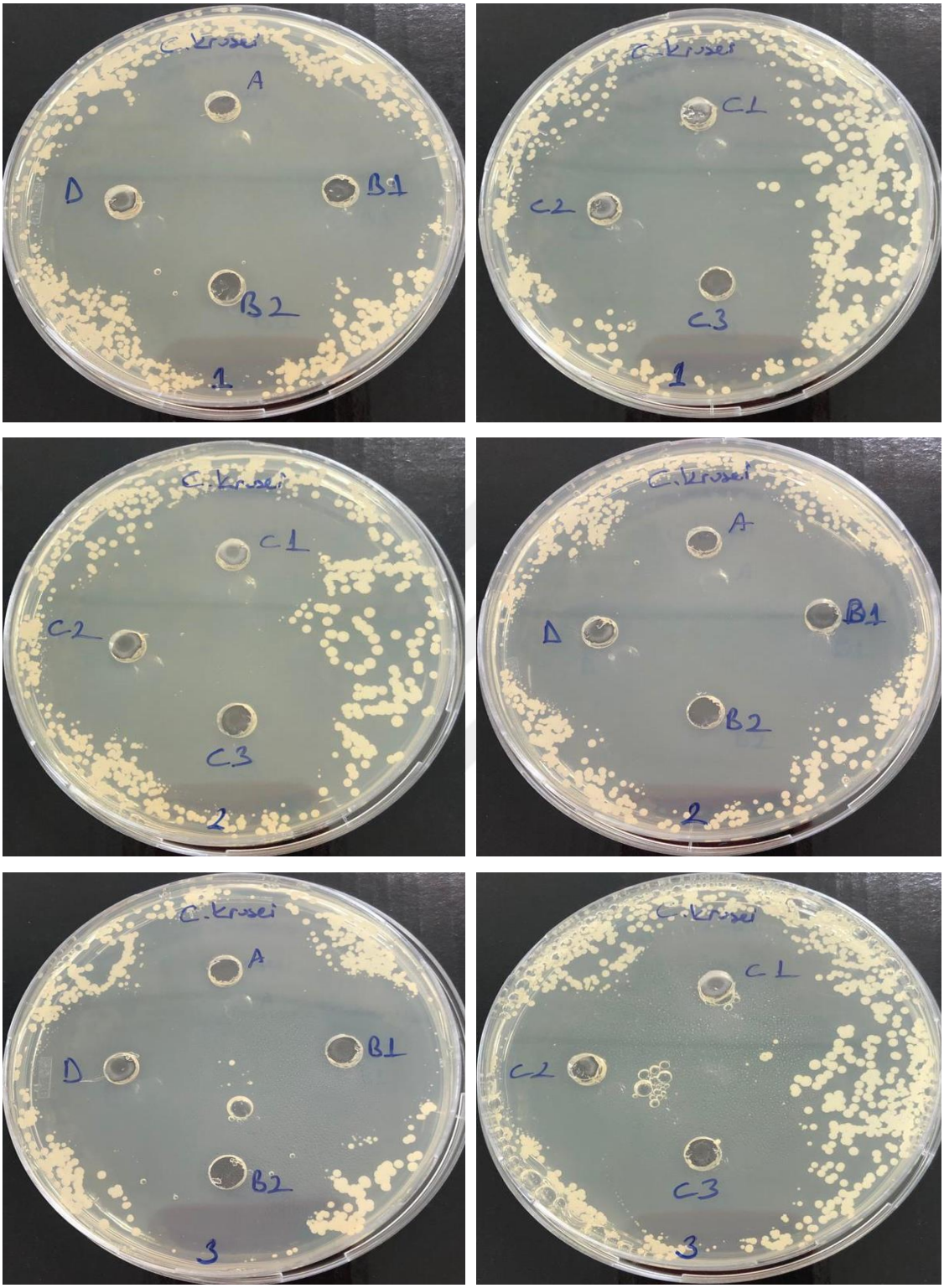


Figure 5.49. *Zone views of in situ gel formulations against C. krusei (A: F12.75H05, B_1: C13.5H05MβCD, B_2: C13.5H05HPβCD, C_1: F14, C_2: C14HPβCD, C_3: C14H05MβCD, D: C15MβCD)*

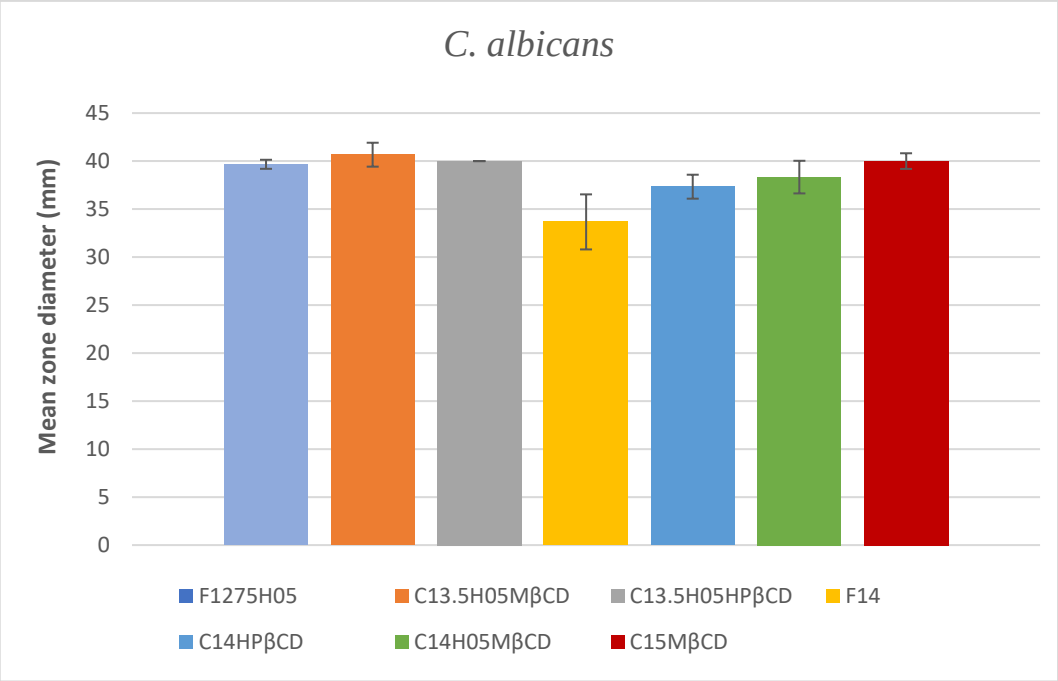


Figure 5.50. *Antimicrobial activity of in situ gel formulations against C. albicans*

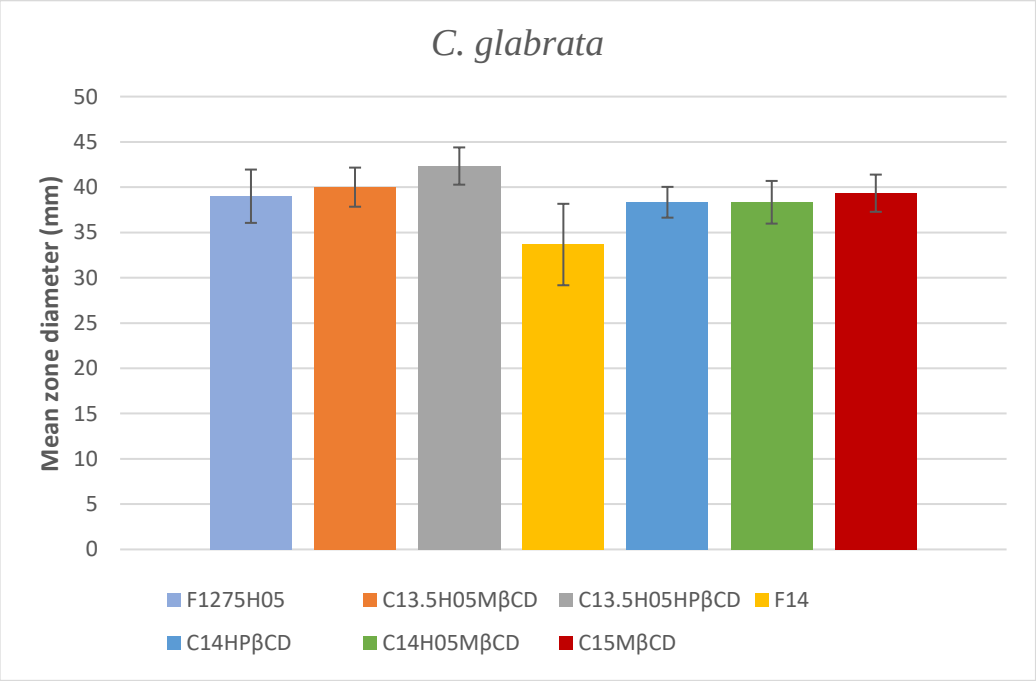


Figure 5.51. *Antimicrobial activity of in situ gel formulations against C. glabrata*

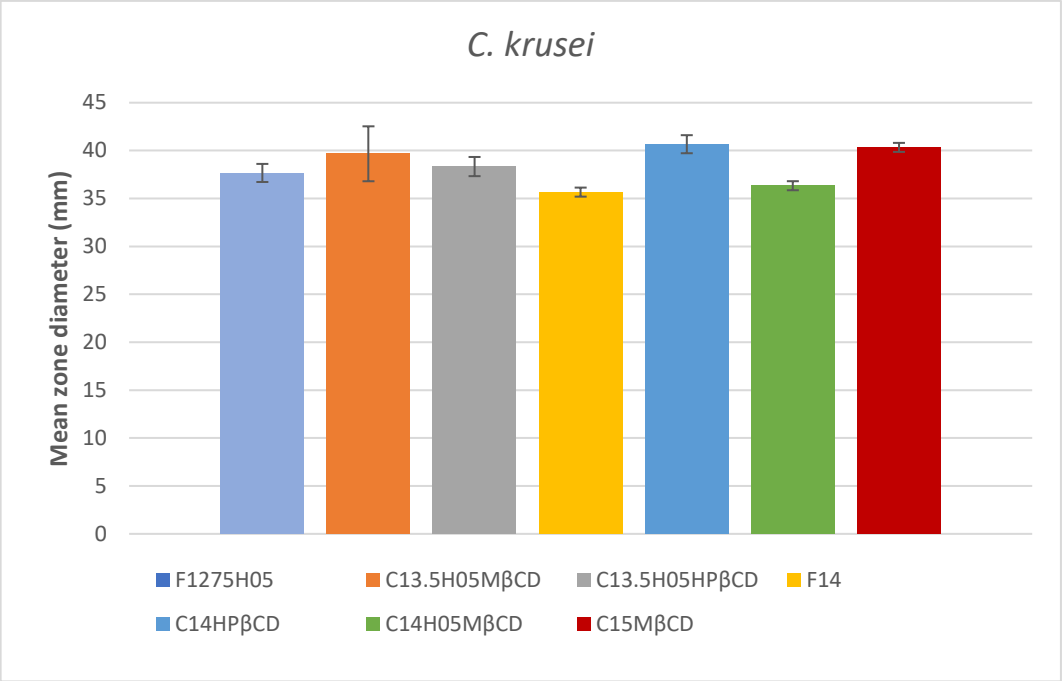


Figure 5.52. *Antimicrobial activity of in situ gel formulations against C. krusei*

6. DISCUSSION

6.1. Analytical Validation Studies

All the quantification studies in this thesis were carried out by the HPLC method. HPLC has been preferred because of its high sensitivity, rapid to get the results, ease of use and being a method that has been used for many years. In HPLC studies, suitable peak morphology and retention time are obtained by using mobile phase containing methanol:potassium dihydrogen phosphate buffer (0.05M) (50:50, v/v). For the peak morphology and retention time, a flow rate of 0.5 $ml.min^{-}1$ was used in accordance with the properties of the column. The injection volume was determined as 20 µL. Column temperature was set to 40ºC. Based on practical experiments, measurements were made at 210nm wavelength, 250 mm x 4.6 mm x 5 µm C_{18} column was used.

The linearity, accuracy, precision, range, sensitivity, selectivity and stability parameters of the analytical method was evaluated according to the ICH criteria and the method was proven to be safe for active substance quantitation in the used method conditions.

During the validation studies of HPLC quantification method, linearity equation of ISN was determined to be y= 11799.9166x – 65157.5918 (r^2=0.9998). Recovery was calculated in the range of 101.19 - 99.24%. It is stated in the literature that percentage of recovery values should be in the interval in 98-102% (Shabir, 2003). When the precision results were evaluated, it was found that the RSD% values were found for four different concentrations studied were below 2%. For this reason, it was decided that the precision of the method was within the desired ranges and in accordance with the literature (ICH, 1995). LOD was calculated to be 1.3047 $µg.mL^{-1}$ while LOQ was 3.9536 $µg.mL^{-1}$. With the chromatograms obtained as a result of the selectivity analysis, it has been shown that ISN was separated successfully. It has been observed that it is selective for ISN since the system is not affected by other excipients used in the formulations and factors such as dilution solutions. Therefore, HPLC quantification system for ISN was found to be linear, precise, accurate and selective (Yurtdaş-Kırımlıoğlu, 2015).

In order to get accurate results from the analysis, the stability of the analyte in the working conditions should not change. Within the scope of the stability study, in the media (SVF) used in *in vitro* release studies, the working time (96 hours) and the ISN solutions kept at the operating temperature (37 ± 0.5 ° C) at certain time intervals and at the end of 72 hours, no decrease was observed in the initial concentrations of the active

substance. The stability of the active substance has been determined in *in vitro* release medium.

6.2. Formulation of Inclusion Complexes

The natural structure of the CD (native chemically modified, crystalline or amorphous) may be acting a role in drug solubilization (Vyas, 2013). The natural compounds of CD have a low solubility in water. However an important change in aqueous solubility was provided by the alkylation of the free hydroxyl chains of CDs originating from hydroxyl alkyl, methyl and sulfobutyl derivatives (Akbari et al., 2011). In the current work, inclusion complexes were formulated to enhance the solubility and availability of ISN and added to *in situ* gel formulations that provide superiority in the treatment by extending the duration of stay in the area compared to conventional vaginal preparations in vaginal applications.

6.2.1. Phase solubility studies

Phase solubility experiments provide knowledge not only the solubilizing potential but also the apparent stability of the complex by examining the curves of phase solubility (Yurtdaş-Kırımlıoğlu, 2020). The phase solubility graphs are classified into two major categories: A (A_L, A_P, A_N) and B (B_S, B_I) type. A-type diagrams show the generation of soluble inclusion complexes, while B-type diagrams show the complexation that provides poor solubility (Higuchi and Connors, 1965). Although natural CDs generally form B-type graphs owing to their weak aqueous solubility while derivatized CDs (HP-β-CD, M-β-CD) can generate soluble inclusion complexes.

The molar ratios of active substance ISN with β-CD, M-β-CD and HP-β-CD were determined according to the phase solubility diagrams. Determination of equilibrium time performed by adding an excessive amount of ISN in CD aqueous solution (20 mM). After 3 day of analysis, equilibrium time for solubility was determined as 24h for β-CD, M-β-CD and HP-β-CD.

It was observed that the solubility phase diagrams obtained by plotting solubilized amount of ISN for each CD concentration fit the A_L type solubility phase diagram (Higuchi and Connors,1965) (Figures 5.14-5.16). This curve was typical of 1:1 complexation suggesting the generation of water-soluble inclusion complexes between the substrate (ISN) and the ligand (CD derivatives). Moreover, slope was lower than 1

(0.9897, 0.9958 and 0.9897 for β-CD, M-β-CD and HP-β-CD, respectively) indicating that the molar ratio of 1:1 between the host and guest molecule was achieved an inclusion complex formation (Doile et al., 2008).

The apparent stability constants (Ks) were quantified as 430.74 M^{-1}, 1040.49 M^{-1} and 803.52 M^{-1} for β-CD, M-β-CD and HP-β-CD, respectively. The constant values of Ks were within the range of 50-2000 M^{-1} with an average value of 129, 490, and 355 M^{-1} for α-, β- and γ-CD, respectively (Loftsson et al., 2005). The stability constants of M-β-CD and HP-β-CD complexes were importantly higher than the β-CD in accordance with the literature (Arora et al., 2017). The increased performance is ascribed to the increase of inclusion ability and improvement of physicochemical properties of parent CDs (Yurtdaş-Kırımlıoğlu, 2020).

6.2.2. Preparation of ISN/β-CD, ISN/M-β-CD and ISN/HP-β-CD inclusion complexes

The ISN/CD inclusion complexes were developed by two distinct methods such as SD and FD approach. The 1:1 molar ratio was used as regards to the previous phase solubility studies. The inclusion complexes were formulated successfully. In order to identify and evaluate physicochemical and solid state behaviors of ISN/β-CD, ISN/M-β-CD and ISN/HP-β-CD complexes, different procedures (morphology, DSC, FT-IR, ^{1}H-NMR, saturated aqueous solubility and drug content,) have been carried out. The characterization studies of inclusion complexes aimed to investigate the compatibility of pure substances and possible changes in components after formation of inclusion complex by analyzing pure active substance, pure CD derivatives and their physical mixtures.

6.3. Physicochemical Characterization Tests of Cyclodextrin Inclusion Complexes

6.3.1. Morphological studies of inclusion complexes

SEM is applied to identify the surface morphology of the inclusion complexes. Morphological structure of ISN and ISN/CDs inclusion complexes obtained by SEM were shown in Figures 5.17-5.19. The morphological structure of ISN has irregularly shaped particles that are considered to be semi-crystalline structure. β-CD seem as crystals with irregular shaped while M-β-CD seem as amorphous particles of spheres and hemispheres. HP-β-CD seem as a spherical form with interior cavities (Michalska et al., 2017; Yurtdaş-Kırımlıoğlu, 2020). Physical mixture of ISN/β-CD, ISN/M-β-CD and

ISN/HP-β-CD appeared as a collection of the morphology of the parent materials. This indicates that there is no interaction in physical mixtures of ISN and CD molecules (Deng et al., 2016). The FD and SD inclusion complexes seem as disordered and/or spherical particles wherein the original structure of all constituents was lost and dramatic differences in shape were detected and a pronounced association in the solid state is revealed. The ISN/β-CD and ISN/M-β-CD complexes formulated by FD method appeared in a typical morphology with a soft and fluffy structure while ISN/M-β-CD FD complexes seem as more rigid form with different shapes and sizes. On the other hand, the inclusion complexes produced by SD procedure had a spherical shape with smooth surfaces that are the common morphology of spray-dried amorphous products (Figueiras et al, 2015; Srivalli and Mishra, 2015; Yurtdaş-Kırımlıoğlu et al., 2018; Yurtdaş-Kırımlıoğlu, 2020). These microphotographs were consistent with the formation of solid phases with different morphology compare to pure materials suggesting that ISN is totally embedded into the nonpolar cavity of CD derivatives, which confirmed the apparent interaction and generation of the ISN/β-CD**,** ISN/M-β-CD and ISN/HP-β-CD inclusion complexes (Spulber et al., 2008; Tang et al., 2016; Deng et al., 2016).

6.3.2. Thermal analysis of CDs inclusion complexes

DSC data provide information on the structure of the polymer and the physical state of the active substance in the solid dispersions or inclusion complexes (Li et al., 2013). In thermal analyses, DSC is used to determine whether the obtained solid products are a physical mixture or form a complete inclusion complex. DSC thermograms of pure ISN, pure CDs, ISN/CDs physical mixtures and ISN/CDs inclusion complexes are given in Figure 5.20-5.22. The thermogram of ISN showed a typical melting thermogram at 187.5 °C (Figure 5.2) (European Pharmacopoeia 6.0, 2008). Also, β-CD showed one endothermic peak, approximately at 200ºC related to broad endothermic peak (Figure 5.20.B). Pure M-β-CD exhibited two small endotherms; one, revealed at 185 °C according to an irreversible transition phase and the other broad endotherm about at 265°C, indicating water loss. The thermogram of HP-β-CD demonstrated two broad endotherms, one emerged at around 80 °C, which could be related to the release of water molecules from the CD cavity, and the other wide endothermic peak at 270 °C due to the thermal degradation (Güleç and Demirel, 2016; Tang et al., 2016; Li et al., 2018; Yurtdaş-Kırımlıoğlu, 2020). The physical mixture showed a modest overlap of pure ISN by CD

endothermic peaks. In the FD and SD inclusion complexes thermograms expression of complexation were seen in the complete absence of the ISN melting endotherm due to its interaction with cavities of CDs. Complete absence of the melting endotherm of the active agent suggest that the amorphization of the crystalline material in the presence of the amorphous structure and confirms formation of an inclusion complex (Demirel et al., 2011; Olkhovic et al., 2017).

6.3.3. FT-IR analysis of inclusion complexes

FT-IR examinations were suggested as the potential tool to explore the interactions between the components for the complexation. The pure ISN spectrum showed evident several bands at 3059 cm^{-1} owing to aromatic (C-H stretching), 1585 cm^{-1} owing to (aromatic C=C stretching), 1448 cm^{-1} owing to (C=N stretching), peak at 1093 cm^{-1} owing to (C-O-C stretching) and 759 cm^{-1} (aromatic C-Cl stretching) (Figure 5.3) (Ivancic et al., 2016; Abd El-Gawad et al., 2017).

FT-IR spectrum of free β-CD demonstrated notable peaks at 3277 cm^{-1} (for O-H stretching vibrations), 2968 cm^{-1} (for C-H stretching vibrations), 1153 cm^{-1} and 1024 cm^{-1} (for C-H and C-O stretching due to glucopyranose ring) (Figure 5.23b) (Bhargava and Agrawal, 2008; Yurtdaş et al., 2011). The prominent bands at 3397 cm^{-1} (O-H stretching), 2931 cm^{-1} (C-H stretching), 1650 cm^{-1} (H–O–H deformation bands of water molecules), 1154 cm^{-1}, 1085 cm^{-1}, and 1036 cm^{-1} (C-H and C-O stretching) were pertinent to the FT-IR spectrum of free M-β-CD (Figure 24b) (Rachmawati et al., 2013; Chao et al., 2014; Güleç and Demirel, 2016). For HP-β-CD, the wide peak at 3305 cm^{-1} was due to the vibration of the hydrogen-bonded OH moieties. The other distinctive patterns were seen at 2910 cm^{-1} (-CH and-CH_3), 1633 cm^{-1} (H-O-H bending), 1149 cm^{-1} (C-O), 1024 cm^{-1} (C-O-C glucose bending), 846 cm^{-1} (C-O-C of CD rings) (Figure 25b) (Olkhovic et al., 2017; Li et al., 2018; Yurtdaş-Kırımlıoğlu et al., 2020).

The physical mixture spectra included the peaks of both components without shifting or disappearing. The observation of FT-IR spectra of physical mixtures of ISN and CDs molecules showed that no intermolecular interaction between the molecules (Figure 5.23c, 5.24c, 5-25c). For binary systems, to illuminate the alterations of spectra due to formation of inclusion complex, CDs mask, intend to shift or decrease intensity of the prominent peaks of groups that are encapsulated drug in their cavity (Kelemen et al., 2018). The spectra of inclusion complexes exhibited a reduced intensity and shifts in

bands of ISN especially inclusion complexes. The characteristic bands at 1585 cm^{-1} and 1448 cm^{-1} was significantly decreased, indicating that C=C and C=N may have had a intense interaction with hydrogen bond source of CD derivatives. Also, higher wavelength shift was detected in the ISN/M-β-CD (shift to 1597 cm^{-1} and 1455 cm^{-1} and ISN-/HP-β-CD (1590 cm^{-1} and 1452 cm^{-1}) complexes. The C-O-C peaks were overlapped by peaks of CD derivatives. The characteristic peaks of C-Cl were shifted to the upper wavelengths all inclusion complexes, suggesting that the aromatic rings may be located in the CD cores thus this verified the formation of inclusion complexation. In BCD complexes prepared by both SD and FD methods, peak shear values were found lower than the other two CD derivatives (Bhargava and Agrawal, 2008; Yurtdaş et al., 2011; Olkhovic et al., 2017).

6.3.4. ^{1}H-NMR analysis of inclusion complexes

^{1}H-NMR is an easy and efficient technique for evidence of CDs inclusion formation. Interactions between active agent and CD molecules was described as non-covalent bonds such as Van der Waals forces, hydrophobic interactions and hydrogen bonds. If the guest molecule joins the space of the CD, its chemical shifts (δ) in the ^{1}H-NMR spectrum will change (Deng et al, 2016).

Spectrum of ISN showed characteristic peaks of ISN (Figure 5.4). Peaks appeared at 8.79 (singlet, $N^{+}H$), 7.16-7.55 (multiplet, Ar-H, heterocycle H), 5.14-5.16 (triplet, 1H, CH), 4.60 (doublet, N-CH_2) and 4.46 (singlet, Ar-CH_2) belong to the ISN structure (Ji et al., 2011).

^{1}H-NMR spectra of β-CD, HP-β-CD and M-β-CD, before and after complexation were presented in Figure 5.26-5.28. ^{1}H-NMR bands of CD derivatives is consistent with the literature (Schneider et al., 1998). Peak shifts were demonstrated (as ppm) in Table 5.6-5.8. As far as we know, the H-3 and H-5 CD protons are presented in the inner cavity of the CD structure. In addition, H-3 and H-5 protons are near to the secondary and primary face, respectively (Yurtdaş-Kırımlıoğlu, 2020). Notable differences were found in the chemical shifts of the H-3 and H-5 protons as host molecules occurred inclusion complexes with different CD derivatives. For ISN/β-CD inclusion complexes, the shifts for the protons H-3 and H5 were determined as 0.006, 0.0137 and 0.072, 0.0174 for ISN/β-CD FD and ISN/β-CD SD, respectively. In ISN/M-β-CD complexes spectra', the shifts for the protons H-3 and H5 were observed as 0.0253, 0.0464 and 0.0578, 0.0600

for ISN/M-β-CD FD and ISN/M-β-CD SD, respectively. The shifts for the protons H-3 and H5 for ISN/HP-β-CD complexes were determined as 0.0186, 0.0238 and 0.0278, 0.0305 for ISN/HP-β-CD FD and ISN/HP-β-CD SD, respectively.

It is noted that when ΔδH-3 > ΔδH-5, exists partial inclusion of the guest molecule is partly included within the cavity and when ΔδH-3 ≤ ΔδH-5, the complete inclusion appears. Therefore, it can be said that complete inclusion occurred for all inclusion complexes prepared (ΔδH-3 < ΔδH-5 as you can see in Table 5.6-5.8). Moreover, H-3 is close to narrow side, so it was suggested according to the ^{1}H-NMR findings that ISN were located at the center of the β-CD, HP-β-CD and M-β-CD cavities (Chao et al., 2014; Güleç and Demirel, 2016).

6.3.5. Determination of aqueous solubility in distilled water of inclusion complexes

It has been documented that CD molecules are able to improve solubility of the guest molecule (Yurtdaş et al., 2011; Deng et al., 2011). Table 5.9 demonstrates the saturated solubility values of ISN, ISN/β-CD**,** ISN/M-β-CD and ISN/HP-β-CD inclusion complexes prepared by SD and FD method. When the saturated aqueous solubility of pure ISN was determined as 0.5088 ± 0.0062 mg.mL^{-1}, the solubility of inclusion complexes 1.4363 ± 0.1542 mg.mL^{-1}, 2.5684 ± 0.1636 mg.mL^{-1} and 2.0900 ± 0.0757 mg.mL^{-1} for ISN/β-CD FD**,** ISN/M-β-CD FD and ISN/HP-β-CD FD inclusion complexes, respectively. On the other hand, the saturated solubility of inclusion complexes formulated by SD technique was calculated as 1.5613 ± 0.0725 mg.mL^{-1}, 3.8869 ± 0.5084 mg.mL^{-1} and 3.6550 ± 0.1995 mg.mL^{-1} for ISN/β-CD SD**,** ISN/M-β-CD SD and ISN/HP-β-CD SD inclusion complexes, respectively. The solubility was found to be higher in complexes prepared with SD method. This situation was thought to be caused to the smaller particle size and narrower size distribution of SD complexes. Moreover, ISN/M-β-CD and ISN/HP-β-CD complexes displayed about 7-times greater solubility than ISN since M-β-CD and HP-β-CD are more efficient in solubilization than β-CD. The significant increase of the saturated aqueous solubility that obtained with FD and SD complexes has been attributed to the inclusion complexation with amorphous state or reduction of post-complexation crystallinity (Spulber et. al., 2008; Deng et al., 2016; Tang et al., 2017; Yurtdaş-Kırımlıoğlu, 2020).

Depending on the results of the characterization analyses conducted on the inclusion complexes developed by SD and FD method, the decision was made to use the CDs inclusion complexes developed by SD method in preparing *in situ* gel formulations.

6.3.6. Determination of drug content (DC) %

Drug content % of inclusion complexes were presented in Table 5.10. The results were consistent with the theoretical values.

6.4. Formulation of *In Situ* Gels

6.4.1. Formulation development of *in situ* gels

Vaginitis or vaginal candidiasis is a burning of the vagina that can cause discharge, itching, and trouble. The common reason for vaginitis is a variation in the normal equilibrium of vaginal bacteria or an infection. After menopause, lower estrogen levels and some skin disturbances can also cause vaginitis (Sarwal et al., 2019). Therefore, novel antifungal formulations are needed for the treatment of vaginal disorders caused by vaginitis. Vaginal dosage forms require to remain at target sites to obtain desired therapeutic efficacy for a long-term time and this is provided by the *in situ* gel systems (Rençber et al, 2017). Pluronic® F127 is a solution at room temperature (25 °C) which shows as a flowing viscous liquid that can turn into a semi-solid transparent gel at body temperature (37 °C). Toxicity data show that it is well tolerated. Therefore, it is used in preparations for rectal, vaginal, ocular, transdermal and parenteral injection. By extending the duration of the active substance where it is applied, it increases therapeutic effectiveness and decreases the frequency of application (Ruel-Gariepy and Leroux, 2004). Pluronic® F127 in aqueous solution at a concentration of 15% or higher transforms from a low viscosity solution to a semi-solid gel when heated from 4 °C to 23 °C or higher, and this is reversible by the thermo-gelation cooling process. The thermo-gelation phenomenon is characterized by a sol-gel transition temperature. That is, below this temperature, the material is liquid while above this transformation temperature the material is transformed to gel (El-Kamel, 2002). It is a thermosensitive polymer that transform to gel at higher temperature, so cold preparation method was chosen in this study (Rawat et al., 2010). This medicinal preparation for vaginal use contains water which promotes the growth of microorganisms. Thus, it is necessary to use an antimicrobial substance in the formulations. Benzalkonium chloride is the most widely

used antimicrobial preservative in vaginal preparations. In addition, benzalkonium chloride is stable in solution, autoclaving durable, use at low concentration (0.01%) and effective within the used pH range in vaginal formulations (Swietlikowska, 2020). Based on the literature information given above, studies in the development of *in situ* gel formulations were started with pre-formulations consisting of Pluronic® F127 and HPMC components at different w/ w % ratios. As a result of the gelling temperature studies of the prepared formulations without active substance, gelation was observed in the formulations containing F127 or containing F127, 0.5% HPMC and 0.01% benzalkonium chloride. The gelation temperatures varying between 25-43 °C were detected in 20 formulations (Table 5.15). From the gelation temperature measurements, it was observed that the gelling temperature decreased with the addition of HPMC into the formulations. 5 formulations (P12.75H05, P13.5H05, P14, P14H05 and P15) which have the appropriate gelling temperature were selected for further studies.

6.4.2. Formulation and characterization of *in situ* gel formulations containing ISN and ISN/β-CD, ISN/M-β-CD and ISN/HP-β-CD complexes

6.4.2.1. *Gelation temperature of the formulations containing ISN or ISN/CDs* complexes

In situ gel formulations were prepared by adding ISN, ISN/βCD, ISN/MβCD and ISN/HPβCD complexes containing 1% (w/w) ISN to each of the 5 selected formulations (P12.75H05, P13.5H05, P14, P14H05 and P15), and gelation temperatures were determined. The suitable gelling temperature for a vaginal gel is 25-37 °C. The formulations that have a gelation temperature less than 25 °C, the gel maybe formed at 25 °C and induces problems during manufacture and use. The formulations that have a gelation temperature more than 37 °C remain liquid at the body temperature and this causes problems in the release of the active agent in the organism and the retention time would be lower (Rençber et al, 2017). For this reason, the gel transition temperature of our formulations expected to be gel between 25-37 °C was examined using test tube tilting method. In the test tube tilting technique, the temperature rises progressively over time and the phase transition is visually monitored and the gel transition temperature is evaluated. *In situ* gel formulations containing ISN/β-CD complexes were excluded due to its rapid gelatinization at low temperatures below 25 °C.

According to these study, 7 formulations (F12.75H05, F14, C13.5H05MβCD, C14H05MβCD, C15MβCD, C13.5H05HPβCD and C14HPβCD), which have the lowest polymer concentrations and gelation temperature close to ~ 37 °C have been selected for further studies.

6.4.2.2. *pH measurement of the formulations containing ISN or ISN/CDs* complexes

The pH values and gelation temperatures of the prepared formulations were measured (Table 5.13). F12.75H05 has gelation temperature at 33°C with 4.04 pH value, F14 has gelation temperature at 26 °C with 4.50 pH value, C13.5H05-MβCD has gelation temperature at 34 °C with 3.92 pH value, C14H05-MβCD has gelation temperature at 32 °C with 4.18 pH value, C13.5H05-HPβCD has gelation temperature at 30 °C with 4.86 pH value and C14 HPβCD has gelation temperature at 30 °C with 4.37 pH value were selected as optimum formulations.

The pH of the formulations is one of the most significant criteria for vaginal biocompatibility. Vaginal pH ranges approximately 3.5-4.5 pH. Vaginal dosage forms should present compatibility with vaginal pH and, ideally, maintain it or even supported to repair it in case of bacterial vaginitis or menopausal women. The pH values of optimum *in situ* gel systems were determined in the normal pH range of vaginal region. So, our formulations which have in the range of 3.5-4.5 pH can also be ideal for vaginal applications (Sarwal et al., 2019).

6.4.3. Selection of the optimum *in situ* gel formulations

Seven formulations (F12.75H05, F14, C13.5H05MβCD, C14H05MβCD, C15MβCD, C13.5H05HPβCD and C14HPβCD), which have the lowest polymer concentrations, suitable pH values and gelation temperature close to ~ 37 °C have been selected for further studies.

6.5. Characterization of the *in situ* gel formulations

In vitro release, stability and antimicrobial activity tests are planned on the optimum formulations. Before these studies, gelling capacity, swelling, rheological behavior and drug content % analyses were carried out on the optimum formulations.

6.5.1. Gelling capacity

In the prepared formulations within the scope of this thesis, prolonged release is a desired property, which is intended to form gelling at ~ 37 °C and stay at the site of action for a long time. It is impossible for formulations that rapidly lose their gel property to prolong the release of the active ingredient. Likewise, very thick gel-forming formulations, the active substance may not be released at the required time. The rapid gel formation of our formulations at 37 °C and the long durability of the gel is an indication of their ability to release controlled. The gelling capacity was determined by taking a drop from the formulation into vial containing 2 mL of the SVF and kept at 37 °C (Makwana et al., 2016). Gelling capacity assessment was determined that gelation occurred immediately and took a few hours (Table 5.16).

6.5.2. Swelling test

Swelling test was done for 72 hours by using dialysis membrane and closed with dialysis membrane sealer. The gel was weighed before test. The results indicate that the rate of swelling has increased proportionally over time (Table 5.17). At the end of the 72h, swelling rations were found as 118.219%, 184.837%, 201.365%, 169.905%, 166.88%, 195.096% and 142.156% for F12.75H05, F14, C13.5H05MβCD, C14H05MβCD, C15MβCD, C13.5H05HPβCD and C14HPβCD respectively.

6.5.3. Rheological studies

Barnes, Hutton and Walter (1989) stated that while the Newtonian model shows a linear increase in shear stress versus the shear rate of the flowing fluid, the non-Newtonian model shows a parabolic increase in the shear stress against the shear rate. At the same time, the viscosity values of the Newtonian fluid are the same at every point independent of the shear rate. Whereas, in the plastic and pseudoplastic flow defined under the non-Newtonian flow, the viscosity decreases with the shear rate, while the viscosity increases with the shear rate in the dilating flow (Hekimoğlu, 2004).

The examination of the rheological characteristics for the gel-type drug delivery systems would be significant to forecast their *in vivo* behavior (Rençber et al., 2017). The descriptive flow profiles of the *in situ* gel systems were shown in Figure 5.29-30. When the shear stress and viscosity graphs of the prepared formulations against the shear rate drawn at 25 °C were examined, the initial rheograms for optimum formulations, it was

observed that there was a direct increase between shear stress and shear rate at room temperature 25 °C (Figure 5.29). However, as in the newtonian flow, it was observed that the viscosity is not constant and decreases depending on the shear rate. The reason for this was thought to be due to the density created by the powder mass that had to be added to provide the commercial preparation active substance content. There are also studies involved similarly flowing *in situ* gel formulations in the literature (Deshkar et al., 2016).

Also, all formulations demonstrated non-Newtonian pseudoplastic flow at 37 °C as predicted owing to its thermosensitive characteristic. Non-newtonian characteristic flow is indicative of Pluronic® based delivery systems at certain temperatures above sol-gel transition temperature (Chang et al., 2002). The pseudoplastic flow reduces by increasing the shear rate (shear thinning behavior). Our results demonstrated consistency with the literature, with all developed formulations displaying pseudoplastic behavior (shear thinning behavior) at 37 °C (Sarwal et al., 2019). This was mainly due to the fact that Pluronic® being non-ionic PPO triblock copolymers form into micelles at 37 °C. This is owing to the dehydration of polymers chains with temperature. The gel formation was shown as a consequence of micellar enlargement and gel more entangled and viscous at higher concentration of Pluronic®. Because of these micellar entanglements, they cannot be easily separated, which is responsible for the rigidity and high viscosity of gels consists of high Pluronic® content (Jain et al., 1998; İbrahim et al., 2012).

When rheograms of *in situ* gel formulations containing M-β-CD complex were examined, high shear stress and viscosity values were acquired with C15MβCD > C14H05MβCD > C13H05MβCD formulations. This was thought to be related to the increasing Pluronic® percentage. However, in *in situ* gel formulations containing HP-β-CD and pure ISN, the order of high viscosity and shear rate was found to be C13.5H05HPβCD > C14HPβCD > F12.75H05> F14. This is thought to be due to the presence of HPMC, which is used to increase density in order to increase mucoadhesion and residence time into the vaginal region (Sarwal et al., 2019).

The rheological behavior of *in situ* gel systems influence the ease of application and persistence of the vagina. Regarding topical application to the vagina, it is agreed that the optimum rheological patterns of the *in situ* gel systems will regulate the resulting physicochemical characteristics. The existence of HPMC has been shown to have higher elasticity values for HPMC related formulations compared to other formulations.

Increased viscosity of these formulations would be anticipated to improve durability at the application region.

6.5.4. Determination of drug content (DC) %

In situ gels containing ISN and ISN inclusion complexes were prepared at the 2% ratio in accordance with the market preparation. The drug content % of the *in situ* gels were calculated as 0.615%, 0.749%, 0.862%, 0.738%, 0.716%, 0.655% and 0.652% for F12.75H05, F14, C13.5H05MβCD, C14H05MβCD, C15MβCD, C13.5H05HPβCD and C14HPβCD respectively. The values found are slightly less than 1% but are consistent with the literature (Deshkar et al., 2016). The drug contents of the *in situ* gels were within allowable limits and guaranteed dosage uniformity.

6.6. *In Vitro* Release Studies of *In Situ* Gel Formulations

The *in vitro* release research plays a vital role in the development and quality management of drug dosage forms (Yurtdaş-Kırımlıoğlu et al., 2018). *In vitro* release profile of *in situ* gel systems were presented in Figure 5.31-5.32. Formulations F12.75H05, F14, C13.5H05-MβCD, C14H05-MβCD, C13.5H05-HPβCD and C14-HPβCD showed 37.113%, 40.113%, 69.145%, 66.274%, 76.118%, 66.615% and 67.115% ISN release, respectively at the end of the 96h. The release of ISN from *in situ* gel systems was initially higher owing to the burst effect and as the time period got along the gelling effect existed and the release rate was eventually delayed. Formulations bearing inclusion complexes exhibited higher ISN release (C13.5H05-MβCD, C14H05-MβCD, C13.5H05-HPβCD and C14-HPβCD) contrasted to the formulations containing pure ISN (F12.75H05 and F14). This predicted difference was due to higher aqueous solubility of inclusion complexes than pure ISN. In addition, extended release of ISN was already enhanced more than 1.5-2-fold with *in situ* gel systems that contain inclusion complexes, which decreases the required dose for antifungal therapy by improving the bioavailability of ISN (Yurtdaş-Kırımlıoğlu, 2021).

6.6.1. Determination of release kinetics

Many mathematical and statistical criteria have been utilized to examine active agent release data. Fitting *in vitro* release curves to mathematical equations describing drug release as a function of time provides useful evidence on the *in vivo* release activity

of drug delivery systems (Costa and Sousa Lobo, 2001; Zhang et al., 2010). Kinetics of *in vitro* release were examined for *in situ* gel systems bearing inclusion complexes in comparison with the *in situ* gel systems containing pure ISN using DD-solver software. The most common criteria for evaluating kinetic models used as highest r^2, k and model selection criteria (MSC) values and the lowest Akaike information criterion (AIC) values. First-order kinetics, Higuchi, Korsmeyer-Peppas t-lag, Hixson-Crowell, Hopfenberg, Baker-Lonsdale and Peppas-Sahlin models were chosen for DD solver assessment (Table 5.21-5.22).

In vitro release of formulations that are ideally matched to the Peppas-Sahlin model for all parameters. This paradigm offers an integrated process of Fickian (controlled release by diffusion) and non-Fickian release (due to the relaxing of the polymer chain among network relaxation-Case II). It can be concluded that the release of the drug from *in situ* gel systems is regulated by diffusion and case II relaxation period, as defined in the Peppas-Sahlin model, which refers to the induction phase. As can be seen from the results, the release kinetic model was not affected using inclusion complexes in *in situ* gels (Zhang et a., 2014; Yurtdaş-Kırımlıoğlu, 2021).

6.7. Stability studies of *in situ* gel formulations

Pharmaceutical products must maintain the active substance and the formulations in stable state until use and throughout the period of use. Stability studies are extremely important studies as an indicator and assurance that the effectiveness and safety of formulations throughout the shelf life. In this study, gelation temperature, pH, rheological behavior and drug content were measured at 30th, 60th and 90th days. In addition to these analyzes, gelling capacity test was performed on the 90th day of the stability study (Dol et al, 2014).

The results of gelation temperature and gelling capacity study showed that there was no gelation observed in F12.7H05 and F14 formulations stored at 4 °C and 25 °C during 30th, 60th and 90th day. For C13.5H05HPβCD formulation stored at 4 °C and C13.5H05 MβCD formulation stored at 25 °C, no gelation was observed during the 30th, 60th and 90th day. The initial gelling temperatures of the C13.5H05MβCD, C14H05MβCD, C15MβCD and C14HPβCD formulations were found as 34 °C, 32 °C, 34 °C and 30 °C, respectively and gelation occurred immediately and took a few hours. The gelation temperatures of these formulations kept at 4 °C on the 90th day were

determined as 35 °C, 33 °C, 33 °C and 32 °C for C13.5H05MβCD, C14H05MβCD, C15MβCD and C14HPβCD, respectively. The gelation temperatures of C14H05MβCD, C15MβCD, C13.5H05HPβCD and C14HPβCD formulations kept at 25 °C on the 90th day were found as 33 °C, 34 °C, 34 °C and 31 °C, respectively and gelation occurred immediately and took a few hours (Table 5.23-5.24). These results indicate that the C13.5H05MβCD, C14H05MβCD, C15MβCD and C14HPβCD formulations which were kept at 4 °C, remained stable during the period of keeping in the refrigerator. C14H05MβCD, C15MβCD, C13.5H05HPβCD and C14HPβCD formulations kept at 25 °C remained stable during the stability studies period and suitable for vaginal application.

At the end of the 3rd month of the stability studies; the pH value of F12.75H05, F14, C13.5H05MβCD, C14H05MβCD, C15MβCD, C13.5H05HPβCD and C14HPβCD formulations stored at 4 °C were found to be 3.96 ± 0.00, 4.01 ± 0.00, 4.25 ± 0.01, 4.28 ± 0.01, 3.86 ± 0.00, 3.82 ± 0.01 and 3.96 ± 0.01, respectively. The pH value of F12.75H05, F14, C13.5H05MβCD, C14H05MβCD, C15MβCD, C13.5H05HPβCD and C14HPβCD formulations stored at 25 °C were determined to be 4.30 ± 0.01, 4.12 ± 0.01, 4.12 ± 0.01, 4.30 ± 0.0, 3.73 ± 0.00, 3.72 ± 0.01 and 3.75 ± 0.00, respectively. The outcomes of the pH values show that the chemical stability of the formulations does not change (Table 5.27-5.28).

The rheological curves of formulations belong to the stability study were shown at Figure 5.33-5.46. Rheograms taken during the measurements were evaluated in order to monitor the effect of viscosity changes on the rheological behavior of the system. When the shear stress and viscosity graphs of the freshly prepared formulations against the shear rate drawn at 25 °C were examined at 0 day, the initial rheograms for optimum formulations, it was observed that there was a direct increase between shear stress and shear rate at room temperature 25 °C (Figure 5.29). However, as in the newtonian flow, it was observed that the viscosity is not constant and decreases depending on the shear rate. The reason for this was thought to be due to the density created by the powder mass that had to be added to provide the commercial preparation active substance content. In addition, it was stated that viscosity and shear stress values increased in rheograms taken at 37 °C and pseudoplastic flow was observed. According to the gelation temperature and gelling capacity results, F12.75H05 and F14 formulations kept at 4 °C and 25 °C did not show gelling properties at 37 °C at the stability period. When the rheograms of these formulations were examined, it was thought that the non-gelation after the 1st month was

due to the viscosity not significantly increasing against shear rate. Similarly, it was assumed that the viscosity of C1305HPβCD stored at 4 °C and C1305MβCD stored at 25 °C did not increase significantly due to the shear rate and therefore did not show gelation.

It was decided that the amount of active agent in the formulations did not change significantly during the stability test period (Table 5.29-Table 5.30).

As a result of stability studies, it was decided that among the formulations stored at 4 °C C13.5H05MβCD, C14H05MβCD, C15MβCD and C14HPβCD formulations and among the formulations stored at 25 °C, C14H05MβCD, C15MβCD, C13.5H05HPβCD and C14HPβCD formulations remained stable during the stability test period.

6.8. Antimicrobial Efficacy Testing of *In Situ* Gel Formulations

In the antimicrobial activity test, comparison of inhibition zone diameters measured after 24 hours of incubation, inhibition zone diameters were noted for substance concentrations and standard substance (Figure 5.47-5.52). The antifungal effectiveness of the *in situ* gel systems containing pure ISN and ISN/M-β-CD and ISN/HP-β-CD complexes against *C. albicans*, *C. glabrata* and *C. krusei* was investigated. When the data for all three yeast species were plotted and considered together with their standard deviations, it was determined that all yeasts were found to be susceptible for ISN and ISN inclusion complexes loaded *in situ* gels.

Inhibition zone diameters of the *in situ* gel formulations were calculated and presented at table 5.31. Considering the inhibiton zones of all *in situ* gel systems, the highest inhibition zones against *C. glabrata* were found as C13.5H05HPβCD > C13.5H05MβCD > C15MβCD > F12.75H05 > C14H05MβCD = C14HPβCD > F14, respectively. Also, the highest inhibition zones against *C.krusei* were determined as C14-HPβCD > C15MβCD > C13.5H05MβCD > C13.5H05-HPβCD F12.75H05 > C14H05MβCD = > F14, respectively. On the other hand, inhibition zones against *C. albicans* were listed in descending order as follows: C13.5H05MβCD > C13.5H05HPβCD = C15MβCD > F12.75H05 > C14H05MβCD > C14HPβCD > F14. While zone diameters varied between 33 and 40 mm, the least effective formulation was found to be F14 for all yeasts. In general, it can be said that inclusion complex bearing *in situ* gel formulations are more effective than formulations containing pure ISN. As an exception, the F12.75H05 formulation inhibition zones were found as slightly greater than the C14H05MBCD and C14HPBCD formulation for *C. albicans*, the

C14H05MBCD formulation for *C.krusei*, and the C14H05 and C14HPBCD formulation for *C. glabrata*. Besides these small differences, the formulations prepared were found to be significantly different from the F14 formulation in the statistical evaluation ($p < 0.05$).

In situ gel systems tested did not reduce the effectiveness of ISN. On the other hand, placebo systems do not have any antifungal activity, as supported by the experimental results, inhibition zone formation was not observed.

7. CONCLUSION

According to the data obtained from the whole study, the following results have been acquired.

The validation study results obtained from the linearity, accuracy, precision, sensitivity, selectivity and stability parameters were found to be in accordance with the analytical validation criteria in the ICH guidelines and the method could be used safely in the study.

The phase solubility studies curves obtained with β-CD, HP-β-CD and M-β-CD, fit the A_L type solubility phase diagram. According to the molar calculations and slope values for the prepared inclusion complexes, the molar ratios at which active substances and CDs can be complexed were found as 1:1 for all used CDs. According to the apparent stability constants (Ks), the inclusion ability and physicochemical properties for ISN/HP-β-CD and ISN/M-β-CD were higher contrasted to ISN/β-CD complexes.

According to inclusion complexes characterization studies results (SEM, FT-IR, DSC and ^{1}H-NMR), ISN/β-CD, ISN/M-β-CD and ISN/HP-β-CD inclusion complexes were successfully prepared in solid form by lyophilization and spray-drying method.

Aqueous solubility studies indicate that the complexes formulated with SD method are more soluble in distilled water than the complexes prepared by FD method. Furthermore, ISN/M-β-CD and ISN/HP-β-CD complexes displayed about 7-times greater solubility than pure ISN since M-β-CD and HP-β-CD are more efficient in solubilization than β-CD.

The inclusion complexes prepared with SD method showed drug content values greater than the complexes prepared with FD method. Therefore, inclusion complexes developed by SD method were selected for *in situ* gel systems.

In the pre-formulation studies conducted for the development of *in situ* gel formulations, Pluronic® F127, 0.5% HPMC and 0.01% benzalkonium chloride were used in formulating the preparations. According to the gelation temperature measurements, 5 formulations were selected to formulating ISN/CDs *in situ* gel systems (P12.75H05, P13.5H05, P14, P14H05 and P15).

After adding ISN, ISN/β-CD, ISN/M-β-CD and ISN/HP-β-CD complexes in the amounts calculated for the addition of 1% w/w active ingredient to the pre-formulations, it was determined that the desired gelling properties could be achieved with 7

formulations (F12.75H05, F14, C13.5H05MβCD, C14H05MβCD, C15MβCD, C13.5H05HPβCD and C14HPβCD).

The gelation temperature for the 7 formulations (F12.75H05, F14, C13.5H05MβCD, C14H05MβCD, C15MβCD, C13.5H05HPβCD and C14HPβCD) ranges from 26 °C to 34 °C.

The pH measurements for the 7 formulations (F12.75H05, F14, C13.5H05MβCD, C14H05MβCD, C15MβCD, C13.5H05HPβCD and C14HPβCD) ranges from 3.92 to 4.86.

According to the results of the preliminary formulation studies, formulations with codes F12.75H05, F14, C13.5H05MβCD, C14H05MβCD, C15MβCD, C13.5H05HPβCD and C14HPβCD have appropriate physical appearance, pH, gelling capacity and gelation temperature values. It has been decided to continue with *in vitro* release, stability, antimicrobial efficacy studies with these formulations.

According to the results of *in vitro* release studies, formulations containing inclusion complexes (C13.5H05-MβCD, C14H05-MβCD, C13.5H05-HPβCD and C14-HPβCD) showed higher ISN release than formulations containing pure ISN (F12.75H05 and F14). This predicted difference was due to higher aqueous solubility of inclusion complexes than pure ISN. In addition, extended release of ISN was already enhanced more than 1.5-2-fold with *in situ* gel systems bearing inclusion complexes, which decreases the required dose for antifungal therapy by improving the bioavailability of ISN.

In vitro release of formulations fit best to Peppas-Sahlin model in which diffusion and erosion (Fickian and non-Fickian release) take place together in the release and the release kinetic model was not affected using inclusion complexes in *in situ* gels.

According to the stability studies carried out at 25 °C and 4 °C of the *in situ* gelling formulations, F12.75H05, F14 formulation kept at 4 and 25 C, C1305HPβCD kept at 4 °C and C1305MβCD stored at 25 °C lost their ability to gelation during the stability test period. The amount of active agent in the formulations did not change significantly during the stability test period and pH values show that the chemical stability of the formulations does not change.

It was reported that viscosity and shear stress values increased in rheograms taken at 37 °C and pseudoplastic flow was determined. When the rheograms of these formulations (F12.75H05, F14 kept at 4 °C and 25 °C, C1305HPβCD stored at 4 °C and

C1305MβCD stored at 25 °C) were examined, it was thought that the non-gelation after the 1st month was due to the viscosity not significantly increasing against shear rate. Similarly, it was assumed that the viscosity of did not increase significantly due to the shear rate and therefore did not show gelation.

The results of the stability test can be summarized as follows: the formulations stored at 4 °C C13.5H05MβCD, C14H05MβCD, C15MβCD and C14HPβCD formulations and among the formulations stored at 25 °C, C14H05MβCD, C15MβCD, C13.5H05HPβCD and C14HPβCD formulations remained stable during the stability test period.

In comparing the antimicrobial activities of *in situ* gel formulations prepared with ISN/M-β-CD, ISN/HP-β-CD and pure ISN against *C. glabrata*, *C.krusei* and *C. albicans*, it was found that C13.5H05HPβCD formulation has the higher efficiency against *C. glabrata*, C14-HPβCD has the higher efficiency against *C.krusei* and C13.5H05MβCD has the higher efficiency against *C. albicans*. F14 formulation obtained the lowest antimicrobial activity efficacy seen by all the yeasts that were used.

It is obvious that the results will promote to the development of *in situ* gel systems loaded with ISN/HP-β-CD and ISN/M-β-CD complexes with the expected effects in the efficient antifungal therapy. Conclusively, *in situ* gel systems containing ISN/HP-β-CD and ISN/M-β-CD inclusion complexes come across as a promising delivery system for vaginal use of ISN can be used as a preferable alternative to traditional creams because of their advantages.

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