DEVELOPMENT AND ASSESSMENT OF KETOCONAZOLE INTRAVAGINAL THERMOSETTING HYDROGEL FORMULATIONS

by

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ABSTRACT

Imidazole compounds are commonly used as antifungal therapies and ketoconazole was the first broad spectrum orally active azole identified and registered. However, the risks of hepatotoxicity and drug interactions following systemic delivery and absorption of ketoconazole outweigh the therapeutic benefits and ketoconazole was therefore discontinued as first line systemic antifungal therapy in many countries. Although not yet banned in South Africa, the South African Medicine Formulary has ceased to recommend the use of ketoconazole for systemic treatment. Topical use of ketoconazole is, however, regarded as safe following extensive human use as low systemic absorption occurs following topical administration. Vulvo-vaginal candidiasis is a yeast infection that affects a large number of women, some of whom present with several infections annually. The topical treatment options for vulvo-vaginal candidiasis include the use of vaginal tablets, capsules, ovules and creams administered as a single dose or one to three times daily for three to fourteen days either alone or in combination with another dosage form depending on the regimen. Administration of the dose nightly is recommended for most vaginal creams and ovule formulation due to leakage and the uncomfortable feel of the dosage form if administered during the day. A thermosetting gel that remains in the vagina following administration and prolongs the release of ketoconazole from a once daily dose would be a useful addition to the arsenal for intra-vaginal antifungal therapy. Thermosetting gels would be more comfortable to administer as the gel would set in a form similar to naturally occurring mucous in the vagina and, if formulated with a low pH, irritation of the sensitive and fissured tissue would be minimised. A further benefit would be that once set the gel would loosely take on the anatomical shape of the vagina.

A simple, precise, accurate, reproducible and sensitive stability-indicating reversed phase-high performance liquid chromatographic method using ultraviolet detection for the quantitation of ketoconazole was developed and validated. The method was specific and was applied to the determination of ketoconazole in commercial and experimental formulations in addition to samples from degradation studies and *in vitro* release testing. Product performance characteristics of commercial products were investigated with the goal to provide a strategy for the development of a novel intra-

vaginal gel in the shortest possible time. Characterisation of Xolegel®, Kez® shampoo and Ketazol® cream included an evaluation of pH, viscosity and assay, in addition to spectroscopic and thermal analysis, to identify ideal characteristics of topical products that could be used as targets during formulation development of the gel. An *in vitro* release method was developed and validated for precision and accuracy and the *in vitro* release profiles of commercial ketoconazole products were compared using analysis of variance, model dependent and independent approaches. Ketoconazole release data from test gel manufactured during formulation development were investigated to obtain information about the relationship between formulation content and drug release.

Poloxamers marketed as Pluronic® and Lutrol® are synthetic non-ionic tri-block copolymers that consist of hydrophobic propylene oxide and hydrophilic polyethylene oxide blocks, which in solution interact to exhibit thermo-reversible behaviour. *In situ* forming hydrogels consisting of poloxamers, more specifically poloxamer 407, are activated following a temperature stimulus and undergo a sol to gel transition. This approach was used to produce a thermosetting vaginal gel that would exhibit a long residence time in the vagina with an associated enhancement of therapeutic efficacy. Ketoconazole-excipient compatibility was investigated during preformulation studies using spectroscopic and thermal analysis to enable the selection of excipients best suited for the production of a novel dosage form prior to formulation development activities. No obvious interactions between ketoconazole and excipient were observed and ketoconazole was found in an amorphous form when in combination with polysorbate 80 and poloxamers. A two-level factorial design was used to produce solvent systems with different amounts of polysorbate 80, citric acid and ethanol to identify a vehicle in which ketoconazole exhibited optimum solubility and at a pH that would be least irritating to the vaginal mucosa with a low content of excipients. The optimised vehicle consisted of 4% m/v citric acid, 1.5% v/v polysorbate 80 and 9.5% v/v ethanol made up to 50 g with citrate-phosphate buffer adjusted to pH 5.0, resulted in a vehicle of pH of 3.5 in which 71.41 mg of ketoconazole was dissolved per mL.

A Central Composite Design was used to evaluate compositions for the modulation of viscosity of the thermosetting dosage form such that it was a liquid at 22 ºC that rapidly formed a stiff gel when heated

to 37 °C (intra-vaginal temperature) using different amounts of the poloxamer grades 407, 188 and 237. Thermosetting gels containing 2% m/v ketoconazole were manufactured using specifications generated using the Central Composite Design and the viscosity at 22 °C and 37 °C, solution to gel transition time, potency and ketoconazole release at 24, 48 and 72 hours investigated. Contour and three-dimensional response surface plots and mathematical relationships with target ranges set for responses were identified and with the aid of Central Composite Design the optimisation of a desirable thermosetting gel was achieved. The optimised composition included 16% m/v poloxamer 407, 10% m/v poloxamer 188 and 6% m/v poloxamer 237 in the gel that was used as the basis for further optimisation studies. The low ketoconazole release for ketoconazole observed indicated that the poloxamers had formed a gel matrix that sustained the release of ketoconazole and would therefore ensure that once daily administration of the gel was possible. The sol-gel transition test may be used as a simple and cheap alternative to viscosity testing for thermosetting formulations when expensive viscometers and rheometers are unavailable and was successfully used for this purpose.

Ketoconazole is photolabile and is prone to degradation in aqueous solutions. The hydrophobic core of micelles formed in these dosage forms are believed to shield ketoconazole molecules and improve stability in aqueous solutions and acidic gels. The thermosetting gel optimised for poloxamer content was subjected to a further Central Composite Design in which sodium metabisulphite content and vehicle pH were investigated. The length of storage was used as a numeric variable and storage condition as a categoric variable at two levels to monitor the stability of the gels. The formulations were investigated at sample times of 0, 1, 2, 4 and 8 weeks at 5 °C, 25 °C and 40 °C. The use of a Central Composite Design facilitated an understanding of the interactions between input variables and their impact on the responses analysed including ketoconazole content, release at 24, 48 and 72 hours, gel pH and viscosity at 22 °C and 37 °C. Design of Experiments may be used as a rapid cost effective tool for an overall assessment of the stability of novel topical dosage forms. However, a more thorough assessment of stability may be required for product registration. Ketoconazole was found to be unstable in the acidic thermosetting gels despite the addition of antioxidant. The gels in liquid form at 5 °C and

25 °C have a low number of micelles for ketoconazole incorporation and therefore additional optimisation studies would be required to enhance the shelf-life of this product.

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STUDY OBJECTIVES

Vulvo-vaginal candidiasis (VVC) is a common yeast infection known as vaginal thrush and is a muco-cutaneous disease [1]. Repeated VVC (RVVC) may result in as many as three infections annually and highly invasive infections can affect vital organs [1-3]. VVC is identified by symptoms such as the production of a white 'cottage cheese-like' discharge accompanied by inflammation and pruritus of the vaginal mucosa [4]. Treatment for VVC includes systemic or topical therapy with imidazole antifungal or polyene agents over a few days or systemic treatment over several weeks for difficult to treat infections [1]. Ketoconazole (KZ) is a broad-spectrum orally active antifungal used to treat invasive and non-invasive fungal infections [5] that has largely been discontinued for oral use due to hepatotoxicity and has been replaced with azole derivatives that exhibit fewer adverse effects [6]. Topical KZ is considered safe as low systemic absorption is observed and therefore could be included in a novel technology [7-9]. The development of novel KZ formulations may result in a safe and cost effective solution to the treatment of VVC and a thermosetting vaginal gel may maximise the benefits of KZ therapy when treating VVC. A gel administered as a liquid that coats the vaginal mucosa and forms a rigid gel rapidly at body temperature may increase the contact time of KZ in the vagina. A prolonged residence in the vagina with sustained release of KZ may prevent the recurrence of and limit invasive VVC infections. An aqueous gel would be soothing to the irritated, sensitive and fissured vaginal mucosa. RVVC may also be more effectively managed due to the availability of an alternative addition to the therapeutic arsenal to treat VVC.

The objectives of this study were to:

1. Develop and validate a Reversed Phase-High Performance Liquid Chromatographic method with Ultraviolet detection for the accurate and precise quantitation of KZ in topical pharmaceutical formulations and release testing.
2. Characterise commercial KZ products to obtain information about their properties and composition.
3. Develop and validate a simple extraction and *in vitro* release testing method to precisely and accurately measure release from topical formulations.
4. Conduct preformulation studies using spectroscopic and thermoanalytical methods to investigate the compatibility of KZ with excipients in binary mixtures.
5. Use response surface methodology (RSM) for the optimisation of an acidic vehicle, the manufacture of the vaginal gels using a cold method, thermosetting behaviour and poloxamer content with a minimum number of studies.
6. Assess the potential of RSM for stability assessment of novel topical dosage forms under accelerated and conventional conditions of an optimised poloxamer content gel of different pH and sodium metabisulphite content and analysing responses using 3D surface methodology and polynomial equations.
7. Conduct stability assessments of the gel formulations generated using design of experiments following storage at 5 °C, 25 °C and 40 °C for 1, 2, 4 and 8 weeks.

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ABBREVIATIONS

% RSD	Percent relative standard deviation
2D	Two-dimensional
2FI	Two-factor interaction
3D	Three-dimensional
ACN	Acetonitrile
Ala	Alanine
ANOVA	Analysis of variance
API	Active Pharmaceutical Ingredient
As	Peak asymmetry factor
BBD	Box Behnken Design
BCS	Biopharmaceutics Classification System
BHT	Butylated hydroxytoluene
BST	Black standard temperature
C3	Carbon-3
CA	Carbamazepine
CACYP51	*Candida albicans* CYP51
CCC	Central Composite Circumscribed
CCD	Central Composite Design
CCF	Central Composite Face-centred
CCI	Central Composite Inscribed
cGMP	Current Good Manufacturing Practices
CI	Confidence interval
CL	Clotrimazole
CMC	Critical micelle concentration
CMT	Critical micelle temperature
CV	Coefficient of variation
DC	Dendritic cells
df	Degrees of freedom
DHP	Disodium hydrogen phosphate
DoE	Design of experiments
DSC	Differential Scanning Calorimetry
EDTA	Ethylenediamine tetraacetic acid
EMA	European Medicines Agency
FDA	United States Food and Drug Administration
FDS	Fraction of the design space
FTIR	Fourier Transform-Infrared
Gln	Glutamine
GLP	Good Laboratory Practices

GRAS	Generally Recognized as Safe
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HETP	Height Equivalent to a Theoretical Plate
His	Histidine
HIV/AIDS	Human Immunodeficiency Virus/ Acquired Immunodeficiency Syndrome
HPLC	High Performance Liquid Chromatography
ICH	International Conference on the Harmonisation of Technical Requirements for the Registration of Pharmaceuticals for Human Use
Ile	Isoleucine
IR	Infrared
IS	Internal standard
IVIVC	*In vitro-in vivo* correlation
IVRT	*In vitro* release testing
K	Release rate constant
k'	Retention factor
KZ	Ketoconazole
L127	Lutrol® F127/Poloxamer 407
L68	Lutrol® F68/Poloxamer 188
L87	Lutrol® F87/Poloxamer 237
LCST	Lower critical solution temperature
Leu	Leucine
LOD	Limits of detection
log $P_{o/w}$	N-octanol water partition coefficient
LOQ	Limits of quantitation
LSD	Least significant difference
MeOH	Methanol
Met	Methionine
min	Minute
m/m	Mass per mass
m/v	Mass per volume
NaOH	Sodium hydroxide
NK	Natural killer cells
NLC	Nanostructured lipid carriers
NMR	Nuclear Magnetic Resonance
N_p	Number of theoretical plates
OECD	Organisation for Economic Cooperation and Development
OR	Ornidazole
PAMAM	Polyamidoamine
PBS	Phosphate buffered saline

PEO	Polyethylene oxide
Phe	Phenylalanine
PPO	Propylene oxide
PR	Propranolol
R^2	Correlation coefficient
RP-HPLC	Reversed-phase High Performance Liquid Chromatography
Rs	Resolution
RSM	Response Surface Methodology
RVVC	Repeated vulvo-vaginal candidiasis
SAMF	South African Medicines Formulary
SD	Standard deviation
SEM	Scanning Electron Microscopy
Ser	Serine
SF	Sulphafurazole
SLN	Solid lipid nanoparticles
SM	Sodium metabisulphite
ST	Sulphathiazole
SUPAC	Scale-Up and Post Approval Changes
SVF	Simulated vaginal fluid
Tf	Peak tailing factor
UCST	Upper critical solution temperature
USA	United States of America
USP	United States Pharmacopoeia
UV	Ultraviolet
UV-Vis	Ultraviolet-Visible
VIF	Variance inflation factor
VP	Verapamil
v/v	Volume per volume
VVC	Vulvo-vaginal candidiasis
YPD	Yeast extract, peptone dextrose medium
λ	Wavelength

CHAPTER ONE
KETOCONAZOLE

1.1 Introduction

Vulvo-vaginal candidiasis (VVC) is a multifaceted disease that occurs at least once in the lifetime of 70-75% of women and has become a global health problem [1]. VVC recurs in 40–50% of women and 5–8% women suffer from repeated VVC (RVVC) that results in more than three infections annually [2,3]. New-born infants, pregnant, diabetic and women exposed to long term use of broad spectrum antibiotics are susceptible to VVC and/or RVVC. Uncomplicated VVC can be treated over a few days with topical or systemic regimens, but RVVC requires extensive systemic therapy over many weeks [1].

Candida is a yeast-like fungus and is the most common cause of VVC. It is commonly found in the reproductive and gastrointestinal mucosae in healthy individuals and can cause local muco-cutaneous disease in addition to invasive infections that may affect vital organs. *Candida albicans* is the most pathogenic organism, of the species, to humans. It is a dimorphic fungus that exists and propagates through the blastophore phenotype and can transform into yeast, hyphae and pseudo-hyphae according to the environment. As it interacts with human cell membranes an immune response is triggered, resulting in symptoms such as inflammation and pruritus amongst others [4].

The common symptoms of VVC include but are not limited to vaginal irritation, production of a white viscous creamy to floccular discharge ('cottage cheese-like') with no milky or fermentation odour, burning, pruritus and swelling. Redness and fissuring of the vulva may also occur with minor bleeding from the fissures. The pH in the vagina remains within the normal range of 3.5 to 4.5 during VVC infection [2]. Treatment of VVC may require the use of azole- containing therapeutic compounds.

Azole compounds provide synergistic therapy to the natural immune response of the body to eradicate infection [4]. Antifungal treatment of VVC includes the use of imidazole antifungal agents such as ketoconazole (KZ), clotrimazole, miconazole and tioconazole or triazole compounds such as fluconazole and itraconazole or polyene agents such as nystatin [1].

KZ is a second-generation azole molecule and was the first broad-spectrum compound to exhibit good oral bioavailability for use in the treatment of invasive and non-invasive fungal infections [5,6,7]. Following extensive research and commercial use KZ was withdrawn from the market in some countries and/or the labelling was amended due to hepatotoxicity, endocrine dysregulation and drug interactions that were evident following oral administration of the molecule. The use of KZ for the systemic treatment of fungal infection has largely been superseded by the use of newer azole derivatives that

exhibit fewer adverse effects [8]. VVC may however be treated using topical KZ due to limited systemic absorption following topical application of KZ [9], whilst exhibiting sufficient potency to treat non-invasive simple candidiasis safely and effectively [10].

Research and development of novel drugs and products is expensive and time consuming [11]. An efficient and cost effective use of existing resources may result in the development of a novel formulation containing KZ for topical delivery to the vagina. In this way adverse effects would be limited, thereby maximising the potency and efficacy of KZ against VVC. Furthermore there is a need for the development of a cost effective topical treatment for VVC that can be accessed by all women. The development of a novel KZ vaginal gel formulation was therefore undertaken in these studies.

1.2 Physicochemical properties

1.2.1 Description

KZ was discovered in 1976 by Janssen Laboratories in the United States of America (USA) and was approved for commercial use and distribution in 1981 [12,8]. Ketoconazole or ketoconazolum has the chemical name 1-acetyl -4- (4- (2- (2, 4-dichlorophenyl) r-2- (1H-imidazole-1ylmethyl) -1, 3-dioxolan-4-yl) methoxy) phenyl) piperazine [13,14,15]. The molecular and mono-isotopic masses of KZ are 531.44 [16] and 530.15 [17] and the chemical structure of KZ is shown in **Figure 1.1**. KZ exists as a white or off-white odourless powder [18,19]. The CAS registry number is 65277-42-1 [19,20].

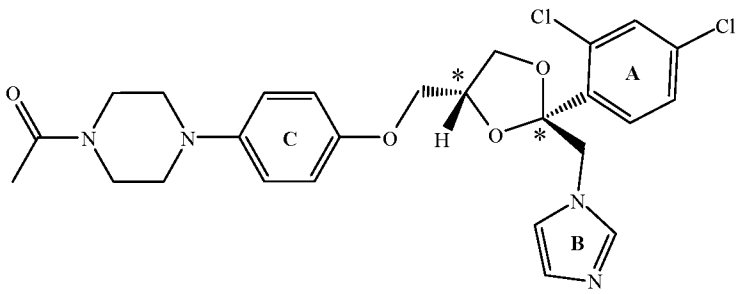


Figure 1.1 Chemical structure of KZ (MM = 531.44) [21].

KZ is a synthetic imidazole exhibiting antifungal, anti-seborrheic and steroid synthesis inhibitory effects. KZ is commonly administered via the oral and topical route and has been formulated into tablet, cream, gel, shampoo and ophthalmic dosage forms [22].

1.2.2 Stereochemistry

KZ is an optically active organic compound containing two chiral carbons as indicated with asterisks in **Figure 1.1**. KZ exists in the cis- and trans-configuration **(Figures 1.2 and 1.3)** and is generally marketed as a racemic mixture predominantly made up of the cis-enantiomer [20,23,24] and the trans-isomer which exhibits lower activity [10]. The trans-isomer is sometimes considered an impurity formed during synthesis. An impurity is defined as any constituent found in an API or formulation that is neither part of the API nor any of the excipients used in a formulation [24]. During synthesis of KZ the cis- and trans-isomers are separated through crystallization [10,20,25].

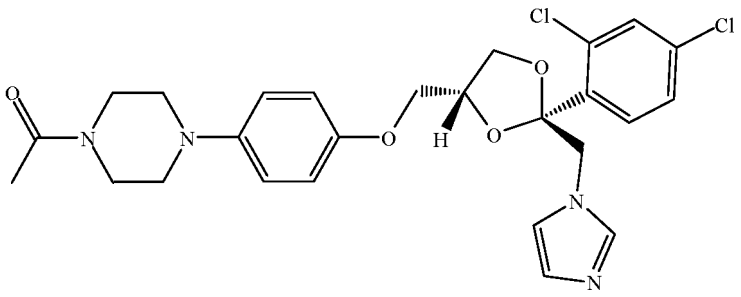


Figure 1.2 Cis-isomer of KZ.

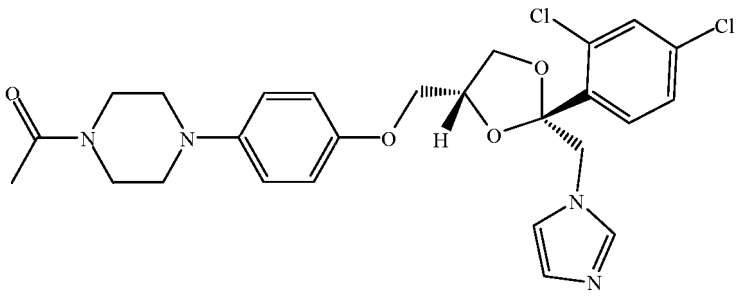


Figure 1.3 Trans-isomer of KZ.

1.2.3 Melting point

The melting point of KZ is 146 °C [19,20,26,27].

1.2.4 Biopharmaceutics Classification System

KZ is a Class II drug according to the Biopharmaceutics Classification System (BCS) [28]. The BCS classifies drugs according to solubility and permeability, and BCS class II compounds are defined as low solubility and high permeability compounds [28,29]. The bioavailability of BCS class II drugs is restricted by their low solvation rate [28,30,31,32]. *In vitro-in vivo* correlations (IVIVC) are generally acceptable for BCS Class I and II drugs [30]. The high permeability of Class II compounds results in a high absorption rate, but lower than that observed for Class I drugs. Absorption occurs over extended periods of time [28]. In some cases, due to the development of an acceptable IVIVC, bio-waivers that

exempt applicants from the need to conduct expensive bioequivalence studies have been granted. This results in a decrease in the costs associated with conducting *in vivo* studies and limits the exposure of healthy human volunteers to drugs delivered from experimental dosage forms [33].

1.2.5 Solubility

KZ is almost insoluble in water [13] and is freely soluble in dichloromethane, is soluble in methanol and is sparingly soluble in ethanol [14].

1.2.6 Dissociation constant

KZ is a dibasic compound with pKa values of 2.94 and 6.51 [7,13,34]. KZ is therefore more soluble in acidic media and will exhibit enhanced dissolution and absorption from media of acidic pH [35,36].

1.2.7 Partition coefficient

The n-octanol water partition coefficient (log $P_{o/w}$) of KZ is 3.78, suggesting that the molecule is lipophilic [27,37].

1.2.8 Optical rotation

KZ is optically active, with an optical rotation of -0.10 to +0.10 (t = 20) in methylene dichloride at 289 nm (sodium D line) [20].

1.2.9 Heavy metals

KZ has a maximum of 20 ppm heavy metal content [18].

1.2.10 Sulphated ash

The sulphated ash test establishes the amount of inorganic impurities in an organic compound [14] and 1.0 g of KZ produces a maximum of 0.1% sulphated ash [18].

1.2.11 Loss on drying

A maximum mass of 0.5% is lost from 1.0 g KZ on drying at 105 °C in an oven [18].

1.2.12 Auto-ignition temperature

KZ is not flammable under normal atmospheric conditions and has an auto ignition temperature of 520 °C, at which it releases poisonous chlorine and nitrogen oxide fumes on decomposition [27].

1.2.13 Nuclear Magnetic Resonance spectroscopy

KZ was characterized by Nuclear Magnetic Resonance (NMR) spectroscopy using a 600 MHz Bruker™ spectrometer (Fällanden, Zürich, Switzerland). The ^{1}H and ^{13}C NMR spectra generated are shown in **Figures 1.4** and **1.5** respectively.

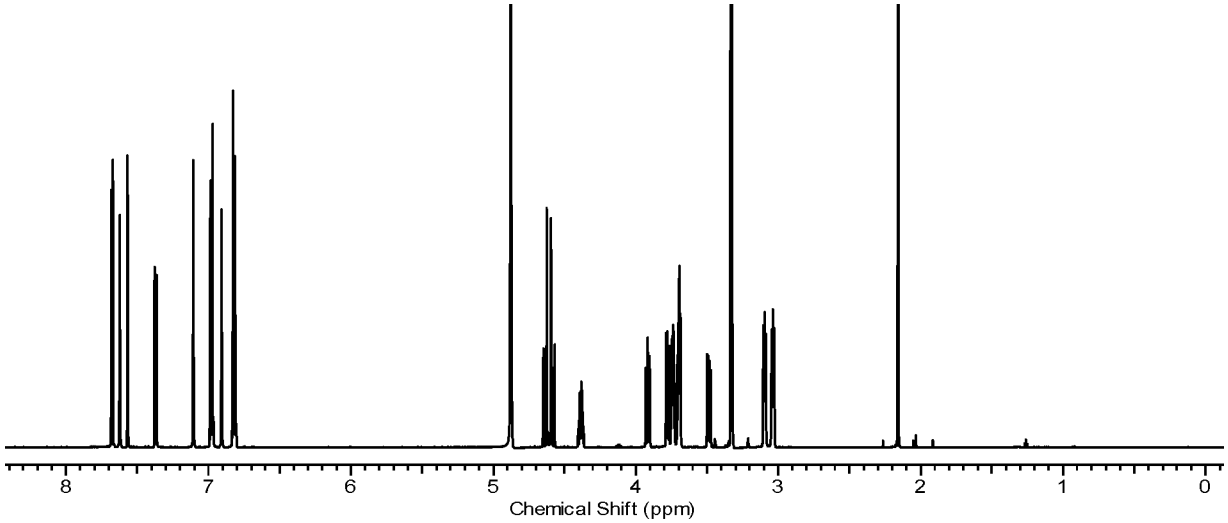


Figure 1.4 ^{1}H NMR (600 MHz, CD_3OD) spectrum of KZ.

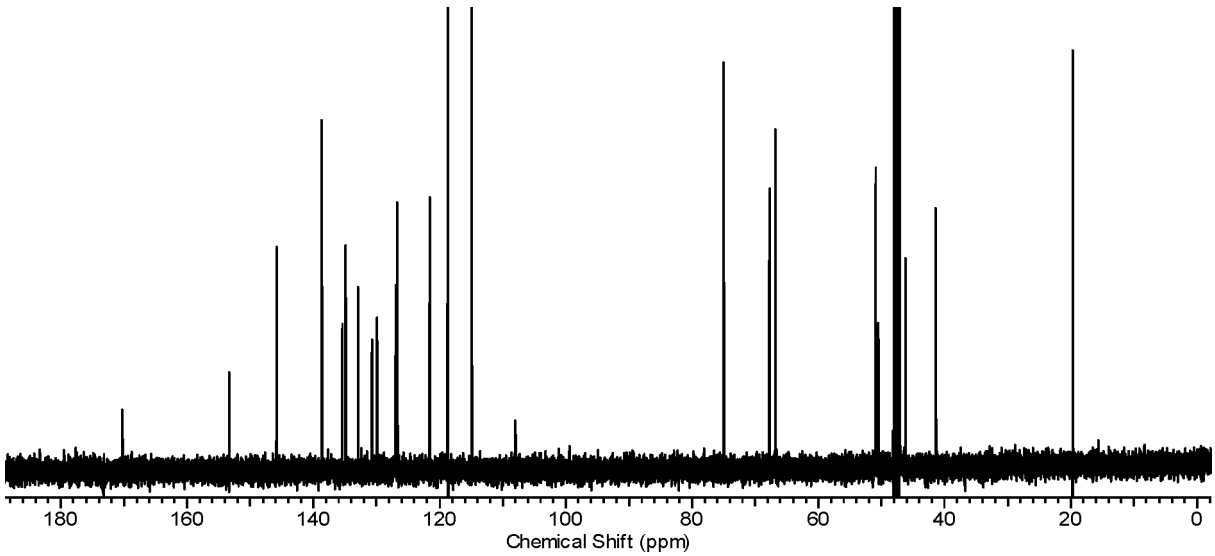


Figure 1.5 ^{13}C NMR (150 MHz, CD_3OD) spectrum of KZ.

The ^{1}H NMR spectral features shown in **Figure 1.4** indicate the presence of protons in the region δ 6.5-8.0 that are associated with aromatic rings A, B and C (**Figure 1.1**) of KZ. Five sets of methylene protons and one methane proton are observed between δ 3.0 and δ 5.0, with a methyl signal displayed up-field close to δ 2.0. From the ^{13}C NMR spectrum (**Figure 1.5**) 22 carbons were detected with four duplicate carbons resulting in the presence of a total of 26 carbons. The ^{1}H and ^{13}C NMR data generated

were compared to publish data and were found to be consistent with the NMR spectra reported for KZ [38,39].

1.2.14 Ultraviolet absorption spectrum

Ultraviolet (UV) detection in combination with high pressure liquid chromatography is commonly used for pharmaceutical analysis of dosage forms. Optimum absorption is achieved at the wavelength of maximum absorption of a compound. The UV absorption spectrum of KZ shown in **Figure 1.6** was generated using a Model GBC 916 UV-Vis double beam spectrophotometer (Melbourne, Victoria, Australia) at a scan speed of 600 nm/min over the wavelength range of 190 - 280 nm.The spectrum was generated using a 20 μg/mL solution of KZ dissolved in acetonitrile and the lamda max was established to be 200 nm.

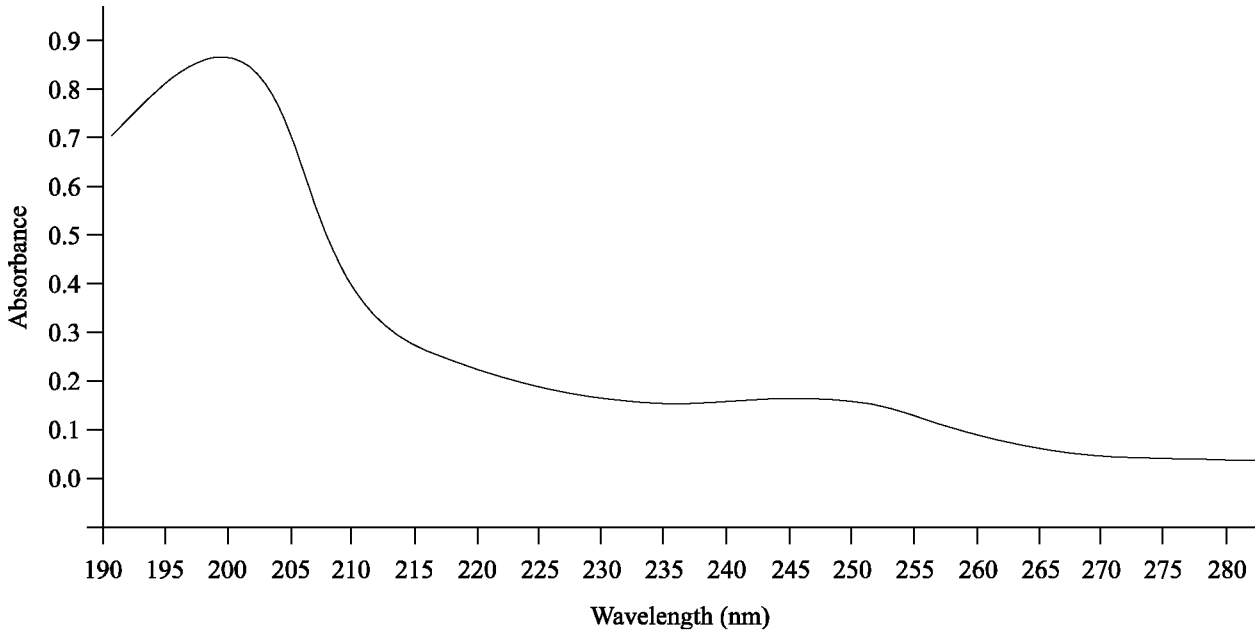


Figure 1.6 UV absorption spectrum of KZ in acetonitrile.

1.2.15 Infrared spectroscopy

Infrared (IR) spectroscopy is used for structural elucidation and identification of compounds. Functional groups in molecules absorb IR radiation and resonate at distinctive frequencies that can be determined using IR spectroscopy [40]. The Fourier Transform-Infrared (FTIR) spectrum of KZ powder is shown in **Figure 1.7.** It was generated using a Perkin Elmer® Spectrum 100 FTIR ATR spectrophotometer (Beaconsfield, Buckinghamshire, England) without additional sample preparation. The scanning range was between 4000 and 650 cm^{-1} and the resolution was 4 cm^{-1}. The band at 1643.53 cm^{-1} is due to a carbonyl group and between 1001.40 and 1241.57 cm^{-1} demonstrates the presence of aliphatic and cyclic ethers, respectively [41]. The IR spectrum displayed in **Figure 1.7** was compared to that reported and was found to be consistent with reported IR spectrum of KZ [42,41].

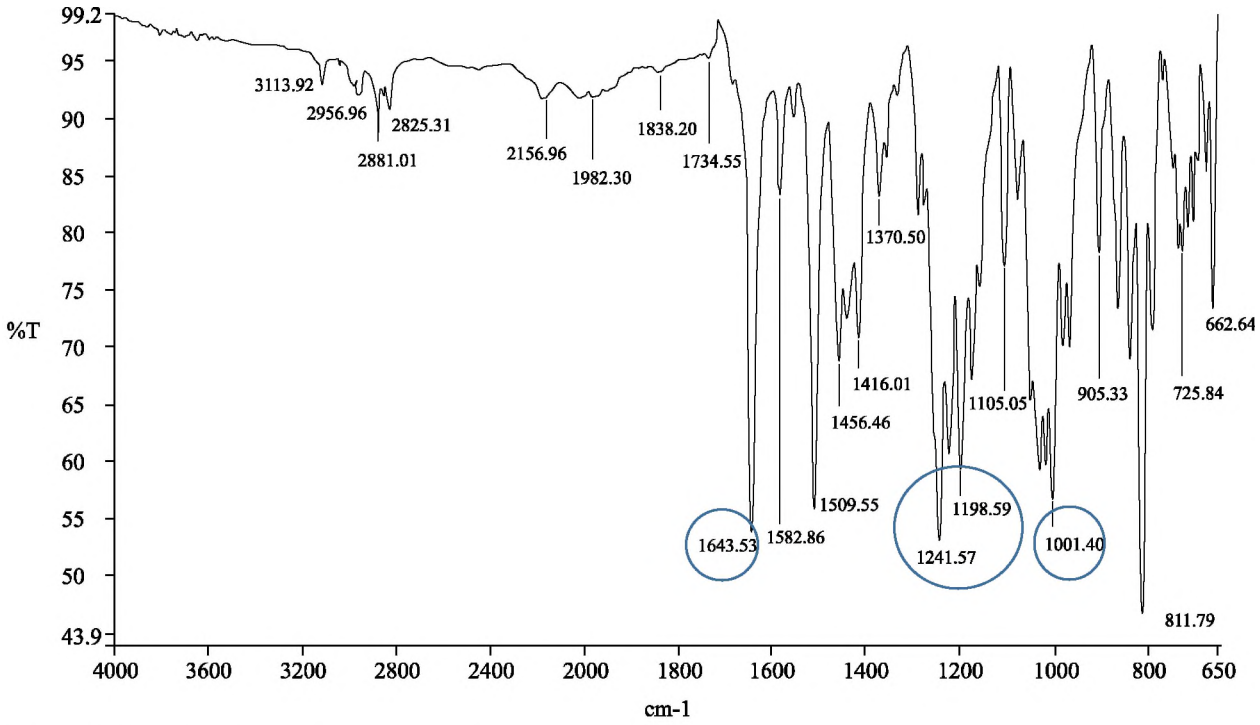


Figure 1.7 IR absorption spectrum of KZ.

1.2.16 Differential Scanning Calorimetry

The melting point of KZ was established using a TA Model Q100 Differential Scanning Calorimeter fitted with a RCS (90) refrigerated cooling system (New Castle, Delaware, USA). Approximately 2.5 to 5 mg KZ was weighed into an aluminium pan that was then hermetically sealed with an aluminium disc. An empty aluminium pan was used as a reference. The Differential Scanning Calorimetry (DSC) scan was generated by heating the sample from 30 °C to 445 °C at a rate of 10 °C/min. The system was cooled to 22 °C with a liquid nitrogen purge at a flow rate of 100 mL/min. The data were displayed and analysed using TA Universal Analysis 2000 software (New Castle, Delaware, USA).

The resultant DSC thermogram is shown in **Figure 1.8,** and reveals a sharp melting endotherm at 151.80 °C, which is similar to the reported melting point of KZ [17,18,23,24]. The enthalpy of reaction was 85.14 J/g and the onset temperature 142.21 °C. The wide exothermic peak observed at 369.14 °C shows the onset of thermal decomposition of KZ at 338.07 °C and a peak width of 53.59 suggests that several degradation products may be formed during heating.

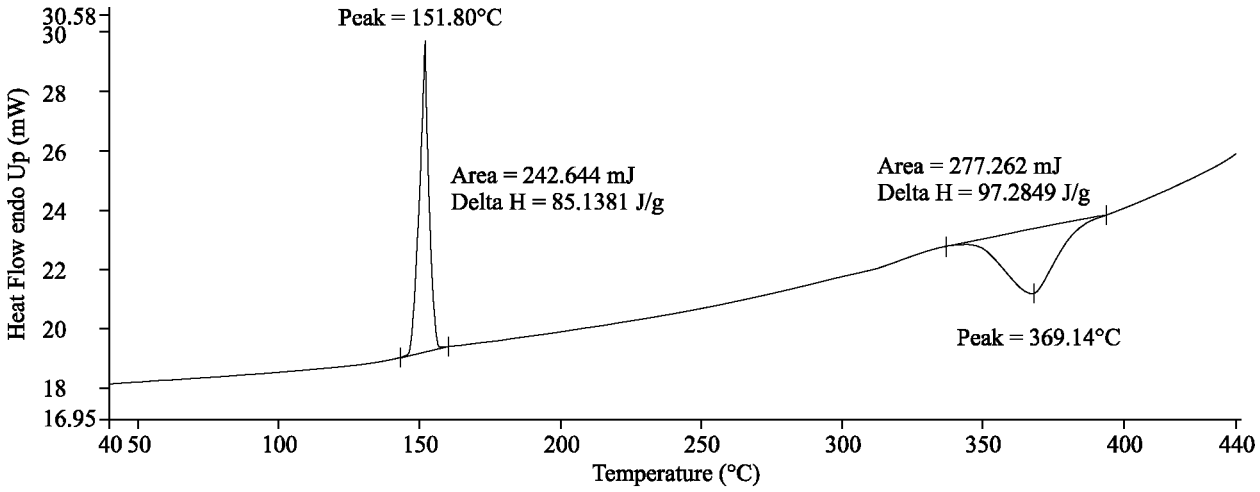


Figure 1.8 DSC thermogram of KZ.

1.3 Stability of KZ

The stability of KZ in aqueous solution was investigated over the pH range 1.2 to 10 and revealed that KZ was most stable at pH 4. The activation energies of KZ in alkali and acid were 14.32 and 13.67 kcal/mol, respectively. The study revealed that the degradation of KZ in solution was dependent on the pH and temperature of the test system [43]. In another study the decomposition of KZ in solution via acid and base catalyzed hydrolysis and oxidative conditions confirmed the presence of the same hydrolytic and oxidative degradation products. KZ powder was found to be stable when exposed to heat and photolytic conditions [38]. The degradation products of KZ are described in **§ 2.7.1** of this thesis. KZ lotion was subjected to forced degradation studies and KZ was observed to undergo acid hydrolysis, oxidation and photolytic degradation, and was found to be stable when incubated in a basic solution at 75 °C for 6 hours [44]. The stability of KZ is reported in **§ 2.7.1** *vide infra*.

1.4 Synthetic pathway

KZ synthesis (**Figure 1.9**) commences with a reaction between 2, 4-dichloroacetophenone and glycerol to form a ketal structure using an acylation or alkanoylation reaction (stage I). The ketal undergoes radical bromination to form a bromoalcohol (stage II) after which the bromoalcohol undergoes esterification/benzoylation with benzoyl chloride (stage III) to form a benzoate ester. The cis- and trans-isomers are then separated through crystallization. During the reaction at stage IV of the synthesis an imidazole residue is added to the benzoate ester to form an imidazole benzoate. Hydrolysis of the ester group of the imidazole benzoate occurs with sodium hydroxide (stage V) to form an alcohol. Tosylation occurs at the alcohol functional group and cis-tosylate is formed (stage VI). The cis-tosylate is reacted with acetyl phenol in the presence of sodium methoxide to form cis-ketoconazole (stage VII) [10,20].

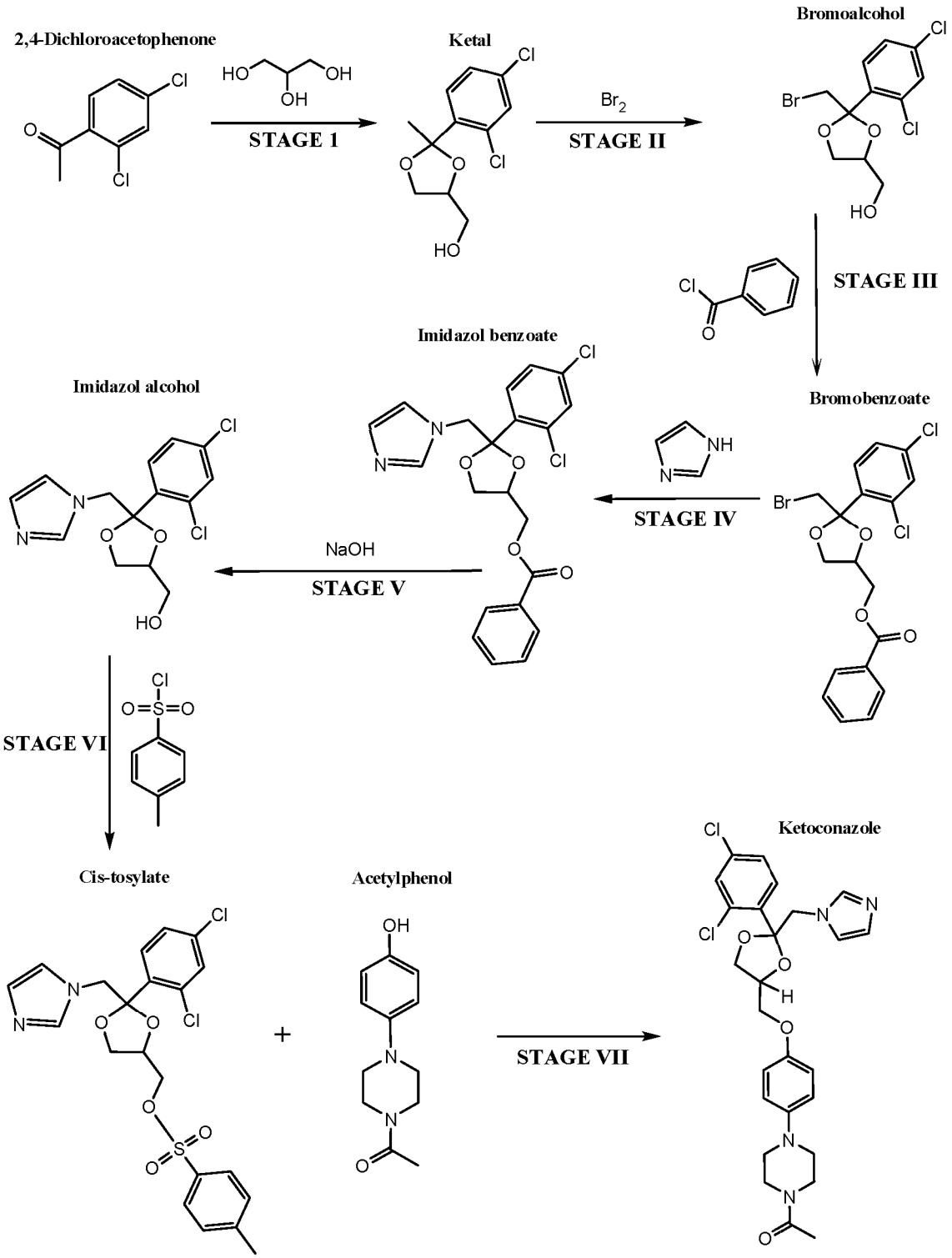


Figure 1.9 Synthesis of KZ adapted from [10].

1.5 Structure activity relationship

The antifungal activity of azole compounds depends on the presence of an imidazole or triazole heterocyclic aromatic five-membered ring [5]. The nitrogen in the imidazole ring binds to the heme group of the lanosterol demethylase enzyme resulting in inhibition of fungal membrane sterol biosynthesis (**§ 1.6.1**). The active site of lanosterol 14 α-demethylase enzyme in *Candida albicans* CYP51 (CACYP51) has four binding pockets, *viz.* S1, S2, S3 and S4. Site S1 represents the site at which hydrophilic hydrogen bonding occurs with Gln309 (glutamine), His310 (histidine) and Ser312 (serine) amino acid sequences and studies have revealed that the hydroxyl group located at carbon-2 interacts with S1 [45]. The S2 pocket is located above the heme ring, is hydrophobic and imidazole or triazole compounds generally bind to the S2 pocket via a coordination bond with the iron located in the heme molecule. Pocket S3 is a narrow hydrophobic region of the enzyme at which the dichloro-phenyl group in KZ interacts with the Ala114 (alanine), Phe126 (phenylalanine), Leu139 (leucine), Ile304 (isoleucine) and Met306 (methionine) amino acid residues. Consequently the imidazole ring, dichloro-phenyl and hydroxyl group at carbon-2 are necessary structural components that impart the activity of ketoconazole. The S4 compartment binds to carbon-3 (C3) side chains in azole compounds through hydrophobic hydrogen bonding. The C3 side chains differ in different azole analogues and for adequate binding to the hydrophobic S4 pocket to occur the side chain should be hydrophobic with a hydrogen bond donor/acceptor. The presence of a C3 side chain with a more hydrophobic functional group will result in strong hydrophobic and Van Der Waals interactions with the CACYP51 enzyme but will also reduce water solubility and bioavailability. The additional hydrogen bonding capability as a result of the presence of a hydrogen bond donor/acceptor will result in better affinity for the CACYP51 enzyme [45]. More active azoles are generally aligned and oriented with the configuration of the CACYP51 enzyme, whereas less active compounds do not fit or bind perfectly to the enzyme [45].

Azole compounds are known to interact with human CYP51 that contains Ile379 instead of Ser378 found in fungal cells, giving rise to side effects normally associated with administration of azole compounds. Derivatives that bind to Ser378 via hydrogen bonding may be more selective for CACYP51 than the commercially available azoles and would very probably exhibit fewer side effects [45].

The addition of a 2,4-dibromo (A) and 2,3,4-trichloro (B) in place of 2,4-dichloro substituent on the phenyl group at the 2-position of the dioxolane group as shown in **Figure 1.10** produced compounds with *in vivo* activity comparable to that of KZ [10].

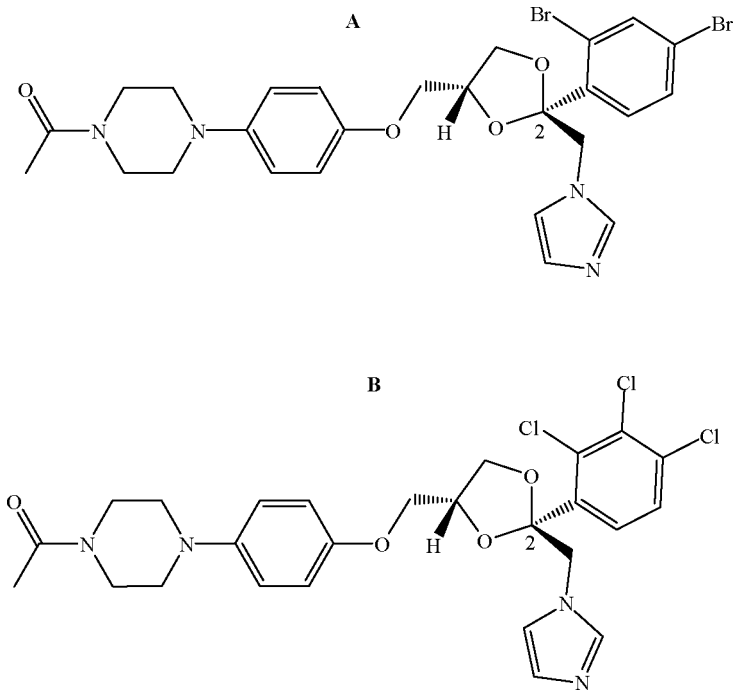


Figure 1.10 2, 4-dibromo (A) and 2, 3, 4-trichloro (B) analogues of KZ [10].

A triazole derivative of KZ exhibited lower *in vivo* potency than KZ [10]. The acyl group of KZ (R-C=O) was reduced to form alkyl groups and several imidazole and triazole analogues that exhibited potent topical activity against non-invasive fungal infections, but low oral activity compared to KZ. The imidazole ring when substituted with a triazole ring results in the formation of derivatives with greater oral and topical potency than KZ. Terconazole was synthesized in this manner (**Figure 1.11**). The regions of the terconazole molecule that differ from KZ are highlighted with arrows.

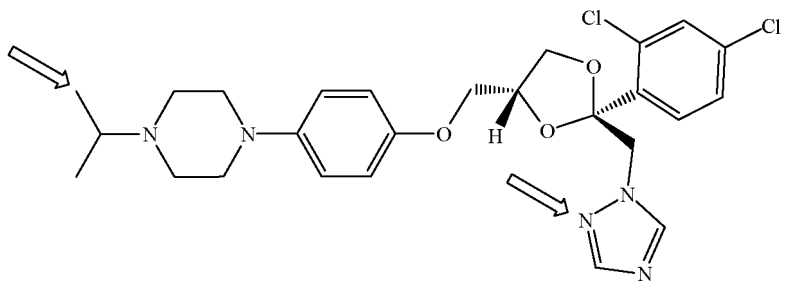


Figure 1.11 Structure of terconazole [10].

Substitution of the imidazole ring with a triazole ring in addition to substitution of the acyl ring with a phenyl ring bearing substituted oxo-heterocycles in the para position of that ring produced derivatives

with better oral bioavailability than KZ. One of the compounds produced in this manner is aryl-triazolone or itraconazole (**Figure 1.12**), which exhibited activity following oral administration in *in vivo* models for dermal candidiasis, dermatophytosis and vaginal candidiasis in guinea pigs and rats [10].

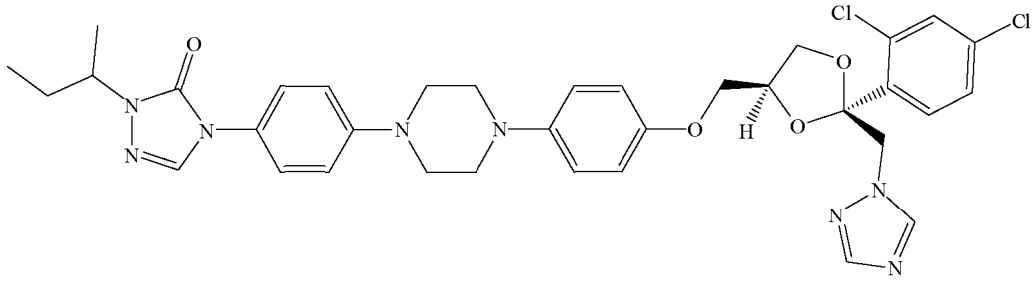


Figure 1.12 Structure of itraconazole [10].

1.6 Clinical pharmacology of KZ

KZ is a clinically effective fungistatic that inhibits the growth of fungi without killing them and at high doses is fungicidal to pathogens [46]. The use of topical and systemic routes of KZ reveals equivalent efficacy, but the systemic route often results in a higher incidence of adverse effects, the severest of which is hepatoxicity [47,48,49,50]. The FDA does not recommend the oral use of KZ as a first line treatment of fungal infections due to the development of the more effective and less harmful triazole compounds such as fluconazole, itraconazole, posaconazole and voriconazole [51,52]. Novel azole compounds such as fluconazole and itraconazole display lower toxicity and better efficacy when delivered orally, however KZ may be a more cost effective compound [53] for use in the developing world.

1.6.1 Mechanism of action of KZ

Lanosterol is converted to ergosterol in the presence of the lanosterol 14 α-demethylase enzyme in *Candida albicans*. Azole compounds bind competitively to the active site of the lanosterol demethylase enzyme and competitively inhibit the endogenous lanosterol substrate via heme group ligation, hydrogen bonding, π-π stacking and hydrophobic interactions within the heme environment of the enzyme [54]. The substrate is shielded and oxygen binding is prevented [5], inhibiting demethylation of lanosterol and thereby preventing the biosynthesis of ergosterol. The depletion of ergosterol and the build-up of 14 α-methylated sterols and lanosterol in fungal cells results in weakening of the fungal membrane integrity, after which the loss of crucial intracellular elements may occur and consequently inhibition of fungal cell growth is observed [3,55].

KZ also inhibits the biosynthesis of triglycerides, phospholipids, fungal oxidative and peroxidative enzyme activities that result in hydrogen peroxide accumulation in fungal cells, after which deterioration of organelles and eventually cell apoptosis occurs [16]. During the budding process and

Candida albicans proliferation, the blastophore changes into a filamentous mycelium, resulting in germ tube formation thereby facilitating adhesion to human epithelial cells. KZ inhibits blastophore transformation into invasive mycelia [55].

1.6.2 Clinical indications

The oral administration of KZ was reviewed in July 2011 following a request by France under Article 31 of Directive 2001/83/EC in the Official Journal of the European Communities [56,57]. Article 31 allows for the review of a request for marketing approval of a drug that is required for the wellbeing of society. The European Medicines Agency (EMA) was asked to assess the risk-benefit of KZ when administered orally to determine whether marketing authorisation of KZ products should be retained or suspended across the European Union. In July 2013, the EMA committee for Medicinal Products for Human Use recommended a suspension of the marketing authorisation for oral KZ formulations throughout the European Union. This recommendation was endorsed and made legally binding by the European Commission effective throughout the European Union as from October 2013 [56,57,58]. The decision was based on the fact that liver injury risk from taking KZ orally is greater than the benefit derived when treating fungal infections, in addition to the increased availability of additional antifungal treatments [59,58]. Topical KZ formulations can still be used as low systemic KZ absorption is observed from topical KZ formulations. Oral KZ is available for off-label use in patients with Cushing's syndrome but will be accessible under controlled conditions so as not to leave these patients without access to treatment [58].

In 2013 FDA also announced that the oral use of KZ as first line treatment was not recommended unless all other treatment approaches had failed due to an elevated risk of hepatoxicity and adrenal gland failure following oral administration of the compound [52,60]. This warning applies only for orally administered KZ. Topically applied KZ can be used as it exhibits negligible systemic availability.

Orally administered KZ can be used to treat systemic mycotic infections, chronic and recurrent vaginal candidiasis that is unresponsive to topical treatment, serious chronic infections of the skin, hair and nails caused by sensitive dermatophytes, lesions over large areas of skin that penetrate into deeper dermal layers, hair and nails when topical treatment is ineffective, serious mycoses of the gastro-intestinal tract unresponsive to treatment, serious chronic mucocutaneous candidiasis, blastomycosis and coccidioidomycosis, pulmonary, oral and disseminated histoplasmosis, chromomycosis and paracoccidioidomycosis. Oral administration of KZ tablets should not be used to treat fungal meningitis as KZ is poorly distributed and does not enter the cerebrospinal fluid [61,62].

Topical KZ is used for the treatment of infections caused by yeasts of the *Pityrosporum* species that result in pityriasis versicolor, seborrheic dermatitis and pityriasis capitis (dandruff) and is also used for

the prophylactic treatment of seborrheic dermatitis, the treatment of dermatophyte infections of the skin such as tinea corporis, tinea manus and tinea pedis, cutaneous candidiasis and tinea versicolor [63,64,65].

1.6.3 Dose and routes of administration

The recommended oral dose of KZ for treating chronic and recurrent vaginal candidiasis in adults is 400 mg dosed daily for 5 days. KZ is no longer recommended for systemic use in the South African Medicines Formulary as KZ is less effective and more toxic than available triazole analogues [66].

A 2% m/m KZ cream (20 mg/g) applied twice daily, with treatment continued for a few days after healing of lesions and/or when all symptoms disappear is sufficient to ensure the resolution of Candida infections. The 2% m/v KZ shampoo or liquid formulations are lathered onto the affected area(s) and removed following 3 - 5 minutes exposure. For the treatment of seborrheic dermatitis topically applied 2% m/m KZ is used twice a week for approximately 2 - 4 weeks. For the treatment of tinea versicolor topically applied 2% m/m KZ is used once daily for 5 days [66]. Xolegel® a topical gel formulation containing 2% m/m KZ and currently unavailable in South Africa, can be obtained as a prescription drug in USA as a treatment option for VVC. Xolegel® should be applied once daily to the affected area(s) for up to 2 weeks [65].

Intravaginal formulations recommended for use in the South African Medicines Formulary (SAMF) for the treatment of candidiasis include nystatin vaginal tablets, clotrimazole (vaginal cream, single dose cream, vaginal tablets and a Duopak® combination), econazole (vaginal cream, ovules and single dose ovules), fenticonazole (cream, ovules, single dose ovules), miconazole (cream, vaginal capsules, Combipak® combination treatment and single dose capsules) and these are listed in **Table 1.1** [66]. The SAMF states that vaginal tablets should be inserted deep into the vagina and are recommended for use at night, especially where the treatment regimen requires once daily dosing. Applicators are provided for most vaginal products, particularly if semi-solid formulations are to be used. It is good practice to treat both partners for candidiasis to prevent re-infection [67,66].

Table 1.1 Intravaginal products listed in the SAMF for the treatment of candidiasis in South Africa [67,66].

API	Product	Strength	Indications and dose
Nystatin	Canstat® vaginal tablet	20 mg (100000 units)	Insert 1 tablet high into the vagina using applicator once or twice daily for 14 days
Clotrimazole	Canesten® vaginal cream	10 mg/g (1% m/m)	5 g (1 applicator full) cream is inserted into the vagina at night or twice daily for 6 to 12 days
	Normospor® vaginal cream		
	Canesten® single dose vaginal cream	100 mg/g (10% m/m)	A single dose (0.5 g) is inserted in the vagina.
	Canesten® single dose vaginal tablet	500 mg	Insert tablet deep into the vagina as a single dose.
	Canesten Duopak® vaginal tablet and topical cream	500 mg (tablet), 10 mg/g (1% m/m cream)	Insert the clotrimazole tablet at night, followed by the application of clotrimazole cream 2-3 times daily for 1-2 weeks to the external genitalia.
Econazole	Gyno-Pevaryl® vaginal cream	10 mg/g (1% m/m)	Insert 1 applicator-full (500 mg) of cream into the vagina at night for 2 weeks.
	Gyno-Pevaryl® vaginal ovules	150 mg	One ovule inserted at night for 3 nights.
	Gyno-Pevaryl® Depot single dose ovule		Insert one ovule into the vagina.
Fenticonazole	Lomexin® vaginal cream	20 mg/g (2% m/m)	Insert 5 g cream twice daily for 3 days.
	Lomexin® vaginal ovule	200 mg	Insert one ovule at night for 3 nights.
	Lomexin® single dose ovule	600 mg	Insert one ovule in the vagina as a single dose.
Miconazole	Gyno-Daktarin® vaginal cream	20 mg/g (1% m/m)	Insert 5 g miconazole cream into the vagina at night for 7 nights.
	Gyno-Daktarin® single dose vaginal capsule	1200 mg	Insert capsule into the vagina
	Gyno-Daktarin® Combipak capsule and vaginal cream	400 mg (capsule), 20 mg/g (2% m/m cream)	Insert the miconazole capsule at night for 3 nights and apply 20 g miconazole cream to the vulva twice daily.

1.6.4 Contraindications

KZ should not be used to treat patients with any pre-existing liver disease. KZ is highly bound to plasma proteins and should be used with caution in patients suffering from malnutrition as they exhibit low plasma protein levels and therefore unbound KZ levels will be elevated compared to levels in healthy patients that may result in KZ toxicity [68]. Azole antifungal agents may cause hepatoxicity as they enhance the expression of cytochrome P450 enzymes in the liver and reactive oxygen species levels increase in liver cells resulting in lipid peroxidation and DNA damage [69]. KZ was found to induce expression of CYP1A1 in mice in the presence of benign and malignant liver tumours [69].

KZ may disrupt the endocrine system and impair reproduction, alter sexual differentiation, impair growth and form hormone-dependent cancers. KZ decreases testosterone and cortisol levels in human plasma resulting in gynecomastia and oligospermia in males and irregular menstruation in females. KZ also causes *in vivo* hormonal imbalances as azole compounds disrupt the endocrine system by inhibiting substrate-selective cytochrome P450 enzymes involved in steroid hormone synthesis in mammals [69].

1.6.4.1 Cross-sensitivity

Persons showing hypersensitivity to imidazole compounds may also be sensitive to KZ [15].

1.6.4.2 Pregnancy

VVC is a common occurrence in pregnant women as the elevated estrogen and glycogen levels in vaginal secretions are a carbon source for the development of VVC. Yeast cells adhere more effectively to the vaginal mucosa in the presence of estrogen and colonisation of yeast cells occurs more effectively during hyphal formation and propagation and secretion of aspartyl proteinase and phospholipases [1].

1.6.4.2.1 Systemic use of KZ during pregnancy

KZ is listed as a category C drug by FDA for use in pregnancy. Category C compounds have been shown to exhibit adverse effects in animal foetuses, but insufficient studies have been undertaken in humans [70]. Studies in animals have shown that KZ is teratogenic and results in syndactyly and oligodactyly [16,71]. However the potential benefits may outweigh the risks of use and KZ may be used in pregnant women if other antifungal treatments are unsuccessful [16]. However, in general the use of any orally administered azole compound in pregnancy is contraindicated [72].

1.6.4.2.2 Topical delivery of KZ during pregnancy

The use of topical KZ in pregnancy is permitted throughout pregnancy due to limited systemic absorption of KZ via this route of administration [9].

1.6.4.3 Breast-feeding

KZ is secreted in human breast milk and approximately 0.4% of the systemic concentration of KZ following oral administration of KZ directly to infants, was found in breast milk following oral administration of KZ to the mother [73,71]. This low level of KZ has not been assessed for safety in infants and the benefits of breast-feeding might compensate for potential adverse reactions due to low exposure to KZ. However as insufficient studies have been undertaken to prove the safety of KZ, it should be used with caution, if at all, by nursing mothers.

1.6.4.4 Paediatric patients

Low systemic absorption of KZ has been observed in infants following topical application of 2% m/m KZ cream and the KZ concentration is considered insignificant compared to the concentrations of KZ observed following oral administration to adults. The systemic dose-dependent side effects usually associated following oral administration of KZ are therefore unlikely to occur when the compound is administered topically [74,75,76].

1.6.4.5 Geriatric patients

Dosage adjustment of oral KZ is not specifically indicated in geriatric patients. Adverse effects from drug interactions may occur due to polypharmacy prescribing in elderly patients. An age-related change that occurs in the gastrointestinal tract is elevated gastric pH, and if the pH is above 5 the absorption of KZ may be affected [77]. The safety of topically applied KZ in geriatric patients has not yet been proven and therefore it should be used with caution in these patients as a consequence of the thin skin often associated with this population group [13,70,15].

1.6.4.6 Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome patients

There is a high occurrence of Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome (HIV/AIDS) amongst adults in South Africa [78]. Patients suffering from HIV present with suppressed immune systems and are susceptible to opportunistic infections caused by viruses, bacteria, parasites and/or fungi [79]. AIDS patients also exhibit reduced gastric acid secretions resulting in gastric hypochlorhydria [76], thus an acidic beverage (such as carbonated water or cola) should be taken with KZ to ensure better absorption of the drug [76]. KZ is effective as primary prophylaxis for the treatment of cryptococcal meningitis in AIDS patients [80] and due to its low toxicity compared to amphotericin B can be used for the treatment of chronic pulmonary histoplasmosis, a severe infectious disease often observed in patients with HIV and/or AIDS. High relapse rates have however been reported with KZ use in pulmonary histoplasmosis patients [81]. Nausea and vomiting in AIDS patients is relatively common following oral administration of KZ, therefore administration with food may reduce the incidence of these side effects, but the absorption of KZ may be impaired and the extent of absorption is often dependent on the type of meal eaten [79]. Interactions between KZ and HIV protease inhibitors

may occur if the medications are taken concomitantly. A serious side effect of KZ is hepatoxicity, and this phenomenon is responsible for a number of interactions with other drugs [52]. KZ must be used with caution in patients with HIV when treating opportunistic fungal infections. The health of the patient should be established prior to administration of KZ. The benefits of oral administration of KZ outweigh the risk of serious side effects in such patients and alteration of doses in conjunction with data from therapeutic drug monitoring is recommended to avoid preventable complications of KZ use [79].

1.6.5 Drug interactions

Azole compounds can inhibit liver cytochrome P450 enzymes that are implicated in phase I metabolism of xenobiotics [69]. Oral administration of KZ may result in potentially harmful drug interactions with concomitantly administered medications due mainly to the severe impact of KZ on the liver and adrenal glands [82]. Orally administered KZ should not be used with terfanadine and astemizole [62] as these compounds are histamine H1-receptor antagonists and therefore conversion to the desalkyl and hydroxyl metabolites is reduced due to cytochrome P450-3A4 inhibition. The reduction of conversion results in ventricular arrhythmias that may be fatal [83,83,84,85]. Simvastatin, lovastatin and atorvastatin are metabolized by CYP3A4 mediated HMG-CoA reductase inhibitors that may result in skeletal muscle toxicity if these compounds are co-administered with KZ. The metabolism of these statin compounds is decreased by KZ and high concentrations of the agents in the plasma may result in myopathy or rhabdomyolysis [86]. In contrast, topically applied KZ is considered safe for use and has not yet been shown to exhibit any drug related interactions.

1.6.6 Adverse reactions and toxicology

1.6.6.1 Local side effects

Pruritus, stinging, burning, erythema and irritation may occur when KZ is applied topically. Contact dermatitis may occur if the patent is sensitive to components of formulations such as sodium sulphite or propylene glycol [16,87]. An overdose of topical KZ formulation may result in erythema, oedema and/or a burning sensation on the skin [87].

1.6.6.2 Systemic side effects

Nausea, vomiting, gastro-intestinal issues, constipation, headache, dizziness, drowsiness, photophobia, paraesthesia, thrombocytopenia, exanthema or itching have been reported following oral administration of KZ. Less common side effects include anaphylactic reactions, alopecia, urticaria, rash, transient decreased libido, reversible gynaecomastia and oligospermia [62]. Hepatitis may occur following the oral administration of KZ and high doses may result in low testosterone and adreno-corticotropic hormone- induced corticosteroid serum levels [86].

1.7 Clinical pharmacokinetics

1.7.1 Absorption

KZ is rapidly absorbed from the gastrointestinal tract within the first two hours of ingestion following administration of low doses (< 400 mg) to fasting healthy human subjects. Absorption appears to be dose dependent due to pre-systemic elimination and capacity-limited hepatic metabolism and at higher doses (> 400 mg) elimination and metabolism of KZ are saturable processes [88]. The systemic absorption following vaginal delivery of KZ (400 - 1200 mg) in healthy female humans was insignificant compared to systemic absorption of KZ following administration of an oral dose [89]. During treatment of severe seborrheic dermatitis topical KZ (2% m/m) gels administered once daily for two weeks demonstrated insignificant systemic absorption and exposure to KZ [90]. Treatment with a topical 2% m/m KZ formulation resulted in circulation levels of KZ below the limit of detection of several analytical methods [90,91].

KZ dissolves in the acidic contents of the stomach and is converted to a hydrochloride salt prior to absorption [92,93]. Gastric acidity is therefore an important factor for the absorption of KZ following oral administration. Concomitant administration of KZ with antacids, ranitidine, cimetidine and anti-muscarinic agents that reduce gastric acid secretion therefore result in a decrease in the solubility and bioavailability of KZ. If concomitant administration of KZ with drugs that reduce the acidity of the stomach is essential, administration of KZ should take place at least two hours prior to treatment with the other molecules [92,94]. Patients suffering from achlorhydria should ingest an acidic beverage such as Coca-Cola® classic (pH 2.5) or orange juice (pH 3.8) to regulate the absorption of KZ from oral dosage forms [95,94,76]. The bioavailability of KZ is lower in immuno-compromised or -deficient patients, possibly due to gastric hypochlorhydria that is often observed in patients such as those presenting with AIDS. The use of a dilute hydrochloric acid solution may also assist to regulate the absorption of KZ [76].

Insufficient studies have been undertaken to prove whether food has an effect on the absorption of orally administered KZ. One study showed that the absorption of KZ decreased significantly following a high carbohydrate and low fat breakfast, whereas in another study it was demonstrated that KZ taken immediately before a breakfast of unknown fat or carbohydrate content resulted in an increase in systemic absorption of KZ [95,96].

1.7.2 Distribution

KZ is 84-99% bound to plasma proteins, most specifically to albumin [92,35]. In human adults KZ has been isolated from urine, bile, sebum, saliva, synovial fluid, cerumen and cerebrospinal fluids following oral administration [97]. In rats, following the oral administration of KZ, elevated concentrations were isolated from liver, pituitary and adrenal glands, moderate levels from lungs, kidneys, bladder, bone

marrow, myocardium, teeth and several other glandular tissues and low levels from the brain and testes [92].

1.7.3 Metabolism

KZ is partly metabolised to form inactive metabolites in the liver through oxidation and degradation of the imidazole group and through oxidative O-dealkylation and aromatic hydroxylation of the piperazine rings [92,5].

1.7.4 Elimination

Elimination of KZ following oral administration is biphasic and the initial half-life is two hours, whereas the terminal half-life is approximately eight hours [15]. The main route of elimination of KZ and its metabolites is through excretion in the faeces via the bile duct [92]. In a study in which healthy fasted adults were given 200 mg KZ orally, approximately 70% of the dose was excreted, of which 57% was eliminated in the faeces within four days of administration and between 20-65% of the drug was eliminated unchanged. Approximately 13% of KZ was eliminated in the urine within four days of dosing, of which 2-4% was unchanged [13].

1.8 Conclusion

In comparison with other azole drugs, KZ is a cost-effective treatment for fungal infections. It exhibits adequate efficacy for the topical treatment of non-invasive fungal infections. KZ is a BCS Class II drug for which IVIVC would be acceptable to regulatory agencies as evidence of efficacy. KZ may be considered for a biowaiver for bioequivalence studies, therefore dissolution or *in vitro* release testing (IVRT) may be used to monitor KZ release from test and/or commercially available products *in lieu* of clinical studies.

KZ is a lipophilic compound with pKa values of 2.94 and 6.51. It is insoluble in water and requires an acidic environment to ensure effective dissolution and absorption. KZ auto-ignites at 520 °C and emits toxic gases during thermal decomposition. Stability studies and thermal analysis of KZ should therefore be undertaken in a fume hood or in enclosed containers for safety. The wavelength of maximum absorption is 200 nm and this would be suitable for HPLC analysis, however the high energy of radiation of this wavelength makes selective UV detection challenging. The IR absorption spectrum was used to identify the major functional groups present in KZ, and these data will be used in preformulation studies to evaluate potential drug-excipient interactions prior to formulation development experiments. The DSC thermogram of KZ confirmed the melting point of KZ and will also be used in preformulation assessment of potential KZ-excipient interactions.

KZ is relatively safe for topical application but should be used with caution when administered orally, due to the high incidence of adverse effects and toxicity associated with this route of delivery. During formulation studies with KZ, sodium sulphite (an anti-oxidant), if required, should be used sparingly as it may cause contact dermatitis. Alternative anti-oxidants may have to be considered for inclusion.

There is a need for a cost-effective vaginal formulation containing KZ to treat yeast infections. A formulation containing KZ that is easy to administer and spreads readily to coat the vaginal mucosa, or results in an increased contact time in the vagina, unlike conventional vaginal formulations, would be a useful addition to the therapeutic arsenal to treat candidiasis. In addition a prolonged therapeutic effect may prevent the recurrence of yeast infections. The formulation should act locally in the vagina and exhibit little or no systematic absorption. Furthermore there should be no leakage of the formulation from the vagina and any damage to the sensitive tissues of the vagina should be avoided.

The aim of this research study is to develop a formulation for the intra-vaginal delivery of KZ in an *in situ* thermosetting KZ gel and to develop and validate a stability- indicating HPLC method of analysis of KZ to be used for the assessment of quality, stability and *in vitro* release of KZ during formulation development studies.

CHAPTER TWO
RP-HPLC METHOD DEVELOPMENT AND VALIDATION

2.1 High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) can be used to analyse a wide range of compounds including ionic species, macromolecules, natural products, polymers, molecules containing a number of functional groups and high molecular mass compounds [98]. The major types of HPLC separations that are available include reversed-phase HPLC (RP-HPLC) that is most commonly used in the pharmaceutical sciences. In RP-HPLC analytes are separated by interaction with non-polar packing particles of the stationary phase and the polar nature of the mobile phase. The non-polar column particles are usually octadecyl (C18) bonded groups bound onto a silica backbone support [99].

Silica/bridged ethylene hybrid, 2-ethylpyridine, aminopropyl, diol and amide are examples of support materials used as the stationary phase in normal-phase chromatography, while pentafluorophenyl, phenyl, C8 and C18 based backbones are used as the stationary phase for reversed-phase chromatography. Pentane, hexane, heptane, xylene, toluene, diethyl ether, dichromethane, chloroform, acetone, dioxane, tetrahydrofuran, methyl tert-butyl ether, ethyl acetate and dimethyl sulfoxide are examples of mobile phase solvents used for normal-phase chromatography whereas acetonitrile, isopropanol, ethanol, methanol and water are solvents that are appropriate for use in reversed-phase chromatographic separations [100].

2.2 Methods of detection

A wide range of detectors are used for RP-HPLC analysis including UV and fluorescence detectors as they are able to measure absorption and emission properties of an analyte in a sample without interference from the mobile phase [101]. A UV detector was selected for the analysis of KZ in these studies and the rationale will be explained in **§ 2.3** *vide infra.* The maximum wavelength of KZ was obtained as described in **§ 2.4** *vide infra.*

2.3 Analysis of ketoconazole

KZ bulk API, in pharmaceutical dosage forms and biological fluids, has been analysed using UV-Visible (UV-Vis) spectrophotometry [102], spectrofluorimetry, titrimetry [103], potentiometry [102], reversed-phase chromatography [102,103] and more recently using high-throughput approaches [104]. The preferred method of analysis for the quantitation of KZ described in the United States Pharmacopoeia is RP-HPLC [105]. The development and validation of a rapid, selective, robust, precise and accurate RP-HPLC method for the analysis of KZ in semi-solid dosage forms is the focus of this research.

A variety of samples such as tablets [106,107], creams [106-108], shampoos [106,109,110], emulsions [111], capsules [112], liquids [113], plasma [114], serum [115,116], stratum corneum [117] and KZ API [118] have been analysed for KZ content using RP-HPLC (**Table 2.1**) and the most commonly used organic modifiers include acetonitrile (ACN) [106,110,112,114-117] and methanol (MeOH) [107-109,111,113, 118]. Acetonitrile is often paired with a phosphate buffer with pH ranging from 4.0 to 8.0 [106,110,112,114-117] and MeOH was paired with a solution of ammonium acetate in water (pH = 5.5) [107-109,111,113]. Solutions of KZ were also analysed in a study using a simple mobile phase comprised of MeOH and water [118]. These published data were used to select the initial conditions for the analysis of KZ using RP-HPLC in these studies.

The addition of diethylamine [106,115,116], mono-isopropylamine [109], trimethylamine [108,111] and di-isopropylamine [113] to the mobile phase to limit KZ peak tailing has been reported. The purpose of this practice is to prevent or limit silanolphilic interactions by masking the residual silanol groups of the stationary phase from KZ by adding a competing amine compound to the mobile phase. Alternatively peak tailing of KZ may be minimized using base-deactivated columns [119].

Hypersil®[106,108,115] and LiChrospher® [107,109,111] columns are the most common phases used for the analysis of KZ. In studies reporting the use of Hypersil® C18 columns both ACN [106,115] and MeOH [108] were used as organic modifiers and with LiChrospher® C18 [107,111] and LiChrospher® C8 [109] phases MeOH was used as the organic modifier. The Hypersil® C18 columns were preferred for these studies due to published literature showing the use of mobile phases comprised of both ACN and MeOH. The column ultimately selected and used is described in **§ 2.5.7** *vide infra*.

The wavelengths at which the studies were conducted ranged between 225-260 nm and the most common flow rate reported was 1.0 mL/min [108-112,116,118]. The IS reported were all azole containing compounds and included terconazole [115], itraconazole, econazole and miconazole [116].

Table 2.1 RP-HPLC methods used for the analysis of KZ.

Sample Matrix	Column	Mobile Phase	Flow Rate, mL/min	λ, nm	Retention Time, min	Internal Standard	Reference
Tablets, Creams, Shampoo	Hypersil® ODS, 200 x 4.6 mm, 3 µm	A: ACN, B: 20 mM disodium hydrogen orthophosphate, A:B (60:40 v/v), adjusted pH of 4.0, and addition of 0.2% v/v diethylamine	1.5	232	4.2	-	106
Shampoo	LiChrospher® 100 C8, 150 x 4.6 mm, 5 µm	A: Monoisopropylamine in MeOH (2:500 v/v) B: Ammonium acetate-water (1:200 m/v), A:B (70:30 v/v), adjusted pH of 5.5 with acetic acid	1.0	225	4.0	-	109
Emulsions	LiChrospher® 100 C18, 125 x 4 mm, 5µm	A: Triethylamine in MeOH (1:500 v/v), B: Ammonium acetate solution in water (1:200 m/v), A:B (75:25 v/v)	1.0	225	3.9	-	111
Blood plasma	XTerra® MS C18, 4.6 x 100 mm, 5 µm	A: 0.05 M disodium hydrogen orthophosphate, B: ACN, A:B (50:50 v/v), adjusted to pH of 6.0 with glacial acetic acid	1.5	240	2.04	-	114
Capsules	Symmetry® C18, 250 x 4.6 mm, 5 µm	A: ACN, B: 0.025 M potassium dihydrogen phosphate, A:B (58:42 v/v), pH of 4.5 adjusted with potassium hydroxide	1.0	240	5.0	-	112
Tablets, Creams	Lichrosorb® C18, 250 x 4.0 mm, 5 µm	A: MeOH, B: Ammonium acetate 0.5% A:B (80:20 v/v)	2.0	225	2.66	-	107
Serum	Hypersil® ODS, 100 x 3 mm	A: Water, B: ACN, A:B (55:45 v/v), pH of 8.0 adjusted with orthophosphoric acid and addition of 500 µl/L diethylamine	0.6	254	5.0	Terconazole	115
Cream	Hypersil® C18, 150 x 4.6 mm, 5 µm	A: Triethylamine in MeOH (1:500 v/v), B: Ammonium acetate solution in water (1:200 m/v), A:B (75:25 v/v)	1.0	225	-	-	108
Stratum corneum	Whatman® C18, 46 x 125 mm, 5 µm	A: ACN, B: 0.01 M potassium phosphate, A:B (65:35 v/v) adjusted to pH 6.0	0.7	254	8.6	-	117
KZ drug	Waters® C18, 5 x 200 mm	MeOH:water (74:26 v/v)	1.0	239	-	-	118
Shampoo	Alltima® C8, 250 x 4.6 mm, 5 µm	A: ACN, B: 0.01 M sodium dihydrogen phosphate, A:B (45:55 v/v), pH of 4.0 adjusted with 1.0 M phosphoric acid	1.0	250	8.7	-	110
Shampoo	Discovery® C8, 50 x 4.6 mm, 5 µm	A: ACN, B: 0.01 M sodium dihydrogen phosphate, A:B (45:55 v/v), pH of 4.0 adjusted with 1.0 M phosphoric acid.	1.0	250	1.3	-	110
Serum	Nova-pak® C18, 100 x 8 mm, 4 µm	A: ACN, B: 0.01 M ammonium acetate, A:B (65:35 v/v) with the addition of 0.5% diethylamine	1.0	260	4.73	Itraconazole Econazole Miconazole	116
Oral liquids	Bakerbond® C18, 250 x 4.6 mm, 5 µm	A: Diisopropylamine in MeOH (1:500 v/v), B: Ammonium acetate solution (1:200 v/v), A:B (70:30 v/v)	3.0	225	3.10	-	113

2.4 Ultraviolet spectroscopy

The studies listed in **Table 2.1** all report the use of UV-Vis detectors for the analysis of KZ and therefore UV detection was selected for detection for the analysis of KZ. UV-Vis detection for HPLC preferably uses the wavelength of maximum UV absorption of a compound to ensure that optimum sensitivity for the analysis can be achieved. The UV-Vis absorption spectrum of KZ generated using a Model GBC 916 UV-Vis double beam spectrophotometer at a scan speed of 600 nm/min over the wavelength range of 190-280 nm (**Figure 1.6**) and a 20 µg/mL solution of KZ dissolved in ACN reveals the lamda max as 200 nm. The UV-cut off for the constituents of the mobile phase (acetonitrile, methanol and phosphate buffer) is ≤ 200 nm [120] therefore a UV of 205 nm was used for RP-HPLC analysis so as to avoid the interaction of solvent peaks with the KZ and internal standard peaks.

2.5 Method development

2.5.1 Chemicals and reagents

All reagents were at least of analytical reagent grade and were used without further purification. KZ was purchased from Oman Chemicals and Pharmaceuticals LLC (Buraimi, Al Buraimi, Sultanate of Oman) and verapamil (VP), the internal standard, was purchased from Nicholas Piramal India Ltd. (Mumbai, Maharashtra, India). ACN 200 far UV Romil-SpS™ Super Purity solvent was purchased from Microsep® (Port Elizabeth, Eastern Cape, South Africa), Minema® phosphoric acid 85% v/v was purchased from Spellbound Laboratory Solutions (Port Elizabeth, Eastern Cape, South Africa). Merck Univ AR® sodium hydroxide (NaOH) pellets were purchased from Saarchem® (Johannesburg, Gauteng, South Africa). A number of internal standards including clotrimazole, DL-propranolol and ornidazole were purchased from Sigma-Aldrich® (St. Louis, Missouri, USA) and sulphathiazole and sulphafurazole, obtained from Maybaker (Pty) Ltd. (Port Elizabeth, Eastern Cape, South Africa) were tested prior to selecting VP as the IS of choice. Forced degradation studies were undertaken using 32% v/v hydrochloric acid (HCl) purchased from B and M Scientific (Cape Town, Western Cape, South Africa) and hydrogen peroxide (H_2O_2) was obtained from Allied Drug Company Ltd. (Durban, Kwazulu-Natal, South Africa). A Millipore® Milli-RO 15 water purification system (Milford, Massachusetts, USA) comprised of Organex-Q®, Super-C carbon, and two Ion-X® ion-exchange cartridges were used to produce HPLC grade water following initial purification by reverse osmosis.

2.5.2 Instrumentation and analytical conditions

The HPLC system used was comprised of a Model 2695 Alliance Separations® Module, a Model 2487 Waters® Dual wavelength absorbance detector and data was captured and analyzed using Waters® Empower 2 data acquisition software (Milford, Massachusetts, USA). The stationary phase was a Phenomenex® Hyperclone 5 µm ODS (C18) 120 Å 150x4.6 mm i.d. column purchased from Separations (Johannesburg, Gauteng, South Africa). A Branson® B12 sonicator (Danbury, Connecticut, USA) was to sonicate samples during solution preparation. All formulations and chemicals were weighed using an

AG135 Mettler Toledo® top-loading analytical balance (Port Elizabeth, Eastern Cape, South Africa). A Scientific Industries® Model G-560E Vortex Genie-2 mixer (Bohemia, New York, USA) was used to agitate solutions to produce homogenous samples prior to analysis.

2.5.3 Sample preparation

Correct sample preparation for HPLC analysis is vital to ensure the accuracy and precision of a method [121]. The sample to be analysed must be homogenous in order to ascertain the concentration of the analyte in solution. HPLC samples are usually prepared in calibrated A-grade glassware specifically produced for quantitative analysis. Furthermore, the number of manipulations and dilutions should be kept to a minimum to minimize sample loss and the introduction of errors. An appropriate mixing method should be used to dissolve samples in solvents and ensure thorough mixing of the sample. The solvent used to prepare stock solutions for HPLC analysis should preferably be the mobile phase or a solvent weaker in polarity than the mobile phase to ensure that the peak shape is retained for accurate analysis [121]. KZ is a poorly soluble compound and it was necessary to use ACN to dissolve the molecule. Careful manipulation of the stock solution was therefore necessary so as to avoid evaporation of ACN and dilutions using a 50:50 ratio of ACN: water were prepared by weighing the stock solution into volumetric flasks for better precision.

2.5.4 Preparation of stock solutions

Standard stock solutions of 1000 µg/mL KZ and 120 µg/mL VP were prepared on a daily basis by accurately weighing approximately 100 mg of KZ into a 100 mL A-grade volumetric flask and 12 mg VP into a different 100 mL A-grade volumetric flask. The stock solutions were made up to volume with ACN and sonicated for 5 minutes or until the KZ had fully dissolved. Analytical standards were prepared from the KZ stock solution by serial dilution to produce solutions of 0.50, 1.00, 2.50, 5.00, 10.00, 20.00, 50.00, 60.00, 100.00, 120.00 and 150.00 µg/mL. A 1:1 ratio of the internal standard solution and the KZ solution were then transferred into an amber vial that was sealed and the contents mixed using a Model G-560E Vortex Genie-2 mixer. A 0.1 M sodium hydroxide solution (NaOH) was prepared by dissolving 200 mg sodium hydroxide pellets in 50 mL HPLC-grade water, using a Branson® B12 sonication bath.

2.5.5 Preparation of mobile phase

The mobile phase used for column efficiency testing was prepared by measuring 650 mL of ACN and 350 mL of HPLC grade water into A-grade measuring cylinders prior to transferring the solvents into a 1000 mL Schott Duran® glass bottle (Bestenheid, Wertheim, Germany). The mobile phase for all other HPLC analyses was comprised of ACN: phosphate buffer in different ratios. The molarity of the buffer and pH was investigated and the final HPLC method required a mobile phase comprised of ACN: phosphate buffer (pH = 6.0) in a 50:50% v/v ratio. The mobile phase was prepared using a 1000 mL A-

grade measuring cylinder to measure the volume of HPLC grade water and an Eppendorf® Research 100-1000 µL micropipette was used to measure 680 µL 85% v/v phosphoric acid that was made up to 1000 mL to produce a 0.05 M buffer. The pH of the buffer was measured using a Crison® Instruments S.A. GLP 21 pH meter (Barcelona, Catalonia, Spain) and was adjusted to 6.0 using a 0.1 M solution of NaOH. A 50:50% v/v ACN: phosphate buffer (pH = 6.0) was mixed in a 2000 mL Schott Duran® conical flask (Bestenheid, Wertheim, Germany) using a Labcon® Laboratory Magnetic Stirrer-hotplate (Maraisburg, Gauteng, South Africa). The mobile phase was prepared daily and kept in a 2000 mL Schott Duran® laboratory glass bottle. The addition of a competing amine, e.g. diethylamine, to the mobile phase was not necessary as the peak tailing estimated from the resultant KZ peaks were within the range permitted for peak tailing (peak asymmetry, As and peak tailing factors, Tf ≤ 2) as accepted by the FDA [14,122,123] as described in § **2.5.11** *vide infra*.

2.5.6 Degassing

Degassing of mobile phases is important to remove dissolved gasses, specifically oxygen and nitrogen, as these may result in bubble formation between the solid-liquid interface in the column and/or detector and may affect the performance of the method and detector [124]. Vacuum filtration is usually sufficient to degas mobile phases for isocratic separations. This technique usually removes approximately 60% of dissolved gas in a mobile phase and this approach is considered good practice for the generation of consistent and reliable results using HPLC [125]. Degassing systems can also filter particles that might be present in a solvent and serves as an additional precaution to avoid damage to the solvent delivery module, injection needle and/or stationary phase [124].

The mobile phase was degassed under vacuum using an Eyela® Aspirator-degasser A-2S (Bunkyo-ku, Tokyo, Japan) and filtered through a 0.45 µm Millipore® Corporation HVLP Durapore membrane filter (Milford, Massachusetts, USA).

2.5.7 HPLC column

HPLC stationary phases are fine silica based materials that are packed into stainless steel columns. The packing materials are held in the column with porous end frits of either 0.5 or 2.0 µm pore size located at each end of the column [126]. HPLC stationary phases are manufactured as normal- or reversed-phase, ion-exchange and size-exclusion chromatographic columns. Approximately 80% of HPLC separations are performed using RP columns [126] and column performance including for example limit of detection and sensitivity is dependent, in part, on the length and internal diameter of the column. System and method parameters such as flow rate, back pressure, void volume and sample capacity are also phenomena that are a consequence of column dimensions. Pharmaceutical analyses using short columns of small internal diameter are preferred as the resultant separation is rapid and demonstrates the requisite sensitivity with suitable resolution for the analysis of simple sample mixtures when

compared to longer columns. In addition lower volumes of solvent are used during short analytical run times, often rendering the use of these columns cost effective [126].

Silica based stationary phase backbones are generally used as the basis for column packing material as they demonstrate good chromatographic performance. RP chromatography makes use of mono-chlorosilane functionalities bonded to silanol groups to produce a hydrophobic environment within the stationary phase. The remaining unbound silanol groups are highly adsorptive and may be end-capped with smaller dimension silane functional groups to reduce silanol activity rendering the column more stable [126,127]. The RP packing materials can operate effectively only between pH 2 and pH 8. Different chromatographic separations require the use of different types of stationary phases that are able to function under other conditions according to their chemistry [128]. In addition the smaller the particle size of the packing material the better the efficiency of the column and ultimately the resolution between the peaks of the analyte(s) of interest.

A review of the literature (**Table 2.1**) revealed that Hypersil® columns have been widely used for the analysis of KZ. Hyperclone™ stationary phases offer similar separation qualities and performance and are more cost effective than Hypersil® columns [129]. A Phenomenex® Hyperclone 5µm ODS (C18) 120 Å 150x4.6 mm i.d. column was selected and used for these studies.

2.5.7.1 Column heaters

In general most HPLC separations are performed at ambient temperature. The Biopharmaceutics Research Laboratory in the Faculty of Pharmacy at Rhodes University is air-conditioned and the ambient temperature is 22°C. Therefore it was not deemed necessary to control the temperature or insulate the column further. It is known that chromatographic separations can be enhanced if the column temperature is regulated and maintained at a constant temperature controlled to at least half a degree Celsius [124]. Chromatographic separations of large molecules are more affected by changes in column temperature than smaller molecules. The viscosity of mobile phases decreases slightly as temperature is increased and its diffusivity concomitantly increases [130]. Fluctuations in the retention time of some potential internal standards were observed in initial experiments. The impact of column temperature on the separation of KZ and VP was therefore monitored and assessed as described in § **2.5.14.5** *vide infra*.

2.5.7.2 System suitability testing

New columns should be tested prior to use so as not to rely solely on the test results from the manufacturer of the column [131] and it is recommended that the columns be tested regularly in order to monitor the performance and condition of the stationary phase [132]. Columns were tested every six months and replaced if the column failed the performance test. Column performance was measured by establishing the number of theoretical plates, height equivalent to a theoretical plate, inter-peak

resolution factor (Rs), peak asymmetry factor (As), peak tailing factor (Tf) and capacity factor for the column, and have been analysed as described in **§ 2.5.10 - § 2.5.12** *vide infra*.

2.5.8 Distribution of analyte between the mobile and stationary phases

The distribution of an analyte of interest in a sample between the mobile and stationary phases should theoretically be in equilibrium [133]. The partition coefficient is a quantification of the equilibrium and can be estimated using **Equation 2.1.**

$$\text{Partition coefficient} = \frac{\text{Concentration of analyte in the stationary phase}}{\text{Concentration of analyte in the mobile phase}} \qquad \textbf{Equation 2.1}$$

The retention time, t_R, is defined as the time between sample injection and the appearance of the peak of the analyte of interest when it elutes from the column. The retention time of the mobile phase is referred to as t_M and any peak that is evident from the mobile phase is termed the solvent front.

2.5.9 Capacity factor

The retention factor, k' is also referred to as the capacity factor and is the migration rate of the analyte through a column with reference to the void volume of the column which is the time required for components that are not retained by the column to elute.

A capacity factor <2 is deemed not suitable due to poor reproducibility of retention times and therefore values between 2 and 10 are considered acceptable [134], however complex mixtures may result in capacity factors>10. The capacity factor, k' can be calculated from a chromatogram using the retention time for retained compounds, t_R and the retention time for compounds that elute in the void, t_M using **Equation 2.2**.

$$k' = \frac{(t_R - t_M)}{t_M} \qquad \textbf{Equation 2.2}$$

Where,

k' = capacity factor,
t_R = retention time for retained compounds, and
t_M = retention time for compounds that elute in the void.

2.5.10 Column efficiency

HPLC qualification and performance tests are conducted to assess the efficiency of a column prior to HPLC method development. The number of theoretical plates, As, Tf and Rs, are used as a measure of the performance and quality of a column.

Column testing was performed at 22 °C using a test solution comprised of uracil, acetophenone and naphthalene. The separation of these compounds was achieved on the Phenomenex® Hyperclone 5µm

ODS (C18) 120 Å 150x4.6 mm i.d.column at a flow rate of 1.0 mL/min using a mobile phase of ACN and water in a 65:35 v/v ratio and UV detection at 254 nm following injection of a sample volume of 15 µL.

The uracil, acetophenone and naphthalene peaks obtained from the chromatogram generated were analysed and the number of theoretical plates, height equivalent to a theoretical plate, As, Tf and Rs for uracil, acetophenone and naphthalene were used to establish the efficiency of the column.

The number of theoretical plates, N_p, can be calculated using **Equation 2.3** and is based on the retention time of the peak, t_R and the peak width at half peak height, $w_{\frac{1}{2}}$ derived from a chromatogram containing uracil, acetophenone and naphthalene and are measured as depicted in **Figure 2.1**.

$$N_p = \frac{5.55\, t_R^2}{w_{\frac{1}{2}}^2} \qquad \textbf{Equation 2.3}$$

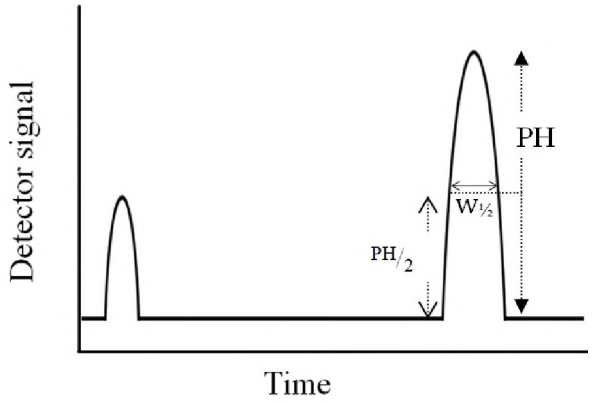


Figure 2.1 Schematic representation of the measurements taken to calculate HETP.

In this instance, the mean N_p for the three test compounds used was 8938 plates, which satisfies the limit of N_p>2000 plates for a column that is considered efficient and can be used for HPLC method development and validation according to FDA guidelines [134].

The number of theoretical plates or the plate height, also referred to as the Height Equivalent to a Theoretical Plate (HETP) of a column, is a measure of column efficiency [121,134]. The more efficient a column the higher the number of theoretical plates and the HETP can be calculated using **Equation 2.4**. The HETP was established to be 0.00168. The short theoretical plates allow for more plates to fit in the column length and this translates to a higher column efficiency.

$$\text{HETP} = \frac{\text{Length of column}}{\text{Number of theoretical plates}} \qquad \textbf{Equation 2.4}$$

2.5.11 Peak tailing

Gaussian peaks tend to be uncommon in chromatographic separations and most analyte responses tend to exhibit some degree of tailing which can be evaluated by calculating the As and Tf using **Equations 2.5** and **2.6** respectively [122].

The As is calculated by measuring the width of the front half of the peak measured at 10% peak height (a) and the width of the back half of the peak measured at 10% peak height (b) as depicted in **Figure 2.2**.

$$As = \frac{b}{a}$$ **Equation 2.5**

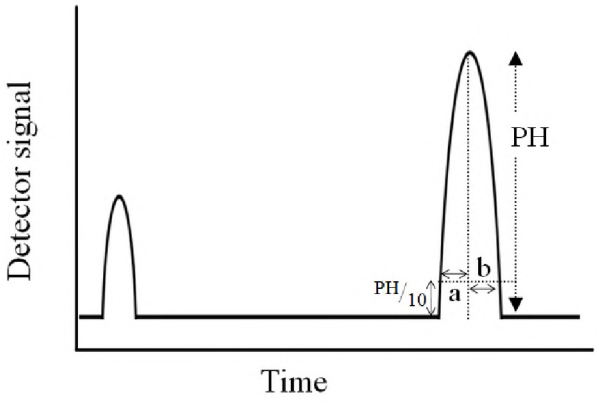


Figure 2.2 Schematic representation of the measurements required to calculate the As.

The USP suggests calculating the Tf using **Equation 2.6** and by measuring the width of the back half of the peak at 5% peak height (c) and the width of the front half of the peak measured at 5% peak height (d) as depicted in **Figure 2.3**.

$$Tf = \frac{c}{2d}$$ **Equation 2.6**

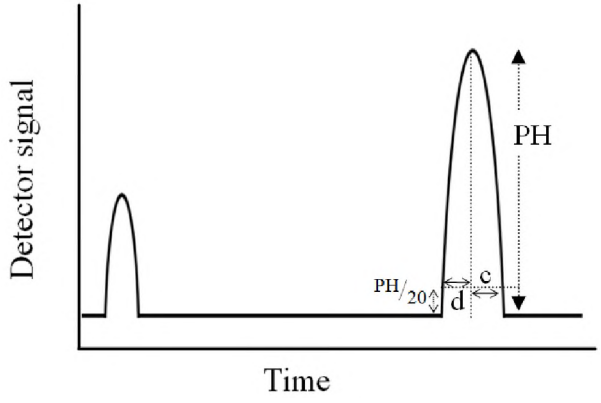


Figure 2.3 Schematic representation of the measurements required to calculate Tf.

The two approaches suggested for establishing peak tailing are generally used independently of each other provided that the relevant equation is used consistently as the two equations exhibit slightly different but interrelated values. An As or Tf ≤ 2.0 is considered acceptable by FDA [14,122,123] and this limit was considered appropriate for methods developed in our laboratory. The Phenomenex® Hyperclone 5µm ODS (C18) 120 Å 150x4.6 mm i.d. column used in these studies exhibited a mean As of 1.21, 1.07 and 0.89 and mean Tf of 0.86, 0.59 and 0.45 for uracil, acetophenone and naphthalene respectively, suggesting that the column is suitable for use based on these parameters.

2.5.12 Resolution factor

The resolution factor, Rs is a measure of the separation between two analytes in a sample mixture. The Rs can be calculated using the retention times (minutes), t_x and t_y and the peak widths at half peak height (cm), w½x and w½y using **Equation 2.7** and depicted in **Figure 2.4** [122,135].

$$R_s = 1.18\frac{(t_x - t_y)}{w_{1/2}x + w_{1/2}y}$$ **Equation 2.7**

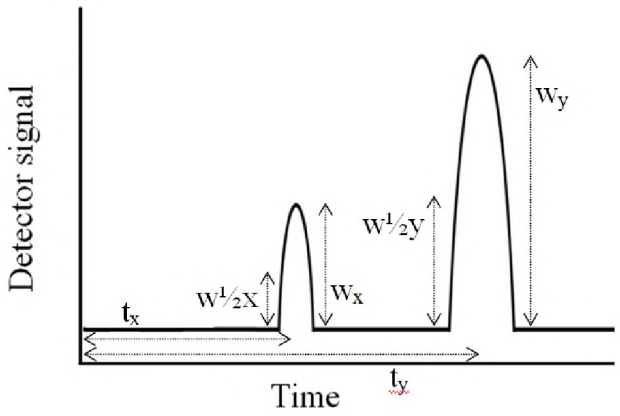


Figure 2.4 Schematic representation of the measurements taken to calculate peak resolution.

A Rs of 16.11 for the separation of naphthalene and acetophenone was recorded and that for the separation of naphthalene and uracil was 22.83. The separation between acetophenone and uracil yielded a Rs of 6.65. In all cases the Rs was >2.00 and the column was deemed appropriate for use as a Rs<1.50 is indicative of inadequate separation [121].

2.5.13 Choice of internal standard

An HPLC separation may be improved if an internal standard (IS) is added to samples prior to analysis as the use of an IS has the potential to reduce analytical error. In most cases the IS should to be added prior to sample pre-treatment or preparation in order to compensate for any analyte that may be lost [135]. The IS should have a capacity factor comparable to the analyte of interest if possible.

For the purposes of this method the IS was to be added to samples prior to analysis in order to minimize injection error. The main requirements of an IS are that is must be stable and completely resolved from the analyte(s) of interest and free of interference from sample components, the stationary or mobile phase. The IS should not be part of the original sample and therefore should not be an excipient or component of a formulation that is to be analysed using the HPLC method. In addition the IS should elute close to the analyte of interest without affecting the resolution of the compounds. The solubility in the mobile phase should be similar and it should be available as a high purity material [121].

Several compounds were evaluated as potential IS for this method. The criteria on which the selection of the IS was based included retention time, peak tailing and resolution. The chromatographic conditions used to assess the IS were a flow rate of 1 mL/min, 20 µL injection volume and an ACN: 0.05 M phosphate buffer (pH = 6.0) in a 50:50 v/v ratio mobile phase with the column temperature maintained at 22°C. Solutions of KZ and IS were mixed in a 1:1 ratio prior to RP-HPLC analysis and peak height ratio was used to plot calibration curves for further data reduction.

The chromatographic conditions selected resulted in a resolved peak for KZ peak with minimum tailing (As = 1.80 and Tf = 1.25) and a retention time of between 3 and 10 minutes. Retention times shorter than 3 minutes may result in interference of excipient peaks with KZ during analysis of test formulations. The resolution between the KZ and IS peaks were all within the >2 limit and therefore retention times were used as the main qualifying criteria for the section of an IS. Approximately 20 mg of KZ and 12 mg of the potential IS were accurately weighed and dissolved separately in ACN to produce 100 mL stock solutions from which equal volumes of KZ and IS solution were mixed to obtain 10 mL of a mixture containing 100 µg/mL KZ and 60 µg/mL of the IS. The solution was mixed using a Model G-560E Vortex Genie-2 mixer prior to HPLC analysis. The data summarized in **Table 2.2** reveals that verapamil (VP) performed suitably as an IS (Rs = 6.15, retention time = 9.90) and was therefore selected as the IS. The peak for VP is adequately separated from KZ and the retention time was < 10 minutes. A typical chromatogram in which the separation of KZ and VP is depicted is shown in **Figure 2.5**.

Table 2.2 Retention time and Rs factor for potential IS.

Compounds	Retention time min	Time between KZ and IS min	Retention time between 3 and 10 mins	Time between retention times ≥ 2 minS	Rs
Sulphafurazole (SF)	1.96	3.91	No	Yes	8.15
Sulphathiazole (ST)	1.74	4.10	No	Yes	8.92
Propranolol (PR)	5.11	0.69	Yes	No	0.81
Carbamazepine (CA)	2.61	3.23	No	Yes	7.87
Clotrimazole (CL)	15.08	9.01	No	Yes	8.99
Ornidazole (OR)	2.03	3.89	No	Yes	10.33
Verapamil (VP)	9.90	2.93	Yes	Yes	6.15

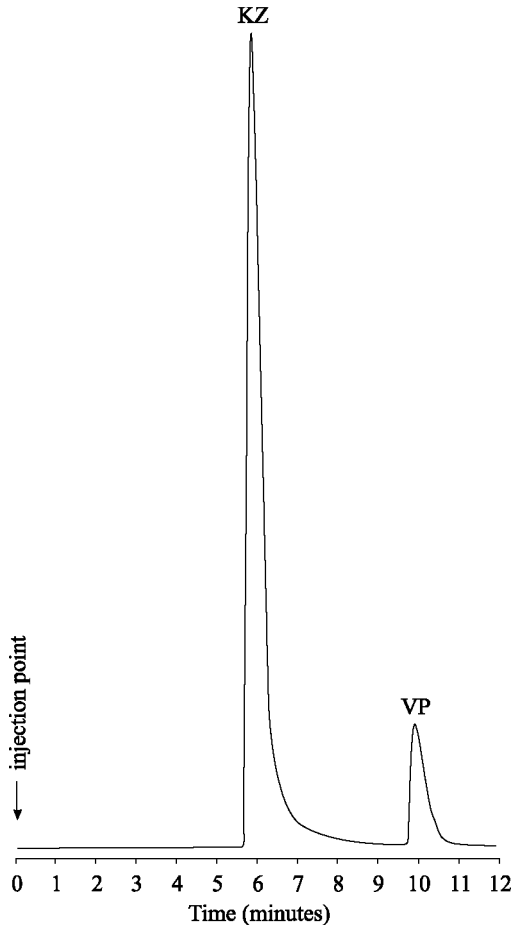


Figure 2.5 Typical chromatogram showing the separation of 100 µg/mL KZ and 60 µg/mL VP using a mobile phase composition of ACN: 0.05 M phosphate buffer (pH = 6.0) in a ratio of 50:50 v/v.

2.5.14 Selection of critical parameters for RP-HPLC and robustness testing

Parameters that are critical for the proper functioning of a RP-HPLC system and analytical method that result in an adequate Rs, As and Tf include flow rate, mobile phase composition, temperature, injection volume, molarity and pH of the mobile phase, amongst others.

2.5.14.1 Robustness

The ICH Q2 (R1) guideline defines robustness of an analytical procedure as the ability of the procedure to remain unchanged when small but deliberate deviations in specific parameters of the method are made and is an indication of the reliability of the procedure when it is used [136,137]. In the event that results from robustness studies reveal that changes in specific parameters may result in significant differences in responses then such parameters must be adequately controlled and a precautionary statement in the operating procedure included to indicate this [137]. Robustness can be investigated in three different areas, namely, internal parameters or changes in HPLC-related parameters, external parameters that are similar to reproducibility § **2.6.2.3** and basic parameters such as, for example, stability of samples. Robustness testing encompasses a series of experiments reported in a number of

sections of this chapter and in which internal parameters were investigated during method development and basic parameters such as stability were evaluated during stress testing (**§ 2.7**).

2.5.14.2 Mobile phase selection

ACN: phosphate buffer was selected as mobile phase as discussed in **§ 2.3**. The effect of organic modifier (ACN) composition, effects of phosphate buffer pH and molarity on method performance are discussed in **§ 2.5.14.2** *vide infra.*

2.5.14.2.1 Effect of organic modifier

The purpose of using an organic modifier in the mobile phase is to modify the separation potential of a system through reduction of the polarity of the aqueous phase of the mobile phase in relation to the analyte [138]. A systematic approach was used to identify the most appropriate organic modifier for this separation. The literature review (**Table 2.1**) revealed that MeOH and ACN are the most common organic solvents used for the analysis of KZ. ACN exhibits a low viscosity and polarity and would be the ideal organic modifier for RP-HPLC analysis of KZ [121]. As a starting point an ACN: phosphate buffer and MeOH: water was used and KZ was retained on the stationary phase using an ACN: phosphate buffer mobile phase. The ultimate composition of the mobile phase was further investigated and is reported in **§ 2.5.14.2.2** and **§ 2.5.14.2.3.** Mobile phases comprised of ACN: 0.05 M phosphate buffer (pH = 6) in ratios of 25:75, 40:60, 50:50 and 60:40 v/v were prepared. The values for As, Tf, Rs and retention times for KZ and VP calculated for the separation are listed in **Table 2.3.**

No peaks were observed within 24 minutes when a mobile phase comprising of 25% v/v ACN was used. This suggests that the KZ and VP peaks were not resolved and the trend observed with ACN composition supports this supposition (**Table 2.3**). The retention times when 50% v/v and 60% v/v ACN were used in the mobile phase were < 10 minutes and these mobile phase compositions could therefore be considered for the RP-HPLC analysis of KZ. The As and Tf for the VP peak were at their lowest at 50% v/v ACN and the As calculated for 40% v/v and 60% v/v ACN were not acceptable according to the set As FDA values [14,122,123]. The chromatogram in which the best Rs, As and Tf for KZ and VP peaks were observed when 50% v/v ACN was used in the mobile phase. The resultant parameters for KZ were Rs = 5.11, As = 1.50 and Tf = 1.50 and the As and Tf for the VP peak were 2.00 and 1.25 respectively. These values all fall within the limits recommended by FDA [14,122,123] and applied in our laboratory [135].

The retention times of KZ and VP decrease as the ACN concentration in the mobile phase increases. The log $P_{o/w}$ of KZ = 3.78 [27,37] and log $P_{o/w}$ of VP = 9.1 [139] suggest that the compounds are hydrophobic and have greater affinity for the hydrophobic stationary phase than the aqueous mobile phase [126]. When the ACN in the mobile phase is increased, the affinity of KZ and VP to the mobile

phase increases and is therefore less retained in the column causing a lower retention time. The higher log $P_{o/w}$ of VP suggests greater hydrophobicity as compared to KZ, causing better retention in the column as can be observed in **Table 2.3**.

Table 2.3 Effect of ACN composition on retention characteristics of KZ and VP.

	KZ				VP		
ACN composition % v/v	**Rs**	**As**	**Tf**	**Retention time min**	**As**	**Tf**	**Retention time min**
25	-	-	-	-	-	-	-
40	2.95	1.70	1.36	17.09	3.75	1.60	22.31
50	5.11	1.50	1.50	5.80	2.00	1.25	8.83
60	5.66	1.50	1.00	3.50	3.00	1.50	6.21

2.5.14.2.2 Effect of pH

The summary of published methods in **Table 2.1** reveals that mobile phases with a pH between 4.0 and 8.0 have been used for the analysis of KZ. A mobile phase composition of ACN: 0.05 M phosphate buffer in a 50:50 v/v ratio was used and the pH of the buffer was adjusted to 4.0, 5.0, 6.0, 7.0 and 8.0. The impact on the retention, resolution, tailing and peak shape of KZ and VP when a buffer of different pH was evaluated and these data are listed in **Table 2.4.**

Table 2.4 Effect of buffer pH on retention characteristics of KZ and VP.

	KZ				VP		
Buffer pH	**Rs**	**As**	**Tf**	**Retention time min**	**As**	**Tf**	**Retention time min**
4	3.93	1.57	1.00	5.46	2.00	1.13	8.69
5	3.93	1.00	0.79	5.50	2.33	1.25	9.73
6	5.11	1.71	1.29	5.56	2.00	1.80	9.90
7	11.80	1.67	1.83	5.68	2.00	1.00	12.64
8	16.88	0.60	0.60	6.12	1.80	1.10	17.90

Mobile phases consisting of phosphate buffer of low pH cause the ionisation of the basic KZ and VP molecules which renders them less hydrophobic [140]. The affinity of KZ and VP to the mobile phase therefore increases as pH decreases causing them to be less retained in the column which explains the decrease in retention time of KZ and VP peaks as pH decreases as observed in **Table 2.4**. KZ exhibits pKa values of 2.94 and 6.51 [13] and VP's pKa = 9.04 [141] suggesting that VP is a stronger base than KZ. The retention time of VP is therefore more affected by buffer pH changes than KZ as revealed in **Table 2.4**.

A phosphate buffer of pH 6 in the mobile phase resulted in a separation that exhibited the best resolution and a retention time between 3 and 10 minutes for KZ and VP. The As for KZ and VP with a phosphate buffer of pH 6 was 1.71 and 2.00 and for Tf was 1.29 and 1.80 respectively and that fulfils the criteria recommended by FDA for peak tailing [14,122,123]. The phosphate buffer pH 6 was chosen over the

other pH analysed as the ionisation of VP and KZ was adequate to keep the retention times, Rs, As and Tf, at desirable values and low enough to avoid the dissolution and/or damage of the silica support in the column [142,143].

2.5.14.2.3 Effect of molarity

The buffering capacity of the mobile phase depends on the molarity and pH of the buffer [143]. The effect of buffer molarity on the separation of KZ and VP was investigated using a mobile phase consisting of ACN: phosphate buffer (pH = 6) in a ratio of 50:50 v/v and the molarity of buffer assessed varied between 0.03 and 0.1 M. The separation parameters for the chromatograms are listed in **Table 2.5**.

Table 2.5 Effect of buffer molarity on retention characteristics of KZ and VP.

	KZ				VP		
Buffer Molarity M	**Rs**	**As**	**Tf**	**Retention time min**	**As**	**Tf**	**Retention time min**
0.03	2.44	1.50	1.38	6.59	4.00	2.25	12.83
0.05	5.11	1.71	1.29	6.08	2.00	1.80	9.90
0.07	3.88	2.50	2.25	5.52	5.00	2.00	8.00
0.10	0.40	3.67	2.83	5.47	2.67	2.17	7.64

The retention times for KZ and VP at all molarities except 0.03 M were < 10 minutes and are suitable for RP-HPLC analysis of KZ. The resolution was adequate for all molarities except a 0.10 M buffer and the As and Tf for the 0.05 M buffer were within the limits set for KZ and VP. On the basis of the parameters set a buffer molarity of 0.05 M was selected for all future analysis and a typical chromatogram produced using these conditions is depicted in **Figure 2.5**.

As the buffer molarity increased, an increase in the retention times of KZ and VP was observed. KZ retention times did not reveal significant changes as compared to VP, which is a more basic analyte. At low buffer molarity, the buffer capacity was too low to counteract the changes in overall basicity of the sample due to the presence of the basic KZ and VP analytes and KZ and VP were more retained in the column as they were less ionised in the mobile phase. As the molarity increased, the retention times decreased due to a stronger buffer capacity causing the buffer to remain closer to pH 6 as the molarity of the phosphate buffer increased. This effect was similar to the effect of pH changes on the retention times of KZ and VP as described in § **2.5.14.2.2**.

2.5.14.3 Flow rate

Mobile phase flow rates of 0.4 mL/min, 0.6 mL/min, 0.8 mL/min, 1.0 mL/min and 1.2 mL/min were evaluated for their impact on the separation of KZ and VP using a mobile phase of ACN: 0.05 M phosphate buffer (pH = 6) in a ratio of 50:50 v/v at 22°C. As expected an increase in flow rate resulted in a decrease in the retention time of KZ and VP (**Figure 2.6**). Flow rates of 1.0 mL/min and 1.2 mL/min produced retention times of 5.81 and 4.87 minutes for KZ and 9.23 and 7.63 minutes for VP. A flow rate 1.0 mL/min has been widely used (**Table 2.1**), and produced adequate resolution of 5.11 between KZ and VP with peak tailing ≤ 2.00 (KZ: As = 1.71, Tf = 1.29; VP: As = 2.00, Tf = 1.80) [14,122,123] and therefore a flow rate of 1.0 mL/min was selected for use.

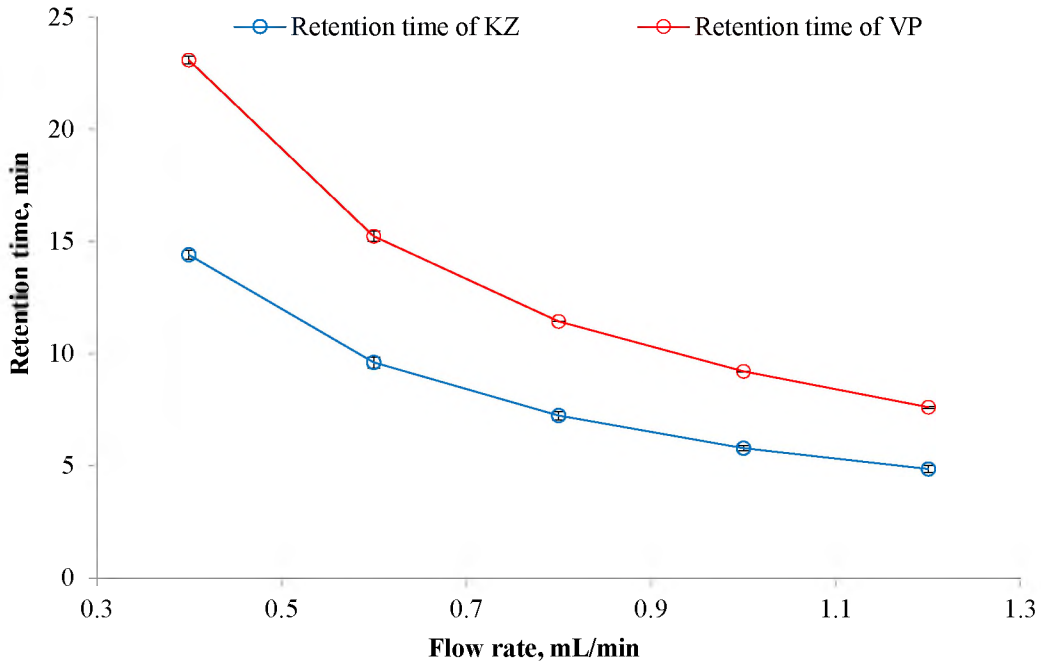


Figure 2.6 The effect of flow rate on the retention time of KZ and VP.

Slower flow rates might be more economical in respect of solvent use; therefore, while the faster flow rate of 1.2 mL/min produced shorter run times, a flow rate of 1 mL/min was chosen as less ACN was required for separation. Flow rates of 0.4, 0.6 and 0.8 mL/min exhibited longer KZ and VP retention times, poor resolution and tailing which were not ideal for RP-HPLC analysis.

2.5.14.4 Injection solvent and volume of injection

Injection volumes of 10, 15, 20, 25, 30 µL were tested to evaluate the impact of injection volume on the separation and chromatographic parameters for these experiments. The results are summarised in **Table 2.6**. An injection volume of 20 µL was selected for use as sufficient sensitivity was achieved without overloading the column [121,144]. Furthermore, a Rs of 5.11, an As of 1.71 and 2.00 for KZ and VP and a Tf for KZ = 1.29 and VP = 1.80 were within the limits set in our laboratory. Different injection volumes did not appear to affect the shape of the KZ and VP peak and no peak splitting or irregular peak shape was observed when 50:50 v/v ACN: water was used as injection solvent. ACN, as injection solvent, and a ratio of 50:50 v/v ACN: water were selected as the mobile phase chosen contained 50:50 v/v ACN: phosphate buffer and using a similar injection solvent composition would not affect chromatographic resolution due to a change in injection solvent strength.

Table 2.6 Effect of injection volume on retention characteristics of KZ and VP.

		KZ			VP		
Injection volume µL	**Rs**	**As**	**Tf**	**Retention time min**	**As**	**Tf**	**Retention time min**
10	6.24	1.00	1.06	6.25	3.00	1.75	9.37
15	6.14	1.43	1.21	6.14	2.20	1.17	9.42
20	5.11	1.71	1.29	6.08	2.00	1.80	9.90
25	4.21	1.71	1.25	6.00	3.50	2.00	9.18
30	3.80	0.50	0.50	6.26	2.00	1.13	9.41

2.5.14.5 Column temperature

The quality of a separation may be enhanced by altering the temperature of the stationary phase and by taking into account the stability of the analyte at the different temperatures. Appropriate conditions for the separation can be selected [138]. The impact of column temperature on the separation of KZ and VP was investigated at 20, 22, 24, 26 and 28°C.

The effect of temperature on the retention time, resolution, tailing and peak shape was obtained by calculating relevant factors such as Rs, As and Tf and these data are summarised in **Table 2.7**. The resultant chromatograms for separations at all temperatures revealed good resolution between KZ and VP. The retention time for KZ was similar at all column temperatures used and no decomposition of KZ or VP was observed during analysis. An ambient temperature of 22 °C was therefore selected for use for all further RP-HPLC analyses.

Table 2.7 Effect of column temperature on retention characteristics of KZ and VP.

	KZ				VP		
Column temperature °C	**Rs**	**As**	**Tf**	**Retention time min**	**As**	**Tf**	**Retention time min**
20	6.20	1.67	1.80	6.06	1.83	2.00	10.53
22	6.15	1.71	1.29	6.08	2.00	1.80	9.98
24	6.44	1.40	1.00	6.04	2.00	1.00	9.71
26	6.29	1.17	1.00	6.06	1.17	0.75	9.45
28	4.72	1.60	1.17	5.98	1.40	0.90	8.57

2.5.14.6 Selection of chromatographic conditions

A typical chromatogram of the separation is depicted in **Figure 2.5**. The capacity factor for KZ was 4.80 and VP was 8.90 and falls within the 2.00 < k'< 10.00 limit [134]. The As for KZ and VP were 1.71 and 2.00 respectively and the tailing factor, Tf, was 1.29 and 1.80 respectively. Visually, the tailing observed for the KZ and VP peaks reveals that they do not interfere with each other and this was confirmed by the calculated Rs of 6.15.

The chromatographic conditions established as optimum for the analysis of KZ are summarised in **Table 2.8** and these conditions were deemed acceptable for the analysis of KZ. The peak height ratio of KZ:VP was used for the quantitative analysis of KZ in all studies.

Table 2.8 Chromatographic conditions for the analysis of KZ.

Column Specifications	Phenomenex® Hyperclone 5µm ODS (C18) 120 Å 150x4.6 mm i.d.
IS	VP
Detection wavelength	205 nm
Flow rate	1.0 mL/min
Injection volume	20 µL
Column temperature	22°C
Mobile phase composition	ACN: 0.05 M phosphate buffer (pH = 6) 50:50 v/v

2.6 Method validation

The quality, reliability and validity of results following the application of an analytical method can be ensured by performing validation experiments prior to the full time implementation of the method [136]. The International Conference on the Harmonisation of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH) established harmonised definitions and terms for analytical validation from as early as 1990 [136]. The FDA has also published validation guidelines including a document relating to analytical procedures and methods validation [145], Guideline on General Principles of Process Validation [146], Pharmaceutical cGMPs for the 21st Century - A Risk-Based Approach [147], Process Validation: General Principles and Practices [148], amongst others. Different compendia and regulators may have different definitions and procedures for validation and the harmonized approach such as those listed in the ICH Q2 (R1) guidelines that provide a list of definitions relating to the validation of a product so as to bridge the understanding of validation procedures internationally[137] is useful. The guidelines should not be used as a checklist and the validation of analytical procedures can be amended depending on the nature of the analyte of interest and the purpose of the procedure [136]. Analytical method validation ensures that an analytical method is suitable for its intended function and it is vital that the objectives of the method and analytical procedure are defined and understood prior to the commencement of validation studies and are applied throughout method

development and optimization experiments [145]. Validation must be undertaken using current Good Manufacturing Practices (cGMP) and Good Laboratory Practices (GLP).

Typical parameters investigated during validation include specificity, linearity, accuracy, precision (repeatability, intermediate precision and reproducibility), range, robustness, limits of quantitation (LOQ) and detection (LOD) [145]. A stability-indicating method requires that changes in the quality of an analyte or a finished product on storage or during manipulation can be detected and therefore the development of such a method requires that forced degradation or stress studies form part of the validation process [145].

The average result of all measurements is an estimation of the true value when normally distributed and the true value can be expected to be located in a specific interval around the average and that interval is known as a confidence interval (CI). A normal distribution is infinite and therefore it is possible to generate data that may deviate significantly from the true value for that information. However, there is a low probability that such data will be generated and therefore the expected range for that data must be confined to a realistic range. The error probability, statistical confidence or statistical significance defines the realistic range and a 95% confidence level is generally used to establish that range [136]. The number of replicate measurements, n, was 6 for these studies and therefore the experimental standard deviation (SD) may be ± 1.05 for a 95% confidence level, whereas the calculated SD may be ± 2.09 [136]. In order to obtain reliable data for the mean and SD, data sets of $n \geq 6$ must be used as the error probability of the SD increases 4.4 fold if the number of replicates used is 3 [136]. The % RSD should be $\leq 5\%$ for an analytical method to be considered accurate and precise [14,123].

2.6.1 Linearity and range

According to the ICH Q2 (R1) guideline, linearity is an indication of the capacity of a method to produce results that are directly proportional to the concentration of an analyte of interest in a sample, within a set range [137] and a calibration curve should be developed using a minimum of five concentrations [136]. The range of an analytical method is the range of values that are contained within the upper and lower concentrations of that analyte that is likely to be found in samples and that demonstrate suitable precision, accuracy and linearity [136]. However, the range can be selected according to the requirements for the analyses to be performed.

Linearity was assessed using eleven standard solutions (n = 6) containing a mixture of KZ and VP in a 1:1 ratio. The calibration standards were prepared as described in § **2.5.4** and were 0.50, 1.17, 2.52, 5.07, 10.31, 20.29, 50.76, 61.04, 101.24, 121.53, 151.30 µg/mL of KZ. The average peak height ratio for KZ:VP were calculated for each concentration and a least squares linear regression best fit line for

peak height ratio versus concentration was plotted and a typical example of a calibration is depicted in **Figure 2.7**.

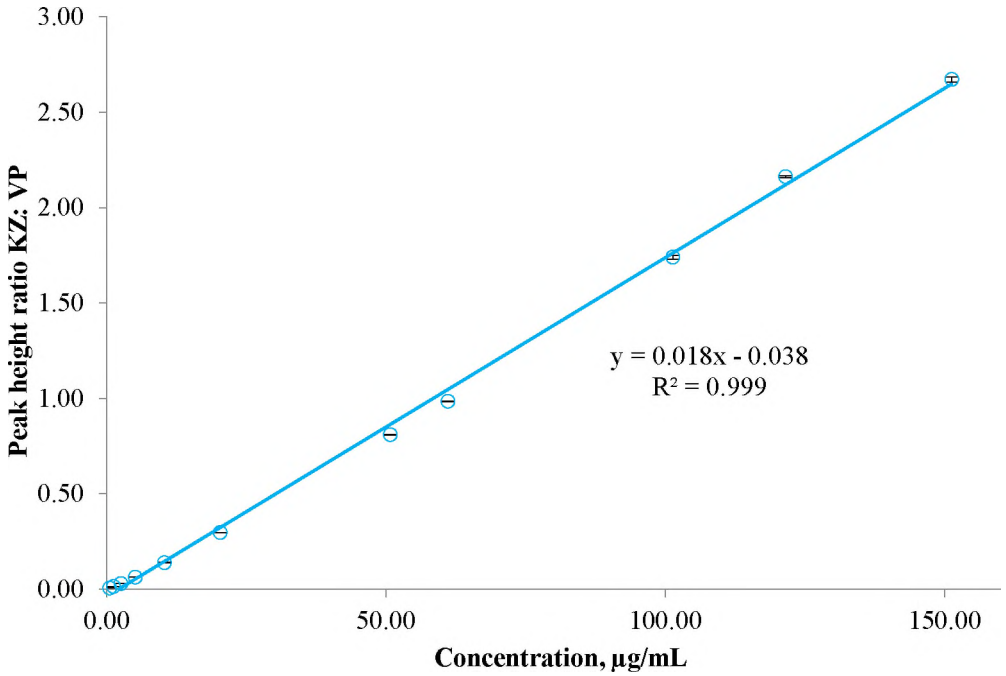


Figure 2.7 Typical calibration curve for KZ over the concentration range 0.50 – 151.30 µg/mL .

The best fit least squares linear regression line revealed there was a direct correlation between the UV absorption of KZ, and the concentration of KZ. The equation for the line was y = 0.018x - 0.038 with a correlation coefficient (R^2) = 0.999 and any value for $R^2 \geq 0.999$ is suitable to declare linearity of a method [149]. The regression plot should produce a y-intercept < 2% of the response or should be as close to zero as possible in order to be assured of linearity [150]. A y-intercept of 0.038 satisfies this criterion for linearity. The error bars representing the SD of the peak height ratio of KZ: VP were small and therefore are not clearly visible in **Figure 2.7**.

All points on the calibration curve revealed an experimental SD $\leq \pm$ 1.05 and a % RSD $\leq$ 5% as summarised in **Table 2.9** indicating that there is good precision.

2.6.2 Precision

The precision of an analytical method is a measure of the closeness of agreement between a series of multiple measurements of the same homogenous sample using set conditions. Precision may be calculated at three levels, *viz.*, repeatability or intra-day, intermediate or inter-day precision and reproducibility [137]. Acceptable precision is based on the SD or variance, % RSD or coefficient of variation and a CI for each stage of precision [136]. Reproducibility or ruggedness by definition is the reproducibility of results obtained from the same samples under different conditions such as analysis

undertaken in different laboratories or using different analysts and/or different instruments [137]. The tolerance for the SD was set at ≤ ± 1.05, % RSD ≤ 5% and % recovery = 100 ± 5%.

Table 2.9 Intra-assay data for a typical calibration curve.

Theoretical Concentration µg/mL	Average Peak Height Ratio (KZ: VP) n = 6	SD	% RSD
0.50	0.0298	0.0002	3.38
1.17	0.0138	0.0003	2.36
2.52	0.0060	0.0002	0.80
5.07	0.0628	0.0001	0.14
10.32	0.1394	0.0002	0.11
20.29	0.2961	0.0004	0.12
50.76	0.8096	0.0010	0.13
61.04	0.9847	0.0020	0.20
101.34	1.7404	0.0094	0.54
121.53	2.1632	0.0049	0.23
151.30	2.6723	0.0123	0.46

2.6.2.1 Repeatability or intra-day precision

Repeatability refers to the precision of a method established under specific experimental conditions over a short time interval. Intra-day precision is recommended to be assessed by analysing three different concentrations of KZ in triplicate or by analysing the samples at the maximum concentration at least six times [137]. RP-HPLC analysis was performed by a single analyst in one laboratory using the same HPLC system on the same day. Six replicates (n = 6) were analysed at three different concentrations, *viz.*, low, medium and high within the range of the calibration curve and the results are summarised in **Table 2.10**. Solutions of 30 µg/mL , 80 µg/mL and 130 µg/mL KZ concentration were accurately prepared in 25 mL A-grade volumetric flasks as described in **§ 2.5.4**. A 5 mL aliquot of the KZ solutions was mixed with 5 mL of a 120 µg/mL VP and analysed by RP-HPLC. The values for SD, % RSD and % recovery were calculated and it was established that the data were within the set limits and therefore the method exhibits adequate intra-day precision.

Table 2.10 Intra-day precision for KZ.

Theoretical Concentration µg/mL	Actual Concentration µg/mL	SD	% RSD	% Bias	% Recovery
30.70	30.60	0.00120	0.25	-0.33	99.67
81.83	80.82	0.00606	0.43	-1.24	98.77
131.61	132.17	0.01050	0.45	0.43	100.43

2.6.2.2 Intermediate or inter-day precision

Intermediate precision refers to variations in data that may occur in the same laboratory [137]. Inter-day precision experiments were performed daily on three consecutive days at three concentration levels, *viz.*, low, medium and high for KZ (n = 6) using similar concentrations to those used for the repeatability studies (**§ 2.6.2.1**). The results of inter-day precision studies are listed in **Table 2.11**. In all cases the SD $\leq \pm$ 1.05, % RSD $\leq$ 5% and the % recovery values were 100 $\pm$ 5% indicating that the analytical method exhibited adequate intermediate precision.

Table 2.11 Inter-day precision data for KZ.

	Theoretical concentration µg/mL	Actual concentration µg/mL	SD	% RSD	% Bias	% Recovery
Day 1	30.70	30.60	0.0012	0.25	-0.33	99.67
	81.83	80.82	0.0061	0.43	-1.24	98.77
	131.61	132.17	0.0105	0.45	0.43	100.43
Day 2	30.49	30.10	0.0005	0.15	-1.29	98.71
	80.79	79.23	0.0037	0.35	-1.94	98.06
	130.22	132.19	0.0051	0.28	1.52	101.52
Day 3	30.70	30.17	0.0012	0.26	-1.73	101.76
	81.83	80.44	0.0061	0.43	-1.69	101.72
	131.61	131.92	0.0105	0.45	0.24	99.77

2.6.2.3 Reproducibility

Reproducibility refers to the precision of experiments performed in different laboratories or with different analysts or with different equipment [137] and is normally undertaken when method transfer is required for the purposes of standardising the method in different environments. All experiments were performed in the same laboratory and by the same analyst for these studies and therefore establishing method reproducibility was not required. The ICH guidelines also stipulates that reproducibility studies are not required when the laboratory developing the method of analysis is the same laboratory in which the analyses are to be conducted [137].

2.6.3 Accuracy

The accuracy of an analytical procedure is defined as the closeness of agreement between a value that is established as a conventional true value of an accepted reference and the value obtained following analysis [136]. Accuracy is sometimes also referred to as trueness or lack of bias [136,137]. The % bias was set to ≤ ± 5% for accuracy and % bias was calculated using **Equation 2.8**.

$$\% \text{ Bias} = 100 \frac{\text{True value} - \text{measured value}}{\text{True value}} \qquad \textbf{Equation 2.8}$$

Three different concentrations of KZ , *viz.*, low, medium and high within the calibration range, were prepared as for precision studies (**§ 2.6.2**) and injected (n = 6) onto the chromatographic system. The SD, % RSD and % bias for these studies are listed in **Table 2.12**.

Table 2.12 Accuracy data for KZ.

Theoretical Concentration µg/mL	Actual Concentration µg/mL	SD	% RSD	% Bias
31.80	30.61	0.02000	0.82	-3.89
80.77	81.82	0.00901	0.25	1.28
130.00	131.95	0.06010	0.67	1.48

The % bias established from accuracy experiments were all ≤ ± 5% indicating that the analytical method is accurate.

2.6.4 Limits of quantitation and detection

The limit of quantitation (LOQ) is required for part of the calibration curve as it is the lowest amount of an analyte that can be precisely and accurately measured using the stated analytical conditions. The limit of detection (LOD) is the lowest amount of an analyte that can be detected but not quantified with the necessary precision and accuracy [136]. The LOQ and LOD must be elucidated when validating an analytical procedure as described in the ICH Q2 (R1) guideline [137].

There are three approaches to calculating the LOQ of a method, *viz.*, through visual evaluation, use of the signal-to-noise ratio or the evaluation of SD of response and slope of the response [137]. The visual and SD approaches were used for the validation of this method. Visual evaluation is generally undertaken when non-instrumental chromatographic separation is performed; however, it is also possible to use this approach for instrumental chromatographic analyses. Analysis of samples containing known low concentrations of the analyte(s) of interest was undertaken and the lowest concentration at which the analyte was able to be quantitated with the necessary precision and accuracy was selected as the LOQ [137]. Precision and accuracy are measured using % RSD and a concentration of 0.50 µg/mL of KZ responded with a % RSD = 3.38% which is < 5.00% and was therefore deemed

acceptable as the LOQ [136]. The LOD is calculated by convention as 0.3 x LOQ [137] and was established as 0.165 μg/mL KZ for this separation

2.7 Stress testing

The instability of analytes affects many aspects in the pharmaceutical environment including analytical method development, formulation development, packaging, storage conditions, shelf-life determination, drug safety, toxicology, process and manufacturing parameters, absorption, distribution, metabolism, and excretion in addition to requiring consideration of environmental issues. The aforementioned are of concern during product development and information relating to the stability of an API was vital to the successful development of a product for this research [151].

Stress testing or forced degradation studies are undertaken to establish the specificity of an analytical method and to ensure that it is stability-indicating [152]. Such studies may also be useful for the prediction of potential stability related problems and the identification of degradation pathways and products [151].

There are no detailed and/or specific guidelines as to how to conduct stress testing studies and research scientists therefore design their own approaches to induce degradation of analyte(s) of interest, taking into account the type of formulation to be developed and tested using the analytical method and consequently flexibility and scientific judgement are required when designing and undertaking stress testing investigations [151].

A list of the recommended stress tests that may be performed on drug substances and products is summarised in **Table 2.13**. Thermolytic, hydrolytic, oxidative and photolytic degradation were performed on KZ solutions and are comprehensively described in **§ 2.7.1 - § 2.7.4**.

Table 2.13 General recommendations for stress testing of drug substances and products [152,153].

	Drug substance		Drug product	
Condition	**Solid**	**Solution/suspension**	**Solid/semi-solid**	**Solution**
Acid/base hydrolysis	-	x	-	optional
Oxidative	optional	x	x	x
Photolytic	x	optional	x	x
Thermal	x	-	x	x
Thermal/humidity	x	-	x	-

x = recommended tests; optional = suggested for certain compounds; - = not required

It was observed that the degradation of KZ results in the formation of a solution that is red in colour [109] and it is clear that a colour change is therefore a good indication KZ has degraded in solution and this result may therefore be used as an initial visual assessment of instability during stress studies.

2.7.1 Thermolytic degradation

Thermolytic degradation occurs due to bond breakage or pyrolysis following exposure of a compound to elevated temperatures [151]. The ICH Q1A (R2) guideline suggests that thermolytic degradation can be investigated by exposing an analyte to temperatures that are increased by 10°C increments above accelerated stability test temperature conditions of 40°C [154]. Stock solutions of KZ (200 µg/mL) were exposed to temperatures of 50, 60, 70, 80, 90 and 100°C respectively (n = 3) by refluxing for four hours with the aid of a Colora® Model NB-34980 Ultra-Thermostat water bath (Lorch, Hesse, Germany). Samples (5 mL) of each solution were removed when the temperature of the solution had cooled to 22°C. The aliquot was then mixed with 5 mL of a 120 µg/mL VP solution prepared as described in § **2.5.4** prior to analysis. The % recovery of KZ was established and quantitated using RP-HPLC analysis and the results are depicted in **Figure 2.8**.

Approximately 0.09 ± 0.53% and 5.06 ± 1.13% degradation of KZ was observed at 50°C and 60°C and when exposed to 100°C approximately 82.43 ± 0.78% KZ was recovered after 4 hours of heating. This suggests that solutions of KZ can be heated up to 50°C for a maximum of 4 hours during analysis, formulation and/or storage without the risk of excessive degradation of KZ.

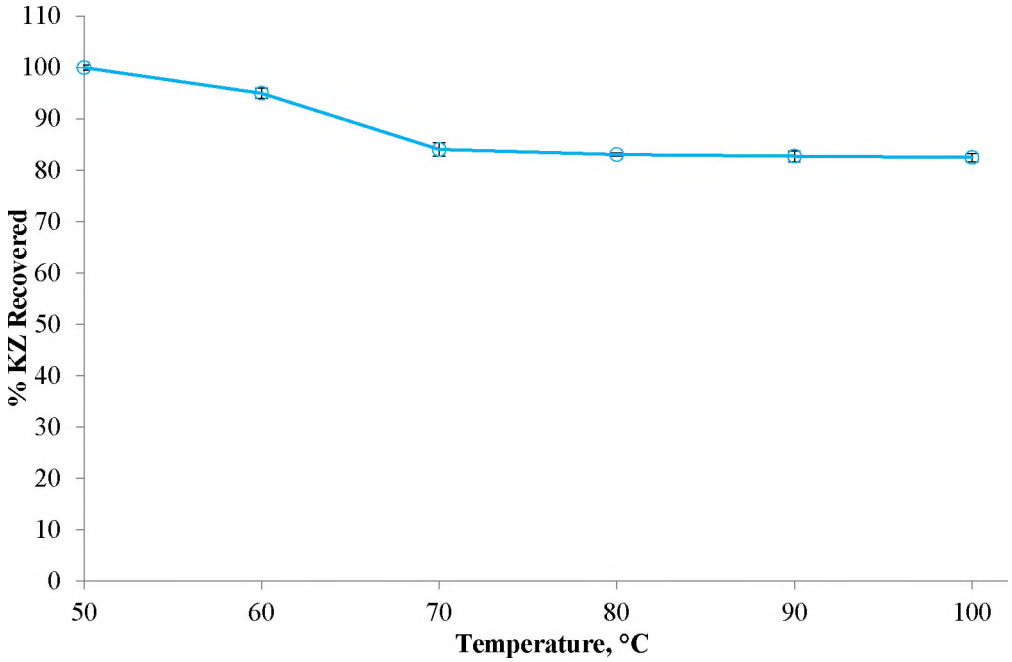


Figure 2.8 KZ recovered following exposure in solution to temperatures of 50, 60, 70, 80, 90 and 100°C (n = 3).

2.7.2 Hydrolytic degradation

2.7.2.1 Acid hydrolysis

Acid hydrolysis of KZ was undertaken performed by exposing 10 mL of a solution of a 200 µg/mL solution to 10 mL of 0.1 M, 0.5 M, 1.0 M, 1.5 M and 2.0 M HCl in water at 22°C for one hour. An estimation of the stability of KZ in acidic solution would be valuable since the intention was to produce an acidic gel that was similar to the intravaginal environment for the administration of KZ [155]. Following exposure for one hour, 5 mL samples were removed and mixed with 5 mL of a VP solution prior to RP-HPLC analysis. The % KZ recovered was calculated following quantitation using RP-HPLC. Approximately 50.09 ± 0.43% and 17.28 ± 0.65% KZ was recovered when 0.1 M and 2.0 M HCl was used and the data are depicted in **Figure 2.9**. KZ degradation in 0.1 M HCl has also been reported [38].

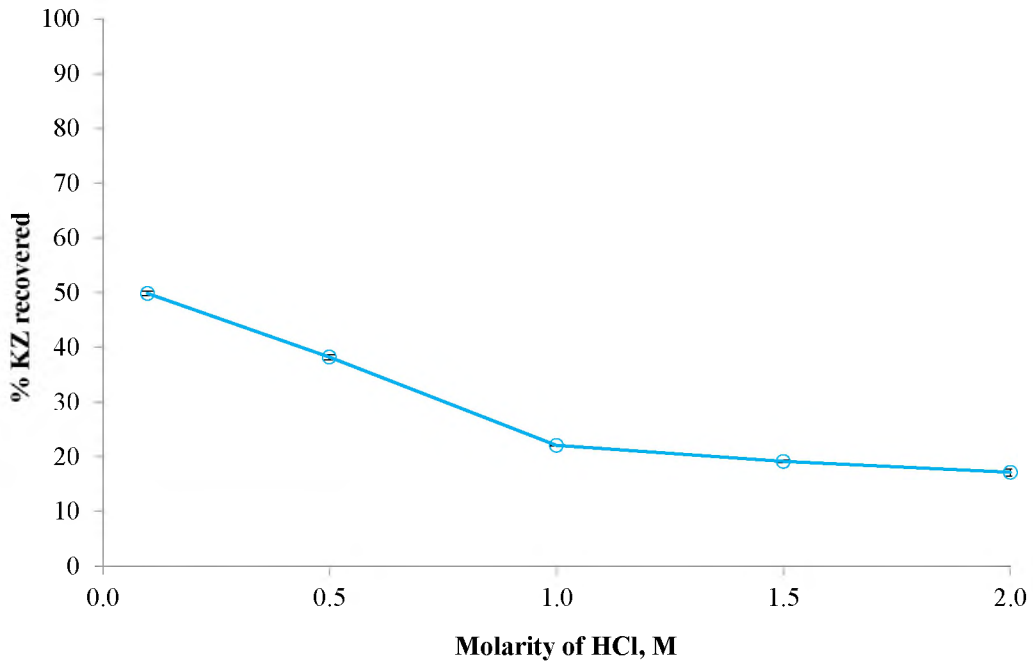


Figure 2.9 % KZ recovered following exposure to 0.1 M, 0.5 M, 1.0 M, 1.5 M and 2.0 M HCl for one hour.

An unknown peak at a retention time of 1.42 minutes in all chromatographic runs and the peak height for this response increased as the molarity of the HCl solution added was increased. This degradation product may be 1-(4-phenyl) piperazine (MM = 489.39) that has been identified [38] using LC-MS, NMR and HPLC. The molecular structure of KZ and the degradation product that forms due to hydrolysis of the carboxylic acid functional group are described in **§ 1.2.1, Figure 1.1** and **Figure 2.10.** The degradation product was reported by Mhaske et al., [38] and was not isolated and identified in these studies.

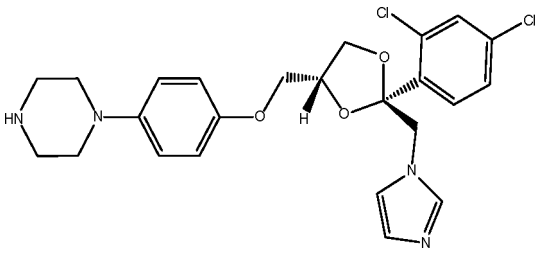
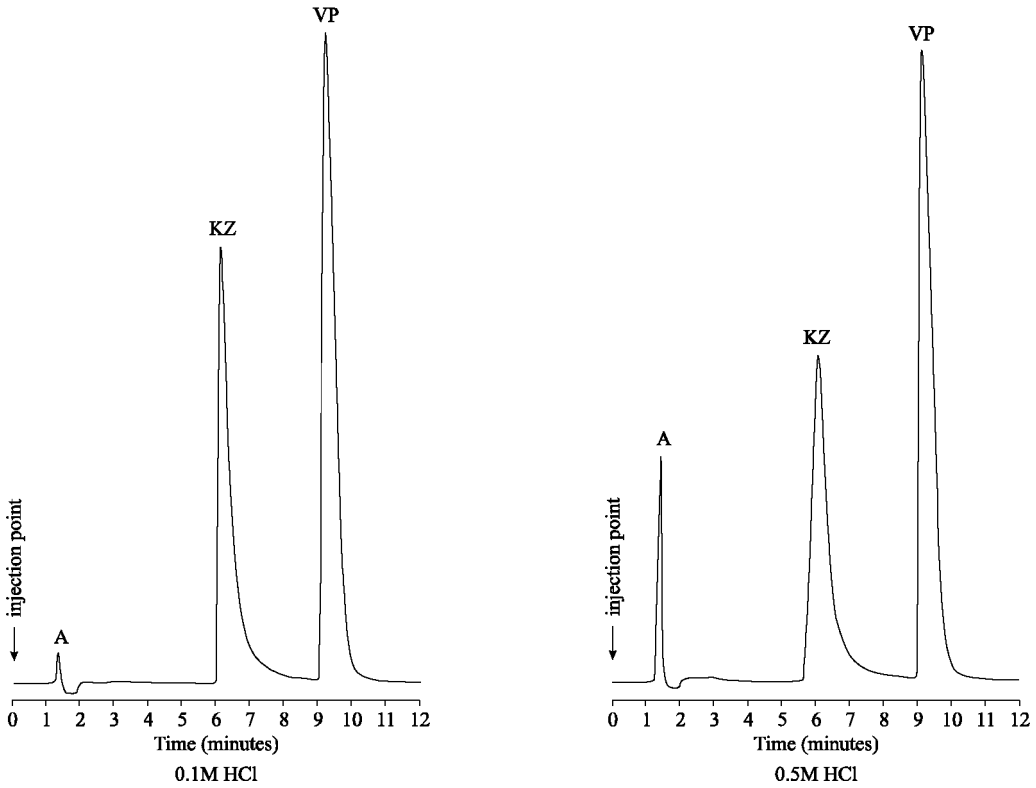


Figure 2.10 Degradation product of KZ isolated following acid hydrolysis (MM = 489.39) [38].

The chromatograms generated following acid hydrolysis studies of KZ are depicted in **Figure 2.11**. The extra peak (labelled A) which was formed does not interfere with the peaks for KZ or VP and therefore it is unlikely to interfere with the quantitation of KZ. It is clearly evident that as KZ degraded in solution and as the concentration of HCl was increased additional products that were unresolved formed and this is evident due to the presence of a distorted peak for KZ observed in **Figure 2.11**.



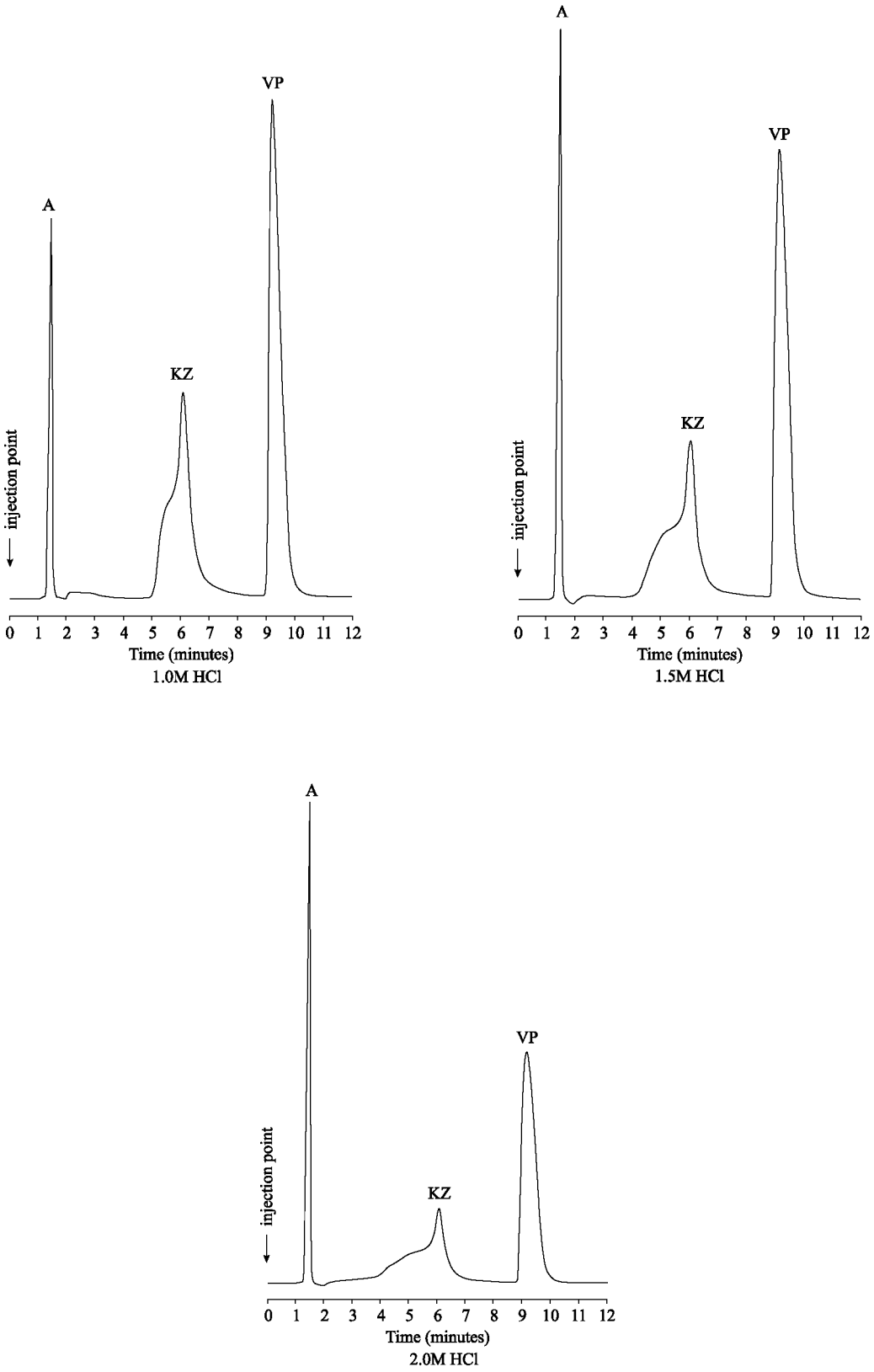


Figure 2.11 Chromatograms generated following acid hydrolysis of KZ in solutions of different molarity.

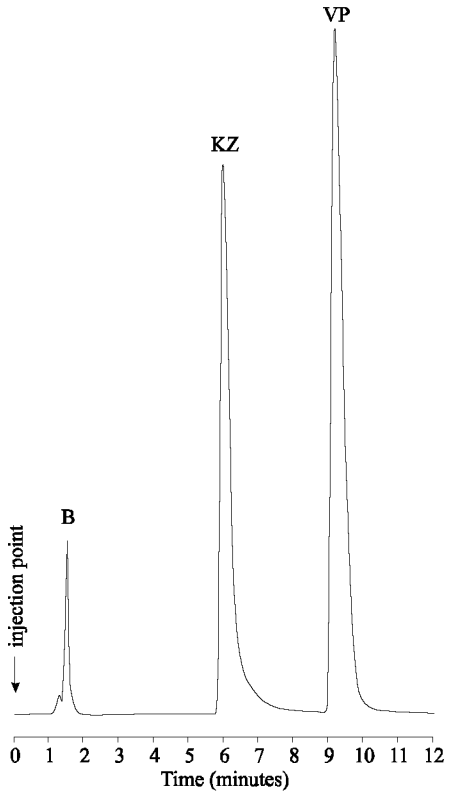
2.7.2.2 Base hydrolysis

In order to study base hydrolysis of KZ, 0.1 M of NaOH was added to KZ solutions (200 μg/mL) that were then maintained at 22°C for one hour. The % KZ recovered was 55.50 ± 0.65% and two overlapping peaks were observed at retention times of 1.29 and 1.50 minutes and are likely to be degradation products. The same hydrolytic degradation product is expected for acid and base hydrolysis [38]. Additional experiments were performed using more concentrated NaOH solutions; however, two-phase systems appeared to form due to a separation between the ACN and concentrated base [38]. The phase separation phenomenon was observed when 0.5 M, 1.0 M, 1.5 M and 2.0 M NaOH was used but not when 0.1 M NaOH was added to the solutions. Approximately 5 mL of each of the aqueous and organic phases were harvested using a Pasteur pipette and then added to 5 ml VP solution prior to analysis using RP-HPLC. The retention times of the resultant degradation products are listed in **Table 2.14** and the chromatograms are depicted in **Figure 2.12**.

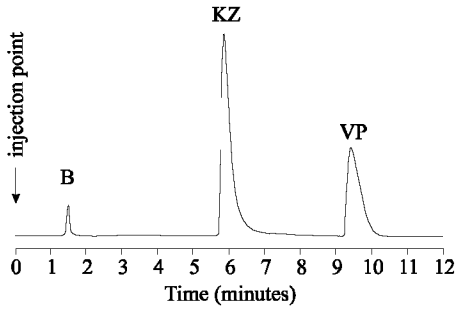
Table 2.14 Retention time of degradation product(s) following exposure of KZ to alkali hydrolytic conditions.

NaOH concentration M	Peaks resolved in the organic phase min	Peaks resolved in the aqueous phase min
0.1	1.45, 1.50	No phase separation
0.5	1.45, 1.50	1.51, 1.50
1.0	1.50, 2.02	1.50, 2.02
1.5	1.50	1.38, 1.60
2.0	1.50	1.36, 1.51

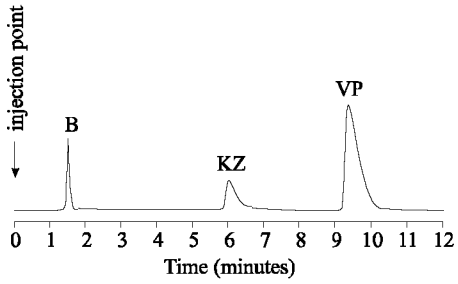
Analysis of the aqueous and organic phases when phase separation occurred revealed that when 0.1 M, 0.5 M and 1.0 M NaOH was added KZ was found in the organic and aqueous phases whereas when 1.5 M and 2.0 M NaOH was added it was only present in the organic phase. A degradation peak (peak C) was observed at two minutes when the aqueous phase was injected and this peak increased dramatically in size when 1.5 M and 2.0 M NaOH solutions were used. The large amount of this degradation product may be an indication of the complete degradation of KZ in the aqueous phase when 1.5 M and 2.0 M NaOH was added; therefore no KZ was observed in these samples.



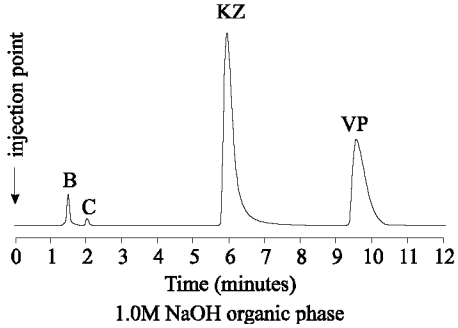
0.1M NaOH



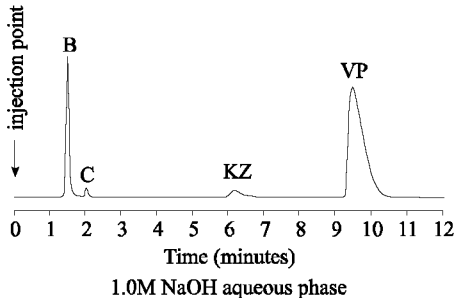
0.5M NaOH organic phase



0.5M NaOH aqueous phase



1.0M NaOH organic phase



1.0M NaOH aqueous phase

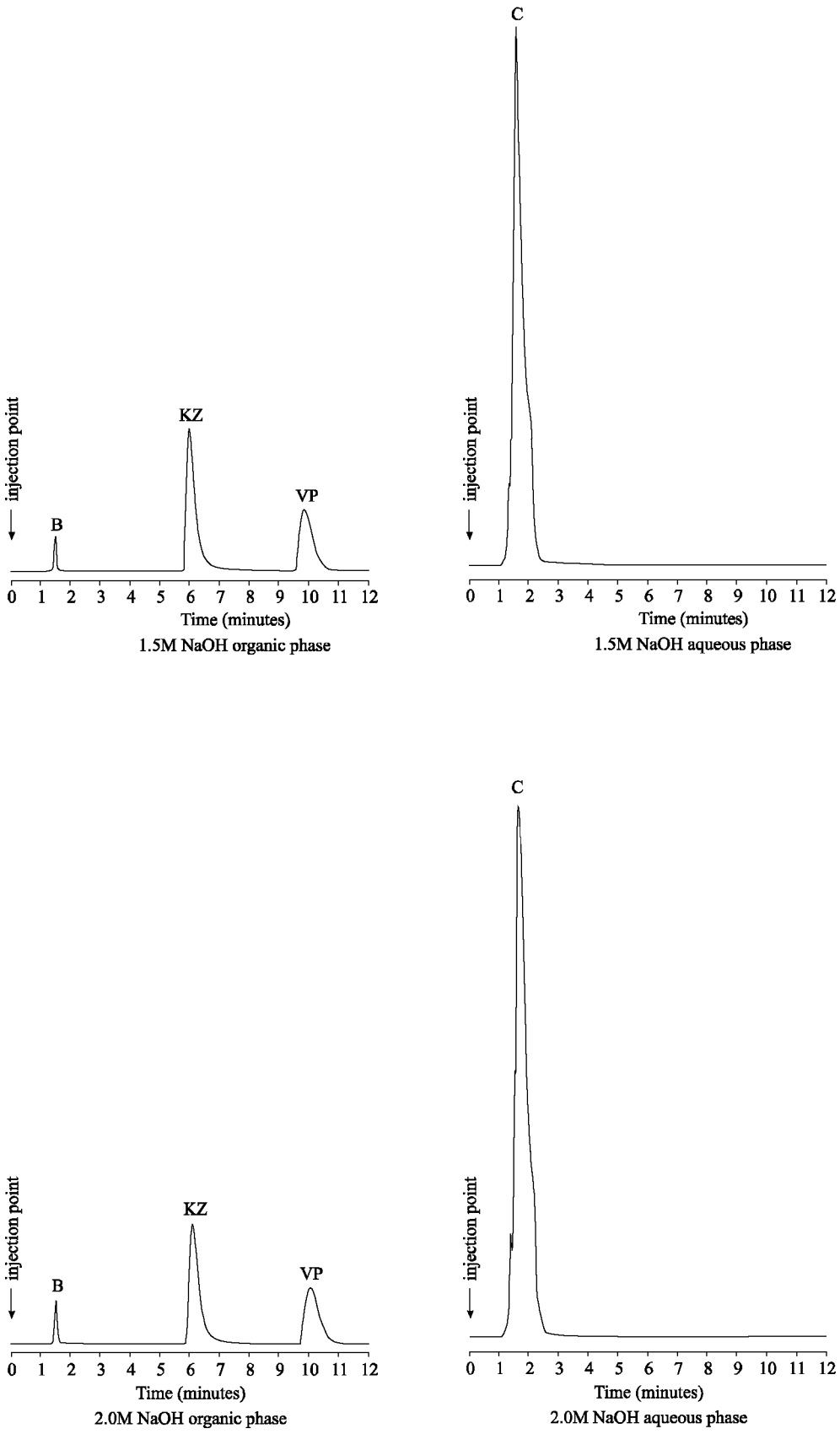


Figure 2.12 Chromatograms generated following alkali hydrolysis of KZ with NaOH solutions of different concentration.

2.7.3 Oxidative degradation

Oxidative degradation of KZ was assessed using hydrogen peroxide (H_2O_2) solutions of 5% v/v, 10% v/v, 15% v/v, 20% v/v and 30% v/v concentration. KZ solutions were exposed for one hour at 22°C after which 5 mL samples were collected using glass Pasteur pipettes and mixed with 5 mL VP solution prior to HPLC analysis. The results of oxidative degradation studies are depicted in **Figure 2.13**. Approximately 63.67 ± 0.40% and 53.92 ± 0.66% of KZ was recovered when the compound was exposed to 5% v/v and 30% v/v H_2O_2 respectively. This data is useful in ensuring that the analytical method is conducted in such a way to minimise degradation of KZ during analysis. The stabilility of KZ stock solutions was therefore monitored over a few days as depicted in **§ 2.8** following the conclusion that KZ is susceptible to oxidation from this set of data. These values also suggest that an antioxidant may be required in KZ formulations in which the drug is solubilised in order to limit oxidation of the molecule.

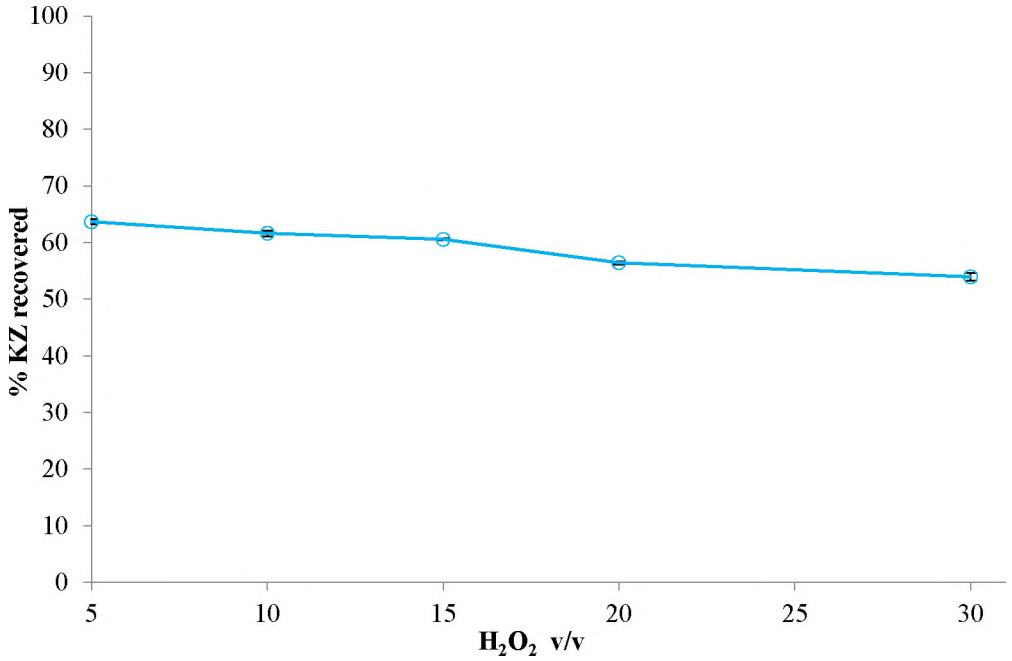


Figure 2.13 KZ recovered following oxidative degradation of KZ in solution.

The degradation product 1-[4-(4-phenyl)-4-oxidopiperazin-1-yl]ethanone (MM = 547.43) that is an N-oxide (**Figure 2.14**) has been reported to be produced when oxidation of KZ occurs [38] and this compound may be present in the chromatograms depicted in **Figure 2.15**. This degradation product was not identified as this was not within the scope of these studies.

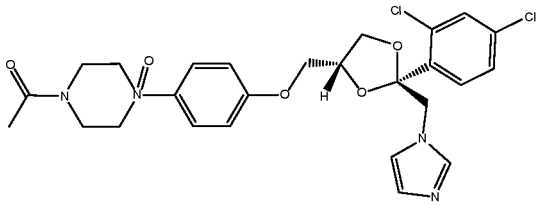
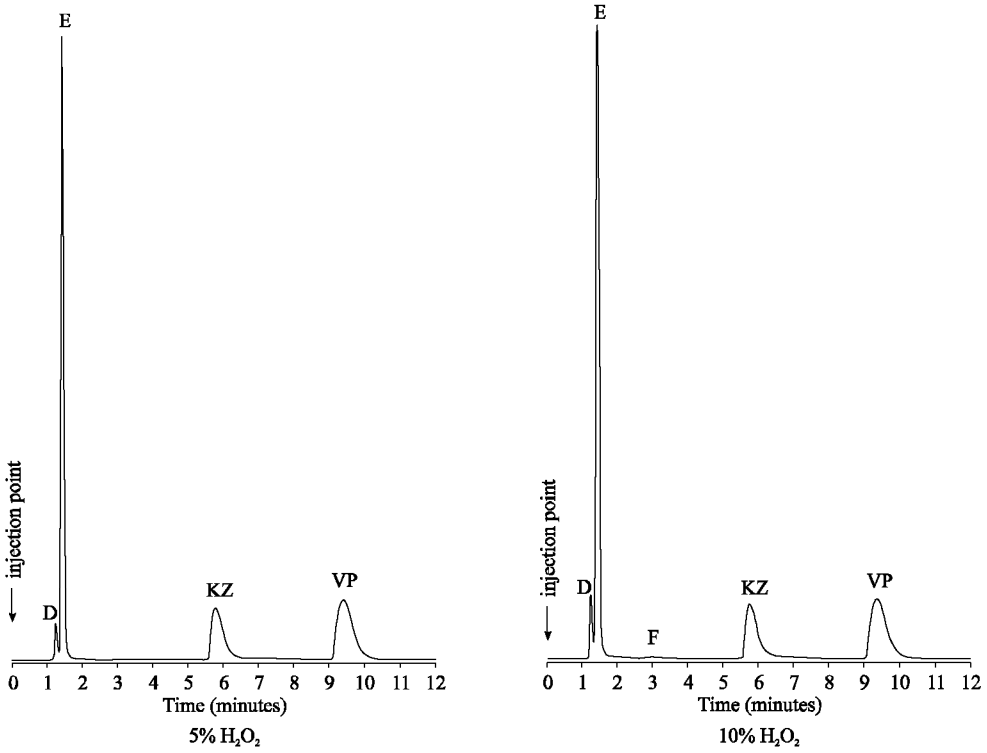


Figure 2.14 Oxidative degradation product of KZ (MM = 547.43) reported by Mhaske et al., [38].

Peak E that was observed in chromatograms following oxidation of KZ with H_2O_2 was a prominent degradation product and may be 1-[4-(4-phenyl)-4-oxidopiperazin-1-yl]ethanone. A smaller peak (peak D) was observed in all chromatograms and peak F was observed when H_2O_2 concentrations ≥ 10% v/v were used for degradation studies.



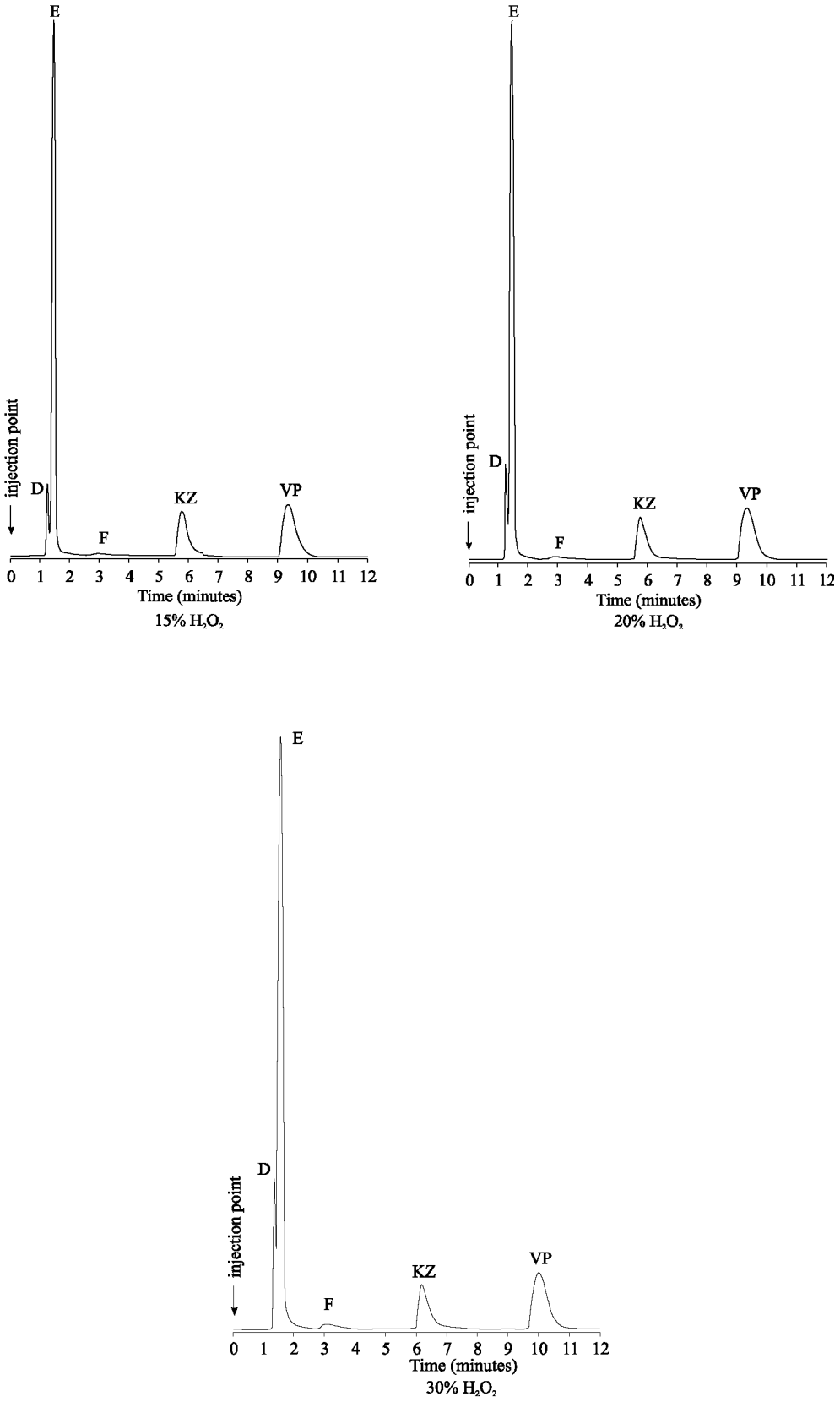


Figure 2.15 Chromatograms obtained following oxidation of KZ in solutions with different concentrations of H_2O_2.

2.7.4 Photolytic degradation

KZ is stable when exposed in the solid state to light [38]; however, in solution degradation occurs resulting in a significant decrease in antifungal activity of the compound [156]. Photodegradation of KZ in solution was undertaken by exposure to artificial sunlight for 1, 2, 4, 8, 12, 24 and 36 hours. A stock solution of KZ (200 µg/mL) was placed in an Atlas® Suntest CPS+ chamber (Linsengericht, Hesse, Germany) and was subjected to 550 W/m^2 and black standard temperature (BST) of 40°C using Filter G, coated quartz glass as the base filter and a special window glass plus solar ID65 as an additional filter. The use of Filter G simulates solar radiation behind a 6 mm glass window and it is recommended for use for pharmaceutical product testing in the ICH Q1B guideline [157]. The KZ solution that had been exposed to light appeared as a dark shade of red [109]. It was allowed to cool to 22 °C after which a 5 mL sample was mixed with 5 mL VP solution prior to RP-HPLC analysis. The % KZ recovered following exposure to sunlight for 1, 2, 4, 8, 12, 24 and 36 hours is depicted in **Figure 2.16**. Approximately 65.42 ± 0.88%, 64.23 ± 0.87%, 38.68 ± 0.52%, 22.58 ± 0.30%, 8.54 ± 0.12%, 5.70 ± 0.66% and 0.16 ± 0.73% of KZ was recovered clearly indicating that KZ is indeed photolabile [38].

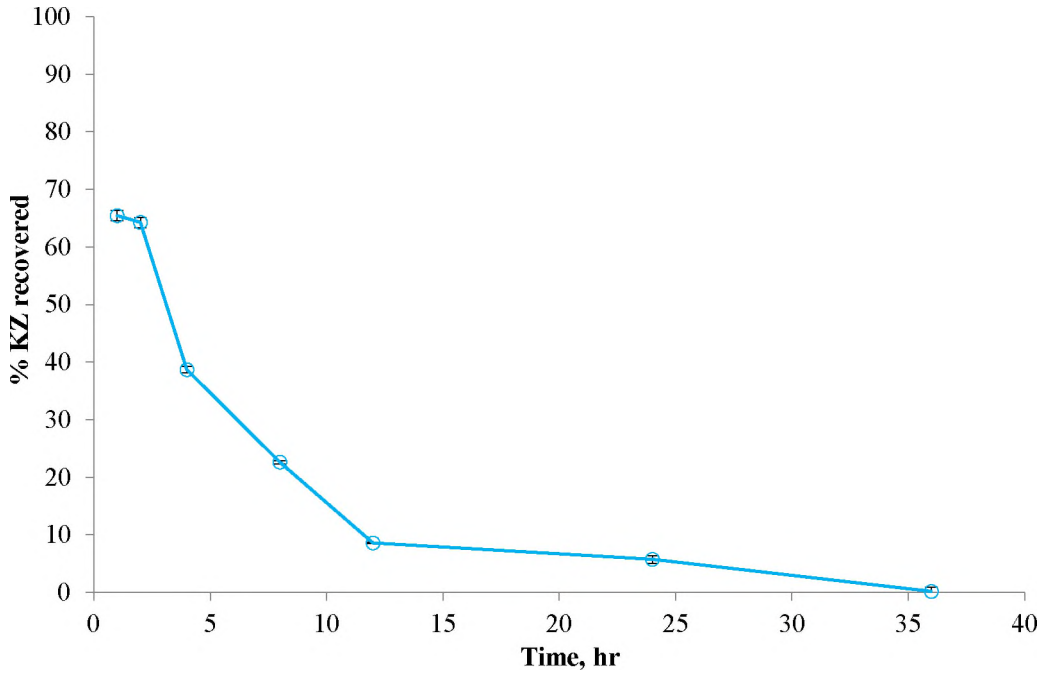


Figure 2.16 KZ recovered following exposure to artificial sunlight for 36 hours.

The two main photodegradation products of KZ are 1-[4-(4-phenyl)piperazin-1-yl]ethanone (I) (**Figure 2.17**) and 1-[4-(4-phenyl)piperazin-1-yl]ethanone (II) (**Figure 2.18**) and these compounds were identified following HPLC-MS and NMR analysis [156].

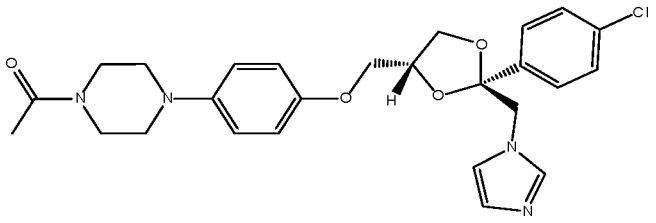


Figure 2.17 Photo-degradation product (I) of KZ.

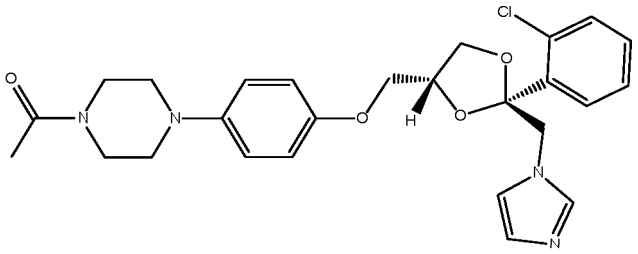
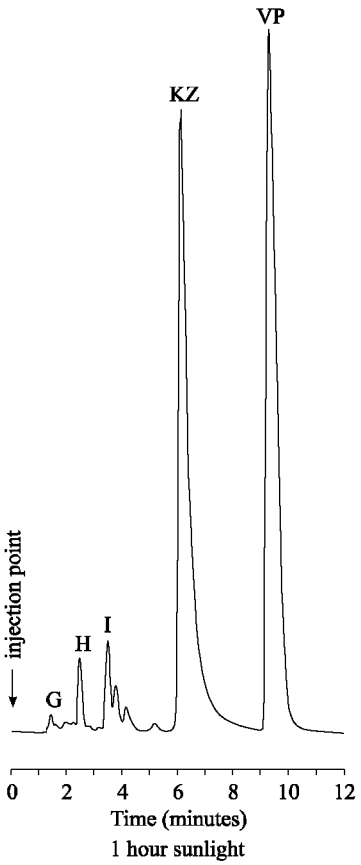
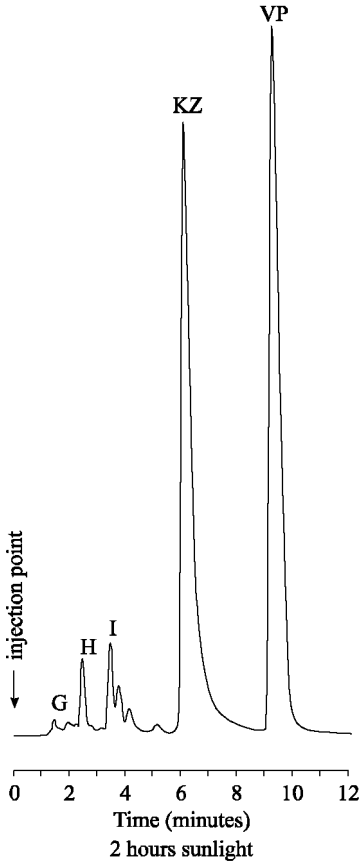


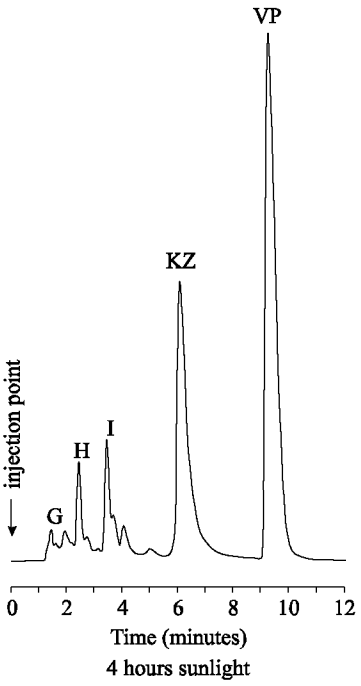
Figure 2.18 Photo-degradation product (II) of KZ.

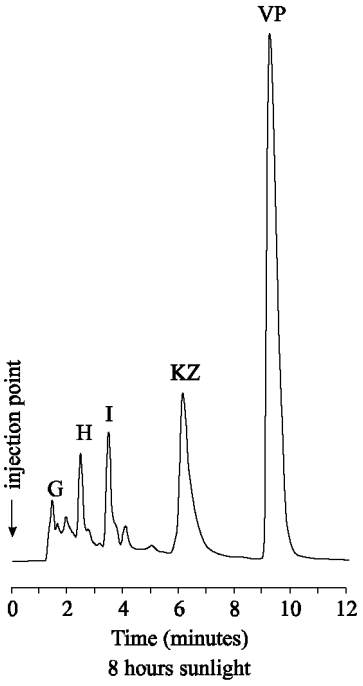
Compounds I and II may be any of peaks G, H, I or J observed in the chromatograms depicted in **Figure 2.19**. Peak G increases in height as KZ degrades and Peak J is observed after 24 hours and may be a degradation product of peak I, which decreased in size from 12 hours.

It is evident that KZ must be protected from light once it is in solution for RP-HPLC analysis and during formulation and product development and assessment studies. Aluminium foil was used to wrap all containers in which KZ was stored and amber vials and bottles were used to prepare samples for RP-HPLC analysis and during stability studies. These strategies were used to limit exposure of KZ to light.


VP
KZ
injection point
H
I
G
0 2 4 6 8 10 12
Time (minutes)
1 hour sunlight


VP
KZ
injection point
H
I
G
0 2 4 6 8 10 12
Time (minutes)
2 hours sunlight


VP
KZ
injection point
H
I
G
0 2 4 6 8 10 12
Time (minutes)
4 hours sunlight


VP
KZ
injection point
H
I
G
0 2 4 6 8 10 12
Time (minutes)
8 hours sunlight

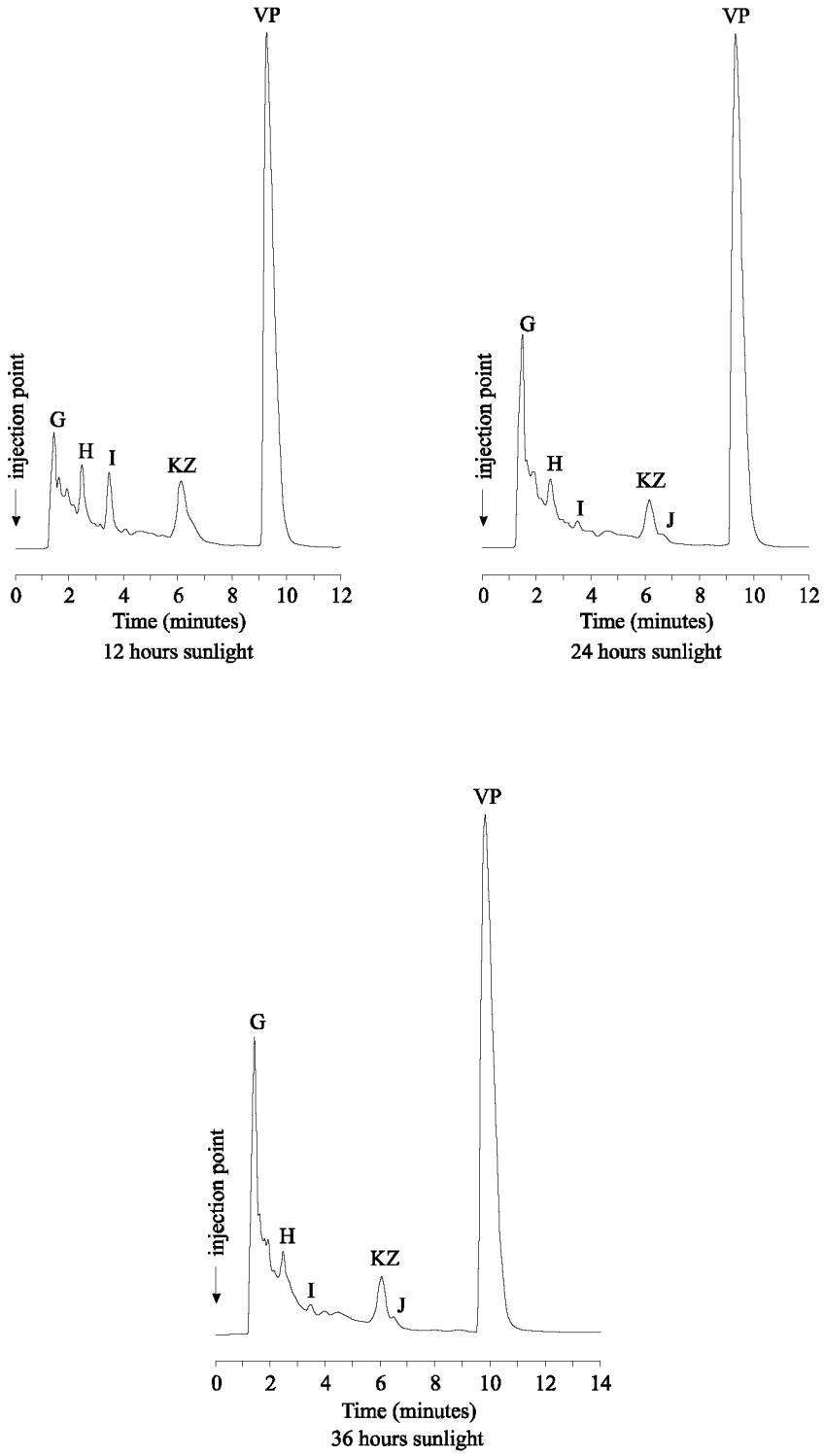


Figure 2.19 Chromatograms depicting KZ following exposure to artificial sunlight for 36 hours.

2.8 Stability of analyte

Solutions of KZ (200 µg/mL) were assessed for stability following storage in amber vials in the refrigerator (4°C), on the bench (22°C) and in the auto-sampler (26°C) for a maximum of 8 days prior to analysis using the validated RP-HPLC. Samples were analysed after 1, 2, 3, 4, and 8 days' storage and the % KZ recovered following storage and analysis is depicted in **Figure 2.20**. The amount of KZ recovered following storage in the refrigerator and the bench were similar and therefore are almost overlapping lines in **Figure 2.20**. An overall minimum of 96.54% KZ was recovered from samples stored in the auto-sampler for 8 days indicating that KZ is fairly stable over 8 days under the three storage conditions. These data indicate that stock solutions of KZ did not need to be prepared on a daily basis and were manufactured and kept for a maximum of 4 days in the refrigerator before being discarded.

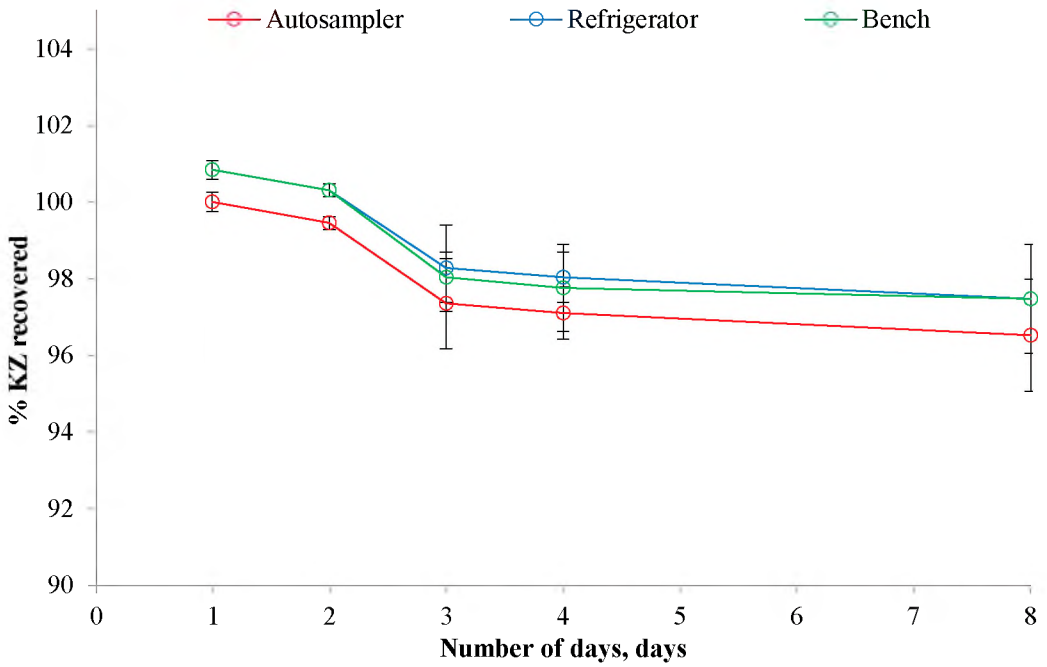


Figure 2.20 Stability of KZ in solution following storage in the refrigerator, on the bench and in the auto-sampler for 8 days.

2.9 Conclusions

A simple rapid stability-indicating RP-HPLC method with UV detection at 205 nm was successfully developed, optimised and validated with respect to accuracy, precision, selectivity, linearity and robustness. This method is applicable for quantitative analysis of KZ in pharmaceutical dosage forms using VP as an IS. Method development studies included assessment and selection of a suitable stationary phase, IS, mobile phase composition, buffer molarity and pH, flow rate, injection volume and column temperature. A Phenomenex® Hyperclone 5µm ODS (C18) 120 Å 150x4.6 mm i.d. column was selected for use as it is similar to the widely used Hypersil® C18 columns reported in the literature as suitable for the analysis of KZ using RP-HPLC. VP was selected as the IS as it exhibited similar

analytical properties to KZ and the adequate Rs exhibited between KZ and VP and satisfactory As and Tf observed under the analytical conditions investigated.

The separation of KZ and VP was achieved using an optimized mobile phase composition of a binary mixture of ACN and 0.05 M phosphate buffer of pH 6.0 in a 50:50 ratio and a flow rate of 1.0 mL/min. These chromatographic conditions resulted in retention times of 6.08 and 9.90 minutes for KZ and VP respectively with a total run time for sample analysis of 12 minutes which can analyse a large number of samples over a short time. The buffer molarity and pH affected the retention times of KZ and VP, justifying the need to optimize the phosphate buffer with respect to molarity and pH. The method was found to be linear in the range of 0.50 – 151.30 μg/mL and was found to have a correlation coefficient of 0.999. Solutions of KZ in ACN were found to be stable for a maximum of 8 days following storage at 4°C, 22°C and 26°C. These validation data reveal that the analytical method developed was linear, precise, accurate and sensitive. This method is arguably the first one reported using a Phenomenex® Hyperclone 5μm ODS (C18) 120 Å 150x4.6 mm i.d. column as stationary phase and ACN: 0.05 M phosphate buffer (pH = 6) 50:50 v/v as mobile phase without using an additive such as diethylamine to minimise KZ peak tailing.

KZ was stable to heat at temperatures of up to 50°C for a maximum period of 4 hours. KZ was found to be photo-labile and susceptible to oxidation when exposed to H_2O_2. Approximately 50% of the KZ content degraded in the presence of 0.1 M HCl and NaOH for one hour. The degradation peaks observed on chromatograms during analysis of samples from stress studies did not interfere with the peaks for KZ or VP except when samples that had been directly exposed to sunlight for 24 and 36 hours were evaluated and a peak, *viz.*, J, was seen to elute close to the KZ peak without concealing that response. The stability data obtained in these studies will be useful for analysis of samples during formulation development studies when producing a thermosetting KZ vaginal gel. The RP-HPLC method was stability-indicating and specificity was achieved as degradation peaks do not conceal KZ and VP responses during analysis.

CHAPTER THREE
CHARACTERISATION OF TOPICAL KZ FORMULATIONS

3.1 Introduction

One of the initial steps in generic drug product development is the characterisation of a reference or innovator product and these studies, also known as reverse engineering studies, may accelerate the formulation development process for generic products [158,159]. Characterisation studies involve the quantitation of a critical excipient composition in addition to the solid state characterisation of an API [159-161]. Commercial success with generic products often depends on the speed at which the product is developed and marketed following expiration of restrictive patents held by the owner of the innovator product [162]. Generic products require proof of therapeutic equivalence, i.e. pharmaceutical equivalence and bioequivalence with an innovator product prior to regulatory approval being granted [159]. In contrast the drug product development process for novel API and/or formulations is often lengthy and costly. The development of generic dosage forms may, on average, take three years when compared to the development of novel products which may often take in excess of seven years [163-165]. Some generic product development strategies may be used to accelerate the development of novel formulations. Therefore it is advantageous when developing a novel formulation to evaluate the composition and performance of commercial KZ containing formulations and incorporating suitable attributes into a novel formulation for use at a different target site or for the development of a different type of dosage form [158].

In general innovator products make use of the most stable polymorphic form of a drug so as to avoid transformation into less stable forms during manufacture. Generic companies tend to use the same polymorphic form of a drug as that used in an innovator product in order to achieve similar dissolution and stability performance. FTIR and DSC analyses are techniques used to characterise innovator products in order to identify the polymorphic form of the drug used in the commercial product. However, this is not a simple process, as interference from other excipients may make this a challenge. RP-HPLC analysis aids the identification of formulation composition; however, characterisation remains a challenge due to complex compositions [166]. During the development of a generic product maintaining the type of emulsifier, hydrophilic-lipophilic balance and solvent-to-emulsifier ratio as close to that of the innovator product may assist in obtaining a better quality generic product that exhibits the same performance characteristics as an innovator product [167]. Consequently, pH and viscosity studies were strategies used to assess commercially available KZ products in order to establish product performance characteristics and to elucidate product composition. The characterisation of commercially available KZ products with a goal to develop a novel topical KZ formulation forms the basis of this chapter and the application of this strategy was thought to be less time consuming than commencing the developmental phase without any information about registered KZ formulations.

The ICH Q2 (R1) guideline defines specificity as the capacity of a method to accurately and precisely detect an analyte in the presence of degradation products, impurities and/or excipients [137]. If the specificity criteria are not met, supporting analytical procedures may have to be undertaken. There are three steps to ensuring specificity, *viz.*, identification of the analyte, purity tests and assay to ensure that criteria for specificity of an analytical procedure are met. The specificity of the validated RP-HPLC method was further confirmed by analysing chromatograms generated following the assay of KZ in commercially available dosage forms.

3.2 Characterisation of Xolegel®, Kez® shampoo and Ketazol® cream

Commercially available KZ products in South Africa include Adco-Dermed®, Ketazol®, Kez®, Nizcreme®, Nizoral® and Nizshampoo®. Nizovules® and Nizorelle® have been discontinued or are not actively marketed [15]. In the USA, Extina®, Ketodan®, Nizoral® and Xolegel® are commercially available KZ products [15]. Xolegel® and Ketazol® cream were purchased from pharmacies in the USA and South Africa and characterized for the purposes of generating product information for use in the development of a novel thermosetting ketoconazole vaginal gel formulation. Kez® shampoo was purchased from a local pharmacy and characterised to gather information about liquid KZ gel formulations. A combination of this information was considered valuable for developing a thermosetting vaginal gel that is liquid at 22°C. The viscosity of the commercial formulations at 22°C and 37°C, pH and assay were investigated. In addition DSC and FTIR spectroscopy was used to evaluate the commercial products and the IVRT of the products was undertaken.

3.3 Description of commercial products

Kez® shampoo and Ketazol® cream are commercially available as over the counter (OTC) products in South Africa and are classed as Schedule 1 medicines, whereas Xolegel® is only available as a prescription drug in the USA.

3.3.1 Xolegel®

Xolegel® is manufactured by Aqua Pharmaceuticals (West Chester, Pennsylvania, USA) and contains 2% m/m equivalent to 20 mg/g ketoconazole USP, 34% v/v dehydrated alcohol, ascorbic acid, butylated hydroxytoluene, citric acid monohydrate, glycerine, hydroxypropyl cellulose, polyethylene glycol 400, PPG-15 stearyl ether, propylene glycol, FD&C yellow No 6 and FD&C yellow No 10. Xolegel® is a translucent, clear amber coloured gel [168] that is indicated for the treatment of seborrheic dermatitis in immune-competent adults and children older than 12 years of age [169].

3.3.2 Kez® shampoo

Kez® shampoo is manufactured by PharmaDynamics (Pty) Ltd (Westlake, Cape Town, South Africa) and contains 2% m/v equivalent to 20 mg/g ketoconazole, 0.3% m/m imidurea and PEG-7 glyceryl

cocoate, sodium laureth sulphate, disodium laureth sulphosuccinate, PEG-120 methyl glucose dioleate, cocamide DEA, lauryldimonium hydroxypropyl hydrolysed collagen, sodium hydroxide, sodium chloride, hydrochloric acid concentrate, purified water and erythrosine C.I. 45430 [170]. The product is a fluorescent pink coloured gel used for the treatment of fungal infections of the scalp that present with flaking, scaling and itching associated with dandruff [63,171].

3.3.3 Ketazol® cream

Ketazol® cream is manufactured by Aspen Pharmacare Ltd (Port Elizabeth, Eastern Cape, South Africa) and contains 2% m/m equivalent to 20 mg/g ketoconazole, 0.2% m/m sodium disulphite and 0.4% m/m imidurea. The formulation is a white o/w cream [64] that is indicated for the treatment of skin conditions such as tinea corporis (ring worm), tinea cruris (jock itch), tinea pedis (athletes foot), tinea vesicolor and/or yeast infections of the integumentary system [171].

3.4 Materials, equipment and methods

3.4.1 Viscosity measurement

Viscosity is one of the key parameters to be monitored when evaluating thermosetting gels and was a primary consideration in these studies. Approximately 25 g of each of Xolegel®, Ketazol® cream and Kez® shampoo were weighed into 50 mL beakers and the viscosity measured (n = 3) at 22°C using a Brookfield® DVI+ Viscometer (Middleborough, Massachusetts, USA) fitted to a helipath stand. The gels were then placed into a Colora® Model NB-34980 Ultra-Thermostat water bath for 10 minutes and the viscosity measured at 37°C (n = 3) at an operating speed of 5.8 rpm using spindle D S94 with a helical path of 10 seconds.

3.4.2 pH

The pH of each of the commercial products was measured using a Model GLP 21 Crison® Instruments pH meter. The pH meter was calibrated on a daily basis using Crisolyt-A Electtrolita (3M KCl with AgCl) at pH 4.0 and Crison® pH 7.00 standards. Following calibration, the pH of each formulation was measured (n = 3) by inserting the electrode into 25 g of product and reading the pH after 30 seconds. After each reading the probe was rinsed with distilled water and dried, after which an additional reading was taken. This procedure was followed so as to avoid cross contamination of samples and to ensure accuracy of the pH readings.

3.4.3 Ketoconazole assay

Analysis was undertaken in order to quantitate the amount of KZ in Xolegel®, Kez® shampoo and Ketazol® cream and the cartoon depicted in **Figure 3.1** is visual schematic representation of the approach used for the assay of each product.

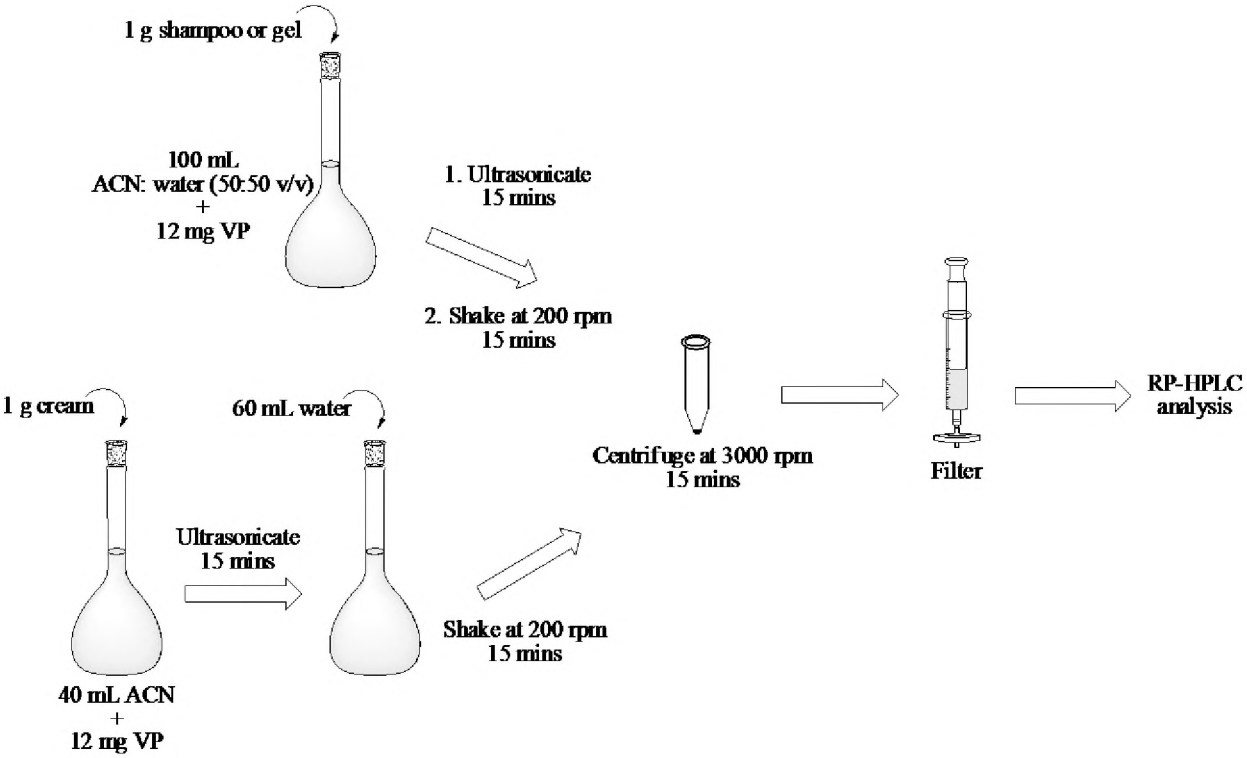


Figure 3.1 Assay procedure for the analysis of commercially available KZ products.

Approximately 1 g of the gel or shampoo and 12 mg VP were accurately weighed into an A-grade volumetric flask and dissolved in 100 mL of an ACN: water mixture in a 50:50 v/v ratio and sonicated using a Branson® B12 sonicator for 15 minutes prior to shaking with the aid of a Junior Orbit® Shaker (Melrose Park, Illinois, USA) at 200 rpm for a further 15 minutes. A 10 mL aliquot of the mixture was pipetted from the mixture and transferred into a 10 ml Kimax® centrifuge tube (St. Louis, Missouri, USA) and centrifuged at 3000 rpm using a Damon® Model HN-SII centrifuge (Dunstable, Bedfordshire, England) for 15 minutes after which the supernatant was filtered using a Millipore® 0.45 µm PVDF hydrophilic syringe filter (Milford, Massachusetts, USA) attached to a 10 mL Neomedic® plastic syringe (Rickmansworth, Hertfordshire, England). The plastic syringe and syringe filters did not leach materials that interfered with the analysis and this was confirmed by evaluation of the chromatograms of samples that were found to contain only the peaks for KZ and VP. A 1.5 mL aliquot of the filtrate was then transferred to a 2.0 mL Waters® amber glass screw top vial (Milford, Massachusetts, USA) and analysed using a validated RP-HPLC method. The analysis of Ketazol® cream commenced with a slightly different initial step (**Figure 3.1**) in which approximately 1 g of cream and exactly 12 mg VP

were accurately weighed into an A-grade volumetric flask to which 40 mL of ACN was added. The mixture was then sonicated for 15 minutes after which 60 mL HPLC grade water was added to the mixture and the solution shaken for 15 minutes using a Junior Orbit® Shaker at an agitation rate of 200 rpm. The oily component of the cream separated and the supernatant was removed using a Pasteur pipette and filtered using a 0.45 µm PVDF hydrophilic syringe filter attached to a 10 mL plastic syringe. Approximately 1.5 mL of the filtrate was transferred into a 2.0 mL amber screw top glass vial and analysed using the previously described RP-HPLC method.

KZ is a hydrophobic compound that is unlikely to bind to the hydrophilic PVDF syringe filter membrane. In order to ascertain if this was indeed the case, a simple experiment was conducted to establish whether KZ was likely to bond to the membrane during filtration. If KZ did bind to the membrane this would be reflected as lower recovery than the % RSD. A 5 mL aliquot of ACN and water in a 50:50 v/v ratio was passed through a 0.45 µm PVDF hydrophilic syringe filter that had been used after which the filtrate was spiked in a 1:1 ratio with a 120 µg/mL VP solution prior to analysis using RP-HPLC. Any KZ that may have been bound to the filter would have dissolved in the ACN: water mixture as it is very soluble in this solvent mixture. The resultant chromatograms revealed that no KZ was present at concentrations higher than the LOD providing evidence that KZ does not bind to the 0.45 µm PVDF hydrophilic syringe filter membrane and therefore the filters were considered suitable for use for all analytical testing of KZ formulations.

3.4.3.1 Validation of the extraction procedure

The method used to extract KZ from formulations must be validated to ensure accuracy, reliability and reproducibility of the test procedure [165]. The ICH Q2 (R1) guideline states that, similar for analytical procedure validation, assay procedures may and should also be validated for precision, accuracy, specificity, linearity and range [137]. In this case the extraction procedure was validated for repeatability (intra assay precision) and accuracy using the experimental approaches described in **§ 2.6.2.1** and **§ 2.6.3.** The limits for these parameters were set at ± 1.05 for experimental SD, ± 2.09 for the calculated SD, ≤ 5% for the % RSD, 100 ± 5% for the % recovery for the extraction procedure to exhibit repeatability, and the % bias was set at 5% for establishing the accuracy of the method [14,123,136].

3.4.3.1.1 Repeatability (intra assay precision)

In order to establish repeatability of the extraction procedure, replicate samples (n = 6) were analysed at three different concentrations, *viz.*, low, medium and high, within the range used for the calibration curve. Approximately 0.30 g, 0.80 g and 1.30 g Xolegel®, Kez® shampoo and Ketazol® cream were

weighed and extracted and assayed using the extraction procedure depicted in **Figure 3.1** and the results are described in **§ 3.5.3**.

3.4.3.1.2 Accuracy

The accuracy of the extraction procedure was assessed by analysing the % recovery of known amounts of KZ that had been added to each of the commercial products. An appropriate amount of KZ (18 mg) was added to 0.30 g of the commercial formulations to produce a label claim of 120% of the amount of KZ expected in each formulation. Samples were assessed in replicate (n = 6) and the accuracy of the extraction procedure established.

3.4.4 Characterisation using Infrared Spectroscopy and Differential Scanning Calorimetry

FTIR was undertaken to evaluate the commercial products as described in **§ 1.2.15** using a Spectrum 100 FTIR ATR spectrophotometer without any additional sample preparation. The FTIR spectra for Xolegel®, Kez® shampoo and Ketazol® cream were compared to the spectrum that was generated for pure KZ.

A Model Q100 Differential Scanning Calorimeter fitted with a RCS (90) refrigerated cooling system was used to undertake DSC analysis using the procedure described in **§ 1.2.16**. Approximately 2.5 to 5.0 mg of each of Xolegel®, Kez® shampoo and Ketazol® cream were weighed into an aluminium pan that was hermetically sealed to avoid splashing of formulations with an aluminium disc. An empty aluminium pan was used as the reference. The thermogram was generated by heating the sample from 30 to 445 °C at a heating rate of 10 °C/min and then the system was cooled to 22 °C with a liquid nitrogen purge at a flow rate of 100 mL/min prior to additional sample analysis. Similar to analysis of the FTIR spectra the DSC thermograms of the three commercial KZ formulations were compared to that for pure KZ and the results of these analyses are reported in **§ 3.5.4** *vide infra*.

Characterisation studies using FTIR spectroscopy and DSC were performed to obtain information about the crystal structure and solvate form of KZ within commercial formulations as changes in crystal characteristics can influence bioavailability, chemical and physical stability that may affect the function of a dosage form [172]. For example, *in vitro* KZ release might be affected as the crystal structure and solubility of KZ within the formulation change. This information will be used to identify an approach for the incorporation of KZ in the thermosetting vaginal gel to be developed in these studies.

3.5 Results

3.5.1 Viscosity

The results of viscosity determination are summarized in **Table 3.1** and reveal that the viscosity of all products was greater when measured at 22°C than at 37°C for all products. In addition Ketazol® cream was more viscous than the other products at these temperatures.

Table 3.1 Viscosity of commercially available 2% m/m ketoconazole formulations at 22°C and 37°C.

Formulation	Viscosity at 22°C cP n = 3	Viscosity at 37°C cP n = 3
Xolegel®	51953 ± 525	33903 ± 196
Kez® shampoo	21263 ± 202	4138 ± 0
Ketazol® cream	81603 ± 1628	66663 ± 5026

The Arrhenius equation can be used to explain the relationship between viscosity and temperature. When the temperature of a system increases the viscosity of that system may decrease and the activation energy of the system also decreases due to bond breakage [173]. The lower viscosity of the commercial formulations at 37°C can be attributed to the increased kinetic energy of the system at higher temperatures [174].

These products are designed to be applied topically and should therefore exhibit a lower viscosity and better spreadability when applied to the skin surface at 32°C [175-177] as these factors may affect the rate and extent of KZ release and therefore availability to exert a therapeutic response [178,179]. These products also have a low viscosity in hot climates which may lead to changes in the stability of formulations and that may result in poorly reproducible drug delivery, *in vivo*. However the viscosity of the gel and cream (≥ 10000 cP) formulations suggest that these formulations will remain in a semi-solid state at 37°C although they may be slightly more fluid. The shampoo is a semi-solid at 22°C and exhibits a viscosity ≥ 10000 cP; however, at 37°C the shampoo flows more readily and exists in the liquid form with a viscosity of 4138 cP. This phenomenon should not pose a problem for use in hot climates as the shampoo is generally used with water and requires to be lathered onto the scalp and therefore the semi-solid structure of the formulation should not adversely affect the usability of the product. This approach does not apply to thermosetting gels which should exhibit an increase in viscosity as the temperature the product is exposed to increases [180-186]. There may be an issue should the thermosetting vaginal gels be used in very hot climates as the liquid may well become semi-solid and exhibit reduced flowability when intended to be inserted into the vagina. Therefore the impact of climate on formulation behaviour should be considered when developing thermosetting gel products.

3.5.2 pH

Of all formulations, Ketazol® cream exhibited the highest pH of 7.38 whereas Xolegel® had a pH of 6.12 and the data for all products are summarized in **Table 3.2**. These pH values are adequate for use on the surface of the skin surface as the pH of the skin is normally between 4.0 and 6.0 [187,188], consequently the products will be less irritating to the skin. Vaginal pH ranges between 3.5 and 4.5 and therefore the development of a thermosetting vaginal gel formulation should include an assessment of pH in this range so as to avoid the potential for irritation of the vaginal mucosa. The pH of the commercial products may have been selected as KZ is stable under pH conditions between 6.0 and 7.5.

Table 3.2 pH of commercially available 2% m/m ketoconazole formulations.

Product	pH n = 3
Xolegel®	6.12 ± 0.01
Kez® shampoo	6.35 ± 0.006
Ketazol® cream	7.38 ± 0.006

3.5.3 Assay

The assay results summarized in **Table 3.3** reveal that all commercial formulations tested exhibited a recovery of 100 ± 5% for KZ and complied with the limits set in most official compendia and in our laboratory. This indicates that the products are of suitable quality and that the extraction procedure used for the assay is adequate to characterize the potency of test and reference formulations. The extraction procedure was validated for repeatability and accuracy and these data are described in **§ 3.5.3** *vide infra*.

Table 3.3 Assay results for commercially available 2% m/m commercial KZ products.

Formulation	KZ recovered %	Assay content % m/m
Xolegel®	103.81 ± 0.54	2.07 ± 0.011
Kez® shampoo	99.47 ± 0.72	1.99 ± 0.014
Ketazol® cream	101.02 ± 1.53	2.02 ± 0.031

The chromatograms generated following HPLC analysis of the commercial products are depicted in **Figures 3.2 – 3.4** and it is clear that the method demonstrates specificity for KZ as no excipient peaks present interfered with the peaks for KZ and VP. There is a small excipient peak (X) that was retained at 7 minutes as shown in **Figure 3.3** in the chromatogram for Kez® shampoo that is relatively close to KZ whereas the chromatograms for Xolegel® and Ketazol® cream are free of any extraneous peaks and the method is therefore specific for KZ in all formulations tested.

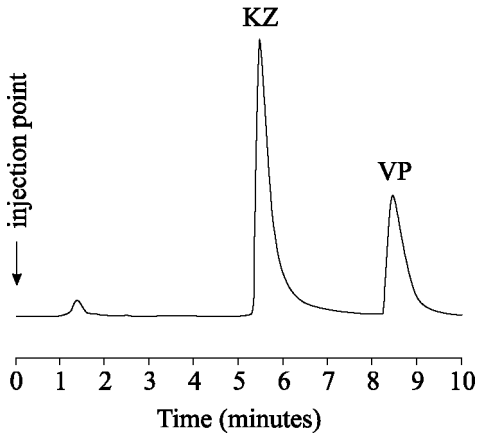


Figure 3.2 Chromatogram for the assay of Xolegel®.

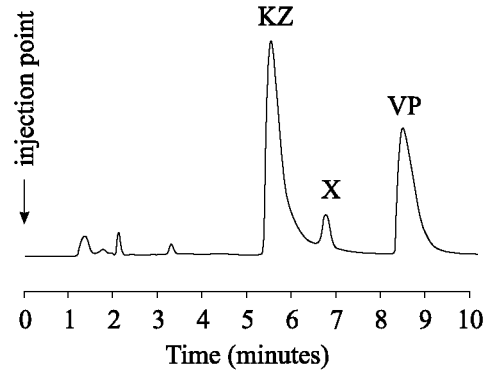


Figure 3.3 Chromatogram for the assay of Kez® shampoo.

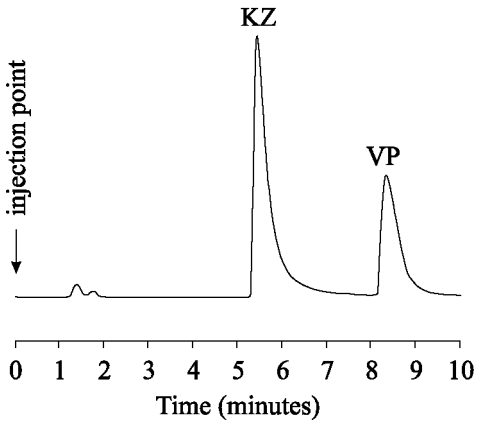


Figure 3.4 Chromatogram for the assay of Ketazol® cream.

3.5.3.1 Repeatability (intra-assay precision)

The values for SD, % RSD and % recovery of KZ following extraction from Xolegel®, Kez® shampoo and Ketazol® cream are listed in **Table 3.4** and these data were within the tolerance limits set **(§ 3.4.3.1)** and therefore the extraction method for the analysis of KZ in semi-solid formulations is repeatable and exhibits good intra-assay precision.

Table 3.4 Repeatability data for the assay of KZ in Xolegel®, Kez® shampoo and Ketazol® cream.

Formulation	Theoretical Concentration µg/mL	Actual Concentration µg/mL	SD	% RSD	% Recovery
Xolegel®	30.00	28.67	1.05	3.66	104.64
	80.75	80.12	0.39	0.49	100.79
	130.40	133.07	1.24	0.93	97.99
Kez® shampoo	31.57	32.89	0.52	1.58	95.99
	80.84	80.23	0.14	0.17	100.76
	130.50	132.88	0.84	0.63	98.21
Ketazol® cream	31.20	30.95	1.23	3.97	100.81
	80.94	81.5	1.06	1.30	99.31
	131.27	133.82	0.16	0.12	98.09

3.5.3.2 Accuracy

The % bias was used as a measure of accuracy and the % bias established from accuracy studies summarised in **Table 3.5** were all ± 5%, thereby indicating that the extraction procedure for KZ from commercial products is accurate. The thermosetting vaginal gels developed in these studies can therefore be accurately analysed for KZ content during formulation development, product validation and stability studies.

Table 3.5 Accuracy results for KZ analysis.

Formulation	Theoretical concentration µg/mL	Actual Concentration µg/mL	SD	% RSD	% Bias
Xolegel®	30.38	31.15	0.13	0.42	2.47
Kez® shampoo	30.02	30.43	0.64	2.10	1.35
Ketazol® cream	30.55	30.89	0.30	0.97	1.10

The data for precision and accuracy of the extraction of KZ from commercial semi-solid products reveals that the procedure is suitable for the analysis of gel, shampoo and/or cream formulations and will be suitable for the analysis of KZ in thermosetting gels that were to be manufactured in these studies.

3.5.4 Characterization of commercial products using Fourier Transform-Infrared Spectroscopy and Differential Scanning Calorimetry

The FTIR absorption spectrum for pure KZ and each of the commercial products investigated are depicted in **Figure 3.5** and reveal the presence of a broad peak at 3000-3600 cm^{-1} for all three spectra. This vibration represents O-H functional group stretching within an alcohol or phenol functionality. The narrow peaks observed at 2800 cm^{-1} may well be due to alkyl C-H band stretching. The broad band observed at a frequency of 1650 cm^{-1} was only present in the FTIR spectrum of the shampoo and cream and may be due to the amide C=O stretch from imidurea, a preservative which was only present in the shampoo and the cream. The narrow low intensity peaks observed at a frequency of 1500 cm^{-1} may be due to C=C aromatic bending and the narrow peak observed at a frequency of 1050 cm^{-1} was most prominent in the spectrum for Xolegel® and may be due to a =C-H alkene bend. Overall the gel, shampoo and cream FTIR spectra reveal the presence of KZ and there is little likelihood of an interaction between the excipients and KZ within these dosage forms.

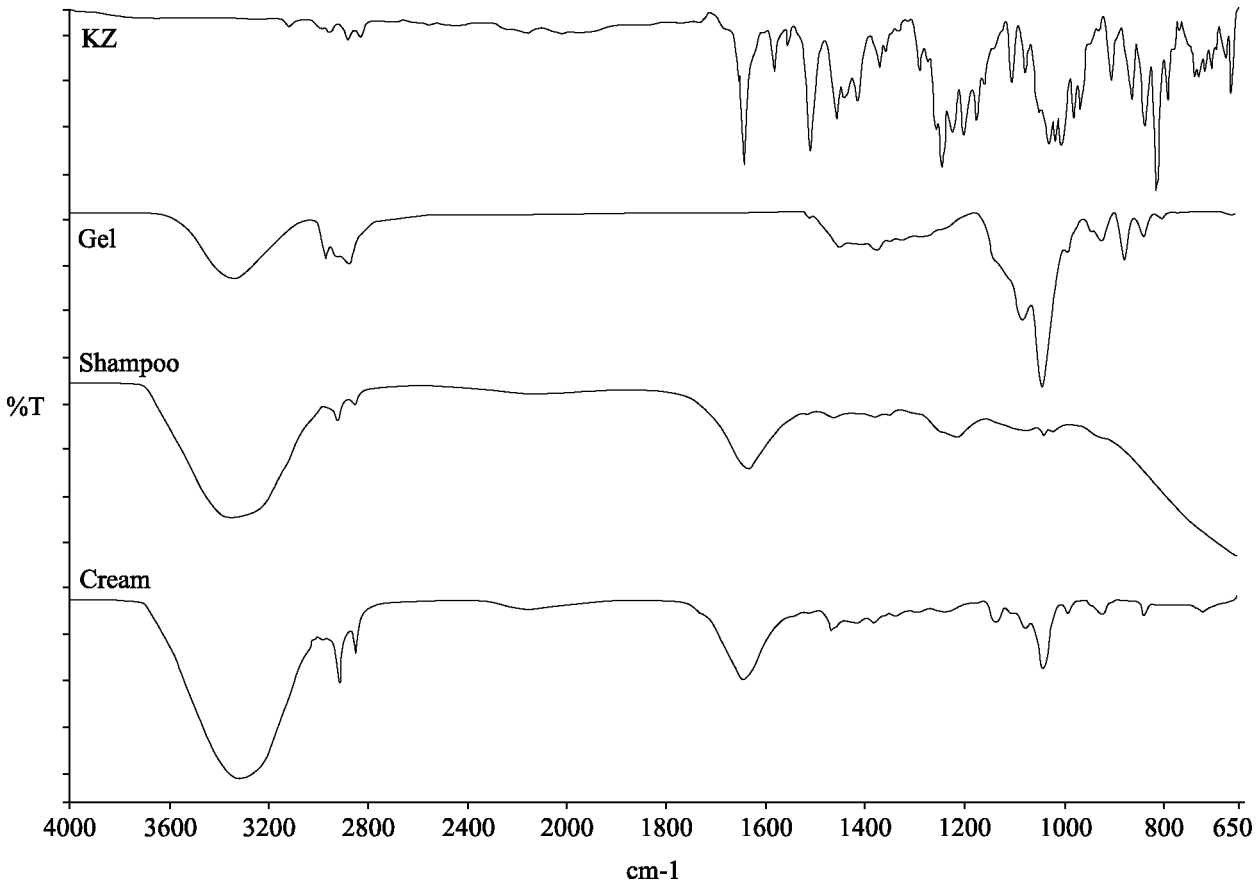


Figure 3.5 FTIR absorption spectra for pure KZ, Xolegel®, Kez® shampoo and Ketazol® cream.

DSC thermograms of pure KZ and commercial products are depicted in **Figure 3.6** and clearly indicate endotherms that represent the evaporation of water from the gel, shampoo and cream formulations at 100.32 °C, 106.19 °C and 101.53 °C respectively. The high intensity of this endotherm observed for Kez® shampoo is more than likely due to the higher water content of the shampoo. A detailed summary of the enthalpy, area and type of peak observed in the thermograms is summarised in **Table 3.6.**

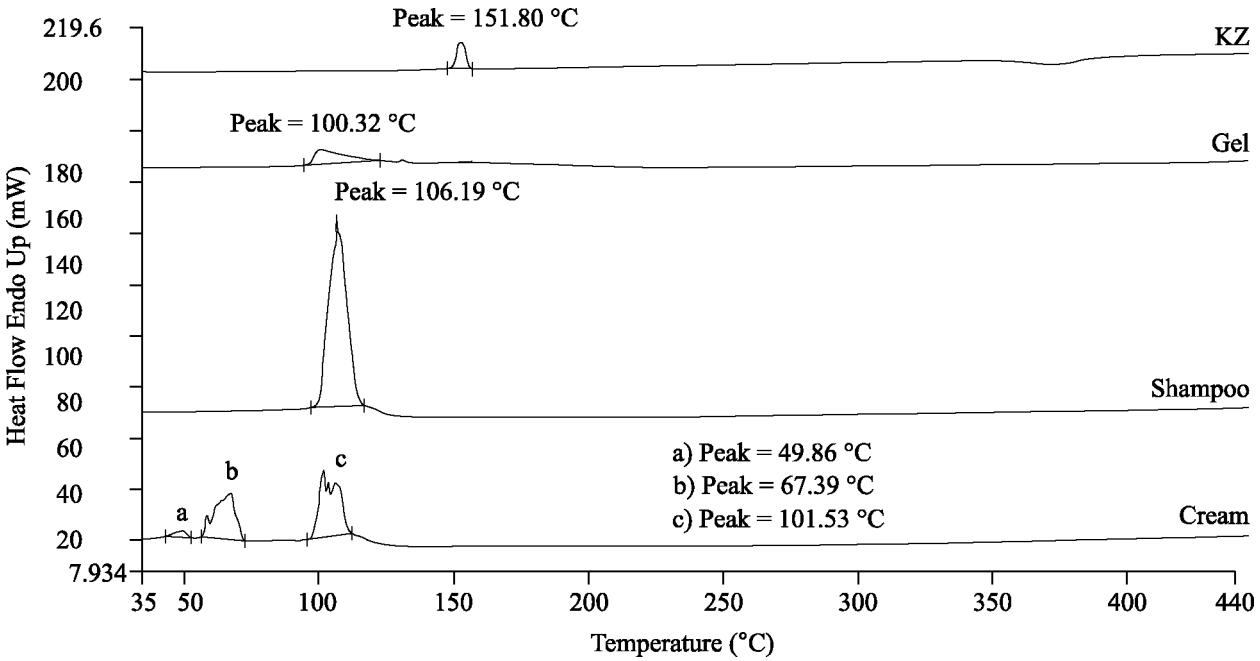


Figure 3.6 DSC thermograms for KZ, Xolegel®, Kez® shampoo and Ketazol® cream.

Table 3.6 Interpretation of DSC thermograms for KZ, Xolegel®, Kez® shampoo and Ketazol® cream.

Product	Temperature °C	Enthalpy J/g	Area mJ	Type of peak
KZ	151.80	85.14	242.64	Sharp endotherm
	369.14	97.28	277.26	Broad exotherm
Xolegel®	100.32	93.15	419.17	Broad endotherm
Kez® shampoo	106.19	953.11	3185.29	Sharp endotherm
Ketazol® cream	49.86 (a)	33.74	86.26	Broad low endothem
	67.39 (b)	370.89	948.36	Minimum of 1 sharp and 3 broad overlapping endotherm
	101.53 (c)	542.27	1386.59	Minimum of 2 sharp and 1 broad 3 overlapping endotherms

Ketazol® cream is a biphasic o/w emulsified system and the thermogram for the cream revealed the occurrence of three endothermic events that are, in turn, a combination of events as indicated by overlapping peaks and that are more than likely an indication of different components of the cream and may reflect melting endotherms at 49.86 °C (a), 67.39 °C (b) and evaporation of water from the aqueous phase at 101.53 °C (c). The endotherm observed at 49.86 °C (a) may be due to the melting of cetyl alcohol (49 °C) [189], steryl alcohol (59.00 °C) and/or sorbitan stearate (53.00 to 57.00 °C) which are

excipients used in the manufacture of Nizoral® cream and may also have been used in Ketazol® cream but are not reported [190,191].

The FTIR absorption spectra were used to establish whether differences, if any, may exist between the commercial products. The spectral data suggest that the shampoo and cream may contain similar excipients. Further experiments must be undertaken to identify the excipients used in each formulation but such studies do not form a major component of this research. The thermosetting gel that was to be developed and manufactured would also be subjected to DSC and FTIR analysis and these data would be compared to the FTIR spectra and thermograms generated for the Xolegel®, Kez® shampoo and Ketazol® cream. Such analyses may provide an indication of the nature of KZ inclusion, i.e. whether it is in the crystalline or amorphous form within the dosage forms [41].

The main purpose of undertaking DSC studies was to identify similarities in the composition of the gel and shampoo and the products seemed to behave similarly in respect of their melting behaviour. The cream on the other hand appeared to exhibit three distinct melting regions one of which was similar to that observed for the shampoo and gel. The melting point observed at approximately 100 °C was similar in all three thermograms and is more than likely due to the evaporation of water from the formulations. A melting endotherm for KZ was not observed in the DSC thermogram for the formulations as KZ may be present in an amorphous form that is not detected using DSC [41]. The response also suggests that KZ is completely dissolved in all formulations and that none of the formulations existed as solid dispersions as an endotherm for the melting point of crystalline KZ would have been observed in the thermograms had this been the case.

3.6 Conclusions

The viscosity at 22 °C and 37 °C, pH and assay of Xolegel®, Kez® shampoo and Ketazol® cream were assessed so as to collect information about these products to identify the ideal characteristics for use during the formulation and development of a thermosetting vaginal gel. The viscosity decrease observed at higher temperatures is opposite of what would be expected for a thermosetting gel dosage form and the decrease may well be due to an increase in the kinetic energy of the system or alteration of the physical properties of the viscosity enhancer used in the formulations [174]. The pH of the topical commercial products were all within the range of pH that would be expected exhibit the lowest degree of irritation to human skin. Consequently vaginal gel formulations should be at a pH that does not irritate the vaginal mucosa and tissues.

An extraction procedure was developed and used to measure KZ content in formulations and was found to be precise and accurate with a % recovery of 100 ± 5% for KZ. The procedure was successfully validated for repeatability or intra-assay precision and accuracy with SD = ± 2.09, % RSD ≤ 5% and %

bias $\leq \pm$ 5% [14,123,136]. The extraction procedure will be applied to the analysis of KZ in thermosetting vaginal gels during reverse engineering and formulation development studies.

The characterisation of Xolegel®, Kez® shampoo and Ketazol® cream using FTIR and DSC analysis have arguably been performed for the first time using this approach. Information about the properties of KZ in these formulations were elucidated and it was found that KZ may be present in the amorphous form. KZ in an amorphous form will be present in the thermosetting vaginal gels so as to enhance stability as it has been effectively used in commercial products. It was concluded that Ketazol® cream may contain similar excipients to Nizoral® cream, *viz.*, cetyl alcohol, steryl alcohol and sorbitan stearate through evaluation of the endotherms present in the DSC thermogram for Ketazol® cream. These are excipients that are generally used in cream formulations; however, this data alone cannot be used to categorically state that Ketazol® cream may be a complete copy of Nizoral® cream. The FTIR spectra suggest that there is no indication of interactions between KZ and other excipients in each of the three commercial formulations and this was an expected outcome as the registration of these products would not have been permitted had compatibility and accelerated and long term stability not been proven prior to market authorisation. The extraction of individual excipients and further characterization studies using Raman spectroscopy, thermogravimetric analysis, NMR, mass spectroscopy, optical microscopy and X-ray diffraction may prove useful for the elucidation of the exact composition of commercial formulations but are not within the scope of research for these studies. Sufficient information was obtained from the characterisation studies for Xolegel®, Kez® shampoo and Ketazol® cream to undertake preformulation and eventually formulation development of a novel thermosetting vaginal gel that contains KZ.

CHAPTER FOUR
DEVELOPMENT AND VALIDATION OF AN *IN VITRO* METHOD FOR THE ASSESSMENT OF KZ RELEASE FROM SEMI-SOLID DELIVERY TECHNOLOGIES

4.1 *In vitro* release testing

Dissolution and IVRT are widely used in the pharmaceutical industry for product development and quality testing purposes. These tests are also useful to measure the time taken for the complete recovery of an API during assessment of drug release from experimental dosage forms. Dissolution tests are mainly used to assess API release from solid oral formulations and have recently become popular for testing modified release solid oral formulations. However, the emergence of novel dosage forms has required the approach to the assessment of drug release to be modified. The term 'dissolution testing' is commonly used when assessing the release of an API from oral formulations whereas for topical and/or transdermal drug delivery technologies this test is referred to as *in vitro* or drug release testing [192].

Drug release from vaginal formulations is generally estimated using *in vitro, in vivo* and/or *ex vivo* models [193]. *In vivo* studies make use of live animals or humans whereas *in vitro* release studies require the use of a dissolution test apparatus with or without artificial membranes and *ex vivo* studies involve the use of excised animal or human tissues to assess the release of an API from formulations under investigation [194]. *In vivo* studies are essentially the gold standard of proof of performance as they are realistic models; however, there are ethical concerns and some biological drawbacks when using live animals for testing. *Ex vivo* testing is a cheap and viable alternative to conducting *in vivo* experiments that can be easily set up and are readily controlled from an experimental perspective [195]. Ketoconazole is a BCS Class II compound and is therefore a poorly soluble and highly permeable molecule for which it is possible to develop an IVIVC for regulatory and other purposes [30]. Furthermore, by reducing the need for *in vivo* studies, the risk of unnecessary exposure of healthy human and animal subjects to the drug are avoided. *In vitro* and *ex vivo* testing are vital during pre-clinical formulation testing and can facilitate an understanding of transport mechanisms by which an API may be transported across membranes [195]. *In vitro* and *ex vivo* release models that have been designed for the assessment of semi-solid vaginal delivery technologies are valuable for monitoring and assessing the release of an API from different dosage forms and to quantitate changes in release when excipients, the viscosity and/or manufacturing processes are altered [196]. However, these models may not be a measure of the bioavailability of a drug from such technologies unless an IVIVC has been established for that model [197].

Franz diffusion cells are a commonly used vertical diffusion cell apparatus applicable for development of biorelevant *in vitro* performance tests for assessing release from vaginal formulations intended for

topical delivery [196,198-205] and a schematic representation of a typical Franz diffusion cell system is depicted in **Figure 4.1**. The Franz diffusion cell test system is not a compendial apparatus for IVRT of topical semi-solid formulations, but vertical diffusion cell testing of semi-solid dosage forms has been suggested as an appropriate test system in the United States Pharmacopoeia (USP) pharmacopoeial forum [206]. The United States Food and Drug Administration (FDA) has published a Guidance for Industry on non-sterile semi-solid dosage forms for Scale-Up and Post Approval Changes (SUPAC) for *in vitro* release and *in vivo* bioequivalence testing. The SUPAC document contains specific guidelines for *in vitro* methods making use of Franz diffusion cell test systems [207].

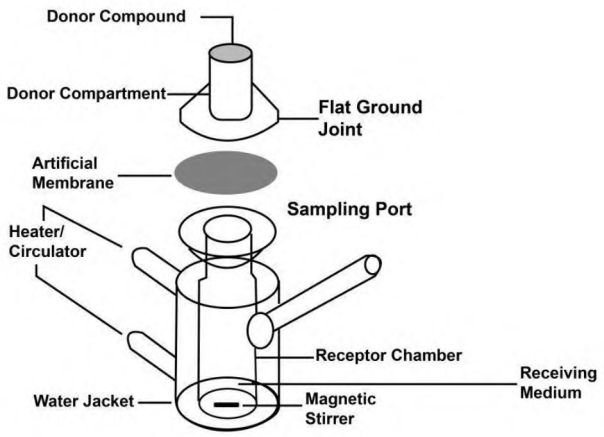


Figure 4.1 Schematic representation of a Franz diffusion cell apparatus, adapted from [208].

IVRT using Franz diffusion cells has been performed on topical KZ formulations [209-215]. The low aqueous solubility **(§ 1.2.5)**, high molecular weight **(§ 1.2.1)** and high log $P_{o/w}$ **(§ 1.2.7)** of KZ may negatively influence the potential for penetration of KZ into and through the vaginal mucosa and therefore poor *in vitro* release or flux may be observed [92,216]. The high log $P_{o/w}$ value for KZ of 3.78 results in high permeability and affinity of KZ for the lipid bilayers that are located in the vaginal mucosa and the compound is unlikely to partition from the vaginal mucosa and therefore exhibits low systemic distribution and low release into receptor fluids that may be used for IVRT [27,37,165,217].

The use of *in vitro* and *ex vivo* testing approaches with Franz diffusion cells was undertaken to evaluate and/or predict KZ transport across the vaginal mucosa and, of the models tested, one was identified and selected for use to evaluate KZ release from commercial semi-solid dosage forms. Following the selection of target experimental parameters, a Franz cell diffusion test method for the evaluation of commercially available KZ formulations was validated for precision at two levels, *viz.*, repeatability and intermediate precision [218] in order to distinguish the individual *in vitro* release profiles observed for Xolegel®, Kez® shampoo and Ketazol® cream. These data were then used to establish a target profile for the assessment of the thermosetting vaginal gels to be developed in these studies. In this respect, in

addition to precision studies dissolution or *in vitro* release procedure, validation should also comply with the Validation of Analytical Procedures and the Dissolution Procedure described in the ICH guidelines and in relevant USP monographs and chapters [206,208].

4.2 Selection of receptor fluid

When conducting *in vitro* or *ex vivo* tests using Franz diffusion cells it is vital that sink conditions are maintained in the receptor compartment during all studies. To facilitate the attainment of sink conditions the receptor medium may contain alcohol or a surfactant depending on the solubility of the API under investigation [192]. The Scientific Committee on Consumer Safety of the European Commission published a document stating that saline or buffered saline solutions may be used as a receptor fluid for hydrophilic compounds and serum albumin, alternate solubilisers or emulsifiers in amounts which do not impair the integrity of the membrane used and the analytical procedure may be included when evaluating the release of lipophilic compounds [219]. In addition the API should be stable in the receptor fluid and the medium should as far as possible reflect physiological conditions and pH. According to the Organisation for Economic Cooperation and Development (OECD) Guideline 428 or EC B.45 document on percutaneous absorption, the components of the receptor medium should be compatible with the formulation and must meet the solubility criteria for the compound under investigation [219,220]. Surfactants such as polysorbate 20 have also been used as a modifier to enhance the solubility of test compounds [221]. When using natural membranes such as human or animal skin, the lower layers of the skin may be modified due to interactions of the membrane with the receptor medium. Examples of such interaction include precipitation of skin proteins or dissolution of endogenous compounds that may ultimately result in a change in the diffusion barrier of the skin for the compound in the test formulation [221].

KZ is a hydrophobic compound that is soluble in weak acids and is sparingly soluble in ethanol [222]. Ethanol is water-miscible and may be a suitable solvent for these studies so as to ensure accurate assessment of KZ release from test formulations [223]. In one study a 0.01 M phosphate buffer of pH 7.4 was used as the receptor medium and cellulose acetate as the barrier membrane to assess KZ release from transfersomal gels from which a maximum amount of KZ of 44.98% was recovered after 24 hours using eucalyptus oil as penetration enhancer and was 16.24% without a penetration enhancer [209]. This relatively low amount of KZ released from the formulation suggests that a solubiliser may be required if the target amount released from commercial products was to be 100%. This high recovery of KZ is crucial if different formulations of thermosetting gels are to be compared as thermosetting gels are expected to exhibit relatively low recoveries over extended periods of time due to retardation and/or control of release. The low solubility of KZ in aqueous solution requires organic solvents or solubilisers to enhance the solubility of KZ in receptor media. A combination of methanol and phosphate buffer are often used as receptor fluids for the evaluation of KZ release from semi-solid formulations [216,224].

Polyethylene glycol, propylene glycol, isopropanol and methanol are used less often to enhance the permeation of lipophilic drugs into receptor fluids [221,225]. Ethanol in phosphate buffered saline (PBS) at pH 7.4 is known to have adequate solubilising effects when used in *in vitro* release studies for lipophilic drugs and concentrations of ethanol ≤ 25% with PBS (pH 7.4) solutions do not appear to affect the permeability characteristics of human skin [226]. A mixture of ethanol and phosphate buffer (pH = 7.4) in a ratio of 1:4 has been used for *in vitro* analysis of KZ [227].

Developmental formulation studies involved the use of a receptor medium that included ethanol and 0.05 M phosphate buffer of pH between 3.5 and 4.5 to mimic the acidic environment of the vagina and to ensure that sink conditions were maintained for the duration of KZ release testing. The choice of an ethanol-based receptor medium was made based on a report in which *in vitro* KZ release was monitored using phosphate buffer as a solvent and ethanol as a co-solvent [227].

4.3 Selection of barrier membrane

4.3.1 *Ex vivo* tissue model

The vaginal mucosa is made up of a stratified squamous epithelium as described in **§ 5.2.1.1** *vide infra.* A barrier to permeation is formed by the outer layer of stratified squamous epithelial cells that protect the underlying tissues of the organ [228]. Formulations that are administered intra-vaginally should ensure penetration of the API through the barrier layers of the organ to guarantee local and systemic distribution and activity. The effect of the barrier on API permeation following release from delivery technologies should be mimicked by membranes that are selected for use in *in vitro* release studies of test formulations [208]. The vaginal barrier is inert and unlike other membranes such as the intestinal mucosa does not exhibit active transport or metabolic activity to facilitate penetration. The barrier function of the tissue is preserved for a period of time, following excision of vaginal tissue [228]. The Scientific Committee on Consumer Safety of the European Commission has reported that porcine skin may be used as an alternative to human skin for permeations studies as it exhibits similar characteristics, although no mention was made about vaginal mucosae [219]. Several e*x vivo* studies using porcine vaginal mucosa and Franz diffusion cells have been published [229-232]. The barrier function of the human vaginal mucosa has been reported to be viable for 12 hours post excision [228] and the advantages and disadvantages of using human [233-235] or porcine [236-240] tissues for *ex vivo* studies are summarized in **Table 4.1**. *Ex vivo* studies usually refer to experimentation performed on tissue while maintaining physiological conditions. In this study, the temperature was maintained at body temperature and a simulated vaginal fluid was placed in the donor compartment and for the purposes of this work, the term *ex vivo* was preferred over *in vitro* when porcine mucosa was mounted on the Franz cells.

Table 4.1 Advantages and disadvantages of using human and pig vaginal mucosa for *ex vivo* studies. Adapted from [228].

Mucosal tissue	Characteristics	Advantages	Drawbacks
Pig vaginal mucosa excised post slaughter	Non-keratinized mucosa with connective tissue Thickness ~ 116 µm Flux of tritiated water = 4667 ppm/cm^2/min[1]	Similarity in structure and function to human vaginal tissue Simple and cheap to procure No ethical approval required	No vascular system therefore limited functional response
Human vaginal mucosa surgically obtained from postmenopausal individuals undergoing hysterectomy	Non-keratinized mucosa with connective tissue. Thickness ~ 150 - 200 µm. Flux of tritiated water = 4155 ppm/cm^2/min[1]	Viable for a maximum of 12 hours following tissue excision Regulatory and ethical approval required for human subjects according to the 'Ethics in Health Research: Principles, Processes and Structures' guidelines and Institutional Ethical Review Boards	Difficult to obtain and there are age specific restrictions Possible contamination with bacteria, fungi and viruses

It is clear from the data listed in **Table 4.1** that the pig vaginal mucosa can be used as a substitute for human tissue for *ex vivo* release studies of KZ as there is a high degree of structural and functional similarity to human vaginal tissue and it is simpler and cheaper to procure porcine than human tissues [228].

Human and animal tissue are non-uniform and often lack homogeneity, resulting in unpredictable variability in the tissues as a consequence of age, race, sexual activity and anatomical region of harvesting [241,242]. The freezing of tissues may also affect the permeability of a membrane to an API and therefore the impact of freezing of tissue was investigated by assessing KZ permeation through fresh and frozen pig vaginal mucosa [195].

Human and animal sourced vaginal tissues require pretreatment prior to use in *ex vivo* studies in order to isolate a layer of mucosa that is free of connective and muscle tissue. The mucosa can be prepared by surgical separation, chemical and/or heat splitting [243-245]. Surgical separation is undertaken by trimming connective and muscle tissues from the mucosa, using a scalpel and surgical scissors to isolate a layer of tissue that exhibits uniform thickness but this approach requires a certain degree of skill to achieve [243,246]. The chemical approach to separation of the mucosal layer from underlying tissues relies on the use of chemicals such as ethylenediamine tetraacetic acid (EDTA) and sodium bromide to detach the underlying tissues from the mucosa by soaking the tissues for up to 24 hours at 7°C in a solution of the splitting agent [247]. Similarly, splitting using heat requires immersion of tissues in a solution of sodium chloride and heating at 60°C for 1-5 minutes, depending on the initial thickness of

the mucosa [248]. Following treatment, the mucosal layer is carefully separated from the underlying tissues and mounted between the donor and receptor compartments of Franz cells for *ex vivo* release testing.

4.3.2 *In vitro* model

Synthetic or artificial membranes may also be used for IVRT and as they are inert, are structurally identical from batch to batch and are readily available, this approach is cost effective when compared to sourcing and using human and animal tissues. Synthetic membranes are selected to simulate, where possible, tissue permeability in addition to ensuring consistency in quality control as they are reproducible [194]. Membranes such as polymethylsiloxane are hydrophobic and mimic the skin and other tissues due to their rate limiting properties [194]. The aim of a dissolution or *in vitro* release study is to compare the release of an API from different batches of the same formulation or different formulation compositions with similar properties and that contain the same excipients in different quantities. However, the use of a rate limiting membrane may not result in the development of a test system that is sufficiently sensitive to discriminate between release profiles and therefore formulation effects may not be detected [249]. Consequently, there is a need to select an artificial membrane that exhibits minimal resistance to API transport and that does not exhibit any binding capacity for the molecule under investigation [75]. The FDA recommends that simple and porous membranes that act as a support and separate the donor and receptor compartments whilst maintaining continuous contact with the receptor medium be used to evaluate the *in vitro* performance of topically applied formulations [207]. Moreover the membranes should be void of any compound that may impact, interfere or modify *in vitro* release test data [75].

4.3.3 Validation of *in vitro or ex vivo* model

The validation of dissolution methods has been reported [75] where the IVRT was validated for repeatability, intermediate precision, effects of dosage strength, composition, process parameters and viscosity on the rate of drug release. The ICH Q2 (R1) guideline indicates that the validation of dissolution procedures has not yet been addressed by the ICH. These tests can be validated using a similar approach to that used for the validation of analytical procedures [137].

4.4 Statistical comparison and mathematical modelling of *in vitro* release profiles

The dissolution of drugs in aqueous fluids may be limited by physical phenomena and the use of mathematical modelling may simplify and permit elucidation of complex drug release mechanism(s), whilst facilitating the quantitation of drug transport, through the development of theoretical concepts that explain the dissolution kinetics of a system [250,251]. The kinetics of drug release from dosage forms may also be investigated using statistical approaches and/or model dependent and independent methods [252]. Three approaches were used to assess KZ release from Ketazol® cream, Kez® shampoo

and Xolegel®. Analysis of variance (ANOVA) was performed using GraphPad® Prism for the statistical evaluation of KZ release. Model dependent analysis applied zero order, first order, Higuchi, Korsmeyer-Peppas and Hixson Crowell models to release data and calculation of the similarity and difference factors formed the basis of model independent analysis.

4.4.1 Statistical analysis

Ordinary one- and two-way ANOVA analyses are undertaken to estimate differences between release profiles through calculation of a p-value, the mean difference, establishment of a 95% CI, use of sum of squares and degrees of freedom (df). Tukey's multiple comparison testing was also performed using the data generated in these studies in order to undertake a more detailed analysis of any differences that may have occurred within data sets.

4.4.2 Model dependent mathematical analysis

4.4.2.1 Zero order model

The application of a zero order model is usually required for the assessment of formulations designed to release a drug in a slow or modified manner and includes transdermal modified release dosage forms [253]. The mathematical description of the zero order model is described in **Equation 4.1** [252].

$$Q_t = Q_0 + k_0 t \qquad \textbf{Equation 4.1}$$

Where,

Q_t = amount of drug released at time, t,
Q_0= the initial amount of drug in solution (generally 0), and
k_0 = the zero order release rate constant with units, $\mu g/cm^2/hr$.

The cumulative % KZ release data was fitted to the zero order model and the goodness of fit evaluated by calculation of R^2 and evaluation of the release rate constant (K).

4.4.2.2 First order model

The first order model first proposed is generally used to describe water-soluble drug release from porous matrix formulations and the mathematical representation of this model is shown as **Equation 4.2** [254].

$$\ln C = \ln C_0 - k_t \qquad \textbf{Equation 4.2}$$

Where,

C = concentration at time, t,
C_0 = the initial concentration of drug,
T = time, and
k = first order rate constant with units, hr^{-1}.

The cumulative % KZ release data was fitted to the first order model and the goodness of fit evaluated by calculation of R^2 and evaluation of K [252].

4.4.2.3 Higuchi model

The Higuchi model is used to describe drug release from non-eroding matrices and is based on the hypotheses that the initial concentration of drug in a matrix is greater than the solubility of that compound in the receptor fluid and that one dimensional dissolution of molecules occurs from the system in which that dimension is smaller than the thickness of the formulation. In addition, the model assumes that swelling and dissolution of the matrix are negligible, that drug diffusion is constant and that sink conditions exist in the receptor fluid [255,256]. A simplified mathematical expression of the Higuchi model is shown as **Equation 4.3** [257].

$$Q = k_H \, t^{\frac{1}{2}} \qquad \textbf{Equation 4.3}$$

Where,

Q = the amount of drug release per unit area at time, t, and

k_H = the dissolution constant with units, $\mu g/\ cm^2/\ hr^{\frac{1}{2}}$.

The cumulative % KZ released data was fitted to the Higuchi model and the goodness of fit evaluated. The elucidation of a linear relationship is indicative that KZ release from the test formulations was controlled by a diffusion process.

4.4.2.4 Korsmeyer-Peppas model

The Korsmeyer-Peppas model is used to describe drug release from hydrophilic polymeric matrix formulations [258] and the mathematical representation of this model is shown as **Equation 4.4** [252]. The diffusional exponent characteristic of the release mechanism is represented by n whose magnitude can describe different release characteristics from a formulation such as Fickian diffusion, case II transport and non-Fickian diffusion [259]. This mathematical model can adequately describe drug release from formulations exhibiting any shape and having different release mechanisms [260]. $n \leq 0.45$ indicates that the release mechanism occurs through Fickian diffusion. Non-Fickian or anomalous drug release is represented by $0.45 < n < 0.89$. $n = 0.89$ indicates case II transport and $n > 0.89$ super case II transport .

$$\frac{M_t}{M_\infty} = Kt^n \qquad \textbf{Equation 4.4}$$

Where,

$\frac{M_t}{M_\infty}$ = the fraction of drug released at time, t,

K = the dissolution constant with units, $\mu g/\ cm^2/hr^n$, and

n = the release exponent.

The Korsmeyer-Peppas model was used to assess the kinetics of KZ release from experimental and commercially available formulations.

4.4.2.5 Hixson-Crowell model

The Hixson-Crowell model or cube root law describes dissolution of a compound parallel to the surface of that compound. In other words, the model assumes that the initial geometrical shape of the dosage form remains constant over time [261] and the mathematical description of this model is depicted in **Equation 4.5** [252].

$$W_0^{\frac{1}{3}} - W_t^{\frac{1}{3}} = kt \qquad \textbf{Equation 4.5}$$

Where,

W_0 = the initial amount of API in the formulation,
W_1 = the amount of API that remains in the formulation at time, t, and
k = a constant that represents the surface to volume relationship with units, $\mu g/\ cm^2/hr^{\frac{1}{3}}$

The KZ release data were fitted to the Hixson Crowell model.

4.4.3 Model independent analysis

Model independent analysis assesses the pair-wise similarity and difference of drug release from formulations using similarity and difference factors as described by Moore and Flanner [262]. Generally this approach requires that release from formulations be compared to a reference formulation. In this case Xolegel® versus Kez® shampoo, Xolegel® versus Ketazol® cream and Kez® shampoo versus Ketazol® cream were compared so as to evaluate the similarity and difference between the in vitro release profiles elucidated for the commercial formulations. The difference factor, f_1 was calculated using **Equation 4.6** [263,264]. An in vitro release profile can be considered similar if $f_1 \leq 15$ [257] and $f_2 \geq 50$ [263,264]. The values for f_1 and f_2 should always lie between 0 and 100.

$$f_1 = \frac{\sum_{t=1}^{n} |R-T|}{\sum_{t=1}^{n} R} \times 100 \qquad \textbf{Equation 4.6}$$

Where,

f_1 = Difference factor,
R = Mean% KZ released from the reference formulation at P time points,
T = Mean% KZ released for the test formulation at P time points, and
n = Number of time points.

The similarity factor, f_2 was calculated using **Equation 4.7**. The similarity factor was adopted by the FDA and EMEA to evaluate similarity of *in vitro* release profiles between formulations [263,264].

$$f_2 = 50 \log\left\{\left[1 + \frac{1}{n}\sum_{t=1}^{n}(R - T)^2\right]^{-\frac{1}{2}} \times 100\right\} \quad \textbf{Equation 4.7}$$

Where,

f_2 = Similarity factor,
R = Mean % KZ released for the reference formulation at P time points,
T = Mean % KZ released for the test formulation at P time points, and
n = Number of time points.

4.5 Materials, equipment and methods

Ketazol® cream, Kez® shampoo and Xolegel® are extensively described in **Chapter 3** and all formulations and chemicals were weighed using an AG135 Mettler® Toledo top-loading analytical balance or a Delta Range® Mettler PM 4600 balance (Urdorf, Zürich, Switzerland). A glass Franz diffusion cell system purchased from Crown Glass Company Inc (Branchburg, New Jersey, USA), consisting of six cells with an average diffusional surface area of 2.06 ± 0.10 cm^2 and a water heater/circulator (Grant instruments Raedene Ltd, Shepreth, Cambridge, United Kingdom) was maintained at 37 ºC. Samples were agitated using a Labotec® shaking water bath (Greenfield, Oldham, United Kingdom).

Double distilled, HPLC grade water was used for all experiments. Analytical grade chemicals and reagents were used for all studies without further modification.

4.5.1 Preparation of receptor solvents

4.5.1.1 Quaternary solvent system

A quaternary solvent system comprised of 1.5 g polysorbate 80, 4.0 g citric acid and 9.5 g absolute ethanol purchased from Aspen® Pharmacare (Port Elizabeth, Eastern Cape, South Africa), PAL Chemicals (Dorking, Surrey, England) and Merck® Laboratories (Wadeville, Gauteng, South Africa) respectively, made up to 50 g with a citrate-phosphate buffer of pH = 5.0 was used for formulation development. The citrate-phosphate buffer consisted of a 50:50% v/v mixture of 0.1 M citric acid and 0.2 M disodium hydrogen phosphate (DHP) [265]. The 0.1 M citric acid solution was prepared by accurately weighing 21.00 g citric acid monohydrate and dissolving in 1000 mL HPLC grade water. Similarly, a 0.2 M DHP buffer solution was manufactured by accurately weighing 28.40 g DHP and dissolving it in 1000 mL HPLC grade water. The mixtures were sonicated until the powders had dissolved. Approximately 500 mL of 0.1 M citric acid and 500 mL 0.2 M DHP were accurately measured and mixed in a 1 L measuring cylinder and the pH was adjusted to 5.0 using a 0.1 M solution of NaOH prepared as described for the citric acid and DHP solutions.

4.5.1.2 Simulated vaginal fluid

Simulated vaginal fluid (SVF) [266] was prepared by dissolving 3.51 g Merck® sodium chloride (Wadeville, Gauteng, South Africa), 1.40 g Merck® potassium hydroxide (Wadeville, Gauteng, South Africa), 0.222 g MacDonald Adams® calcium hydroxide (Johannesburg, Gauteng, South Africa), 0.0180 g bovine serum albumin , 2.00 g lactic acid, 1.00 g acetic acid, 0.160 g glycerol, 0.400 g urea obtained from Sigma-Aldrich® (St. Louis, Missouri, USA) and 5.00 g Saarchem® glucose (Johannesburg, Gauteng, South Africa) in 1000 mL HPLC grade water by sonicating and stirring the mixture using a Branson® B12 sonicator (15 minutes) and a magnetic stirrer (15 minutes).

4.5.1.3 Receptor medium

The receptor medium for all *in vitro* release analyses consisted of ethanol: 0.05 M phosphate buffer (pH 3.5 or 4.5). The fluid composition was varied during method development and the *in vitro* release method identified following development studies was comprised of ethanol and phosphate buffer (pH = 4.5) in a ratio of 50:50.

The receptor medium was prepared using a 1000 mL A-grade measuring cylinder and an Eppendorf® Research 100-1000 µL micropipette to measure 680 µL 85% v/v phosphoric acid prior to making up to 1000 mL with HPLC grade water to produce a 0.05 M phosphate buffer. The pH of the buffer was measured using a model GLP 21 Crison® Instruments pH meter and was adjusted to pH of 3.5 or 4.5 using a 0.1 M solution of NaOH, as required. The receptor fluid containing ethanol and phosphate buffer was mixed in a 2000 mL Schott Duran® conical flask with the aid of a magnetic stirrer . The receptor medium was prepared on a daily basis and was stored in a Schott Duran® laboratory glass bottle. The receptor medium was degassed, under vacuum, using an Eyela® Aspirator-degasser A-2S and filtered using a 0.45 µm Millipore® Corporation HVLP Durapore membrane filter to prevent air bubble formation during testing, particularly when using Franz diffusion cells.

4.5.1.4 *Ex vivo* studies

PBS solution was prepared by weighing 1.83 g disodium hydrogen phosphate dodecahydrate, 0.21 g potassium dihydrogen orthophosphate and 9 g sodium chloride into a beaker and then adding 1000 mL HPLC grade water, sonicating for a minimum of 5 minutes until a clear solution was formed. The 0.03 M disodium EDTA solution was prepared by weighing 1.12 g of disodium EDTA and dissolving it in 1000 mL PBS and sonicating the mixture for 5 minutes.

4.5.2 Release of KZ in receptor fluids of different composition

The solubility of KZ in the receptor medium is an important consideration when selecting the composition of the fluid as it must provide a diffusional sink to facilitate the release of the compound from semi-solid dosage forms [75]. An experiment was performed in order to establish whether a fluid

comprised of ethanol: 0.05 M phosphate buffer was an appropriate receptor medium for diffusion cell studies to assess the release of KZ from Xolegel®, Kez® shampoo and Ketazol® cream. The receptor fluids tested included ethanol and 0.05 M phosphate buffer of pH = 4.5 in ratios of 0:100, 30:70, 40:60, 50:50, 60:40 and 80:20. Approximately 1 g aliquots of each of the products was accurately weighed and placed into 100 ml of the receptor fluid in a volumetric flask, sealed with Parafilm™ and a stopper to ensure that alcohol loss due to evaporation did not occur. The volumetric flasks were mounted in a Labotec® shaking water bath and agitated for 24 hours at a temperature of 37°C [267]. Approximately 10 mL of each solution was poured and transferred into a Kimax® centrifuge tube and centrifuged at 3000 rpm using a Model HN-SII centrifuge, prior to filtration using a Millipore® PVDF hydrophilic syringe filter of 0.45 μm pore size and analysed using the validated HPLC method described in **Chapter 2.**

4.5.3 Selection of membranes

4.5.3.1 Natural membranes

4.5.3.1.1 Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) was used to assess the cross section of the vaginal mucosa obtained from pigs to visualize the layers of tissue, prior to processing for use in *ex vivo* studies. The vaginal mucosa of the pig was initially cut into 1 cm^2 sections using a pair of scissors in a manner to ensure that the inner surface of the mucosa was as smooth as possible. The tissue was soaked in a 2.5% m/v glutaraldehyde in 0.1 M sodium phosphate buffer of pH 7.5 solution overnight at 22 °C in order to lyophilise the membrane. After soaking for 24 hours the membrane was dehydrated by soaking for 5 minutes in 30% v/v, 50% v/v, 70% v/v, 80% v/v, 90% v/v and 100% v/v 95% ethanol sequentially. The mucosa was dried using carbon dioxide purchased from Afrox® (Port Elizabeth, Eastern Cape, South Africa) for approximately 2 hours in an Ashcroft® Critical Point Drying Apparatus (Cape Town, Western Cape, South Africa). The membrane was then mounted onto double sided carbon tape and fixed with colloidal graphite obtained from Agar Scientific (Stansted, Essex, United Kingdom). The membrane was then sputter coated with gold for 12 minutes in a Quorum Q15OR gold coater (Ashford, Kent, United Kingdom). A Vega LMU SEM fitted with a Pfeiffer® Vacuum D-35614 pump (Asslar, Hesse, Germany) and an Oxford Instruments® INCA Penta FETx3 Model 7378 detector (Abingdon, Oxford, United Kingdom) was used to generate images that were subsequently analysed using TESCAN software (Asslar, Hesse, Germany).

4.5.3.1.2 Preparation of natural membranes

The vaginal mucosa from pigs was obtained from the Miller's Abattoir (Uitenhage, Eastern Cape, South Africa). Confirmation that ethical approval was not required for the procurement and use of the mucosa is depicted in **Appendix A**. The underlying connective and muscle tissue was surgically removed and damage to the inner mucosal layer avoided (**Figures 4.2 - 4.4**). The final thickness of the vaginal mucosa

that was harvested was approximately 2 mm to 5 mm . The tissue of the pig vagina and anus depicted in **Figure 4.2 (A)** reveals, depicted with arrows, the presence of the vaginal opening to the right of the figure and the anus on the left of the figure but they cannot be distinguished in **Figure 4.2 (B)**. The tissues obtained from the abattoir were elastic and tough and despite the use of a scalpel and scissors it was not possible to cut the membrane and a sharp kitchen knife was required to excise the anus from the vagina (**Figure 4.3 (A)**) without damage to the vaginal mucosa.

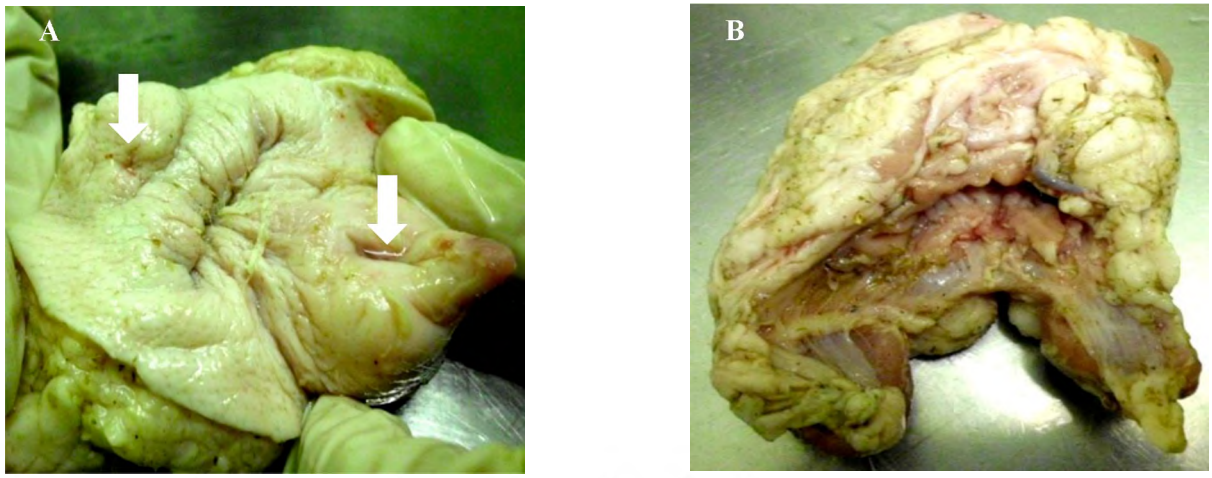


Figure 4.2 Anterior (A) and posterior (B) views of pig vaginal tissues.

The perineum is located between the anus and vagina and is a thin tissue that requires the utmost care during excision and harvesting to avoid any damage to the vaginal mucosa. Following separation of the vagina from the anus (**Figure 4.3 (B)**) the connective and muscular tissue layers were removed using a surgical blade and a pair of scissors until only a thin layer of the vaginal membrane remained.

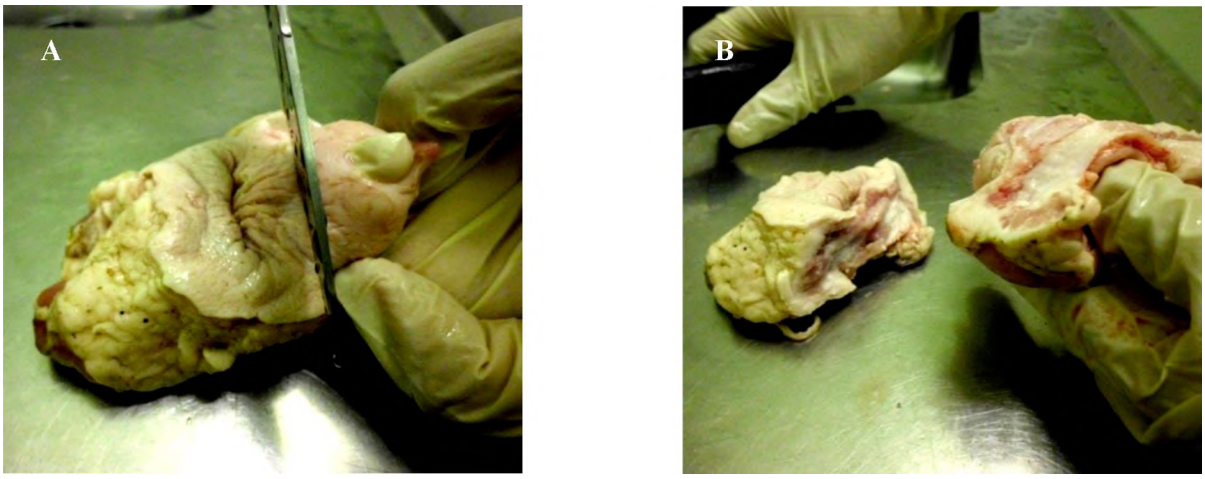


Figure 4.3 Excision of the vagina from the anus using a sharp knife (A) and an example of the excised vagina and anus (B).

The harvested membrane was tubular in shape that was subsequently inverted such that the surface of the vaginal mucosa faced outwards as depicted in **Figure 4.4 (A)** to ensure that the mucosa was not damaged when cut with a pair of scissors (**Figure 4.4 (B)**). The mucosa was washed with distilled water and the membranes were then used for *ex vivo* experiments. The membranes selected for use were mounted onto Franz cells and those not selected were stored flat (**Figure 4.4 (C)**) with the epithelium faced in a dorsal orientation in air tight plastic bags and stored at -20°C and -80°C.

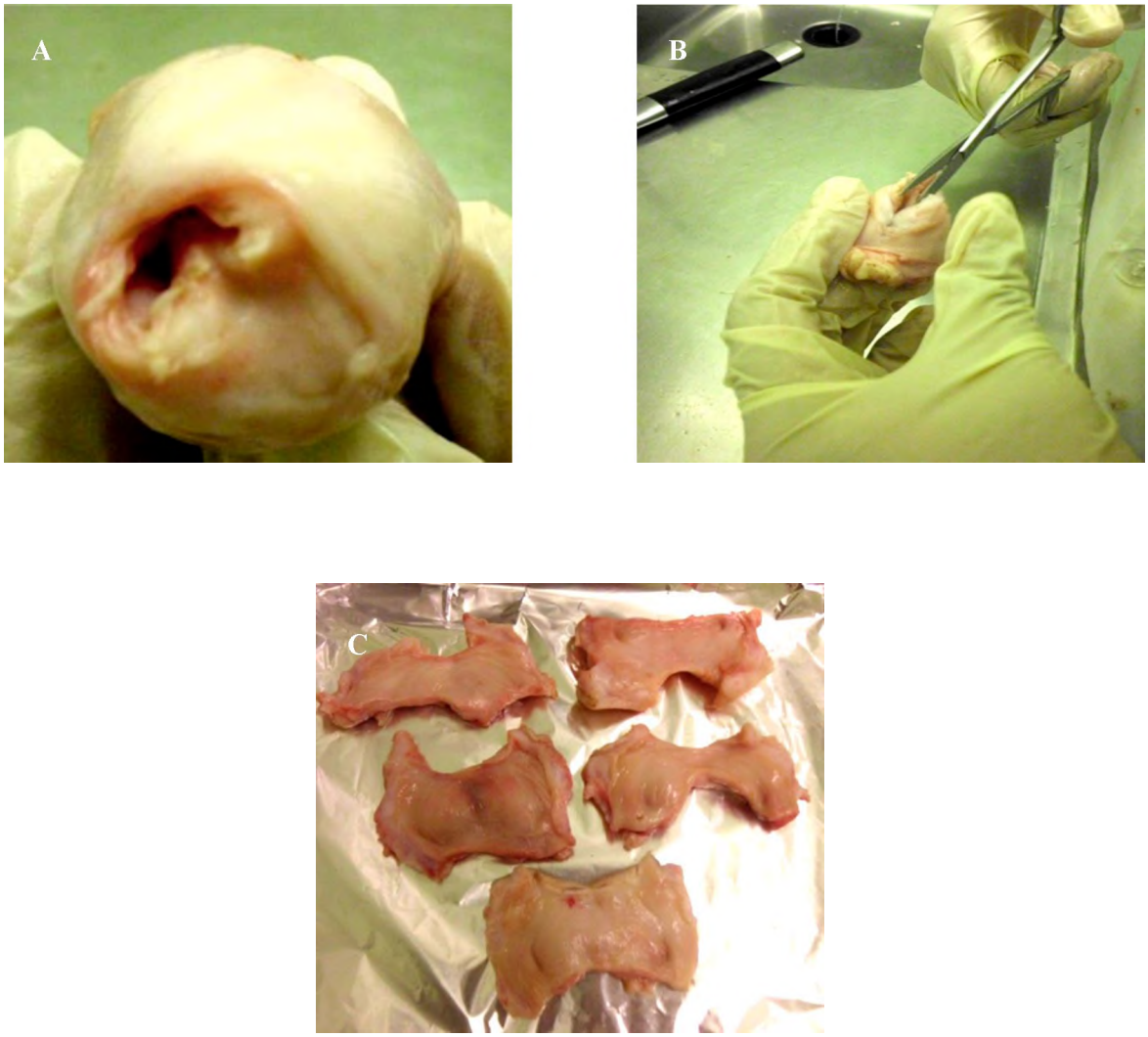


Figure 4.4 Inverted tubular vaginal mucosa (A), opening using scissors (B) and orientation prior to use or storage (C).

Isolation of the vaginal epithelium was initially attempted using chemical splitting with a 0.03 M disodium EDTA purchased from Saarchem® (Johannesburg, Gauteng, South Africa) in PBS solution adjusted to pH 7.4. The vaginal tissue was placed into the splitting solution for 24 hours at 7°C, after which the membrane was removed at 8 hourly intervals to evaluate membrane peeling.

Heat splitting was also assessed as a means of harvesting membranes. A 0.9% m/v sodium chloride solution was prepared by weighing 9 g of sodium chloride crystals and dissolving in 1000 mL HPLC

grade water and heat splitting was performed by immersing the tissue in the solution at 60°C for five minutes or until the mucosa could be peeled from the underlying tissues without tearing. An example of a membrane prepared using this procedure is depicted in **Figure 4.5**.

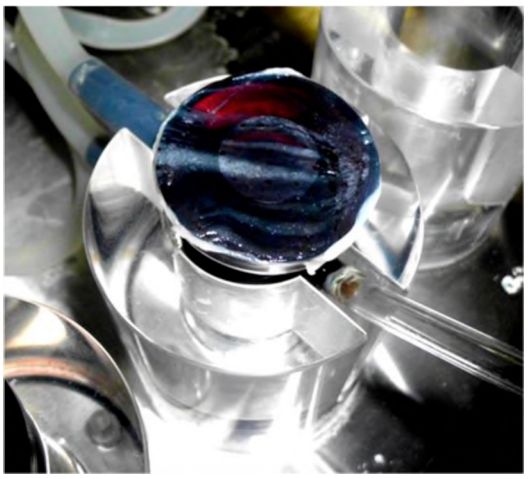


Figure 4.5 Vaginal membrane prepared using heat splitting mounted on a Franz diffusion cell.

Following assessment of chemical and heat splitting it was decided that a surgical approach be used to isolate tissue to be used for release studies. A challenge observed when preparing tissues using heat or chemical splitting was that extremely thin and fragile materials were produced and were difficult to mount on the diffusion cells. Furthermore, the animals from which the tissues were harvested were between 100 and 120 days of age and were therefore considered young. Consequently the membrane that was produced was not thick enough to harvest using chemical or heat splitting but were suitable when processed surgically.

4.5.3.1.3 *Ex vivo* studies to test the integrity of natural membranes

The integrity of membranes stored at -20°C or -80°C was established following storage at the specified temperatures and then performing *ex vivo* release studies after two and six days.

The vaginal mucosae were stored at -20°C in a Model CF 355 KIC® Freezer (Mandeni, Kwazulu-Natal, South Africa) and -80°C in a Model U445 Telstar Igloo Ultra Low Temperature Freezer (Terrassa, Barcelona, Spain) for two and six days post collection and harvesting. The selection of conditions was based on experiments in which the use of pig membranes had been reported [234,268] to establish if storage conditions had an impact on the integrity of the vaginal mucosa after six days. Following the freezing process, the membranes were thawed at 22°C in SVF for one hour prior to mounting on the Franz cells, to minimize tissue damage on mounting between the donor and receptor compartments. The donor compartment was then filled with 2% m/v KZ dissolved in solvent and 1 mL SVF was added after five minutes exposure. SVF was also added to the donor compartment so as to simulate

physiological conditions in the vagina and removal by vaginal secretions so as to render the *in vitro* release test realistic. Following administration, the SVF would be in contact with the thermosetting gel prior to KZ transport into and through the vaginal mucosa. The thermosetting gel manufactured in these studies would form a matrix and remain in the vagina in the presence of vaginal secretions. This technique of adding the SVF to the donor chamber 15 minutes after commencing thermosetting to solidify the gel at 37 ºC in the donor compartment of the cells was adapted from a method in which the washability of muco-adhesive gels was reported when tested at the donor compartment and receptor medium sides [202].

Permeation studies were performed at 37°C with continuous stirring of the receptor fluid at 200 rpm. The receptor fluid was comprised of ethanol and 0.05 M phosphate buffer of pH 4.5 in a 50:50 ratio. Freshly prepared membranes were used as a control for all *ex vivo* studies for comparison of the performance of frozen tissues to freshly prepared membranes.

As part of the testing process the amount of KZ was quantitated to evaluate if the compound was likely to bind to the vaginal membrane during *ex vivo* studies. Binding was assessed by chopping the membrane following completion of the experiment and extracting the KZ using the HPLC and extraction method described for Xolegel® and Kez® shampoo in this thesis.

4.5.3.2 Artificial membranes

An array of synthetic membranes of different pore size were screened for potential use and the properties are summarized in **Table 4.2**. The membranes evaluated included a 8.0 µm Sartorius® cellulose nitrate (Goettingen, Lower Saxony, Germany), 0.8 µm Sartorius® cellulose nitrate (Goettingen, Lower Saxony, Germany), 0.2 µm Whatman® nitrocellulose (St. Louis, Missouri, USA), 0.025 µm Millipore® nitrocellulose (Wadeville, Gauteng, South Africa) and a 0.2 µm Sartorius® cellulose acetate membrane (Goettingen, Lower Saxony, Germany).

Table 4.2 Characteristics of artificial membranes.

Membrane	Manufacturer	Description	Pore Size µm	Thickness µm	Property
Cellulose Nitrate	Sartorius®	White without grid	8.0	130	Hydrophilic
Cellulose Nitrate	Sartorius®	White without grid	0.8	130	Hydrophilic
Nitrocellulose	Whatman®	White	0.2	-	Hydrophilic
Nitrocellulose	Millipore®	White	0.025	-	Hydrophilic
Cellulose Acetate	Sartorius®	White	0.2	-	Hydrophilic

Standard solutions of KZ (20 µg/mL and 100 µg/mL) were prepared prior to filtration as described in **§ 4.5.1.1** and filtered through the membranes (n = 3) prior to analysis to establish the recovery of KZ. Following filtration each membrane was soaked in 5 mL of the quaternary solvent to establish if KZ

had bound to the membrane. Samples of the filtrate were analysed (n = 3) to establish the recovery of KZ and the membrane that displayed minimal binding of KZ was selected for *in vitro* release studies.

4.5.4 Optimisation of receptor medium for *in vitro* release testing

The pH of the buffer and the ratio of ethanol to buffer are important factors to consider when selecting a receptor medium for IVRT [75]. KZ release from Xolegel®, Kez® shampoo and Ketazol® cream (n = 3) was assessed using receptor media of different ratios of ethanol: 0.05 M phosphate buffer at pH 4.5.

The temperature of the diffusion cells was maintained at 37 ± 0.5°C to mimic vaginal temperature [269] and samples were removed for analysis at 1, 2, 4, 8, 12, 20 and 24 hours. The Scientific Committee on Consumer Safety of the European Commission report that IVRT time should be 24 hours and sample removal should occur at regular intervals as exposure time of the membrane to the receptor medium should be consistent with the intended use of the formulation, i.e. a maximum of 24 hours in this case as vaginal formulations will be removed due to normal physiological clearance, daily washing routines, urination or coitus [219].

A 1 g aliquot of test formulation and 1 mL SVF [270] was placed into the donor compartment and the receptor cell was filled with receptor fluid comprised of ethanol and 0.05 M phosphate buffer in ratios of 30:70, 40:60, 50:50, 60:40 or 80:20. The buffer pH used was 3.5 or 4.5 for each study.

The sample volume was 800 µL and the receptor compartment was emptied completely each time, after which the volume was replenished with fresh medium. The cumulative amount KZ released per unit area was calculated for each cell using **Equation 4.8**.

$$Q = \frac{(C_n V + \sum_{i=1}^{n-1} C_i S)}{A}$$ **Equation 4.8**

Where,

Q	= Cumulative amount of KZ released per unit area of membrane, µg/cm^2,
C_n	= Concentration of KZ in µg/mL at nth sampling interval,
V	= Volume of Franz cell,
S	= Sample volume,
$\sum_{i=1}^{n-1} C_i$	= Sum of concentrations of KZ in µg/mL from samples 1 to n^{-1}, and
A	= Surface area of membrane, cm^2.

4.5.5 Validation of *in vitro* test method

Following assessment of the test method, validation was undertaken so as to confirm that the *in vitro* release test would perform in a reliable and reproducible manner. Intra-cell variability or repeatability and inter-day or intermediate precision studies were undertaken and the tolerance level for % RSD for the average cumulative % KZ released from the semi-solid formulations was set at ≤ 10% [145,271].

The amount of KZ released from Xolegel®, Kez® shampoo and Ketazol® cream at all sample points were statistically compared and mathematical modelling was used to analyse and establish if the *in vitro* release test method was able to discriminate KZ release rates between the three commercial formulations.

4.6 Results and discussion

4.6.1 Recovery of KZ in media of different composition

The results of recovery studies using Ketazol® cream, Kez® shampoo and Xolegel® are depicted in **Figure 4.6** and reveal that the % KZ recovered from the formulations in phosphate buffer between 20 to 65% ranged between 95% to 105% and were deemed acceptable according to USP criteria [271]. However, for the analysis of controlled release dosage forms where a low extent of KZ release was expected required the use of a receptor medium in which the highest amount of KZ released would be observed with suitable discrimination of the release between different formulations.

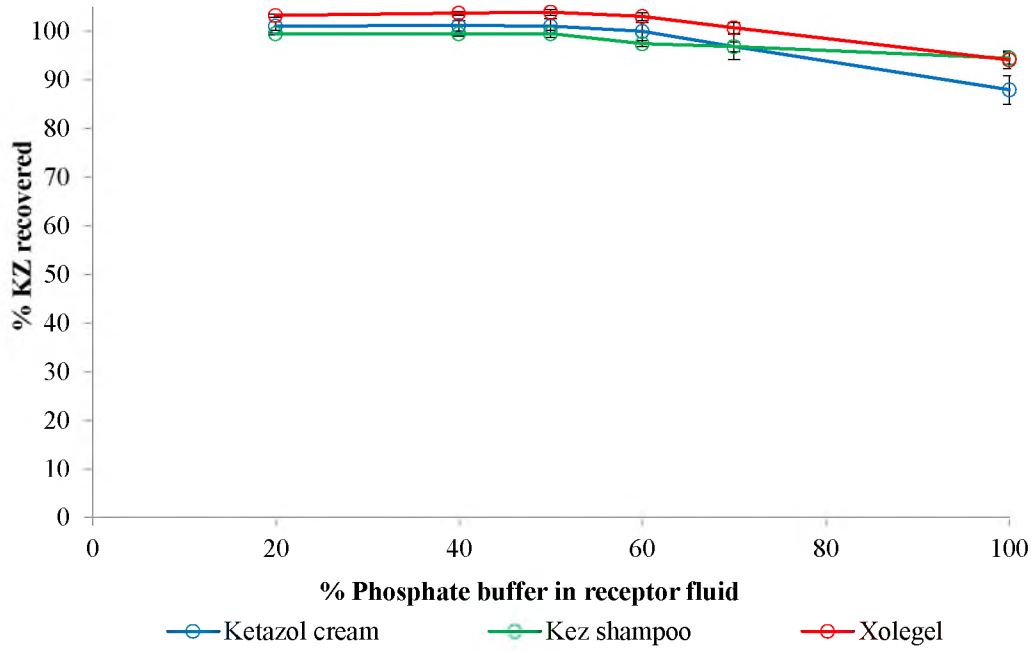


Figure 4.6 KZ recovered from commercially available formulations.

This experiment was used to identify an appropriate receptor medium composition as the experimental conditions when using Franz diffusion cells are different from simple solubility assessments. Consequently preliminary testing was undertaken to establish whether a solvent comprised of ethanol and 0.05 M phosphate buffer of pH = 4.5 would be suitable for assessing release of KZ from semi-solid formulations. In addition different ratios of ethanol and buffer were evaluated and it was established that sink conditions could be achieved and would be suitable to assess KZ transport from the donor compartment through a membrane into the receptor medium. Since the intra-vaginal pH of a human with candidiasis ranges between 3.5 and 4.5, the pH of the receptor medium was tested at 3.5 or 4.5 for each of the ethanol: 0.05 M phosphate buffer ratios evaluated.

The greatest % KZ recovered was observed for Xolegel® which is a water-based gel product that is soluble in ethanol and phosphate buffer. The use of receptor fluid with a large proportion of ethanol resulted in the high % KZ recovered as it is more soluble in ethanol than in phosphate buffer. Ketazol® cream released a relatively high % KZ when receptor fluids of phosphate buffer content between 20% and 60% were used. The amount of KZ released decreased when receptor fluids containing between 70% and 100% phosphate buffer were used and this low recovery may be due to the retention of KZ in the lipid components of the cream. Kez® shampoo revealed constant % KZ recovery when receptor fluids of 20%, 40% and 50% phosphate buffer were used and when the composition of the buffer was increased to 60%, 70% and 100% the % KZ recovered decreased slightly and this is more than likely due to the low solubility of KZ in aqueous media.

4.6.2 Selection of membrane

4.6.2.1 Scanning Electron Microscopy

SEM was used to visualize the microscopic cross-section of porcine vaginal mucosa following harvesting and processing using a surgical processing approach. A typical cross-section of the pig vagina is depicted in **Figure 4.7** and reveals the presence of a ragged surface in which the multiple folds of the mucosa are evident.

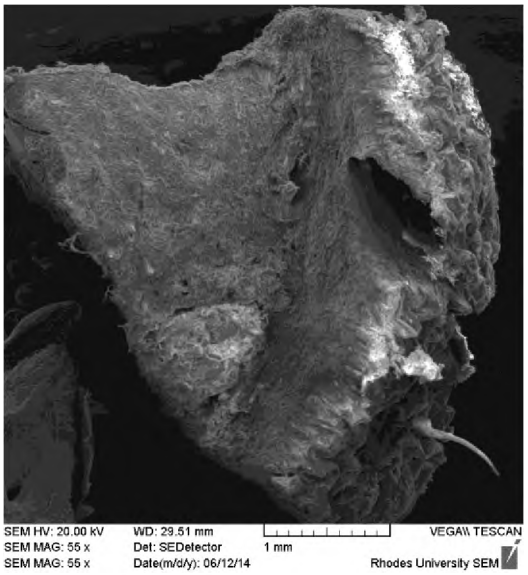


Figure 4.7 Cross section of porcine vaginal tissue prepared using a surgical processing approach.

4.6.2.2 Porcine mucosa

A plot of the cumulative % KZ released versus time following the use of fresh tissue (Day 0) and material that had been frozen for two and six days at -20°C and -80°C are depicted in **Figure 4.8**. The greatest extent of KZ released per unit area at all times was observed for vaginal mucosae that had been frozen at -20°C for two days with a resultant flux of 114.18 $\mu g/cm^2/hr^{1/2}$ at 24 hours. Intermediate release was observed for fresh mucosae and the lowest extent of release was observed for mucosae that had been frozen at -80°C for two and six days. The high degree of variability indicated by large error bars for all experiments suggest that the release profiles generated using fresh and frozen membranes are similar and a two-way ANOVA to test for statistical differences between the release profiles in terms of the flux of KZ though these membranes was performed to confirm this assumption

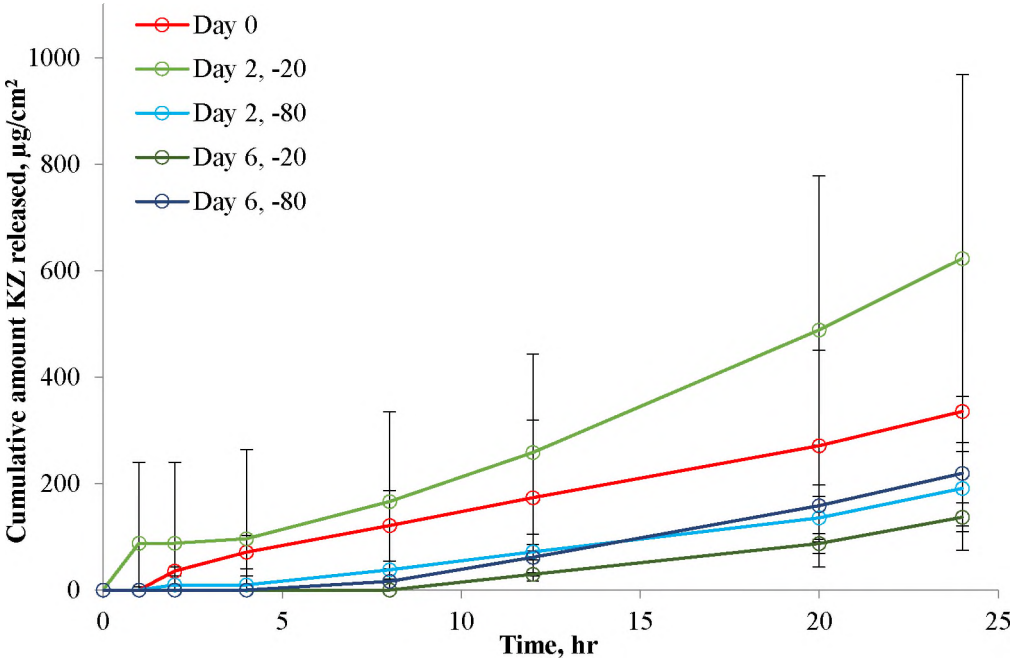


Figure 4.8 Cumulative amount of KZ released per unit area using fresh (D0) and frozen porcine vaginal mucosa.

GraphPad® Prism software was used to perform an ordinary two-way ANOVA on the cumulative % KZ released from a 2% w/v KZ solution using natural membranes (n = 3) that had been stored at -20°C and -80°C for 0, 2 and 6 days with sampling at 1, 2, 4, 8, 12, 20 and 24 hours with an $\alpha = 0.05$ and the results are summarised in **Table 4.3**. It was not feasible to conduct *in vitro* release tests on natural membranes for longer than 24 hours as the membranes denatured and the ability to retain barrier properties was lost [221]. The membranes visibly changed colour after 24 hours and the colour change appeared to reflect a change in the characteristics of the membranes.

In ANOVA, the mean squares represents the difference between sample means and is used to determine whether the factors being compared are significantly different, i.e. the variation between the means of the flux for KZ through membranes sourced from different storage conditions is the mean square for storage conditions and is obtained by dividing the sum of squares by df [272].

Table 4.3 Two-way ANOVA table for the analysis of the integrity of mucosa stored under different conditions.

Source of variation	Sum of squares	df	Mean square	F	% variability	p-value Prob > F	Significant difference
Interaction	12725	24	530	0.619	15.52	0.9034	No
Time	5097	6	850	0.992	6.22	0.4387	No
Storage conditions	9790	4	2447	2.86	11.94	0.0306	Yes
Residual	53966	63	857		-	-	-

The interaction between KZ flux at different times and flux using membranes stored under different conditions accounts for 15.52% of the total variance of these data. However, there is no overall interaction and there is a 90% chance of randomly observing this degree of interaction in an experiment of this nature. The interaction is not considered significant $F = 0.619$, $p = 0.9034$. A non-significant interaction indicates that the main effects can be analysed without considering an interaction. The mean KZ flux through the mucosae following storage under different conditions is not dependent on the time and temperature of storage of the mucosae.

Time accounts for 6.22% of the total variance but has no overall effect and there is a 44% chance of randomly observing an effect this large in an experiment of this magnitude. The effect is considered statistically insignificant $F = 0.992$, $p = 0.4387$. The effect of time on flux was not significant and this indicates that the mean flux through the vaginal mucosae at all times are similar and that sample time is not a factor that influences the flux of KZ through pig membranes.

The flux of KZ through membranes stored under different conditions contributed 11.94% to the total variance observed and therefore storage conditions are considered to have little or no overall effect on KZ flux and there is a 3.1% chance of randomly observing an effect this large (or larger) in an

experiment of this magnitude and this effect is considered extremely significant $F = 2.86$, $p = 0.0306$. The significant difference observed for mean flux as a result of different storage conditions indicates that there is an impact on flux when using mucosae stored under different conditions. Therefore it would be necessary to use fresh vaginal mucosa for all *ex vivo* studies. However, this data included that generated using tissues for all storage conditions such as that stored for two days at -20 °C and six days at -20 °C which are clearly very different as depicted in **Figure 4.8**. Further analysis included the use of Tukey's multiple comparison test as this model computes CIs and the significance of the data analysed. For this analysis, day 0 was set as the reference and the flux of KZ using tissues from other storage conditions were compared to this data. Tukey's test is a powerful tool for pairwise comparisons and the data generated following analysis using Tukey's multiple comparison test are listed in **Table 4.4**.

Tukey's multiple comparison testing revealed that flux through mucosae stored under different conditions was not significantly different to the flux observed for fresh vaginal mucosae with $p \geq 0.05$ at all times except for samples withdrawn at one hour intervals for the tissue stored at -20 °C for two days. This test proved useful for the pairwise comparison of mucosae performance and provided a better indication of the difference in flux when compared to the ANOVA data listed in **Table 4.3**. Therefore no impact on the flux of KZ was observed following permeation through porcine mucosae and the tissue could therefore be harvested and used following storage under any of the conditions investigated without the likelihood of a consequence on the data derived from *in vitro* release studies.

Table 4.4 Tukey's multiple comparison test for two-way ANOVA comparing flux through fresh and frozen vaginal mucosa stored for two and six days.

Tukey's multiple comparison	Mean difference	95% CI of difference	Adjusted p-value	Significant difference	Summary
1 hour					
Day 2, -20 vs. Day 0	74	6.89 to 141	0.0235	Yes	*
Day 2, -80 vs. Day 0	-5.43	-72.5 to 61.7	0.9994	No	ns
Day 6, -20 vs. Day 0	-14.1	-81.2 to 53.1	0.9764	No	ns
Day 6, -80 vs. Day 0	-14.1	-89.1 to 61.0	0.9844	No	ns
2 hours					
Day 2, -20 vs. Day 0	-15.1	-82.3 to 52.0	0.969	No	ns
Day 2, -80 vs. Day 0	-15.1	-82.3 to 52.0	0.969	No	ns
Day 6, -20 vs. Day 0	-15.1	-82.3 to 52.0	0.969	No	ns
Day 6, -80 vs. Day 0	-15.1	-90.2 to 59.9	0.9793	No	ns
4 hours					
Day 2, -20 vs. Day 0	-13.7	-80.8 to 53.4	0.9785	No	ns
Day 2, -80 vs. Day 0	-13.7	-80.8 to 53.4	0.9785	No	ns
Day 6, -20 vs. Day 0	-18	-85.1 to 49.1	0.9427	No	ns
Day 6, -80 vs. Day 0	-18	-93.0 to 57.0	0.9613	No	ns
8 hours					
Day 2, -20 vs. Day 0	7.05	-60.1 to 74.2	0.9983	No	ns
Day 2, -80 vs. Day 0	-7.55	-74.7 to 59.6	0.9978	No	ns
Day 6, -20 vs. Day 0	-17.5	-84.6 to 49.6	0.9482	No	ns
Day 6, -80 vs. Day 0	-7.11	-82.1 to 67.9	0.9989	No	ns
12 hours					
Day 2, -20 vs. Day 0	11.4	-55.7 to 78.5	0.989	No	ns
Day 2, -80 vs. Day 0	-5.5	-72.6 to 61.6	0.9994	No	ns
Day 6, -20 vs. Day 0	-6.62	-73.7 to 60.5	0.9987	No	ns
Day 6, -80 vs. Day 0	-2.29	-77.3 to 72.7	> 0.9999	No	ns
20 hours					
Day 2, -20 vs. Day 0	29.6	-37.5 to 96.7	0.728	No	ns
Day 2, -80 vs. Day 0	-7.37	-74.5 to 59.7	0.998	No	ns
Day 6, -20 vs. Day 0	-8.82	-75.9 to 58.3	0.996	No	ns
Day 6, -80 vs. Day 0	-0.0216	-75.1 to 75.0	> 0.9999	No	ns
24 hours					
Day 2, -20 vs. Day 0	14.3	-52.9 to 81.4	0.9751	No	ns
Day 2, -80 vs. Day 0	-1.99	-69.1 to 65.1	> 0.9999	No	ns
Day 6, -20 vs. Day 0	-3.2	-70.3 to 63.9	> 0.9999	No	ns
Day 6, -80 vs. Day 0	-0.716	-75.7 to 74.3	> 0.9999	No	ns

ns: not significant

A simple study was conducted using vaginal mucosae and diffusion cells to determine whether KZ was trapped or adsorbed onto the tissues and/or membrane. KZ does not appear to adsorb to the mucosal tissue stored under different conditions. The results of studies using porcine vaginal mucosa indicate

that the tissue could be used immediately after harvesting and preparation or following storage for a maximum of six days storage at -20°C and -80°C without the likelihood of a negative impact on the KZ flux. The *ex vivo* studies performed using pig vaginal mucosa exhibited low permeation of KZ into the receptor fluid and the low flux observed was, in part, attributed to the thickness of the membranes following surgical preparation which may have a negative impact on the ability of KZ to permeate through the membrane efficiently.

Another potential reason for the low flux observed with surgically prepared mucosa may be due to the effect(s) of the receptor medium on the integrity of the membrane [221]. Ethanol concentrations ≤ 25% in combination with PBS (pH 7.4) has the ability to preserve the integrity of human skin for *ex vivo* studies [226] and the use of 50% ethanol in the receptor medium may have altered the permeation of KZ through the mucosa over time. Ethanol is used as a permeation enhancer in low amounts. The use of a high ethanol concentration may modify the lower layers of the skin due to precipitation of skin proteins and/or dissolution of lipids in the lipid bilayer of the mucosa resulting in modification of the barrier properties of the mucosa to the diffusion of KZ [221].

However, KZ is a water insoluble lipophilic compound and the use of a high concentration of ethanol in the receptor medium was necessary for sink conditions to be met. Due to the difficulty in preparation and associated challenges with designing reproducible studies natural membranes were therefore not considered ideal for use to assess KZ flux as a high concentration of solvent would be required to produce a receptor medium with the appropriate solution capacity for KZ.

4.6.2.3 Artificial membrane selection

All synthetic membranes tested as potential surfaces for these studies are listed in **Table 4.5**. KZ recovery for artificial membranes was good except for the 0.025 µm Millipore® nitrocellulose membrane when a 20 µg/mL KZ solution was placed in the donor compartment. This particular membrane had the smallest pore size and the % KZ recovered of 94.44 ± 0.87% may have been due to the clogging of pores by micellar aggregates of polysorbate 80 in the solvent system used to dissolve KZ. The surface(s) of polysorbate 80 micelles are dominated by polar hydrophilic groups of the polysorbate 80 molecule and micellar aggregates may have been adsorbed onto the hydrophilic nitrocellulose membrane subsequently blocking of the pores in the membrane and thereby limiting the passage of KZ molecules [273]. The recovery of KZ for all membranes except the 0.2 µm Sartorius® cellulose acetate for a 100 µg/mL KZ solution was lower than that observed for the 20 µg/mL filtrates and this may be due to clogging of the pores or saturation of the membrane with KZ. As KZ passes through filter membranes of small pore size, the pores become smaller as the concentrated KZ solution results in accumulation of KZ and other particles present in the solution and the formation of plaques around the pores causing a decrease in the internal diameter of the pore. As the pore size decreases in diameter, resistance to flow increases until fluid flow eventually ceases [274,275].

Table 4.5 Recovery of KZ from standard solutions following filtration through artificial membranes (n = 3).

Artificial membrane	KZ recovered from 20 µg/mL solution %	KZ recovered from 100 µg/mL solution %
8.0 µm Sartorius® cellulose nitrate	97.02 ± 0.86	94.08 ± 0.21
0.8 µm Sartorius® cellulose nitrate	96.15 ± 0.28	94.23 ± 0.75
0.2 µm Whatman® nitrocellulose	98.39 ± 0.45	91.20 ± 0.19
0.025 µm Millipore® nitrocellulose	94.44 ± 0.87	94.16 ± 0.52
0.2 µm Sartorius® cellulose acetate	97.56 ± 0.31	98.60 ± 0.48

The 0.025 µm Millipore® nitrocellulose membrane filter was not able to accommodate 100 mL of the standard KZ solution, but the filtrate (10 mL) that was obtained was analysed and the % KZ recovered from the membranes following filtration revealed that saturation of KZ on the membrane and the clogging of the hydrophilic nitrocellulose membrane may be the reasons for the low recovery of KZ. The results confirm that the 0.2 µm Sartorius® cellulose acetate filter exhibited the lowest adsorption of KZ and only for the higher concentration test solution. As the highest recovery and lowest extent of binding of KZ following filtration was observed for the 0.2 µm Sartorius® cellulose acetate membrane, it was selected for use in future *in vitro* release studies.

4.6.2.4 Comparison of performance of 0.2 µm cellulose acetate membrane and vaginal mucosa

Statistical analysis was performed on permeation data for KZ using the 0.2 µm Sartorius® cellulose acetate membrane and vaginal mucosa. The two-way ANOVA results table (**Table 4.6**) infer that the interaction between KZ flux at different sample times and using artificial, fresh and frozen natural membranes accounts for 47.04% of the total variance within these data with no overall effect and a 0.01% chance of randomly observing an effect this large in an experiment of this nature. The interaction is statistically significant $F = 20.5$, $P < 0.0001$ and indicates that the relationship between storage conditions and flux is dependent on sampling time and this interaction should be considered when interpreting the main effect(s) in these experiments.

Table 4.6 Two-way ANOVA table for the comparison of KZ flux through cellulose acetate, fresh and stored and natural membranes.

Source of variation	Sum of squares	df	Mean square	F	% variability	p-value Prob > F	Significant difference
Interaction	1720000	30	57421	20.5	47.04	< 0.0001	Yes
Time	446952	6	74492	26.6	12.21	< 0.0001	Yes
Storage conditions	1470000	5	294124	105	40.16	< 0.0001	Yes
Residual	196390	70	2806	-	-	-	-

Sample time accounts for 12.21% of the total variance, has no overall effect and there is a 0.01% chance of randomly observing an effect this large in an experiment of this magnitude. This effect was considered extremely significant $F = 26.6$, $p < 0.0001$ and this denotes that the sample time has an influence on the flux of KZ.

The flux of KZ observed for artificial and natural membranes stored under different conditions contributed 40.16% to the total variance with no overall effect on KZ flux and a 0.01% chance of randomly observing an effect this big in an experiment of this magnitude. The flux observed for the artificial membrane and natural mucosae was considered extremely significant $F = 105$, $p < 0.0001$ and this indicates that there was an impact on flux and a significantly different flux for KZ was observed for the comparison of artificial and natural membranes.

This data was analysed using a Tukey's multiple comparison test and the data are summarized in **Table 4.7**. Pair-wise comparisons were undertaken to better comprehend the source of statistical significance from interaction and main effects such as sample time, artificial and natural membranes type and storage conditions.

Table 4.7 Tukey's multiple comparison test for permeation through a cellulose acetate membrane (control), fresh and stored natural membranes.

Tukey's multiple comparisons	Mean difference	95% CI of difference	Adjusted p-value	Significant difference?	Summary
1 hours					
Artificial Membrane vs. Day 0	1215	1074 to 1357	< 0.0001	Yes	****
Artificial Membrane vs. Day 2, -20	1141	1000 to 1283	< 0.0001	Yes	****
Artificial Membrane vs. Day 2, -80	1221	1079 to 1363	< 0.0001	Yes	****
Artificial Membrane vs. Day 6, -20	1229	1088 to 1371	< 0.0001	Yes	****
2 hours					
Artificial Membrane vs. Day 0	246	104 to 387	< 0.0001	Yes	****
Artificial Membrane vs. Day 2, -20	261	119 to 402	< 0.0001	Yes	****
Artificial Membrane vs. Day 2, -80	261	119 to 402	< 0.0001	Yes	****
Artificial Membrane vs. Day 6, -20	261	119 to 402	< 0.0001	Yes	****
4 hours					
Artificial Membrane vs. Day 0	390	248 to 531	< 0.0001	Yes	****
Artificial Membrane vs. Day 2, -20	403	262 to 545	< 0.0001	Yes	****
Artificial Membrane vs. Day 2, -80	403	262 to 545	< 0.0001	Yes	****
Artificial Membrane vs. Day 6, -20	408	266 to 549	< 0.0001	Yes	****
8 hours					
Artificial Membrane vs. Day 0	302	160 to 444	< 0.0001	Yes	****
Artificial Membrane vs. Day 2, -20	295	153 to 436	< 0.0001	Yes	****
Artificial Membrane vs. Day 2, -80	309	168 to 451	< 0.0001	Yes	****
Artificial Membrane vs. Day 6, -20	319	178 to 461	< 0.0001	Yes	****
12 hours					
Artificial Membrane vs. Day 0	180	38.6 to 322	0.005	Yes	**
Artificial Membrane vs. Day 2, -20	169	27.2 to 311	0.0104	Yes	*
Artificial Membrane vs. Day 2, -80	186	44.1 to 327	0.0035	Yes	**
Artificial Membrane vs. Day 6, -20	187	45.2 to 329	0.0032	Yes	**
20 hours					
Artificial Membrane vs. Day 0	61.1	-80.6 to 203	0.8034	No	ns
Artificial Membrane vs. Day 2, -20	31.5	-110 to 173	0.9865	No	ns
Artificial Membrane vs. Day 2, -80	68.5	-73.2 to 210	0.7172	No	ns
Artificial Membrane vs. Day 6, -20	69.9	-71.8 to 212	0.699	No	ns
24 hours					
Artificial Membrane vs. Day 0	8.18	-133 to 150	> 0.9999	No	ns
Artificial Membrane vs. Day 2, -20	-6.08	-148 to 136	> 0.9999	No	ns
Artificial Membrane vs. Day 2, -80	10.2	-132 to 152	> 0.9999	No	ns
Artificial Membrane vs. Day 6, -20	11.4	-130 to 153	0.9999	No	ns

Statistically significant differences in the flux for KZ were observed when the cellulose acetate membrane and the natural mucosae were compared and was independent of the storage conditions at sample times of 1, 2, 4 and 8 hours, $p < 0.0001$. At 12 hours the flux of KZ through the membranes exhibited an increased p-value, yet was still statistically different with $p < 0.05$. At the 20 and 24 hour

sample times no significant difference in flux of KZ through the membranes, $p > 0.05$, was observed. This data reveals that flux of KZ through the cellulose acetate membrane is large during the first few hours of permeation (1 - 8 hours) and then decreases from 12 hours and eventually is low from 20 to 24 hours. This suggests that KZ permeates rapidly from the donor compartment through the cellulose acetate membrane into the receptor compartment. Therefore the cellulose acetate membrane did not appear to impair permeation of KZ into the receptor medium as the cumulative amount of KZ that was released and that had permeated through the membrane at 24 hours was 4469.43 ± 317.15 μg/cm^2 as compared to 622.76 ± 345.67 μg/cm^2, which was the maximum amount of KZ released through the natural mucosa that had been stored for two days at -20°C. The latter value was the greatest amount of KZ permeation observed through the natural mucosae that were tested (**Figure 4.9**). The cellulose acetate membrane exhibited a flux for KZ that was seven times greater than that observed for the natural tissues.

The cumulative % KZ released observed following permeation studies using a 0.2 μm Sartorius® cellulose acetate membrane, fresh vaginal tissue (D0) and vaginal tissue stored at -20 and -80 for two (Day 2, -20 and Day 2, -80 respectively) and six days (Day 6, -20 and Day 6, -80 respectively) is depicted in **Figure 4.9**. The amount of KZ that had permeated through the artificial membrane was greater than that observed for vaginal tissues. This is, in part, due to the barrier properties of the vaginal mucosa and the method of preparation of the material prior to use (**§ 4.5.3.1.2**). Therefore, due to the low permeability and the time required for the preparation of vaginal tissue it was decided to use a 0.2 μm Sartorius® cellulose acetate membrane for all future permeation studies during formulation development and assessment of thermosetting vaginal gels.

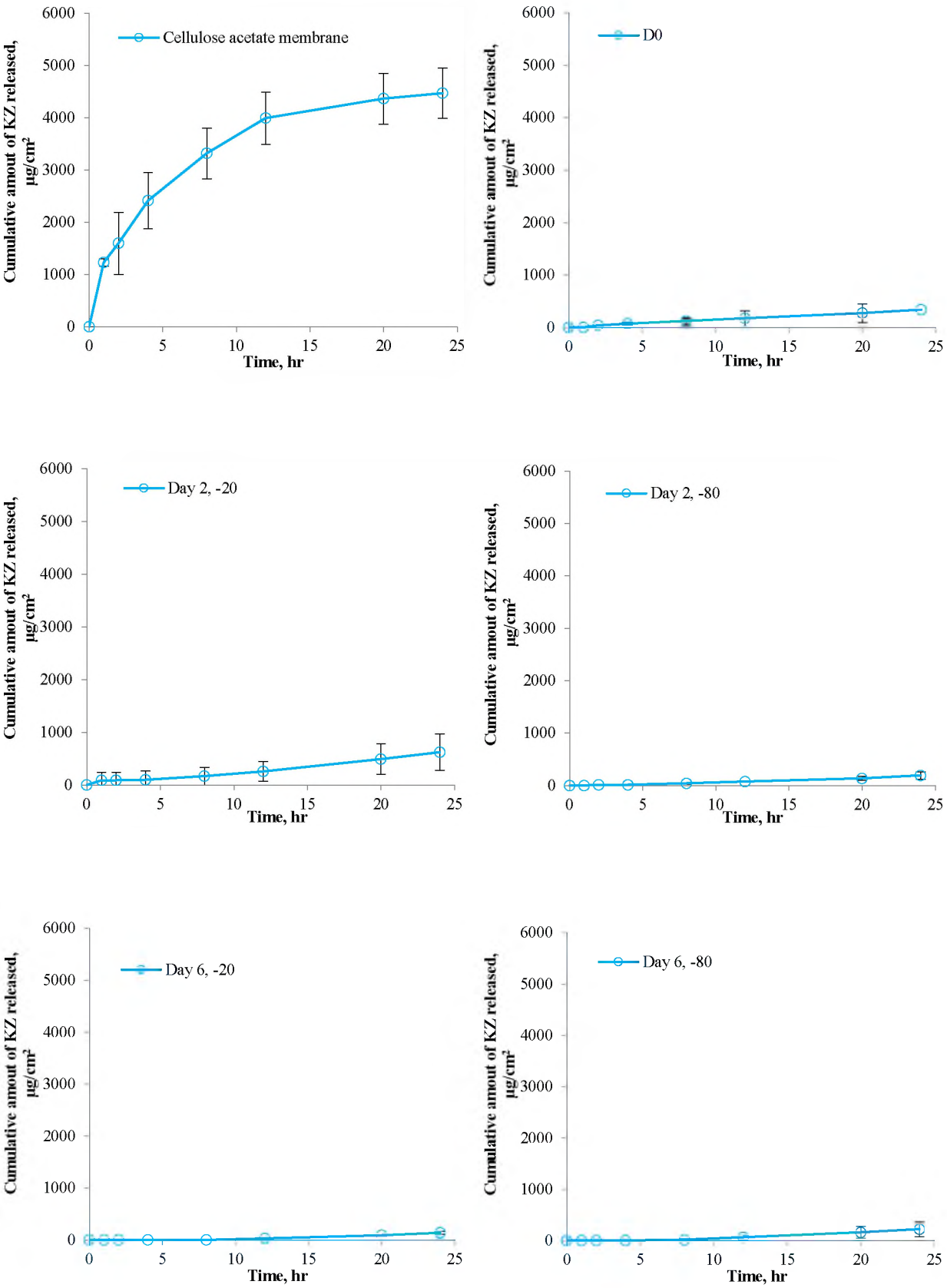


Figure 4.9 *In vitro* release data for KZ permeation through fresh and frozen vaginal mucosa and a 0.2 µm Sartorius® cellulose acetate membrane (n = 3).

4.6.3 Optimisation of the receptor medium for *in vitro* release testing

The receptor medium used in all preliminary studies was comprised of ethanol: 0.05 M phosphate buffer (pH = 4.5) in a 50: 50 ratio after which different ratio compositions and buffer pH were also used to assess KZ release from Xolegel®, Kez® shampoo and Ketazol® cream by conducting *in vitro* release studies with a 0.2 µm Sartorius® cellulose acetate membrane (n = 3).

The cumulative amount of KZ released per unit area from Xolegel® using a receptor medium with a phosphate buffer of pH 3.5 and ethanol in different ratios is depicted in **Figure 4.10.** Testing using a receptor medium with larger volumes of phosphate buffer exhibited lower permeation of KZ and the lowest amount of KZ released after 24 hours was 1128 ± 241.54 µg/cm^2 for the system in which 65% v/v phosphate buffer of pH 3.5 was used. The maximum release of 2419 ± 202.49 µg/cm^2 KZ was observed for the system in which a 35% v/v phosphate buffer of pH 3.5 was used. The pH of 3.5 contributes to the permeation of KZ as the compound is more soluble in an acidic medium. The release profiles observed for the 40% to 60% phosphate buffer (pH 3.5) compositions were comparable and displayed a similar extent of KZ permeation.

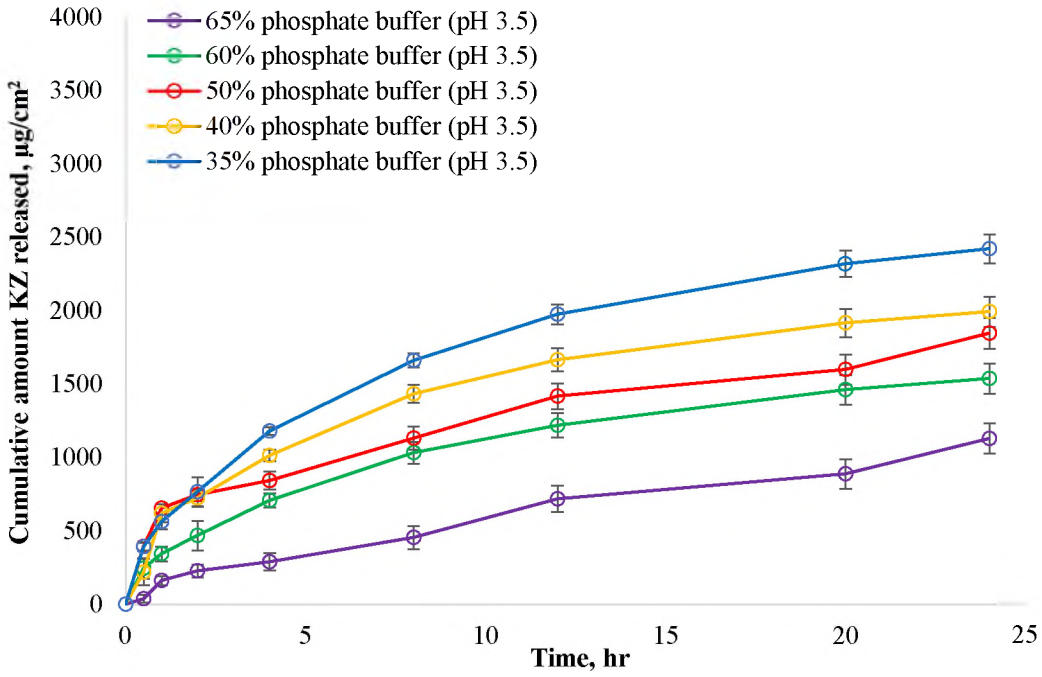


Figure 4.10 Cumulative amount of KZ released per unit area from Xolegel® using a buffer of pH 3.5 in the receptor fluid.

Receptor media in which ethanol and 0.05 M phosphate buffer of pH = 4.5 were used in different ratios were applied to the evaluation of KZ release from Xolegel® and these data are depicted in **Figure 4.11**. The amount of KZ released from Xolegel® was the greatest for fluid in which 65% v/v phosphate buffer of pH 4.5 was used with an amount 1884 ± 135.47 µg/cm^2 of KZ released per unit area which is lower than the maximum amount of KZ released from Xolegel® when a receptor medium with a buffer of pH 3.5 was used. KZ is more soluble in acidic media and this finding may be due to lower permeation of KZ into a buffer of pH 4.5. The error bars for the *in vitro* release study profiles and the proximity of the profiles suggest that the amount of KZ released per unit area from Xolegel® using either 35% or 60% v/v phosphate buffer of pH 4.5 may be similar as it is difficult to distinguish each curve. Consequently a change in phosphate buffer concentration from 35% to 60% may not impact the extent of KZ released during IVRT when receptor media using a buffer of pH 4.5 in the receptor compartment is used.

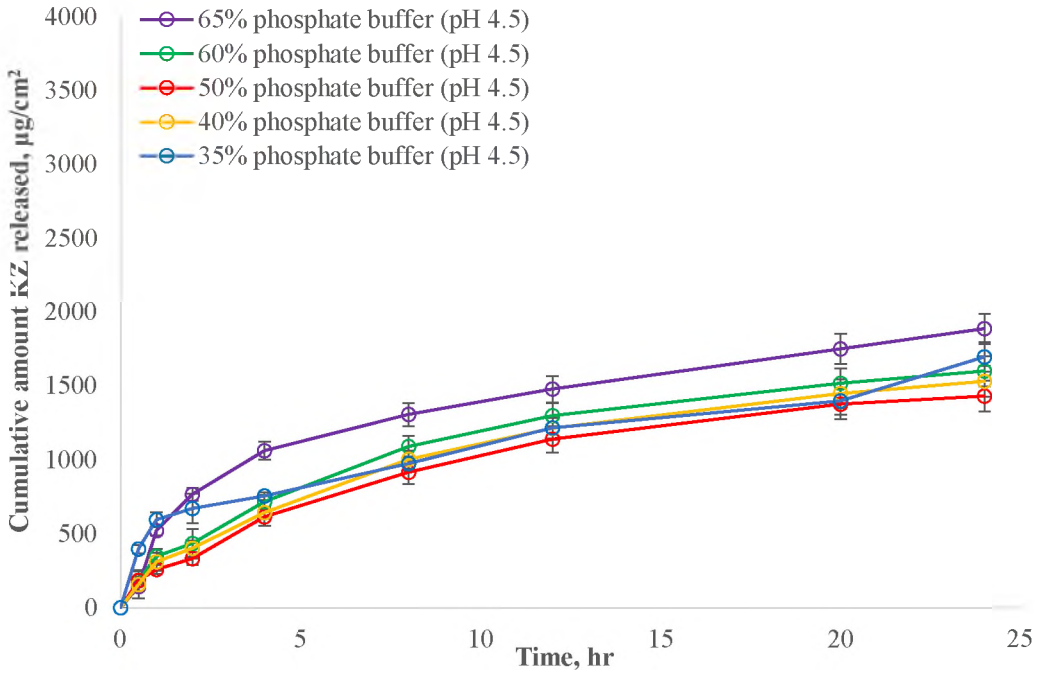


Figure 4.11 Cumulative amount of KZ released per unit area from Xolegel® using buffer of pH 4.5 in the receptor fluid.

The cumulative amounts of KZ released from Kez® shampoo that permeated into the receptor media of different ethanol and 0.05 M phosphate buffer (pH 3.5) in different ratios are depicted in **Figure 4.12**. As observed in the release profiles for Xolegel®, KZ permeated more readily into a receptor medium containing lower volumes of phosphate buffer with the largest t amount of 3190 ± 194.71 µg/cm^2 released at 24 hours when 35% v/v phosphate buffer of pH 3.5 was used in the receptor fluid. The lowest amount of KZ released was 1002 ± 80.35 µg/cm^2 when a 65% v/v phosphate buffer of pH 3.5 was used. The release profiles for Kez® shampoo when a 50% v/v phosphate buffer of pH 3.5 was used revealed that the second largest amount of 2570 ± 97.74 µg/cm^2 KZ was released at 24 hours which in turn was greater than when the largest volume of ethanol was used in this fluid. In this series of release studies 50% v/v phosphate buffer was easily distinguishable from that for the 40% and 60% v/v phosphate buffer (pH 3.5) studies. KZ release from the shampoo was similar for the studies in which a 40% and 60% phosphate buffer (pH 3.5) was used. The low viscosity aqueous Kez® shampoo was likely to release KZ more readily with an associated better permeation when compared to Xolegel®. Xolegel® is a semi-solid dosage form of relatively high viscosity **(§ 3.5.1)** that exhibits lower release than the shampoo as the compound is likely trapped in the gel matrix and is released in a controlled manner.

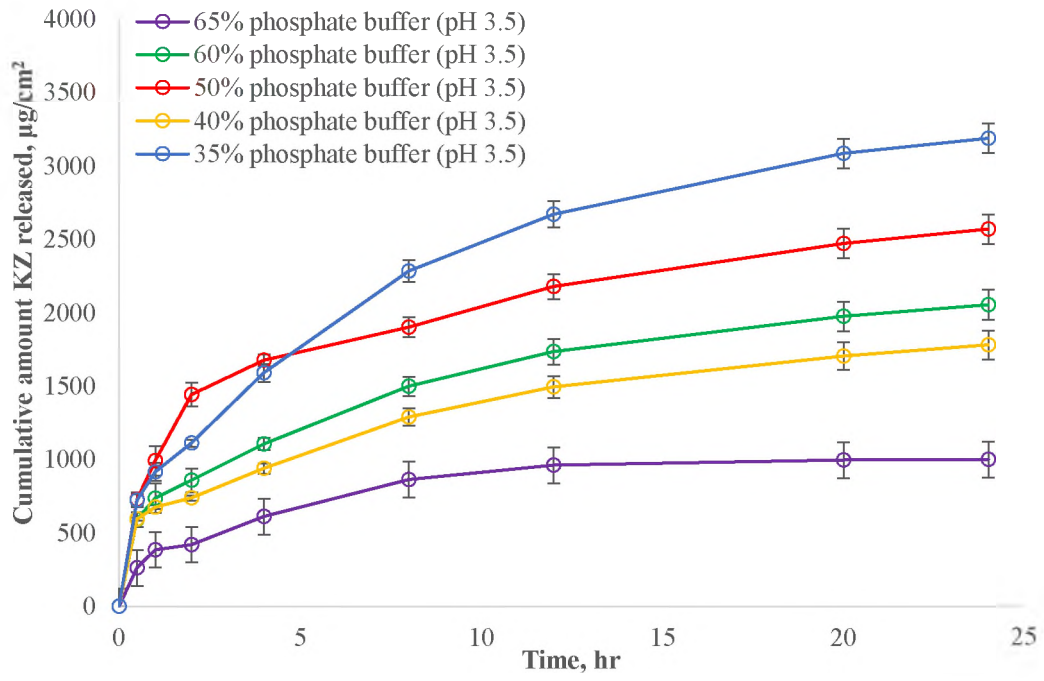


Figure 4.12 Cumulative amount of KZ released per unit area from Kez® shampoo using buffer of pH 3.5 in the receptor fluid.

The cumulative amounts KZ released per unit area into a receptor medium comprised of ethanol and 0.05 M phosphate buffer of pH 4.5 in different ratios from Kez® shampoo are depicted in **Figure 4.13**. The amount of KZ released from Kez® shampoo was greatest for the 0 to 2 hours period when 35% and 65% phosphate buffer (pH 4.5) was used after which KZ permeation reached a plateau at 8 hours for the 35% v/v phosphate buffer composition. The cumulative amount of KZ released into the 35% v/v phosphate buffer (pH 4.5) system at 24 hours was 3738 ± 212.57 µg/cm^2 and for the 65% v/v phosphate buffer (pH 4.5) system was 3091 ± 237.95 µg/cm^2. The release profiles for Kez® shampoo using receptor media of 40% and 60% v/v phosphate buffer were similar with overlapping error bars and were not easily distinguished from each other.

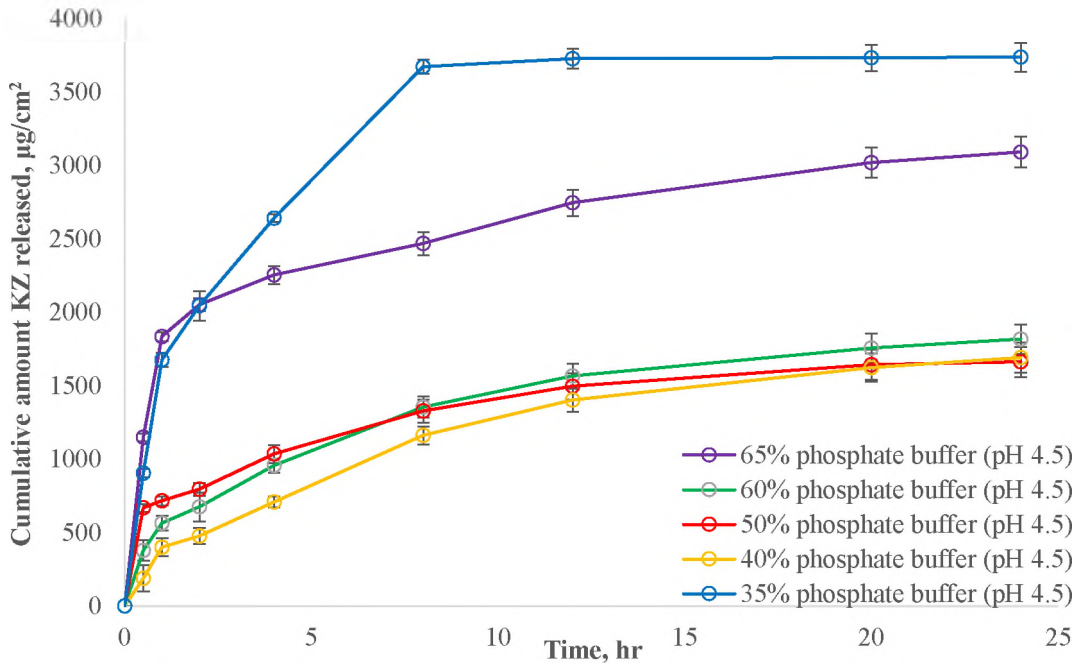


Figure 4.13 Cumulative amount of KZ released per unit area from Kez® shampoo using a buffer of pH 4.5 in the receptor fluid.

The cumulative amounts of KZ released from Ketazol® cream into receptor media of different ratios of ethanol and 0.05 M phosphate buffer (pH 3.5) are depicted in **Figure 4.14**. The release profile for Ketazol® cream using a 35% v/v phosphate buffer (pH 3.5) system was markedly higher than the release observed for the other media tested with an overall amount of KZ released per unit area at 24 hours of 3075 ± 369.43 µg/cm^2 which is comparable to the release profiles observed for Xolegel® and Kez® shampoo. This result is not surprising, as the cream is an o/w emulsion and therefore the miscibility of the receptor fluid with the formulation may increase the surface area for partitioning, resulting in a greater extent of KZ release. The other receptor media assessed did facilitate KZ release due to a lower ethanol content resulting in control of the release of KZ. Buffer contents of 60% and 65% exhibited similar release profiles such that they could not be distinguished from each other. The permeation of KZ into a 50% v/v phosphate buffer (pH 3.5) system was high with rapid release between 0 and 2 hours that plateaued from 4 hours.

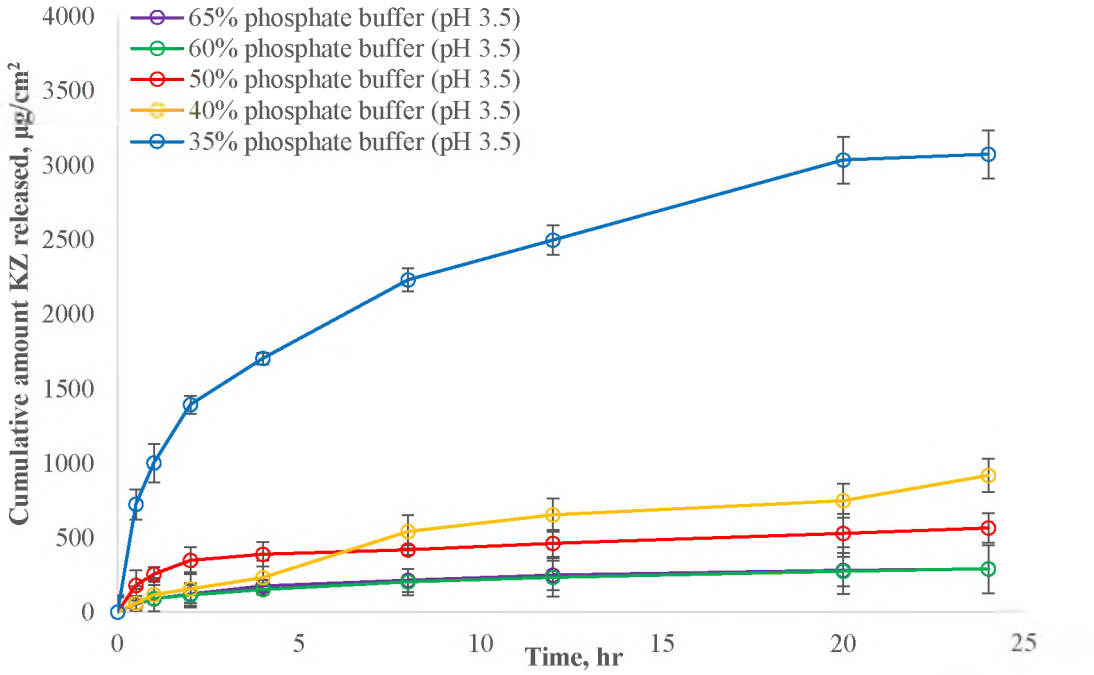


Figure 4.14 Cumulative amount of KZ released per unit area from Ketazol® cream using a buffer of pH 3.5 in the receptor fluid.

The cumulative amounts of KZ released per unit area into receptor media of different ratio compositions of ethanol: 0.05 M phosphate buffer (pH 4.5) from Ketazol® cream are depicted in **Figure 4.15.** The amount of KZ released into a medium comprised of 35% v/v phosphate buffer (pH 4.5) was 2361 ± 113.71 µg/cm^2 and was similar to that exhibited when a receptor medium of the same ratio and a buffer of pH 3.5 was used. The amount of KZ released when a 40% v/v phosphate buffer (pH 4.5) system was used was low between 0 to 4 hours and peaked at 12 hours before reaching a plateau with a total amount of KZ released per unit area of 780 ± 224.58 µg/cm^2.

Low amounts of KZ that were similar and not easily distinguishable from each other were released from the formulation when the other receptor media was used. Hydration of the membrane may have resulted in a low degree of miscibility of the o/w cream emulsion with a receptor medium of pH 4.5 resulting in a greater extent of KZ release into the 40% phosphate buffer (pH 4.5) based receptor medium when monitoring release from the Ketazol® cream.

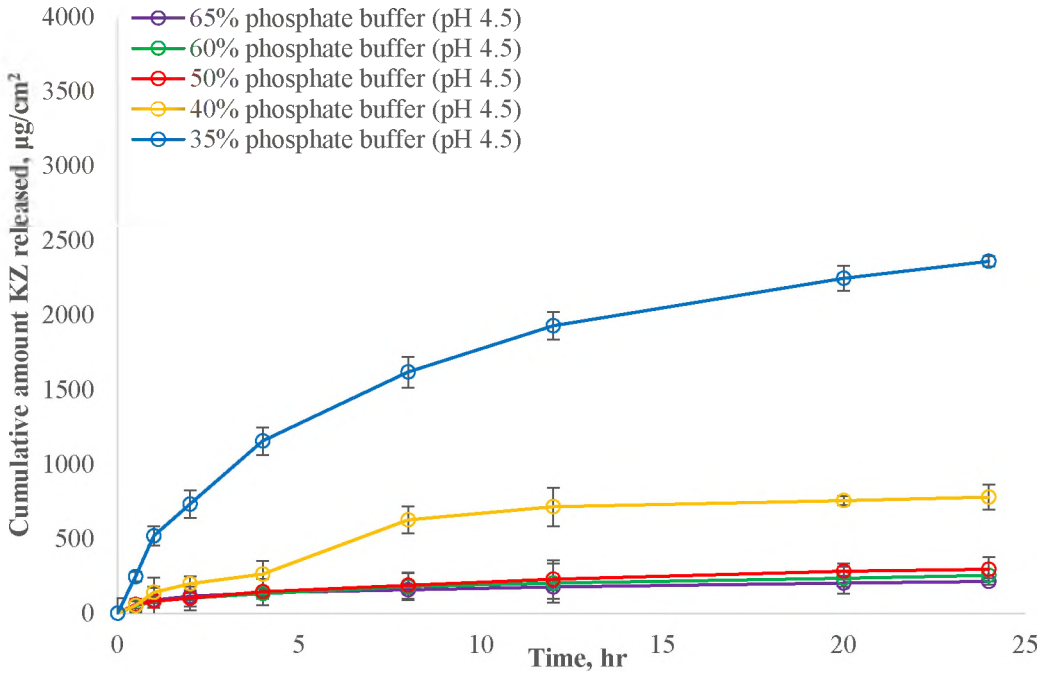


Figure 4.15 Cumulative amount of KZ released per unit area from Ketazol® cream using a buffer of pH 4.5 in the receptor fluid.

The release profiles of KZ from commercial KZ formulations using receptor media of different composition were elucidated and were useful for identifying an appropriate receptor medium for IVRT of the thermosetting vaginal gels. The aim was to identify and select a receptor medium that could be used to distinguish KZ release from the three commercial KZ formulations and not merely that in which the greatest extent of release was exhibited. In addition the maintenance of sink conditions for the duration of the experiments was an important consideration.

Tukey's multiple comparison test was performed on release data in different receptor media at all sample points and the raw data are listed in **Appendix B**. The *in vitro* release profiles for Xolegel®, Kez® shampoo and Ketazol® cream (n = 3) following diffusion studies are depicted in **Appendix C** and are useful for the visual analysis of release profiles when attempting to identify an adequate receptor medium that was balanced between discriminatory capability of the three formulations, bearing in mind that Xolegel® and Kez® shampoo are both aqueous based formulations which may exhibit similar *in vitro* release profiles.

The results of Tukey's multiple comparison testing revealed significant differences at a few sample points and when 35%, 40%, 50% and 60% v/v phosphate buffer of pH 3.5 was used a significant difference in KZ release was observed at a time of 0.5 hours for the comparison of release from Ketazol® cream and Kez® shampoo with p = 0.0386, 0.0181, 0.0053 and 0.0015 for all systems respectively. At 0.5 hours a significant difference in KZ release was observed between the cream and shampoo formulations with p = 0.0059, 0.0075 and 0.0062 for the receptor fluids comprised of 50%, 60% and 65% v/v phosphate buffer of pH 4.5. The receptor medium in which 60% v/v phosphate buffer of pH 3.5 was used resulted in a significant difference in KZ release from Ketazol® cream and Kez® shampoo with p = 0.0309. The release in other receptor media revealed significant differences between KZ release from the cream and the gel or shampoo at 4 hours (40% v/v phosphate buffer pH 4.5 and 60% v/v phosphate buffer pH 4.5), 8 hours (60% v/v phosphate buffer pH 4.5), 12 hours (40% v/v phosphate buffer pH 4.5) and 20 hours (40% v/v phosphate buffer pH 4.5). The significant difference in flux between the cream and aqueous formulations was expected and was visually evident from the *in vitro* release profiles depicted in **Appendix C**. However, the use of only three receptor media resulted in significant differences in flux of KZ for the comparison of between Xolegel® and Kez® shampoo *viz.*, 60% v/v phosphate buffer of pH 3.5, 50% v/v phosphate buffer of pH 4.5 and 65% v/v phosphate buffer of pH 4.5 at the 0.5 hour sample time with p = 0.0285, 0.0269 and 0.0110 respectively. When the *in vitro* release profiles were compared, the cumulative amount of KZ released at 24 hours from Xolegel®, Kez® shampoo and Ketazol® cream were 1536.47 ± 175.65 , 2055.37 ± 240.35 and 290.68 ± 161.86 $\mu g/cm^2$ respectively for the 60% v/v phosphate buffer pH 3.5 system, 1429.74 ± 124.37, 1661.70 ± 119.28 and 294.56 ± 113.71 $\mu g/cm^2$ for the 50% v/v phosphate buffer pH 4.5 system and 1883.68 ± 135.47, 3091.92 ± 172.95 and 212.99 ± 855.42 $\mu g/cm^2$ respectively and 65% v/v phosphate buffer for the pH 4.5 system. Therefore the buffer system in which the *in vitro* release profile exhibited a maximum cumulative amount of KZ released at 24 hours using a receptor medium with the lowest possible amount of ethanol and for which the release of KZ was adequate for quantitation with discrimination for the three formulations tested. Consequently, a 50% v/v phosphate buffer of pH 4.5 that exhibited a high cumulative amount of KZ release for most formulations was selected for use and was validated prior to use in formulation development studies.

4.6.4 Validation of *in vitro* test method

Intra- and inter-day precision studies of the *in vitro* test method were performed to ensure the precision of the method. The % RSD for % KZ released at each sample point was established and a % RSD ≤ 10 was the tolerance level set for the method to be considered precise [271].

4.6.4.1 Intra-cell precision or repeatability

The cumulative % KZ released from each of the three commercial products using separate diffusion cells was used to assess intra-cell variability and these results are listed in **Table 4.8.** These studies were undertaken to ensure the repeatability of the *in vitro* release test and the % RSD at all times was < 10% which suggests the method is precise according to USP criteria [271].

Table 4.8 Intra-cell variability for *in vitro* release data using commercially available KZ products.

Commercial product	Time hr	Cell 1 %	Cell 2 %	Cell 3 %	Mean	SD	% RSD
Xolegel®	0.5	4.56	5.22	4.41	4.73	0.43	9.11
	1	6.71	7.79	6.54	7.01	0.68	9.69
	2	12.80	14.55	12.23	13.19	1.21	9.18
	4	23.82	28.53	25.69	26.01	2.37	9.12
	8	58.77	70.80	65.68	65.08	6.04	9.28
	12	70.21	81.68	84.74	78.88	7.66	9.71
	20	83.92	96.65	95.61	92.06	7.07	7.68
	24	93.20	103.19	101.54	99.31	5.35	5.39
	48	103.33	103.60	103.90	103.61	0.28	0.27
	72	103.66	103.63	103.94	103.75	0.17	0.17
Kez® shampoo	0.5	15.14	15.42	17.98	16.18	1.57	9.67
	1	16.70	17.70	16.84	17.08	0.54	3.18
	2	20.25	19.55	19.27	19.69	0.50	2.56
	4	35.74	30.53	30.69	32.32	2.96	9.17
	8	62.34	70.80	65.57	66.24	4.27	6.45
	12	80.94	81.68	84.02	82.21	1.61	1.96
	20	102.02	96.65	95.79	98.15	3.38	3.44
	24	103.05	102.19	101.42	102.22	0.82	0.80
	48	103.25	102.41	102.09	102.58	0.60	0.58
	72	103.58	103.03	102.94	103.19	0.35	0.34
Ketazol® cream	0.5	1.97	1.72	1.66	1.79	0.17	9.34
	1	2.05	1.89	1.84	1.93	0.11	5.65
	2	2.52	2.68	2.64	2.61	0.084	3.20
	4	6.10	5.29	6.40	5.93	0.57	9.66
	8	10.30	10.77	11.03	10.70	0.37	3.48
	12	15.16	14.97	17.49	15.87	1.40	8.84
	20	29.04	28.13	29.34	28.84	0.63	2.19
	24	30.86	32.01	33.78	32.22	1.47	4.56
	48	45.89	48.99	50.31	48.40	2.27	4.68
	72	61.85	65.59	56.24	61.23	4.71	7.69

4.6.4.2 Inter-day precision

The *in vitro* release of KZ from Xolegel®, Kez® shampoo and Ketazol® cream was assessed using diffusion cells on two consecutive days in replicate (n = 3). The % RSD for the % KZ released on each of the two days was < 10% for all runs **(Table 4.9)** and confirms that the method is precise.

Table 4.9 Inter-day validation of IVRT using commercial KZ products.

Commercial product	Time hr	Day 1 %	Day 2 %	Average	SD	% RSD
Xolegel®	0.5	4.10	4.31	4.21	0.15	3.54
	1	6.44	6.54	6.49	0.072	1.11
	2	11.7	12.23	11.97	0.36	2.98
	4	27.41	25.69	26.55	1.22	4.59
	8	57.04	65.68	61.36	6.11	9.96
	12	78.33	84.74	81.54	4.53	5.56
	20	98.69	95.61	97.15	2.18	2.24
	24	103.06	101.54	102.30	1.07	1.05
Kez® shampoo	0.5	15.54	17.45	16.49	1.35	8.19
	1	16.70	15.98	16.34	0.51	3.11
	2	21.25	24.38	22.81	2.21	9.70
	4	35.74	31.12	33.43	3.27	9.78
	8	62.34	65.76	64.05	2.42	3.77
	12	80.94	83.92	82.43	2.11	2.56
	20	102.02	101.90	101.96	0.086	0.084
	24	103.05	102.79	102.92	0.19	0.18
Ketazol® cream	0.5	1.97	1.74	1.86	0.17	8.90
	1	2.05	1.99	2.02	0.04	2.01
	2	2.52	2.23	2.37	0.21	8.66
	4	6.10	6.64	6.37	0.38	6.05
	8	10.30	9.68	9.99	0.44	4.37
	12	15.16	14.74	14.95	0.29	1.96
	20	29.04	27.61	28.33	1.01	3.57
	24	30.86	30.54	30.70	0.23	0.74

4.6.4.3 Statistical comparison and mathematical modelling of *in vitro* release profiles

Statistical analysis using ANOVA and Tukey's multiple comparison tests and the use of model dependent and model independent modelling have been applied to the comparison of *in vitro* release profiles and were used to assess the release of KZ from Xolegel®, Kez® shampoo and Ketazol® cream. The profiles for mean amount of KZ released from each formulation are depicted in **Figure 4.16** and it is clear that the *in vitro* release test method developed and validated is suitable to discriminate between different formulations that contain 2% m/m KZ .

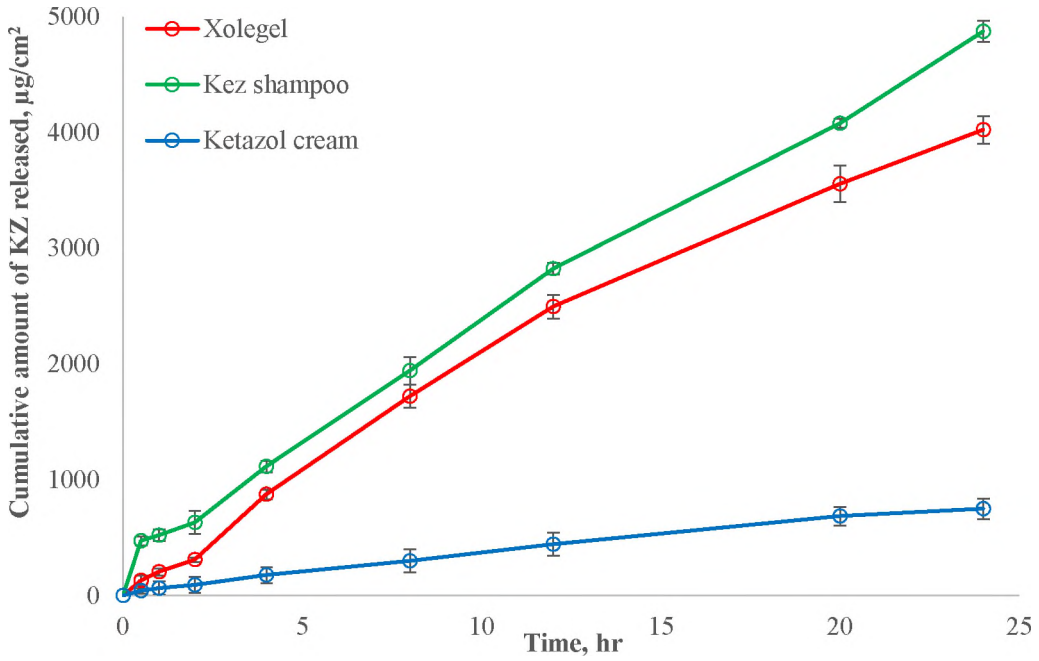


Figure 4.16 *In vitro* release profiles of KZ from Xolegel®, Kez® shampoo and Ketazol® cream using a receptor medium of ethanol and 0.05 M phosphate buffer of pH = 4.5 in a 50:50 ratio.

4.6.4.3.1 Statistical analysis

Two-way ANOVA of the cumulative amount KZ released per unit area from Xolegel®, Kez® shampoo and Ketazol® cream was conducted using GraphPad® Prism in order to establish whether the *in vitro* release method was discriminatory. The ANOVA table for these analyses is summarized in **Table 4.10.**

Table 4.10 Two-way ANOVA table for the analysis of the cumulative amount KZ released from Xolegel®, Kez® shampoo and Ketazol® cream.

Source of variation	Sum of squares	df	Mean square	F	% variability	p-value Prob > F	Significant difference
Time	29100000	7	4160000	6.76	57.06	0.0013	Yes
Formulations	13300000	2	6650000	10.8	26.05	0.0015	Yes
Residual	8620000	14	615531	-	-	-	-

The amount of KZ released and that permeated through the membrane from different formulations contributed 26.05% to the total variance observed and there is a 0.15% chance of randomly observing an effect this large in an experiment of this magnitude. This effect is considered extremely significant $F = 10.8$, $p = 0.0015$. The significant difference observed for the mean amount of KZ released from different formulations indicates that there was an impact on the amount of KZ released from the formulations. The data generated from ANOVA analysis indicate that the release of KZ from Xolegel®, Kez® shampoo and Ketazol® cream were statistically different overall.

Tukey's multiple comparison test for two-way ANOVA was used to obtain a detailed statistical analysis for the comparison of the amounts of KZ released from the formulations at each of the sample times and the data are summarized in **Table 4.11**.

Table 4.11 Tukey's multiple comparison test for two-way ANOVA for the comparison of the cumulative amount of KZ released from Xolegel®, Kez® shampoo and Ketazol® cream.

Tukey's multiple comparisons	Mean difference	95% CI of difference	Adjusted p-value	Significant difference	Summary
1 hours					
Kez shampoo vs. Xolegel	315	-2589 to 3219	0.9566	No	ns
Ketazol cream vs. Xolegel	-141	-3045 to 2763	0.9912	No	ns
Ketazol cream vs. Kez shampoo	-456	-3360 to 2448	0.9117	No	ns
2 hours					
Kez shampoo vs. Xolegel	321	-2583 to 3225	0.9550	No	ns
Ketazol cream vs. Xolegel	-219	-3122 to 2685	0.9789	No	ns
Ketazol cream vs. Kez shampoo	-540	-3444 to 2364	0.8788	No	ns
4 hours					
Kez shampoo vs. Xolegel	241	-2663 to 3145	0.9744	No	ns
Ketazol cream vs. Xolegel	-696	-3600 to 2208	0.8077	No	ns
Ketazol cream vs. Kez shampoo	-937	-3841 to 1967	0.6826	No	ns
8 hours					
Kez shampoo vs. Xolegel	222	-2682 to 3126	0.9783	No	ns
Ketazol cream vs. Xolegel	-1422	-4326 to 1482	0.4278	No	ns
Ketazol cream vs. Kez shampoo	-1644	-4548 to 1260	0.3290	No	ns
12 hours					
Kez shampoo vs. Xolegel	327	-2577 to 3231	0.9533	No	ns
Ketazol cream vs. Xolegel	-2053	-4957 to 851	0.1898	No	ns
Ketazol cream vs. Kez shampoo	-2380	-5284 to 524	0.1164	No	ns
20 hours					
Kez shampoo vs. Xolegel	522	-2382 to 3426	0.8860	No	ns
Ketazol cream vs. Xolegel	-2872	-5776 to 32.4	0.0528	No	ns
Ketazol cream vs. Kez shampoo	-3394	-6298 to -490	0.0217	Yes	*
24 hours					
Kez shampoo vs. Xolegel	850	-2054 to 3754	0.7290	No	ns
Ketazol cream vs. Xolegel	-3274	-6178 to -370	0.0267	Yes	*
Ketazol cream vs. Kez shampoo	-4124	-7028 to -1220	0.0061	Yes	**

Statistical analysis using Tukey's multiple comparison test reveal that the amount of KZ released from Ketazol® cream and Kez® shampoo was statistically different at 20 and 24 hours with $p = 0.0217$ and 0.0061, respectively. The amount of KZ released from the cream and Xolegel® was also found to be statistically different at 24 hours with $p = 0.0267$. At all the other time points the amount of KZ released from each of the formulations was not considered significant, but ANOVA data suggests that an overall significant difference between the amount of KZ released from the formulations exists.

Visual assessment of the release of KZ from the different formulations indicates that the release profiles are different but this was not detected by Tukey's multiple comparison test which could suggest that

statistical analysis might not be sensitive enough when a sample size, such as in this case, of only three replicates are used.

4.6.4.4 Model dependent analysis

Model dependent analysis of the amount of KZ released from Xolegel®, Kez® shampoo and Ketazol® cream was undertaken by fitting the release profiles to zero order, first order, Higuchi, Korsmeyer-Peppas and the Hixson-Crowell cube root law models. The value for R^2, the constant, K and the Korsmeyer-Peppas exponent, n were identified and are reported in **Table 4.12**.

Table 4.12 Model dependent parameters for KZ release from commercial products.

Formulation	Ketazol® cream			Kez® shampoo			Xolegel®		
	R^2	K	n	R^2	K	n	R^2	K	n
Zero Order	0.9927	0.93	-	0.9369	4.33	-	0.9118	4.33	-
First Order	0.8513	0.049	-	0.8383	0.036	-	0.7409	0.051	-
Higuchi	0.9619	4.75	-	0.9853	23.12	-	0.9603	23.17	-
Korsmeyer- Peppas	0.9947	0.26	0.77	0.9668	1.25	0.56	0.9798	0.90	0.85
Hixson-Crowell	0.9952	-0.016	-	0.9532	-0.27	-	0.9824	-0.13	-

The models that best described KZ release from Ketazol® cream and Xolegel® evaluated using R^2 values were the Hixson-Crowell cube root law whereas for Kez® shampoo the release data for KZ was best fitted to the Higuchi model. The R^2 values reported approximated 1 in some of the other models for all three formulations and therefore the release mechanisms of the formulations were elucidated taking into account the dosage form and excipients present in addition to the R^2 value. Models that produced $R^2 < 0.9600$ were excluded for the purposes of this discussion. The goodness of fit for the various models investigated for Ketazol® cream, Kez® shampoo and Xolegel® ranked in order from best to worst was Hixson-Crowell > Korsmeyer-Peppas > zero order > Higuchi, Higuchi > Korsmeyer-Peppas and Hixson-Crowell > Korsmeyer-Peppas > Higuchi.

The Korsmeyer-Peppas model has the capability to predict the transport mechanism of drugs for all types of formulations. In this case a value for the exponent between $0.45 < n < 0.89$ was elucidated for all commercial products indicating that anomalous, non-Fickian diffusion is the mechanism by which KZ is released. Anomalous transport occurs when simultaneous diffusion and erosion of the gel matrix contribute to KZ release. The release of KZ from Ketazol® cream follows a different release mechanism as suggested by the n value generated following fitting of data to the Korsmeyer-Peppas and other models for which the correlation coefficient was close to 1. In this case slow modified release from a cream that forms a porous matrix contributed to KZ release. The water channels within the o/w cream matrix may augment KZ transport into the receptor medium and the two different phases of the cream could be considered a porous matrix. The results of release studies from the cream are, however, best fitted to the Hixson-Crowell model which is indicative that KZ release is mainly driven by drug

dissolution and erosion [253,255,261]. The 50% v/v ethanol content in the receptor medium may cause the lipid phase of the cream to erode over time thereby producing a release profile that best follows this model. The release of KZ from the shampoo is best fitted to the Higuchi and Korsmeyer-Peppas models suggesting that the release of KZ is driven by diffusion due to a potential concentration gradient as the data for the shampoo is best fitted to the Higuchi model [255]. The shampoo contains protein(s) and thickening agents that form a matrix, swells on contact with the receptor medium and permits KZ release via a diffusion controlled process. Release data from Xolegel® was best fitted to the Higuchi, Korsmeyer-Peppas and Hixson-Crowell models indicating that the mechanism of KZ release was driven by KZ dissolution, erosion of the polymer via disentanglement when hydration of the formulation occurred and diffusion to a lesser extent [276]. KZ release kinetics from the commercial products could not specifically be defined by one mathematical model as the data produced are adequately fitted to a number of models. Model dependent analysis in combination with knowledge of formulation differences between delivery technologies in respect of excipients used permit descriptions of possible mechanism of release for KZ from each of the formulations.

4.6.4.5 Model independent analysis

Xolegel® and Kez® shampoo exhibit similar *in vitro* release profiles for KZ when using difference, $f1$ and similarity, and $f2$ factors, and these data are summarized in **Table 4.13**. Values for $f1$ and $f2 \leq 15$ and ≥ 50 respectively are indicative of similarity. When comparing the release of KZ from Xolegel® and Ketazol® cream or from Kez® shampoo versus Ketazol® cream, these were found to be different KZ as the similarity and difference factors for this comparison do not satisfy the tolerance limits for similarity.

Table 4.13 $f1$ and $f2$ values for the comparison of Xolegel®, Kez® shampoo and Ketazol® cream.

Comparison	$f1$	$f2$
Xolegel® vs Kez® shampoo	8	60
Xolegel® vs Ketazol® cream	65	17
Kez® shampoo vs Ketazol® cream	67	16

The FDA and EMEA have adopted the use of the similarity factor, $f2$, when establishing similarity of release between two formulations. It is clear that model independent analysis is reflective of the similarity and difference between *in vitro* release profiles obtained for the different formulations tested, as similarity between Xolegel® and Kez® shampoo can be observed from the *in vitro* release profiles depicted in **Figure 4.16** as can the difference between the cream and other formulations.

4.7 Conclusions

The differences in KZ release observed when fresh and frozen pig vaginal mucosa were prepared using a surgical separation approach were not statistically significant for all comparisons. Therefore, pig

vaginal mucosa may be used immediately following preparation or stored for up to 6 days at -20°C and/or -80°C prior to use for *ex vivo* release studies. The results observed correlate with those reported in which no difference in the barrier properties of fresh and frozen natural membranes were observed [195]. KZ permeation through fresh and stored mucosae into receptor fluids appeared to be controlled and relatively slow suggesting that the tissues exhibit rate limiting properties [221,268,277]. When selecting artificial membranes such as cellulose nitrate, nitrocellulose and cellulose acetate of different pore size, the 0.2 μm Sartorius® cellulose acetate membrane was established as more permeable than the others tested and exhibited minimal adsorption of KZ resulting in better sensitivity for analytical purposes. The pharmaceutical industry is not obliged to undertake *in vivo* studies for product development purposes and rather focuses on the assurance of product consistency during development and marketing and such tests should be discriminatory. IVRT is used to distinguish performance between two different products on the basis of their release profiles [221]. Mucosae such as that of the pig vagina may not be sufficiently discriminatory, due to rate limiting and barrier properties and for the assessment of controlled release formulations in which water insoluble drugs are incorporated. Consequently, an artificial membrane was considered for use and a 0.2 μm Sartorius® cellulose acetate membrane was therefore selected for use in IVRT studies.

A receptor medium in which the maximum amount of KZ was released at 24 hours containing the lowest possible amount of solvent and which did not drastically reduce the extent of KZ released with discriminatory ability in respect of Xolegel®, Kez® shampoo and Ketazol® cream was targeted for IVRT of the thermosetting KZ vaginal gels manufactured in these studies. A receptor fluid of 50% v/v phosphate buffer of pH 4.5 was eventually selected for use and was subjected to validation studies. The % RSD values for the formulations tested during intra- and inter-day precision experiments were ≤ 10% at most time points [271] which was considered acceptable for % RSD for IVRT as indicated in the USP. Therefore, the *in vitro* test method developed in these studies was considered precise and a Tukey's multiple comparison test for intra- and inter-day precision experiments demonstrated similar interactions for the % KZ released when drug release from commercial products was compared in a pairwise manner. Visual assessment of KZ release from Ketazol® cream was significantly different to that observed from the shampoo and gel and the difference may be attributed due to the composition of the cream in which fatty excipients may preferentially solubilize lipophilic KZ. The shampoo and gel are primarily aqueous based formulations in which KZ is dispersed and may therefore facilitate release of the molecule.

Two-way ANOVA and Tukey's multiple comparison tests revealed that KZ release from Xolegel®, Kez® shampoo and Ketazol® cream exhibited significant differences at most time points except at the two later sample times. Statistical analysis using Tukey's multiple comparison tests proved to be insensitive to KZ release data and could not effectively differentiate between the three formulations

possibly due to the low sample size (n = 3) used in these experiments. The mechanism of KZ release may be elucidated using model dependent approaches and this methodology may be used to distinguish the types of formulations tested and the mechanism of delivery using data derived from IVRT. KZ release from Ketazol® cream and Xolegel® was found to follow anomalous or non-Fickian diffusion transport processes suggesting that KZ delivery occurred through both diffusion and erosion of the dosage form. The data from these release studies were best fitted to the Hixson-Crowell model as KZ release mainly occurred through dissolution and erosion from the Ketazol® cream or via polymer swelling and disentanglement for the Xolegel® product. The receptor fluid used was made up of 50% v/v ethanol that may have dissolved some of the excipients in the cream and gel matrices resulting in erosion. Diffusion may have occurred on a smaller scale due to concentration gradients within and external to the formulation. The value for the exponent, n, obtained following fitting of data to the Korsmeyer-Peppas model, revealed that KZ was released from Kez® shampoo via a non-Fickian diffusion mechanism. The release data for the shampoo was best fitted to the Higuchi model suggesting that KZ is predominantly released via a porous matrix comprised of the excipients in the shampoo that undergoes limited or slow erosion. KZ transport is driven by chemical potential and a diffusion process. Model independent analysis was considered the best approach for an overall assessment of KZ release from the commercial KZ formulations and it was established that Xolegel® and Kez® shampoo exhibited similar release patterns whereas Ketazol® cream exhibited very different patterns. Model dependent and independent analysis produced incongruent results for the comparison of KZ release profiles for these three products, but the results of all analyses are useful to elucidate product performance characteristics. The model dependent approach revealed that the mechanism(s) of KZ release from the formulations could be identified although one model could not be selected as a definitive description for KZ release from each formulation. These results have highlighted the fact that each of these products are complex formulations that include materials that have an impact on KZ release.

An *in vitro* release test method established for assessing commercially available KZ formulations was developed and validated for precision and can be used to assess topical formulations for vaginal use. This method can be used to assess product quality and performance of gel formulations intended for vaginal application. Further validation in which the impact of viscosity, changes in composition of formulation, pH of formulation and loading dose may be required [75,278]. The *in vitro* release test will be used to investigate KZ delivery from thermo-sensitive vaginal gel formulations and further investigation should be undertaken to determine the reason for low % recoveries of KZ associated with some artificial membranes.

CHAPTER FIVE
VAGINAL DRUG DELIVERY AND DESIGN OF *IN SITU* THERMOSETTING VAGINAL GEL FORMULATION

5.1 Introduction

The vaginal route of administration has traditionally been used to treat infections of the vagina and vaginitis and has recently gained popularity for consideration as a portal for the systemic delivery of therapeutic compounds [279,280]. The vagina is a complex organ that is dynamic and exhibits frequent changes to the local environmental that may be due to age, pregnancy, menstrual cycle and extent of sexual stimulation [279,281]. Although the efficacy and duration of activity of vaginally administered formulations are affected by environmental changes, intra-vaginal delivery is increasingly popular as it is less invasive than parenteral routes of administration and has the potential to deliver compounds that exhibit low oral bioavailability over extended periods of time [282,283]. The design of an intra-vaginal formulation requires consideration of the anatomy and physiology of the vagina, the structure and physiology of the mucosa, and secretions, in addition to consideration of patient preference [281,284].

Gels are versatile pharmaceutical technologies that can be used to deliver a large number of different therapeutic compounds and can be used in the vagina as they are aqueous formulations with high water and low excipient content that exhibit a low potential for mucosal irritation. The FDA defines a gel as a semi-solid dosage form composed of a gelling agent that when activated results in stiffening of an aqueous solution or colloidal dispersion to form a uniformly distributed liquid that may contain suspended particles [285]. Gels containing the same concentration of an active ingredient as another dosage form may exhibit different performance in terms of application, absorption and release amongst other characteristics [282]. The simplicity of gel formulations and the manufacturing process make such dosage forms excellent alternatives for drug delivery.

5.2 The vagina as a route for drug delivery

The vaginal cavity exhibits a variety of pathologies that may be due to bacterial, fungal, protozoal or viral infection [286-288] and dosage forms for vaginal use may be liquid, semi-solid or solid formulations that are intended to exert local therapeutic effects [289,290]. The vaginal route of drug administration is a promising portal for local and systemic drug delivery due to the fact it exhibits a large surface area for absorption, is a well perfused tissue with a rich blood supply and exhibits permeability to a wide range of compounds [280]. The vaginal route of delivery is currently used to administer microbicides for the prevention of sexually transmitted diseases, drugs to induce labour, spermicides and steroidal compounds for contraceptive purposes [291]. The current trend is to use the vaginal route of delivery as an alternate approach to parenteral delivery for expensive compounds that are susceptible to hepatic first pass metabolism or that may adversely affect the mucosa of the

gastrointestinal tract [287]. The advantages and drawbacks of the vaginal route of delivery are listed in **Table 5.1.**

Table 5.1 Advantages and limitations of the vaginal route of drug administration [284,287,292-294].

Advantages	Limitations
Not affected by hepatic first pass metabolism.	Presence of enzymes, hydrogen peroxide and other vaginal constituents.
Better accessibility when compared to parenteral formulations due to self-administration.	Only for treatment of female patients.
Extensive vascularization for efficient drug transport.	Variability due to changes in hormonal levels during menstruation that include pH, viscosity, amount of mucous secretions and membrane thickness.
Possibility of local and systemic action.	Age-specific variability.
Permeable to large molecules such as proteins and peptides.	Formulations may be easily removed by variable vaginal mucous discharge.
Large epithelial surface area for drug delivery.	
Fewer hepatic side effects from hormonal delivery.	
Low incidence of gastrointestinal tract diseases and side effects.	

5.2.1 Morphology of the vagina

5.2.1.1 Vaginal mucosa

The internal vaginal wall is made up of a mucosal membrane, muscular tissue and an outer serosal layer and the female reproductive tract undergoes morphological changes throughout the menstrual cycle [287]. The vaginal mucosa consists of a stratified squamous epithelium that is separated into superficial, intermediate and basal epithelial layers as depicted in **Figure 5.1** [295].

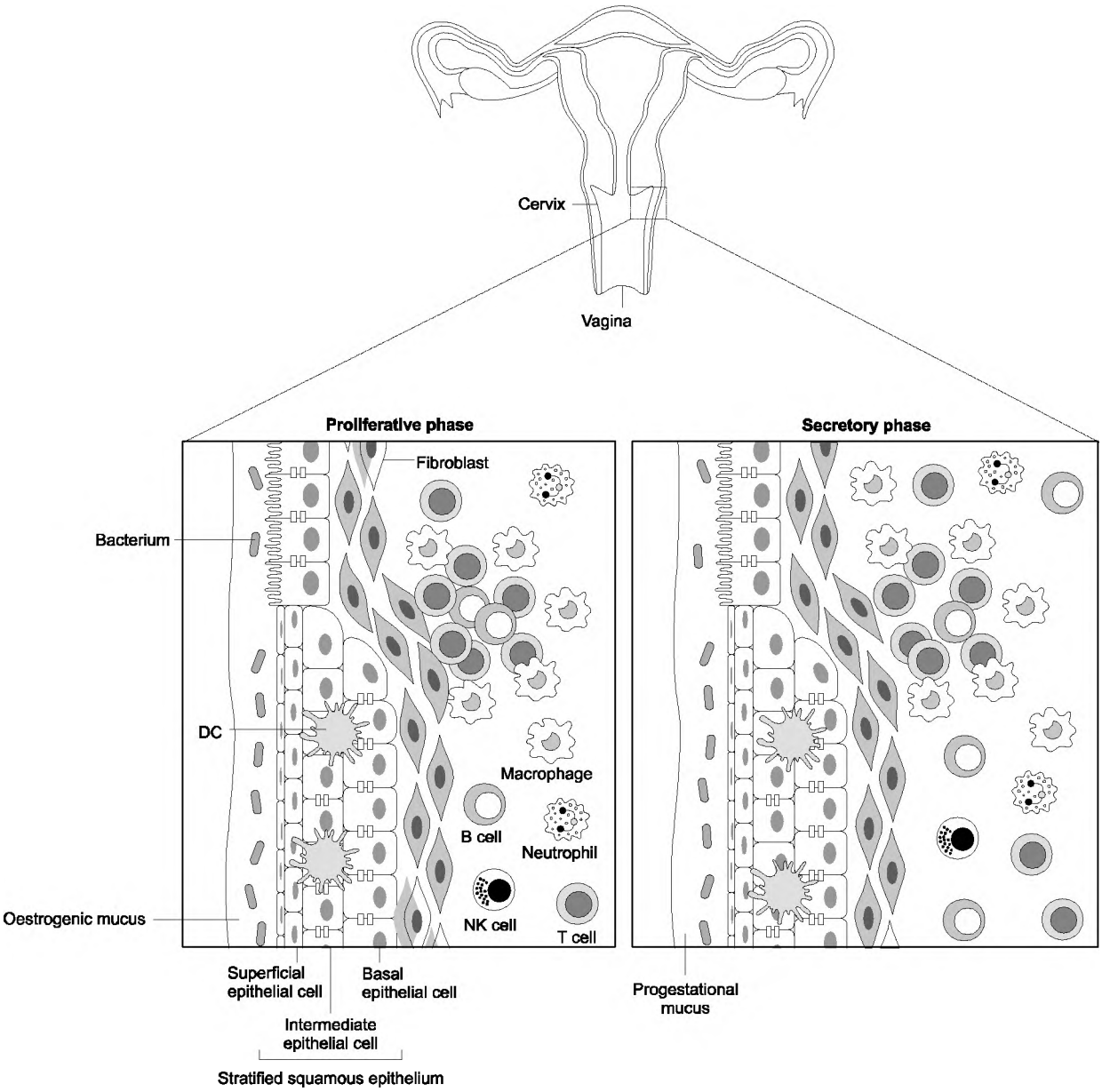


Figure 5.1 Schematic representation of changes in the vaginal mucosa during the proliferative and secretory phases of the menstrual cycle adapted from [295].

The thickness of the epithelial layer of the vagina varies between 200-300 μm according to age, menstruation and hormone levels [286]. The vaginal mucosa, unlike other mucous membranes, does

not contain goblet cells and during the proliferative stage of the menstrual cycle columnar epithelial cells found in the upper part of the female reproductive organs multiply and form glands during the secretory/ovulatory stage of the cycle [287,296]. The epithelial layer of tissue is lined with mucous in the lower reproductive tract and the viscosity varies as hormone levels fluctuate during the cycle. The mucous is generally more viscous during the ovulatory stage of the menstrual cycle. A layer of fibroblasts and immune cells including T-cells, B-cells, macrophages, natural killer (NK) cells, neutrophils and dendritic cells (DC) occur below the epithelium [295]. In addition lactobacilli form part of the natural flora of the vaginal environment and ensure that a low pH is maintained in the vaginal cavity [287].

The vagina is located between the rectum, bladder and urethra and is an S-shaped fibrous muscular collapsible tube [288]. The vaginal lumen is separated from the rectum by a thin membrane called the perineum. Dosage forms placed in the vagina are subjected to pressure from the rectal walls during defecation [297] which may result in expulsion of intra-vaginal dosage forms thereby resulting in uncontrolled drug release. The development of a dosage form to withstand such events is important for intra-vaginal KZ delivery.

5.2.1.2 Vaginal secretions

Vaginal secretions consist of cervical mucous, endometrial discharge, tubal fluids and vaginal transudate in addition to Bartholin and Skene gland secretions [284,286]. The vaginal secretions form a protective layer over the vaginal mucosa and protect deeper tissue layers from infection by pathogens. Sloughing of mucous and cells occurs sporadically and this action flushes and removes adherent microbes [284]. On average, approximately 6 g of vaginal fluid is produced on a daily basis and between 0.5 and 0.75 mL of the vaginal secretions consists of lactic acid, acetic acid, glycerol, urea, glycogen and glucose and is present in the vagina at all times [286]. Lactobacilli produce lactic acid that forms an integral part of the system that maintains homeostasis within the vagina and contributes to the maintenance of the pH between 3.5 to 4.5 normally observed in the human vagina [293]. The acidity of the vaginal fluid prevents microbial growth that may enter the vagina via the perineal membrane [297]. Lactobacilli are gram-positive bacteria, some of which produce hydrogen peroxide that may be a barrier to drug delivery as oxidation of drugs in the vaginal lumen prior to penetration across the vaginal mucosa may occur. In addition vaginal fluids contain enzymes that may result in non-specific (enzyme to drug) binding and/or destabilization of drugs, thereby interfering with therapy [284].

Estrogen secretion results in the production of estrogenic mucous as depicted in **Figure 5.1** and this mucous, although not viscous, is produced abundantly and is a layer through which drug molecules must and can easily diffuse. A thick mucous is produced, albeit in limited quantities due to the activity of progesterone secretions [287]. The use of hormonal, implanted, progestin-only oral and combination

oral contraceptives results in the production of viscous vaginal secretions, the viscosity of which is reduced in the presence of vaginal pathogens [298]. This implies that vaginal secretions have a low viscosity in the presence of *Candida albicans* infection and the use of contraceptives may also affect the distribution of drugs delivered from vaginal formulations. Sexual arousal results in a dramatic increase in the volume of vaginal fluid secreted.

The presence of high concentrations of sodium and chloride ions in the vagina results in an increase in the volume of vaginal secretions that may have an impact on the performance of therapeutic products. The characteristics of formulations may therefore change according to the amount and viscosity of mucous present in the vagina and increased leakage of a semi-solid dosage form from the vagina may occur, thereby decreasing the residence time and bio-adhesion of the formulation and potentially resulting in lower efficacy of treatment. In contrast, an increase in the volume of vaginal fluid secretions may lead to enhanced dissolution of the formulation in the vagina, resulting in increased penetration and absorption of the API into and through the mucosa [284]. During menopause the level of vaginal fluid secretions and glycogen are reduced due to a reduction in hormone levels that results in a decrease in the bacterial count in the vagina and ultimately an increase in the pH in the organ [286]. Vaginal drug delivery systems must therefore be formulated specifically for pre- and/or postmenopausal women and adjusted to an appropriate pH to avoid irritation of the vaginal mucosa whilst taking care to avoid using drugs and/or excipients that might have a negative effect on the natural flora of the vagina.

5.2.1.3 Absorption and distribution from vaginally administered formulations

Following the application of vaginal gel formulations, women have reported experiencing a cold, wet, tingling, itching and/or a burning sensation [299]. The cervix acts as a reservoir for the formulation [284] and the API contained in the formulation dissolves in the vaginal secretions prior to eventually spreading to coat the walls of the vagina. The establishment of a concentration gradient within the vagina facilitates drug release and penetration into and through the epithelial cells of the vaginal mucosa. Drugs that penetrate into the layers of the vaginal cells or permeate into cells results in topical delivery and localized drug activity. Permeation across the vaginal epithelial tissues and through intercellular or para-cellular spaces results in systemic uptake and effects of that drug. Drug transport occurs primarily through the aqueous pores of the mucosal membrane and/or through vesicles or receptors on the mucosa or through lipophilic pathways that exist in the lipid bilayer structure of the membrane [287]. Small lipophilic compounds can penetrate epithelial cells and may be locally distributed in the vagina whereas hydrophilic molecules can diffuse through the aqueous pores in the vaginal mucosa prior to systemic distribution. The effectiveness of vaginal drug delivery is also influenced by the molecular mass, partition coefficient, ionization state, surface charge and chemical properties of the molecule to be delivered. The compatibility of the API with vaginal secretions may impact the stability and bioavailability of the compound following administration. Variations in

hormone levels during the menstrual cycle result in fluctuations of pH and thickness and porosity of the vaginal mucosa, and consequently may result in variable absorption and *in vivo* release profiles of vaginally delivered drugs.

KZ has a large molecular weight (MW = 531.44) and is highly lipophilic (**Chapter 1**), factors that usually hinder the transport of a compound across biological membranes [300]. KZ is a dibasic molecule that is protonated in acidic media and therefore changes in the pH of the environment will change the ionization state of the molecule. KZ will primarily exist in the ionised form in the vagina of healthy fertile females [301]. In any formulation in which KZ is solubilized the dissolution of ionised KZ in acidic vaginal secretions will occur (**Figure 5.2**) thereby facilitating the transport of KZ through aqueous pores in the membrane resulting in systematic delivery of KZ.

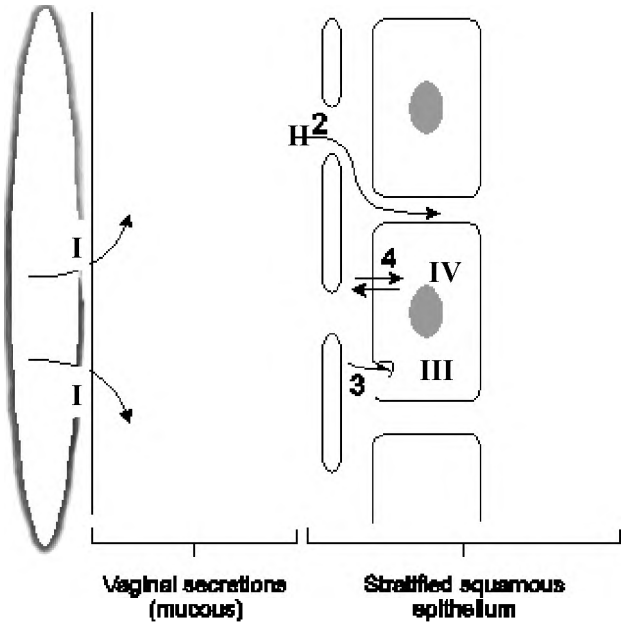


Figure 5.2 Schematic representation of KZ transport in the vagina [295].

The dissolution of KZ in vaginal secretions described in **Figure 5.2** commences with dissolution of the drug in vaginal secretions (I) resulting in a concentration gradient that facilitates localized activity of KZ in the epithelial cells of the vagina. The intercellular transport of ionized KZ occurs (route II) and results in systemic delivery of KZ that can also be absorbed across membranes via vesicular transport (route III) as it is lipophilic and can exert systemic effects. Finally, penetration through the cell membranes due to the existence of a concentration gradient across the membranes may occur via route IV.

The vast network of blood vessels located in the vaginal wall facilitates the systemic delivery of drugs from the lumen of the vagina [297]. However, if KZ was formulated into a specialized formulation such as a thermosetting gel it may be assumed that the formulation and KZ will reside in the vagina for a longer period of time and exhibit extended local activity activated by the presence of a concentration gradient to drive drug delivery. This extended local activity may not translate into systemic absorption of KZ and therefore limit the side effects that are usually associated with systemic delivery of KZ [59]. The low concentration of unionized KZ present in the formulation may result in penetration through cell membranes and result in localized distribution of KZ in the vaginal membrane as depicted in **Figure 5.2** that will result in the death of *Candida albicans*, consequently resulting in an effective local treatment for candidiasis [301,302]. KZ acts by competitive binding to an active site in *Candida albicans* causing disruption of membranes and eventually necrosis of the yeast **(Chapter 1)**. KZ disrupts fungal pathogens soon after administration of a dosage form into the vaginal lumen prior to the fungus binding to the host cells. The natural immune response in the vagina then acts on the fungus that is bound to the host cells.

5.3 Strategies for vaginal drug delivery

5.3.1 Gel dosage forms

Gels are transparent or translucent to opaque, non-greasy dosage forms that may be used for oral, topical, intra-vaginal, rectal, intranasal, ocular and parenteral drug delivery [183,303,304]. Gel dosage forms are useful delivery platforms for the treatment of conditions of mucous membranes and inflamed and/or infected skin as gels have a high water content and are therefore soothing to irritated and/or sensitive skin [303]. Furthermore, gels can be flushed from cavities by natural body fluids such as, for example, vaginal secretions or by gentle rinsing with water.

Gels are semi-solid materials in which the solid component is comprised of a low concentration of molecules that self-assemble and gelate through physical and/or chemical interactions in the presence of a suitable solvent that trap the liquid component within the assembly thereby preventing solvent flow. The solid components self-aggregate to form rods, tubules, fibers and/or platelet-shaped structures [305,306]. Gels are also defined as a swollen network with the cohesive properties of solids and the diffusive transport properties of liquids [183]. Gels can be classified according to the bonds within the gel network, as physical and chemical; the polarity of the liquid phase; the organic solvent in organo-gels; water in hydrogels; and the number of gelling components used, for example, bigels are made of a mixture of oleogels and hydrogels [183,307]. Weak Van Der Waals forces of attraction and hydrogen bonds ensure the retention of the physical structure of gels and strong hydrogen bonds form the basis for crosslinking in chemical bonded gels.

Oleogels are lipophilic solid and liquid mixtures in which a network formed using solid lipid oleogelators entrap a large amount of liquid oil [306,308]. Organogels and hydrogels differ according to the polarity of their external liquid constituents *viz.*, organic solvents in organogels and water in hydrogels [306,307]. Organogels usually contain hydrocarbons and are formulated with non-polar solvents such as hexane, isopropyl myristate, animal and/or vegetable fats, soap bases and polymeric or low molecular weight (≤ 3000 Da) gelating agents [183,306,307]. Hydrogels are manufactured using natural or synthetic homo- or copolymers that form a hydrophilic colloidal network of polymer chains in an aqueous phase and are able to absorb large volumes of water or biological fluids whilst ensuring flexibility that is similar to that observed for natural membranes thereby imparting biocompatible properties to the gel [306]. The introduction of chemical and/or physical cross links ensures that the hydrogels retain a semi-solid structure when in aqueous media and this thermodynamic activity manifests as swelling [309]. Hydrogels can be categorized into amorphous, semi-crystalline, hydrogen-bonded, super-molecular hydro-colloidal aggregates and the description is based on the structure of the gel network [310-312].

5.3.2 Vaginal gels

Mucous is a naturally occurring gel and the use of synthetic gels can be effective for drug delivery due to their similarity to the properties of naturally occurring gels [309]. The ideal vaginal gel must be safe for intra-vaginal placement, be suitable for the effective treatment of the target ailment, be aesthetically appealing, enhance patient adherence, be licensed and be manufactured using a simple and cost effective process [280,282]. Vaginal gels generally consist of one or more API(s), a gelling agent, humectant, preservative and vehicle whereas vaginal solutions contain one or more API(s), a solubilizer, antioxidant and preservative [280].

5.3.3 *In situ* thermosetting gels

Some polymeric materials may swell in response to electromagnetic radiation and/or a physiological stimulus such as pH, ionic strength, temperature [309]. The research undertaken in these studies focused on temperature changes as the stimulus for hydrogel activation.

The vaginal route as delivery site has a number of limitations (**§ 5.2**), one of which is the low vaginal residence time of conventional formulations due to physical removal, i.e. expulsion of dosage form due to pressure from perineum during defecation [297] and/or physiological expulsion that may result in unpredictable drug delivery to the mucosa [286]. A novel formulation approach to tackle short residence times is to use *in situ* or environmentally sensitive gelling systems that solidify at body temperature, with a change in pH or in the presence of ions [313]. *In situ* forming gels are liquids prior to administration and can therefore be accurately and reproducibly administered as compared to

administration of pre-formed gels [314]. This approach can result in increased residence times at the site of activity with a possible enhancement of therapeutic activity.

Covalent or hydrogen bond formation in gels forms chemical and physical crosslinking that results in gelation [100,315] and reversible gels are technologies in which crosslinking bonds are formed, broken and/or modified resulting in changes in gel properties. Permanent gels do not exhibit reversible characteristics due to the strength of the covalent bonds that are present and impart strength to the gel structure [183]. Temperature sensitive hydrogels may be classified as negative thermo-sensitive gels that exhibit an upper critical solution temperature (UCST) or positive thermo-sensitive gels that exhibit a lower critical solution temperature (LCST) and the gels may or may not exhibit thermally reversible behaviour [309]. Gels that exhibit a LCST undergo sol-gel transition as the temperature increases whereas gels that exhibit a UCST undergo gel-sol transitions with an increase in temperature [316].

The gel formulations investigated for the delivery of KZ were thermally reversible and designed to exhibit a LCST that solidified in response to an increase in temperature and that would form a liquid at ambient temperature [317]. Thermo-responsive hydrogels have been widely studied as environment-sensitive polymeric systems [317] and polymers that exhibit thermo-responsive behaviour must be formulated without other compounds and in the hydrogel formulation that does not require any source of heat other than the temperature of the body for the gel to form [317]. Gel formation occurs when the temperature of the polymer changes from ambient to physiological and thermo-responsive gels are therefore easy to apply as they exhibit a low viscosity and are easily spread when in contact with the mucosa prior to gelation. The drug delivered from the thermosetting formulation has the potential to remain in contact with the target organ for longer periods of time than liquid formulations due to gelation of the technology following administration. In addition leakage or loss due to physiological secretions may be minimized and therefore the formulation is not immediately removed from the site of administration. The gel formulation forms a stable and protective layer that can adhere to the surface of the vaginal mucosa and remains intact when pressure is exerted on the vaginal walls or in the vagina such as during defaecation and coitus [286]. Several studies using animals have demonstrated that the thermosetting behaviour of gels is a feasible formulation approach to treat vaginal conditions [318,319]. A literature search revealed that a number of *in situ* gelling formulations have been developed and these data are summarized in **Table 5.2.**

It is clear that poloxamers have been widely used for the preparation of *in situ* gelling formulations and thermo-responsive *in situ* gelling systems appear to be common for the manufacture of vaginal formulations using polymers such as chitosan and Carbopol® 974P.

Table 5.2 Summary of published information relating to the formulation of *in situ* gelling systems.

***In situ* gelling formulation**	**Site of administration**	**API**	**Gelling agent**	**Other excipients**	**Ref.**
Thermo-responsive hydrogel depot	Parenteral	-	Chitosan and glycerophosphate	-	320
pH triggered gelling system	Ocular	Levofloxacin	Carbopol, cellulose acetatephthalate latex	-	314
Temperature dependent gelling system			Poloxamers, tetronics, methyl cellulose	-	
Ion activated gelling system			Gelrite, sodium alginate	-	
Thermo-sensitive mucoadhesive gelling system	Vagina	Curcumin	Poloxamer 407 and Poloxamer 188	HPMC K4M	321
Topical thermo-responsive contraceptive anti-retroviral microbicide	Vagina	Copper-curcumin	Carbopol 974P	Stabilised silver nanoparticles and β-cyclodextrin	322
Thermo-responsive gelling system	Nasal	Celecoxib	Poloxamer 407 and Carbopol 934P	β-cyclodextrin	323
Thermo-responsive floating gel	Stomach	-	Poloxamers, polymer networks of poly(acrylic acid) (PAA) and polyacrylamide (PAAm) or poly(acrylamide co butyl methacrylate)	-	324
pH-sensitive gel	Vagina	Nystatin	Carbopol and polyvinyl alcohol	-	325
Thermo-sensitive gel	Vagina	Nifeviroc	Poloxamer 407 and Poloxamer 188	-	326
Thermo-responsive gel formulations	Vagina	Miconazole nitrate	Poloxamer 407 and Poloxamer 188	-	327
Thermo-sensitive gelling formulations	Vagina	Metronidazole	Poloxamer 407 and Poloxamer 188.	-	328, 329
Thermo-responsive gel formulations	Vagina	Econazole nitrate	Poloxamer 407 and Poloxamer 188.	-	330
Ion activated bio-adhesive gel	Vagina	Clindamycin	Gellan gum.	Chitosan	331

5.3.4 Poloxamers

Poloxamers are commercially marketed by the BASF Corporation as Pluronic® and Lutrol® and for the purposes of these studies the materials were donated by BASF South Africa (Pty) Ltd (Midrand, Gauteng, South Africa). Poloxamers are synthetic non-ionic tri-block copolymers that consist of a central hydrophobic propylene oxide (PPO) block located between hydrophilic polyethylene oxide (PEO) and block copolymers [332-334]. Micelles form when poloxamers are used in concentrations greater than the critical micelle concentration (CMC) in water [335]. Poloxamers are sometimes referred to as PEO-PPO-PEO copolymers and are available in different molecular weights and forms. Pluronic® F127/Lutrol® F127 (Poloxamer 407), Pluronic® F68/Lutrol® F68 (Poloxamer 188) and Pluronic® F87/Lutrol® F87 (Poloxamer 237) were used in combination to produce a vaginal gel that is a free flowing liquid at a room temperature of 22°C and set to form a stiff gel at a temperature of 37°C or body temperature. The Lutrol® F materials are white, coarse powders with a waxy consistency that contain butylated hydroxytoluene (BHT) as an antioxidant. The polymers are soluble in water and in polar and non-polar organic solvents such as acetone and hexane [336].

A low concentration of poloxamer in water results in the formation of mono-molecular micelles that are individual coils or unimers [334,337]. A hydration layer forms around the poloxamer molecules at low temperatures thereby increasing the flowability of the material and produces a liquid gel. Increasing the concentration of poloxamer results in micelle aggregation to form multi-molecular micelle aggregates as depicted in **Figure 5.3 (A)** [183,334,337]. The aggregates form when hydrophobic PPO block copolymers within the core exhibit an affinity for each other and therefore the hydrophobic cores bind together to form a loosely bound aggregate (**Figure 5.3**). As the temperature of the system increases hydrogen bonds that exist between the solvent and hydrophilic chains of the poloxamer are disrupted resulting in hydrophobic interactions between the PPO blocks. The PPO cores have an affinity for hydrophobic cores that result in extensive entanglement of the agglomerates and a transition from a sol to a gel form. Sol to gel transitions occur when liquids that contains the multi-molecular poloxamer forms a rigid gel-like structure through aggregation of the multi-molecular poloxamer components and this results in a decrease in flowability, and with further increases in temperature no flow is possible. Rigid gels that include poloxamer form entanglement-chain matrix like structures as depicted in **Figure 5.3 (B)** due to the formation of weak hydrogen bonds and Van Der Waals forces of attraction [306]. The high concentration of poloxamer in solution (PEO-containing block in a dry state) leads to the formation of surface active micelles and eventually forms an ordered lamellar phase leading to the formation of a liquid lyotropic crystalline phase that occurs as the PEO crystallises to form lamellae [183,338].

This results in the formation of a solid mass of poloxamer with strong hydrogen and Van Der Waals forces of attraction and this mass does not allow free movement of the API or formulation components. The liquid lyotropic crystalline phases do not exhibit thermosensitive behaviour.

Methyl groups in the hydrophobic core of PPO interact with the drug that undergoes solubilisation via Van Der Waals forces of attraction and the oxygen ether groups from PEO blocks interact with water molecules to produce a water soluble system [183,336]. Gelling of the system also depends on the molecular weight of the poloxamer and poloxamers of high molecular weight form gels with low concentrations of the polymer [336].

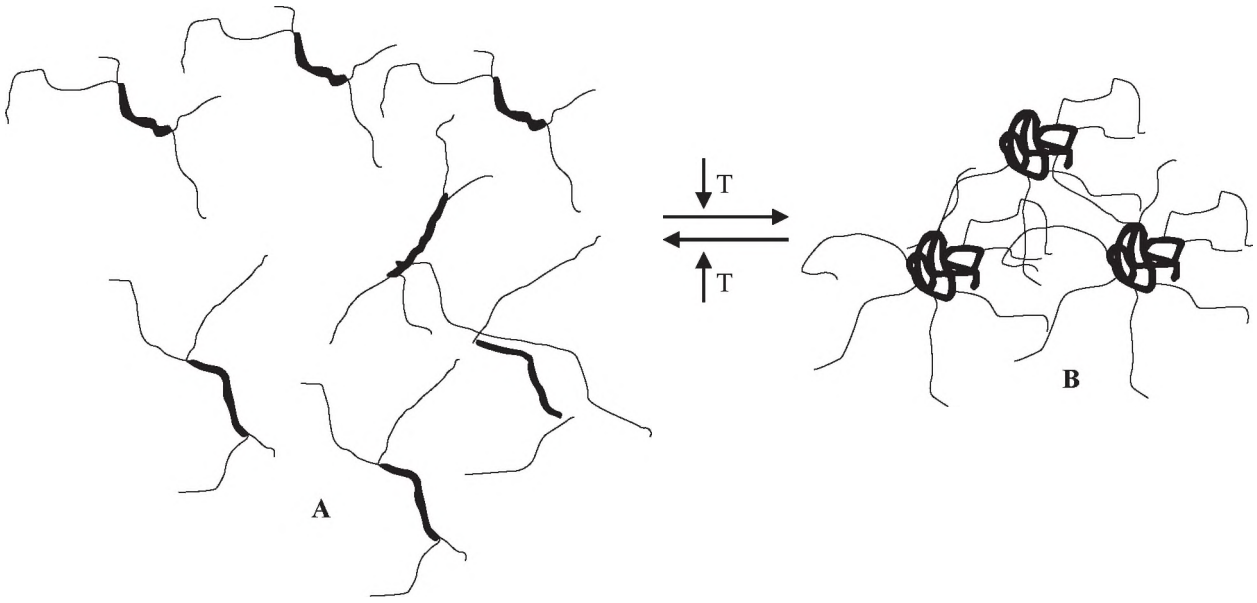


Figure 5.3 Schematic representation of the mechanism of gelation of thermo-reversible poloxamer gels in response to temperature modulation. Adapted from [339].

Lutrol® F127 (L127), Lutrol® F68 (L68) and Lutrol® F87 (L87) have been used as wetting agents, emulsifiers, solubilisers and co-emulsifiers and L127 is also used as a thickening agent, gel former and consistency enhancer [336,340,341]. L127 exhibits thermo-reversible gelation characteristics in concentrations between 16 and 30% m/v in water and is a free flowing liquid at temperatures < 4°C and > 70°C. L127 is more soluble in cold aqueous solution due to higher hydrogen bonding capacity at lower temperatures [183]. Low concentrations of L68 in aqueous solution exhibit Newtonian flow and at higher concentrations non-Newtonian flow is observed [336] and a 20% m/v aqueous solution of L68 exhibits a low viscosity in the temperature range of 15 to 60°C and the maximum viscosity is observed at temperatures between 75 to 80°C [336].

Poloxamers can be prepared using hot or cold manufacturing processes. The cold manufacturing process involves dissolution of the poloxamers in a liquid at temperatures between 4 and 5°C until a clear solution is formed at which point adequate solubilisation of the poloxamers can be considered to have occurred. Water insoluble compounds such as KZ should be dissolved in an organic solvent that forms an homogenous solution on mixing with an aqueous poloxamer solution. The hot manufacturing process

involves the dissolution of poloxamers in a liquid at 70°C. In the case of water insoluble compounds and aqueous liquid components, the compounds are dissolved in an organic solvent initially after which it is mixed with a warm aqueous solution of the poloxamer and both processes yield gels of similar properties [336]. However, the cold process is more widely used as this approach accommodates the inclusion of drugs that are heat labile and also limits the loss of solvents which sometimes results in the formation of materials that exhibit thixotropic effects [336].

5.4 Conclusions

The choice of suitable drug delivery technologies is usually dependent on the physical and chemical characteristics of the drug candidate, the target site of delivery and the requirements of the patient. Existing commercially available vaginal formulations used for the treatment of fungal infections are generally administered as creams, gels, ovules and tablets and these are sometimes inconvenient to use due to leakage of the formulation from the vagina shortly after administration, short residence times in the vagina or they are uncomfortable for the patient due to their solid non-pliable form. Consequently, there is a need for the development of novel formulations that would have minimal or no irritancy to vaginal tissues and that can be easily administered, be retained in the vagina for long periods of time, are water soluble and biodegradable. In addition a dosage form that is naturally removed from the vagina over a period of time and that is so discrete that the patient barely feels it in the vagina would be an advantage. In general intra-vaginal delivery systems should be non-toxic to the natural vaginal environment and the dosage form should be cost effective, readily available, easy to use, be self-administered and require a low dosing frequency [282,342,343].

Care must be taken during product development to design features into the technology to overcome the barriers to KZ delivery in the vagina and include consideration of changes in epithelial thickness, varying amounts of the vaginal secretion and the presence of enzymes and hydrogen peroxide that may impair biological activity of the drug [284]. The penetration of KZ into and across the vaginal epithelium is an important factor that will impact the efficacy and toxicity profiles of any formulation containing the compound [284].

Thermosetting vaginal gels may exhibit advantages as vaginal dosage forms, some of which include their ease of preparation and administration as a liquid that forms a rigid gel of high viscosity at 37°C that prolongs residence time in the vagina. The high water content of hydrogels results in the production of a product of low irritancy to the vaginal mucosa and the flexibility of gel formation at body temperature permits the gel to fit more comfortably in the vagina after administration when compared to rigid solid dosage forms such as a tablets and ovules. The thermosetting gels may therefore be comfortable for the patient thereby potentially enhancing adherence. The water-based gel is biodegradable and can be flushed out of the vagina by a natural sloughing process following treatment.

As the gel dissolves slowly in the vaginal fluids its semi-solid structure erodes and the therapeutic effect of KZ in the vaginal lumen and vaginal mucosa permit effective treatment of *Candida albicans* bound to the host cells. The increased residence time of KZ in the vagina due to the thermosetting behaviour of the delivery technology may also limit the systemic delivery of KZ.

KZ is a potent anti-fungal agent and was used for the development of a novel vaginal formulation. The thermosetting formulation was intended to form a gel rapidly and through mechanical means become fixed inside the lumen of the vagina as soon as the formulation temperature reaches 37 ^{o}C [288]. Gelation would initially occur from the outer most surface of the liquid that is in contact with the vaginal mucosa, resulting in entrapment of the remaining liquid that would not as yet have reached the sol-gel transition temperature. However, within a few seconds the liquid should also undergo gelation, ultimately confining the product within the vaginal cavity to exert a prolonged therapeutic effect [339]. The prolonged residence time of the dosage form in the vagina is likely to facilitate the treatment of recurrent candidiasis and may enhance patient adherence to ongoing therapy [301,302].

The development of multiple formulation types for the treatment of a single disorder has become necessary in order to offer patients therapeutic options that are selected on the basis of personal preference, social, economic, environmental and other considerations [284]. Thermosetting vaginal gels containing KZ were manufactured to develop an alternate technology that would add to the array of anti-fungal treatment options that are currently available so as to provide a treatment approach with a compatible and prolonged release technology for patients with the added benefits that aqueous dosage forms provide.

CHAPTER SIX
PREFORMULATION AND PRELIMINARY STUDIES OF A THERMOSETTING VAGINAL GEL FORMULATION

6.1 Design of experiments

Experiments are performed to answer questions and to obtain significant and relevant information to make informed decisions. It is crucial to conduct a sufficient, but minimal number of experiments so as to be cost effective and to generate reliable and relevant data. The "changing a single variable at a time" or COST approach has been used conventionally [344-346] but is inefficient due to the fact that the interactive effects of and between variables are not readily detected and may lead to the identification of a different optimum solution when one factor is changed at a time. Another disadvantage of COST is that numerous experiments are required to be performed in order to generate sufficient data to make informed decisions. Statistical approaches such as the use of experimental design, and design of experiments (DoE) has become popular due to the fact that changes in the product, process and/or experiment can be monitored simultaneously in a systematic manner [347-351].

DoE is an approach that can be used to develop novel products and processes, improve existing products and processes, optimise the quality and performance of a product, optimise manufacturing processes, screen significant factors, reduce pollution and expenses and to investigate the robustness of products and procedures. DoE is used to achieve three experimental objectives, *viz.*, screening, optimisation and robustness testing, and can be used to highlight the sensitivity of a product or process when minor input factor-related changes are made or occur. In pharmaceutical research independent variables that exhibit significant effects on product quality and/or performance are selected during screening studies and their range in the experimental region is identified in relation to achieving a desired outcome with the aid of prior knowledge and experience [344]. Optimisation studies attempt to establish the ideal conditions for product manufacture and performance by taking into consideration the limitations of input variables or factors and meeting demands of the specific product in terms of responses, quality and patient acceptability. Robustness testing permits adjustment of input factors in order to produce a robust product [347]. DoE generally requires conducting a single standard reference experiment that forms the centre point of a design space and subsequent experiments are planned and are located symmetrically around the reference or centre point, as depicted in **Figure 6.1** [352].

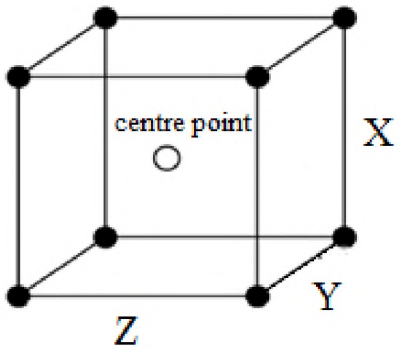


Figure 6.1 Theoretical design space depicting the symmetrical distribution of experimental points around a reference or centre point experiment. Adapted from [347].

6.1.1 Screening

Screening is initial practice in any experimental environment and a DoE may or may not be used for establishing the proposed approach for a screening process. The aim of this methodology is to undertake experiments with a number of input factors or variables in order to determine the influence of the input factors or variables on the outputs or responses for a particular system, if any. In this way, the ranges in which the input variables will ultimately be used are defined and only a few experiments are required in comparison to the number of factors investigated during screening [347,353]. Full or fractional two-level factorial designs are generally used for screening studies due to their efficiency, low number of experiments required and therefore their cost effectiveness [344,354,355].

6.1.2 Optimisation

Optimisation studies involve the use of historical data to predict the output or responses for possible combinations of input variable factors and then elucidate a single or best solution or, in other words, to produce an optimised product formulation and/or process. The optimisation process using DoE should demonstrate the impact that the different input factors exert alone or through interaction on the output or response. Optimisation necessarily requires more experiments than the number of factors to be investigated and some experimental models, such as the Central Composite Design (CCD) [356-362] and Box Behnken Design (BBD) [363-367], are more relevant to optimisation than screening processes as a relatively high number of experiments are required for optimisation [344,368,369].

6.1.3 Robustness testing

The requirements of robustness testing are to establish the range (design space) for input factors within which changes can be made without affecting ultimate product quality and thereby delineating the robustness of the process, and in which small changes in input variable levels result in a negligible change in the relevant product responses. A full-factorial design is recommended for robustness testing

[346,370-373], however the BBD [374], Plackett-Burman [371,375,376] and CCD [375,377] approaches can also be used when comprehensive solutions are required [346,370].

6.1.4 Factorial design

In this chapter, a full-factorial design is described for the optimisation of a solvent system to dissolve KZ as part of formulation development studies. Full factorial experimental designs are the foundation for conventional experimental designs that are used for screening, optimisation and robustness testing [378,379]. The design is referred to as 'full' as all possible combinations of factors are included in the scheme. Two-level full factorial designs are generally used for screening [378,380] due to the relatively low number of experiments required per factor under investigation and the outcome can be improved through the use of composite designs [347] in which centre points are replicated between three to five times in order to account for experimental error and improve the reproducibility of data [381]. When two to four variables are to be investigated full factorial designs are usually preferred whereas if more than four factors are to be evaluated fractional factorial designs are undertaken as fewer experiments are possible [382]. For fractional factorial designs, a fraction of the corner points are investigated and this approach is extensively used for screening and robustness testing [380,383]. A low level value, coded as -1, and high level value, coded as +1, are assigned to factors with a centre point that is coded as 0 [384]. A two-level two factor, also known as 2^2 full factorial design, has a square-shape design space and a three factor two-level or 2^3 full factorial design has a cube-shaped design space [347,380]. A full factorial design with k factors will require 2^k experimental runs and the number of runs required for two, three and four factors in a full factorial design requires 4, 8 and 16 experiments [379,380]. A full factorial design is a feasible research option when investigating up to four variables [379] and when a minimum of five variables are to be investigated fractional factorial and Plackett-Burman designs are preferred, as they require fewer experiments to generate the requisite data [371,375,376]. Investigating five input variables would require eight and 16 experiments for a fractional factorial design of resolution of III and V respectively [379] and two to seven factors evaluated using a Plackett-Burman design would require between 12 and 48 experimental runs [347,385].

6.1.5 Response Surface Methodology

Response Surface Methodology (RSM) studies are generally undertaken following screening so as to evaluate the data generated and identify important and significant factors [380,386]. RSM uses regression models that have finer tolerances when compared to models used during the screening phases of product and process development when using DoE. RSM facilitates mapping of the relationship between critical input factors and key responses. RSM is generally applied to systems in which not more than six factors are to be considered as the number of experiments to be conducted increases dramatically as the number of variables to be considered increase [387]. Statistical designs and other analytical tools are included when designing experiments for data collection using RSM in order to

permit modelling that can elucidate relationships between input variables and the key output responses [388]. An advantage is that the number of responses monitored has no impact on the number of experiments to be performed. Regression analysis is performed to ensure that the best fit model for the data is identified and permits the responses (Y) and input factors (X) to be correlated using mathematical modelling that can be described using the mathematical equation in **Equation 6.1** [388].

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \ldots + \beta_k X_k + \varepsilon \qquad \textbf{Equation 6.1}$$

Where,

β = constants of the coefficients of responses (intercept, linear, quadratic and interaction terms),
k = the number of factors, and
ε = the error.

The best fit model and identified relationships are then presented in graphical and numerical formats that facilitate further examination of the data [387]. In order to optimize formulations in this study, a CCD approach was used as it is useful for optimisation studies [347,384,385,387,389].

6.1.6 Central Composite Design

CCD approach is a symmetrical second-order experimental design derived from a two-level full factorial or fractional factorial model that includes a centre point and several points located at a distance, α, from the centre point [344]. The number of experiments to be performed using a CCD approach can be calculated using **Equation 6.2** [344,390].

$$N = k^2 + 2k + C_p \qquad \textbf{Equation 6.2}$$

Where,

N = number of experiments,
k = number of factors, and
C_p = replication of the centre point.

The number of experimental runs generated from two, three, four and five factors from a CCD are 11, 17, 27 and 45 when three centre point replications are included [390] and α, the distance between the axial points and the centre point, can be calculated according to **Equation 6.3.** Three variables will result in a value for ∝ of 1.68 and α can change according to the type of CCD approach used. Variables are considered at five levels coded as −α, -1, 0, +1, +α and all experiments are conducted using the defined levels [344,390].

$$\alpha = 2^{(k-Cp)/4}$$ **Equation 6.3**

Where,

α = distance from axial point to centre point,
k = number of factors, and
C_p= replication of the centre point.

There are different types of CCD available for use and these include Central Composite Face-centred (CCF) [391], Central Composite Circumscribed (CCC) [388] and Central Composite Inscribed (CCI) designs [347]. The general concept of a CCD model with eight cubic points, six axial points and one centre point is schematically depicted in **Figure 6.2** and CCF, CCC and CCI designs are depicted as two-dimensional (2D) models in **Figure 6.3** [347]. The difference between the CCF and CCC designs is the position of the axial points and the CCI design is a reduced version of the CCC design that is not a widely used model [347,387,392].

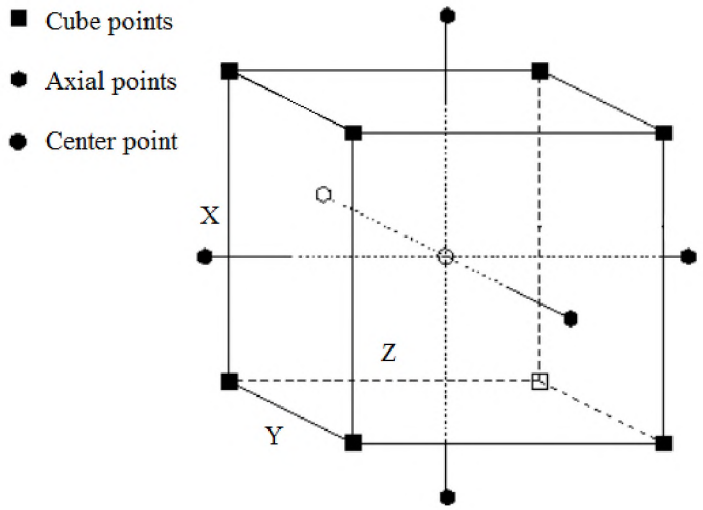


Figure 6.2 Theoretical design space shape of a general CCD model adapted from [347].

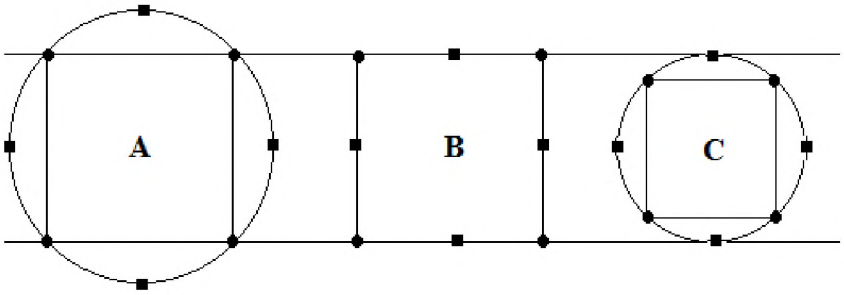


Figure 6.3 Two-dimensional design space models for the CCC (A), CCF (B) and CCI (C) models adapted from [392].

The CCC model consists of three building blocks located in a symmetrical sphere-shaped design space with points from a two-level factorial design, star points located on the factor axes (on the circumference of a circle) and replicated centre points ensure the 'composite' aspect of the design [347]. A CCC model using three factors requires the use of eight corners, six axial and a minimum of three replicated centre points [379]. Five factor levels can be explored using CCC [388] and stronger curvature in the final plots can be used to model any cuboid behaviour of the data [347]. A CCC is performed with the axial points greater than the high and low levels for the input factors, unlike for a CCF in which the high and low values are maintained. Some experiments may not permit the use of values greater than the high and low levels for input variables and the use of a CCF approach would be the preferred model in such cases [391]. In a CCF design the axial points are located on the faces of a cube and the model permits exploration of only three levels for the input factors. The CCF design is inferior to a CCC design as fewer levels of variables can be explored at any time. However, the model is useful for some applications and can be valuable for establishing a quadratic relationship as a number of experiments are conducted. The CCC and CCF models require the same number of experiments to be undertaken and hybrid CCF/CCC models may also be considered if necessary [347,387,392].

6.1.7 Analysis of results using Design Expert®

6.1.7.1 Model fit

Model fitting and mathematical analysis are performed to ensure that statistical models that are developed are adequate to navigate a design space [390]. Model validation of a data set is performed so as to evaluate model fit [393]. Model design evaluation interrogates the statistical properties of a design prior to collection of the data for the responses under investigation. The design can then be evaluated according to the selected order and model. An order is selected according to the terms that should appear in the model and varies according to the requirements of an experiment. If the model fit complies with the validation requirements, the researcher can be confident that the values of individual coefficients in the model approximate to a good degree of covariate effect in the validation settings [393].

The model chi (χ) -square value, also known as lack of fit, is used in Design Expert® and is traditionally used to assess model fit [394-396]. Some discrepancies have been observed over the years regarding the sensitivity of the lack of fit test [397] when small [398] and large [399,400] data sets are used. Therefore, there is no general consensus for the value for the lack of fit df and acceptable values range from 2 [401] to 5 [402].

Power calculations are generally used for continuous response types and factorial designs are used as a measure of the probability of detecting an effect of a specific magnitude. As the number of runs increases, the power of a study increases. The SD of the process indicates the magnitude of the effect. Power calculations are used to judge the capacity of a factorial design and the variance inflation factor (VIF) is a measure of the increase of the variance of the model coefficient due to the lack of orthogonality in the design. The standard error of a model coefficient increases proportionally with the square root of the VIF. VIF is equal to one (1) when the coefficient is orthogonal in relation to the remaining model terms. VIF values > 10 indicate multi-collinearity which leads to poor estimation of coefficients [403]. The multiple correlation coefficient is related to VIF as described in **Equation 6.4**. The coefficient is equal to zero (0) when the design is orthogonal.

$$\text{VIF} = \frac{1.0}{(1-\text{Multiple correlation coefficient})} \qquad \textbf{Equation 6.4}$$

Leverage is referred to as the potential for a design point to affect the coefficients of a model fit according to the position in a design space. Leverage values of 1 indicate that the design point will influence the model. A good model should have leverages < 1 [404] and the addition of runs can reduce the leverage value at each point.

G-efficiency is a term used to compare designs and is a measure of the robustness of that design [405]. G-efficiency is the average prediction variance as a percentage of the maximum prediction variance. G-efficiency is inversely related to maximum variance. Lack of fit runs and replicates reduce the G-efficiency of a model and an acceptable G-efficiency value is $\geq$ 50% [406-408].

6.1.7.2 Analysis of the effects of the response factors on the design model

The Shapiro-Wilk hypothesis test is performed to evaluate the normality of unselected terms on effects plots that include a normal plot, half-normal plot and the Pareto chart in Design Expert® [409]. This test is usually described using a Shapiro-Wilk w-value and a p-value [410]. The null hypothesis is that the unselected terms are normally distributed and, following selection of statistically significant terms, the p-value for the Shapiro-Wilk test will be > 0.10 indicating that the unselected terms follow a normal distribution [409]. The p-value tests the sensitivity of the experiment and a Prob < w is the p-value.

The w-value falls between 0 and 1. Low values for w result in the rejection of normality and a value of 1 is indicative of normality within a data set [411]. There is no standard acceptable w-value reported and values located close to 1 are generally accepted. For the purposes of these experimental investigations values $w \geq 0.90$ were deemed acceptable and considered to indicate the normality of data.

Pareto charts are bar plots that identify critical factors in a model in descending order of significant effects [412]. The Pareto chart is used in conjunction with normal and half normal plots to determine whether the second largest effect observed is significant and if it should be included in the model [413]. Two different t-values are drawn onto the Pareto chart, the Bonferroni or family-wise corrected t and the standard t statistic that represents the individual effects of the test. The Bonferroni limit indicates the lower 50% of observed effects. The aim of using the Pareto chart is to separate large significant, likely to be repeatable effects and small insignificant, likely to be noise effects [414]. The terms located above the Bonferroni limit are mostly likely to be significant and should be included in the model and the terms above the t-limit might be significant and this status can be confirmed through evaluation of normal and half-normal plots.

6.1.7.3 Analysis of model, evaluation of model fit and model graphs for individual responses

Design Expert® included a number of useful statistical data and plots that can be used to analyse the model, evaluate model fit and assist in interpretation and evaluation of the selected model for each response monitored.

ANOVA is performed to calculate adjusted F-values, model p-values, sum of squares, coefficients and an equation for the response under investigation. The ANOVA output is usually described in terms of p- and F- values and the p-value is also Prob > F that is the probability of observing a F-value if the null hypothesis is true. The F-value is used to compare an individual mean square of an experiment to the value for the residual mean square. The probability equals the proportion of the area under the curve for the F-distribution that lies beyond the observed F-value. The F-distribution is determined by the df associated with the variances under comparison. There are no factor effects when the hypothesis is true and a $p < 0.05$ indicates the presence of insignificant model terms whereas a $p > 0.1$ indicates that the model terms are not significant. Significant model terms are considered to have a real effect on the response(s) monitored [415].

Diagnostics such as residual versus predicted, residual versus run, predicted versus actual, Box-Cox for power transformations and residuals versus factor plots are used to evaluate model fitting for each response. Perturbation, one-factor, all factors, interaction, contour and 3D response surface plots

provide a visual display of models in order to assist interpretation and evaluation of the design space. Some of the statistical data and plots will be explained in detail in **§ 6.2.3**.

6.2 Preformulation studies

6.2.1 Introduction

An ideal dosage form would be comprised of an API and a variety of excipients that exhibit no excipient-excipient or excipient-drug interactions [416]. Although clinically inactive, excipients can and do affect all aspects of drug and dosage form performance as they are pharmaceutically active [417]. For example, viscosity modifiers in topical formulations affect the dissolution and bioavailability of drugs. Preformulation studies are a series of experiments undertaken to generate data to enable the selection of excipients that are best suited for use in formulations to be developed and are critical in any dosage form development process. The benefits of conducting preformulation studies include the reinforcement of scientific information for regulatory purposes and guidelines, preservation of resources during formulation development and assessment studies, protecting the community from inappropriate formulations, improving the ultimate quality of formulations, simplification of the application and regulation of novel dosage forms and facilitation of regulatory decision making [418]. Potential physical or chemical interactions between an API and excipients in drug-excipient mixtures can be detected early and decrease the risk of chemical degradation, instability, changes in bioavailability, inefficacy and lack safety when using a formulation following development [419]. The use of thermo-analytical and spectroscopic techniques for undertaking compatibility studies is common during the formulation development phase of product development studies. DSC and FTIR spectroscopy are widely used for preformulation compatibility testing and interaction studies and were applied to the evaluation of compatibility of KZ and potential excipients [416,420,421]. DSC is used to monitor the difference in heat flow that is generated or absorbed by the sample under investigation. The purity and polymorphic nature of a compound can also be elucidated using DSC [422]. The FTIR spectrum of a compound is indicative of bond breaking or formation in drug-excipient mixtures. The aim of compatibility studies is to establish the potential for excipient-drug interactions prior to undertaking expensive product development studies, in this case the development of a formulation of a thermosetting vaginal gel [423].

In order to improve the solubility of poorly water soluble compounds, solubility enhancers are often used so as to ensure that the administration of a therapeutically effective dose is possible. However, the use of high concentrations of solubility enhancers may be harmful to human cells particularly when in contact with sensitive tissues. The vaginal mucosa is a sensitive human tissue located in a well-controlled biological environment and any formulation for use in the vagina must at least be pH balanced with a low volume of solvent and minimal number of excipients to avoid irritation. In this way toxicity would be unlikely and an appropriate amount of preservative and/or antioxidant may also be

necessary to ensure the long term stability of liquid dosage forms [424]. KZ was dissolved in a quaternary solvent system that was adapted from the solvent system designed by Kovács et al., [424]. The solvent system included a buffer, co-solvent and surfactant in which the solubility of KZ was greater than in each of the individual solvents [424].

6.2.2 Differential Scanning Calorimetry and Fourier Transform-Infrared analysis of excipients

Excipients were selected for evaluation based on the composition of commercially available KZ formulations (**Table 5.2**) and those used in the co-solvent system used for KZ proposed by Kovács et al., [424]. Therefore poloxamers L127, L68 and L87 were used to modulate the thermosetting behaviour of the gel, and citric acid, DHP, ethanol and polysorbate 80 were used for the solvent system, and sodium metabisulphite (SM) was included as an antioxidant and to prolong the shelf-life of the gel. No sample preparation was performed prior to DSC and FTIR analysis as size reduction following grinding and/or milling might reduce solvent content and cause shifts in the DSC thermograms [425]. The FTIR spectrum and DSC thermogram for KZ is depicted in **Figures 1.7 and 1.8.** In addition to individual analysis the compounds were mixed in a 1:1 m/m ratio with KZ and these binary mixtures analysed [426]. The thermal compatibility of KZ in the solvent system, in addition to the different grades of poloxamer and SM, was also assessed. Potential incompatibilities were identified by establishing if changes in the melting endotherm of KZ in the presence of excipients had shifted and/or changed [426]. The disappearance and/or shift of the melting endotherm may be due to an interaction between KZ and that excipient; however, this is not a definite measure of incompatibility as the differences may be due to an analytical artefact or due to dilution of the KZ powder [426,427]. Only real time long-term stability studies would be required so as to rule out definitely any incompatibility. In the case of formulations in which excipients and KZ would be subjected to heating during the manufacturing process the DSC studies would also therefore be useful. Nevertheless DSC studies provide a good indication of the reactivity and potential instability of KZ that may arise over the long term and as such major changes in the DSC thermograms of binary mixtures should not be observed [419]. FTIR was used as an additional tool to provide further evidence of potential KZ-excipient incompatibility [427]. Minor changes in DSC thermograms may be acceptable provided that the FTIR spectrum for the combination does not indicate any additional bands when compared to the individual spectrum for KZ and the excipient [427-429]. FTIR spectroscopy was performed on 1:1 m/m mixtures of KZ and each potential excipient and the spectra compared to the spectrum for KZ and the individual excipient in order to check for excipient-drug compatibility [430,431].

6.2.2.1 Excipients

All excipients used in these studies appear in the FDA Inactive Ingredients Guide, are Generally Recognized as Safe (GRAS) and have been used in commercially available vaginal formulations [280,432]. In addition all materials were at least of analytical reagent grade, where appropriate.

6.2.2.1.1 Citric acid

Citric acid monohydrate occurs as colourless crystals or as an anhydrous white crystalline powder. Anhydrous citric acid was obtained from PAL chemicals (Dorking, Surrey, England). Citric acid is a weak organic acid that has three pKa values at 3.14, 4.76 and 6.40 and is generally used in the food, and the cosmetic and pharmaceutical industries [433,434]. Approximately 1.45 mg citric acid is soluble in 1 mL of water at 20 °C and 0.1 M citric acid in aqueous solution at 25 °C exhibits a pH of 2.2 [433]. Citric acid was considered as a suitable acidifier and buffering agent for use in the solvent system required to dissolve KZ [280] and has been used in vaginal formulations in concentrations between 0.3% m/m and 2.0% m/m [280]. The dissolution of KZ in the presence of citric acid was reported to be 8 times higher at pH 6.0 under non-sink conditions, when compared to KZ in solution alone [435]. Therefore, citric acid is a potentially useful candidate for inclusion in a formulation for KZ delivery.

6.2.2.1.2 Disodium hydrogen phosphate

DHP occurs as a white powder (anhydrous) or as colourless crystals (hydrated forms). DHP is commonly referred to as sodium phosphate dibasic in patient leaflets [432] and was purchased from BDH Chemicals Ltd (Poole, Dorset, England). The pKa of DHP is 12.32 and the solubility is 93 g in 1 L of water at 20 °C [436,437]. DHP was used as a buffer component to prepare the citrate-phosphate buffer that was evaluated in these studies. DHP is incompatible with alkaloids, antipyrine, chloral hydrate, lead acetate, pyrogallol, resorcinol, calcium gluconate and ciprofloxacin [438,439].

6.2.2.1.3 Polysorbate 80

Polysorbate 80, also known as Tween 80 or polyoxyethylene 20 sorbitan monooleate, is commonly used in cosmetic, food and pharmaceutical products as a surfactant and solubiliser [440]. Polysorbate 80 was donated by Aspen® Pharmacare (Port Elizabeth, Eastern Cape, South Africa). Polysorbate 80 occurs as a yellow viscous liquid and was used as the solubilizing agent for the development of this formulation as it has been used in other vaginal formulations in quantities between 1% and 15% m/m [280].

6.2.2.1.4 Poloxamer L127, L68 and L87

Poloxamer grades L127, L68 and L87 are also known as Lutrols and were used to modulate the gelling behaviour of the gel. Poloxamers occur as white granules or in the micronized form as white powders and are used in amounts of between 15 and 50% m/m depending on their molecular mass. The general

chemical structure of the poloxamers is depicted in **Figure 6.4**. Poloxamers are comprised of PEO (a) and PPO (b) and the proportions of a and b in commercial materials summarized in **Table 6.1** [438].

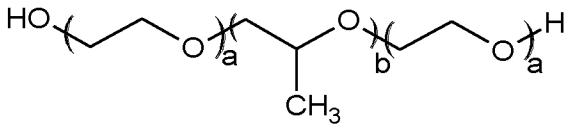


Figure 6.4 General chemical structure of poloxamers.

Table 6.1 Lutrol grade and corresponding block copolymer composition.

Lutrol	Poloxamer	PEO (a)	PPO (b)
L127	407	101	56
L68	188	80	27
L87	237	64	37

6.2.2.1.5 Sodium metabisulphite

SM is commonly known as sodium disulphite or sodium pyrosulfite and was used as an antioxidant. The material was purchased from NT laboratory supplies (Pty) Ltd (Johannesburg, Gauteng, South Africa). SM has been used in topical formulations in concentrations of 0.01 to 1.0% w/v and at a level of 27% w/v in an intramuscular injection formulation [438]. SM has also been used as a preservative and exhibits antimicrobial activity in acidic media [438].

6.2.2.2 Results and discussion

A DSC thermogram represents property changes of a sample as temperature increases [441] in terms of the heat flow associated with those transitions [442]. Crystallisation and melting of KZ in the absence and presence of potential excipients were assessed using DSC. Any changes in the thermogram and/or melting points of materials may suggest changes in the purity of the sample which may be expected in the case of samples in binary or ternary mixtures. Enthalpy changes provide information about the crystal lattice structure of a compound in the sample prior to and after addition of another compound to the sample. Changes in the crystal lattice structure suggests that the compound may be more or less solubilised in the sample under investigation.

The absence of a peak from any compound(s) present in the sample may infer that the compound was completely dispersed in the molten state of the other excipient of lower melting point and is not detected during DSC analysis [443]. Changes in enthalpy or an absence of peaks may suggest that an incompatibility exists between the components of the sample. Therefore, the FTIR spectrum of KZ and sample mixtures was generated in order for any changes in the thermogram to be attributed to either an incompatibility between the compounds or the solubility of the compound in the sample matrix. A decrease in the enthalpy for KZ in the thermogram or the FTIR spectrum of KZ is altered and major or

foreign peaks arise or change and a less crystalline structure of KZ may be present indicating that KZ is partly solubilised in the sample. However, the absence of a peak for KZ in the thermogram and the presence of major frequency bands in the FTIR spectrum without the formation of additional peaks may indicate that KZ is solubilised in the sample. Another consequence could be the complete degradation of KZ; however, following the FTIR spectrum major KZ bands observed indicates that KZ was still present in the mixture.

Evaluation of the thermogram and FTIR spectrum is essential during preformulation studies to detect potential incompatibilities in binary mixtures of KZ and excipients and to identify the impact of individual components prior to use in formulation development studies.

6.2.2.2.1 Citric acid

The DSC thermogram for citric acid was generated as described in **§ 1.2.16** and is depicted in **Figure 6.5**.

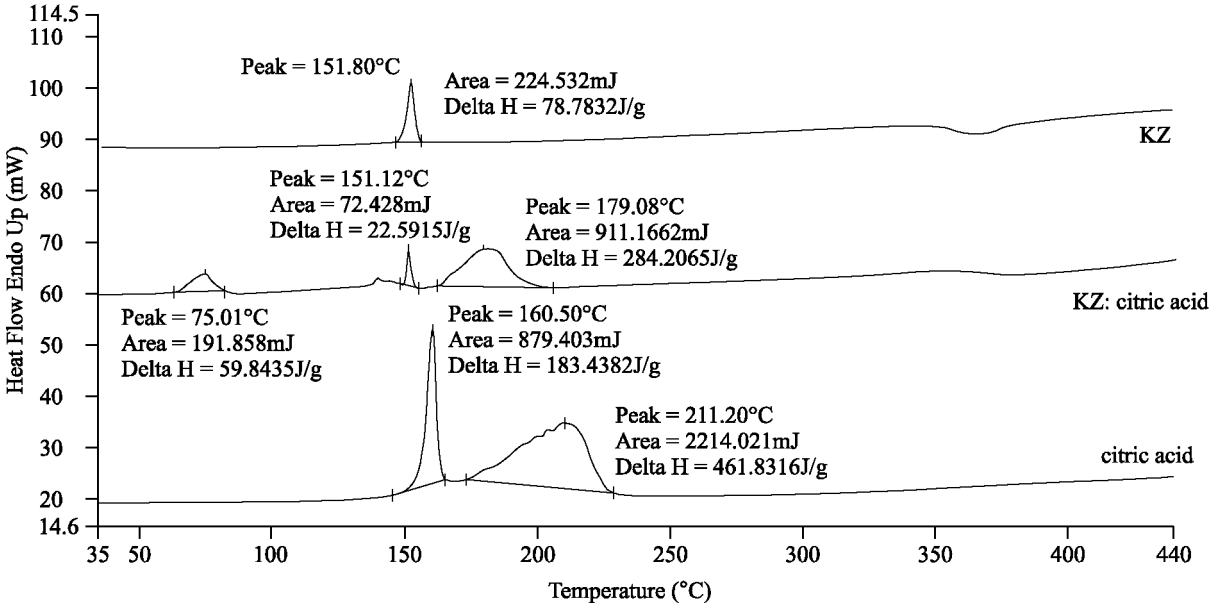


Figure 6.5 DSC thermograms for KZ, citric acid and a 1:1 mixture of KZ and citric acid generated at a heating rate of 10°C/min.

Citric acid melts at 160.50 °C, with the onset of melting at 145 °C indicated by the presence of a sharp endothermic peak with an enthalpy of 183.44 J/g. The broad endothermic peak observed at 211.20 °C with an onset of melting at 170 °C may be due to decomposition of citric acid to form several degradation products as the shape of the peak is ragged and the enthalpy is 461.83 J/g and is similar to previous reports [444,445]. The DSC thermogram generated following analysis of a 1:1 binary mixture of KZ and citric acid reveals the presence of a broad endotherm at 75.01 °C, with an onset of melting of 70 °C. The melting endotherm for KZ can be observed at 151.12 °C which is a slight 0.68 °C shift

with a lower enthalpy of 22.59 J/g. The degradation peak observed for citric acid has also shifted to 179.08 °C with an enthalpy of 461.83 J/g. The difference between the melting peaks of KZ and citric acid is approximately 10 °C and the slight shift observed for the melting endotherms in combination may be due to a thermally induced reaction and/or an interaction between the compounds in a molten state. Acid catalysed degradation of KZ results in the formation of 1-(4-phenyl) piperazine (MM = 489.39). However, as only a low concentration of citric acid will be used in the thermosetting KZ formulation (≤ 5% m/v) and the potential incompatibility of KZ in the presence of citric acid (1:1 m/m) is not necessarily a cause for concern, citric acid may be included provided long term real time stability studies are conducted. Furthermore, the extra peak observed in the thermogram had an onset temperature of melting of 70 °C and as the vaginal gel is designed to set at body temperature and manufacturing would be conducted at low temperatures the possibility of degradation may be minimal during and after manufacture.

FTIR studies were undertaken to further assess the compatibility of KZ and citric acid and augment the data generated from DSC studies. The FTIR spectra for KZ, citric acid and a 1:1 m/m mixture of KZ are depicted in **Figure 6.6**.

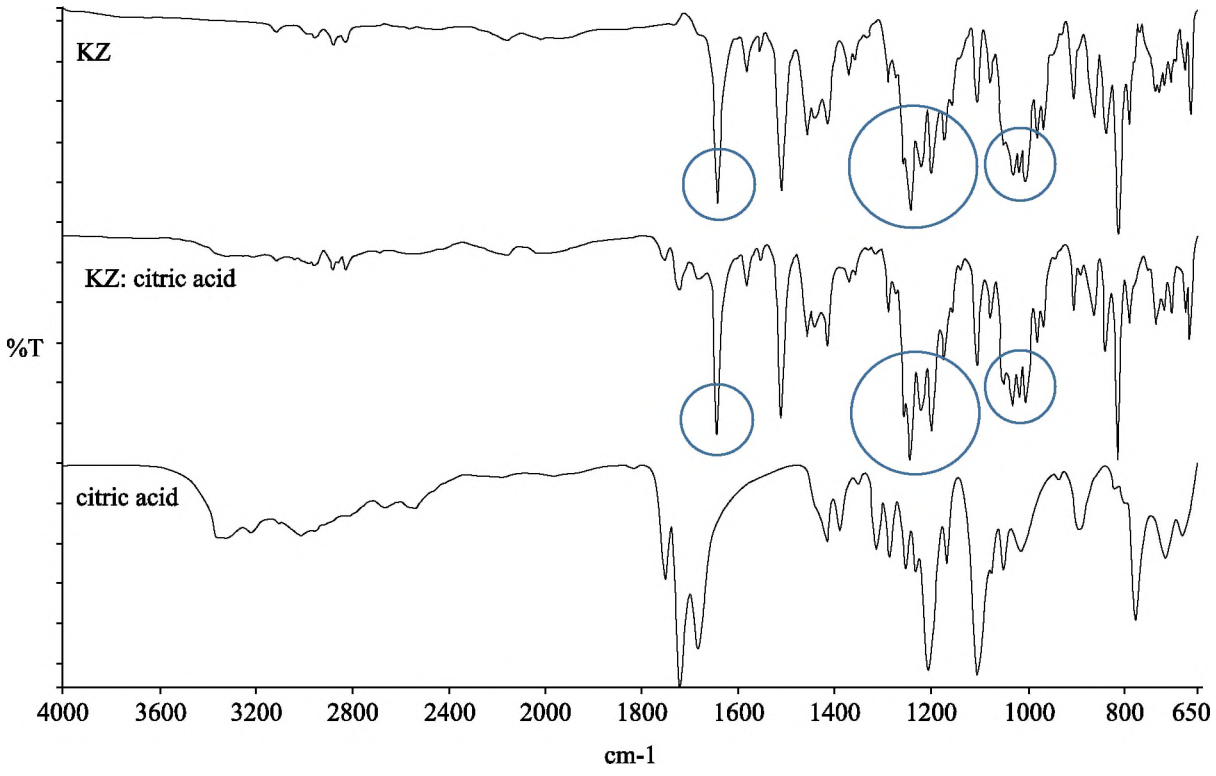


Figure 6.6 FTIR spectra for KZ, citric acid and a 1:1 mixture of KZ and citric acid.

The FTIR spectrum for citric acid reveals the presence of resonance from a wavenumber of approximately 900 cm^{-1} and between 2500 cm^{-1} and 3300 cm^{-1} that represent –OH bends and stretches

associated with carboxylic acid functional groups. The C-H stretch associated with alkane functional groups occurs at 1400 cm^{-1}. The C=O resonance from carboxylic acid functional groups are located between 1600 cm^{-1} and 1800 cm^{-1}. The FTIR spectrum for citric acid was consistent with a previously reported spectrum [446]. No chemical bond shifts were observed for the physical combination 1:1 mixture of KZ and citric acid and the major peaks for KZ were clearly evident in the spectrum. The band observed at a wavenumber of 825 cm^{-1} is due to the C-Cl bond. The bands observed between 1000 cm^{-1} and 1300 cm^{-1} are a result of C-O stretches and aromatic amine C-N stretches respectively [41]. The bands observed between 1400 cm^{-1} and 1500 cm^{-1} are a consequence of C-C aromatic stretches associated with the imidazole, benzene and phenol rings. The sharp peak observed from 1600 cm^{-1} to 1700 cm^{-1} is due to carbonyl C=O stretching. The additional peaks observed between 1700 cm^{-1} and 1800 cm^{-1} are due to the prominent C=O bond stretching associated with the carboxylic acid functional groups in citric acid. It is clear that the data depicted in **Figure 6.6** suggest KZ and citric acid are compatible when assessed as a 1:1 m/m physical mixture. Following the compatibility assessment of 1:1 binary mixtures of KZ and citric acid using DSC and FTIR, the compounds could be used in the same formulation, but these analyses were undertaken in the solid state and the intended formulation is a gel and therefore further studies may be necessary. Citric acid has been used as an excipient in a number of KZ formulations that include Xolegel® [168], foams [447], suspensions [448], shampoos [449], cyclodextrin inclusion complex formulations [450] and in vaginal tablets [451].

6.2.2.2.2 Disodium hydrogen phosphate

DHP is polymorphous and occurs as a crystalline anhydrous material that is hygroscopic and may absorb moisture. The DSC thermogram for DHP depicted in **Figure 6.7** reveals the presence of an endothermic peak at 79.95 °C that may be due to fusion of the dodecahydrate form that has an enthalpy of 103.17 J/g [190,452]. The peak observed at 343.93 °C with an enthalpy of 135.28 J/g may be due to the formation of sodium pyrophosphate [453]. The peak observed at 411.09 °C with an enthalpy of 7.74 J/g may be due to the formation of degradation products. The DSC thermogram for the 1:1 mixture of KZ and DHP reveals the presence of the melting endotherms for KZ and DHP at 151.71 and 76.78 °C and the endotherms at 343.93 and 411.09 °C for the thermogram for DHP were not evident for the mixture. It is possible that an interaction between KZ and DHP may have occurred at higher temperatures. However, endotherm for KZ shifted to a lower temperature by 0.09 °C with an enthalpy of 67.84 J/g and the first endotherm for DHP shifted by 3.17 °C to a lower temperature. These slight shifts may be due to the compounds being analysed in a binary mixture and not analysed alone and therefore this does not necessarily indicate an incompatibility between the two compounds.

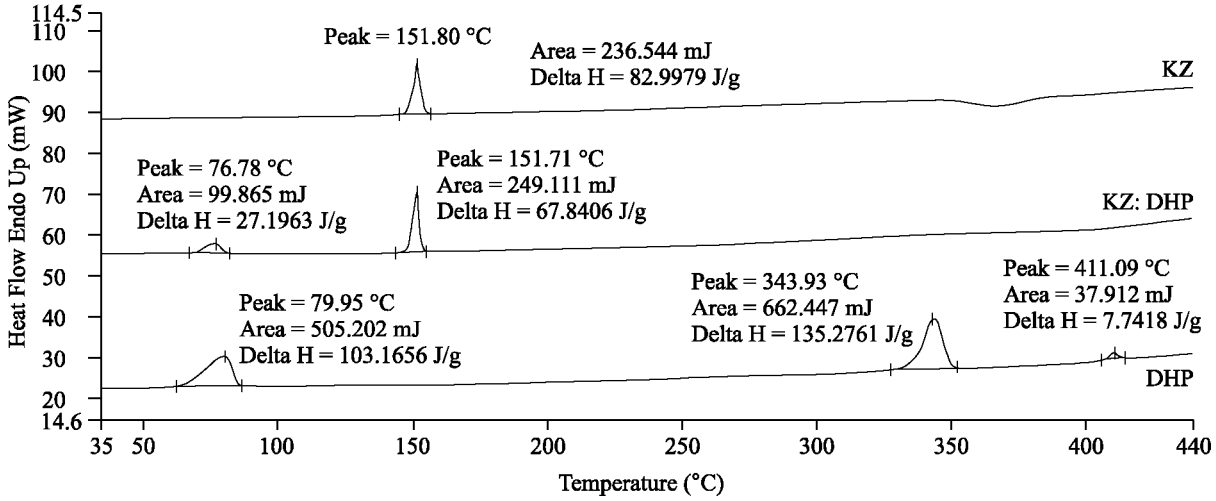


Figure 6.7 DSC thermograms for KZ, DHP and a 1:1 mixture of KZ and DHP generated at a heating rate of 10°C/min.

FTIR analysis was performed on similar samples in order to augment the data generated using DSC and the FTIR spectra for KZ, DHP and a 1:1 mixture are depicted in **Figure 6.8**.

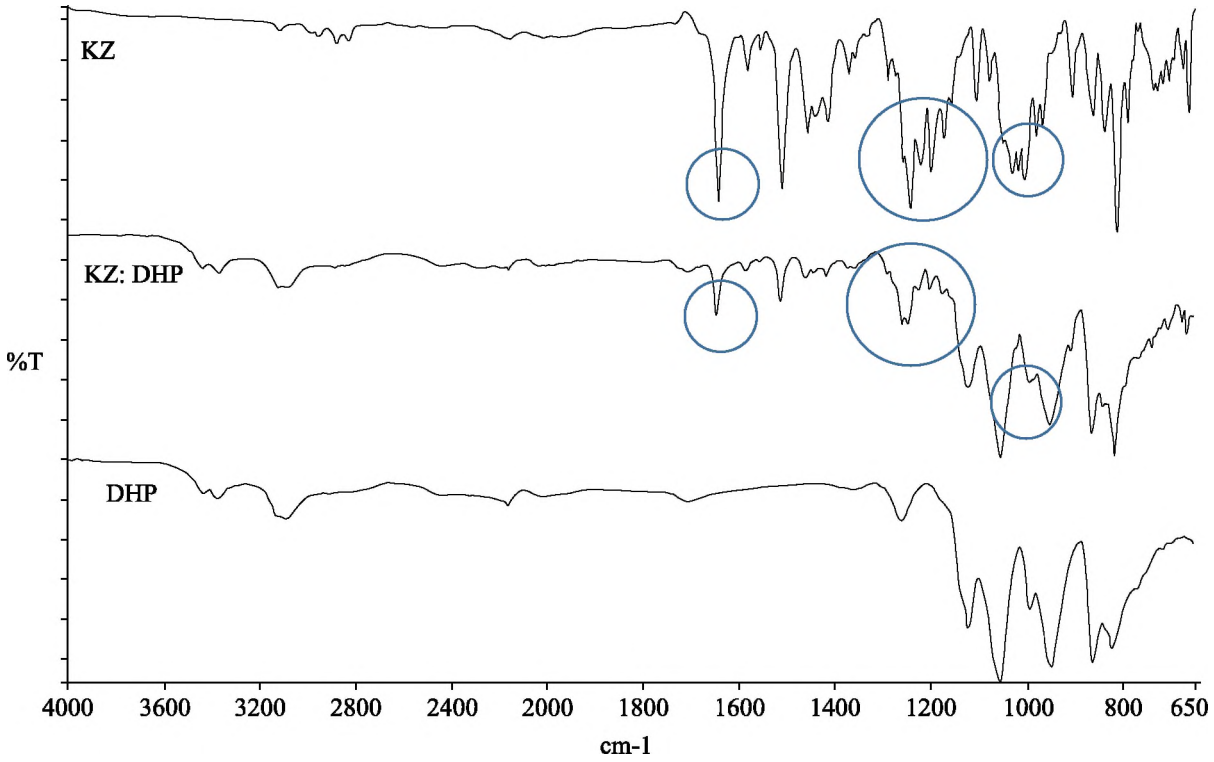


Figure 6.8 FTIR spectra for KZ, DHP and a 1:1 mixture of KZ and DHP.

The peaks due to PO-H bending are observed between 900 cm^{-1} to 1000 cm^{-1}, P-O stretching at 1050 cm^{-1}, P=O stretching at 1250 cm^{-1} and O=P-OH deformation vibrations at 1700 cm^{-1}. In addition P-OH symmetrical and asymmetrical stretching occurs between 2000 cm^{-1} to 2500 cm^{-1} and 3000 cm^{-1} to 3100 cm^{-1} respectively. The FTIR spectrum for DHP is consistent with reported data [454] and for the mixture of KZ and DHP all major peaks for DHP were observed in addition to resonance for a C-Cl group at 825 cm^{-1}, an aromatic amine C-N stretch between 1100 cm^{-1} and 1300 cm^{-1} and for the C=O from the carbonyl group for KZ. The spectrum for the 1:1 mixture reveals the presence of the spectral bands of DHP and they are more prominent than the KZ bands and therefore shield some of the peaks for KZ. No additional peaks were formed suggesting that there is no reaction between KZ and DHP and that the compatibility of KZ and DHP can be assumed until long term real time stability are generated.

6.2.2.2.3 Polysorbate 80

Polysorbate 80 is a liquid and forms a paste when mixed with powdered KZ which may result in KZ existing in an amorphous and/or crystalline form [7,41,455]. The flash point of polysorbate 80 is 149 °C and a small peak is observed at 145.74 °C in the thermogram depicted in **Figure 6.9** [438]. The peaks observed between 375.00 to 420.00 °C in the DSC thermogram for polysorbate 80 may be due to thermal degradation. The absence of a melting point endotherm for KZ in the mixture of KZ and polysorbate 80 may indicate an incompatibility between the two compounds or that KZ changed from a crystalline to an amorphous form and was completely dissolved in the polysorbate 80 [7,41,455,456].

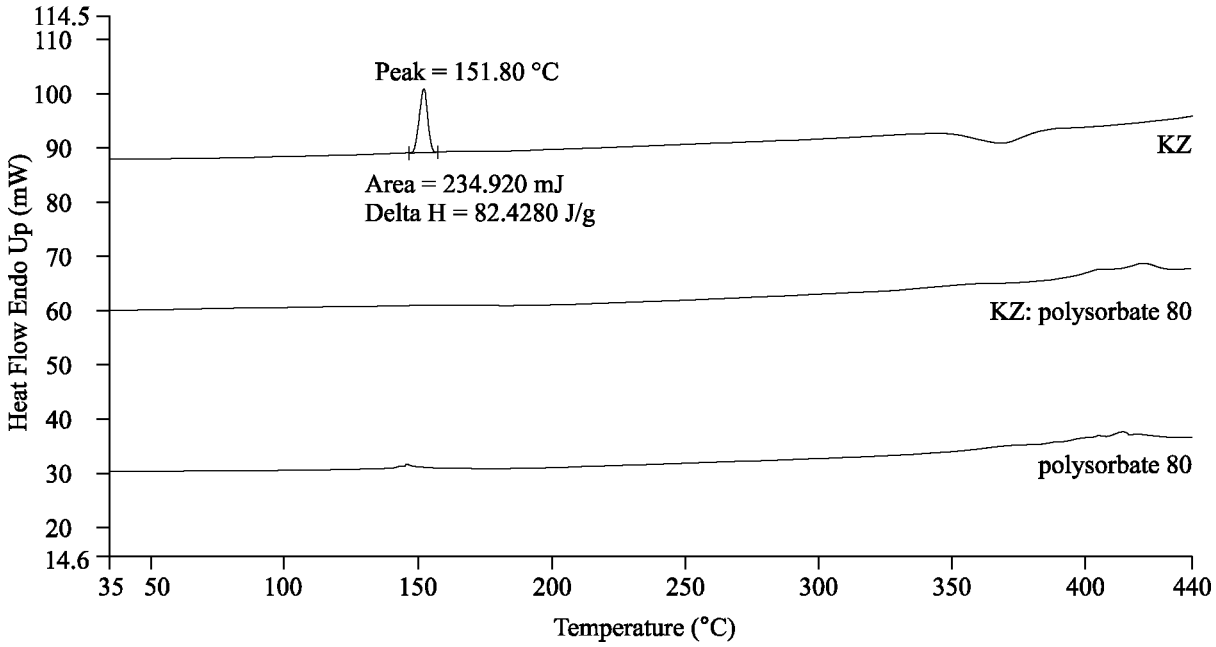


Figure 6.9 DSC thermograms for KZ, polysorbate 80 and a 1:1 mixture of KZ and polysorbate 80 generated at a heating rate of 10°C/min.

The FTIR spectrum for the mixture is a useful tool to establish the presence of KZ in that mixture. The FTIR spectrum for polysorbate 80 depicted in **Figure 6.10** reveals a strong C-O stretch at 1100 cm^{-1} that represents alcohol, ester and ether functional groups. The band at 1700 cm^{-1} is due to a C=O stretch for carbonyl functional groups; the bands at approximately 2800 cm^{-1} are due to the C-H stretch for alkanes and the broad band between 3200 and 3600 cm^{-1} represent –O-H group resonance in the alcohol functional groups. The FTIR spectrum for the physical mixture of KZ and polysorbate 80 reveals the presence of bands for KZ and polysorbate 80. The main signals for KZ are prominent in the spectrum and are due to the resonance of the carbonyl functional group at a wavenumber between 1600 and 1700 cm^{-1}, the C-C aromatic stretch between 1400 cm^{-1} and 1500 cm^{-1} and the aromatic amine functionality between 1200 and 1300 cm^{-1}. Since the main polysorbate 80 and KZ signals are present in the spectrum for a the physical mixture, it can be assumed that no apparent chemical reaction, bond formation or breakage had occurred when KZ and polysorbate 80 were combined.

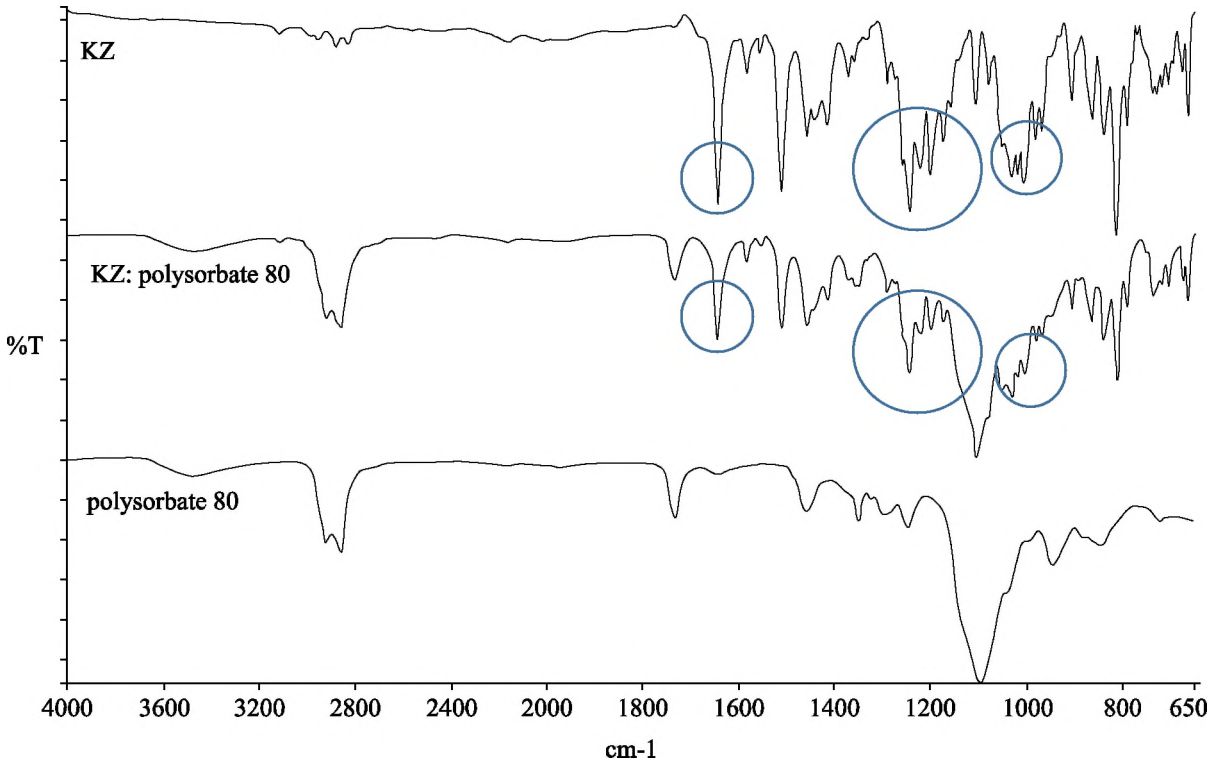


Figure 6.10 FTIR spectra for KZ, polysorbate 80 and a 1:1 mixture of KZ and polysorbate 80.

6.2.2.2.4 Poloxamers

The DSC thermograms for KZ, physical mixtures of KZ and poloxamers and the pure poloxamers are displayed in **Figure 6.11**.

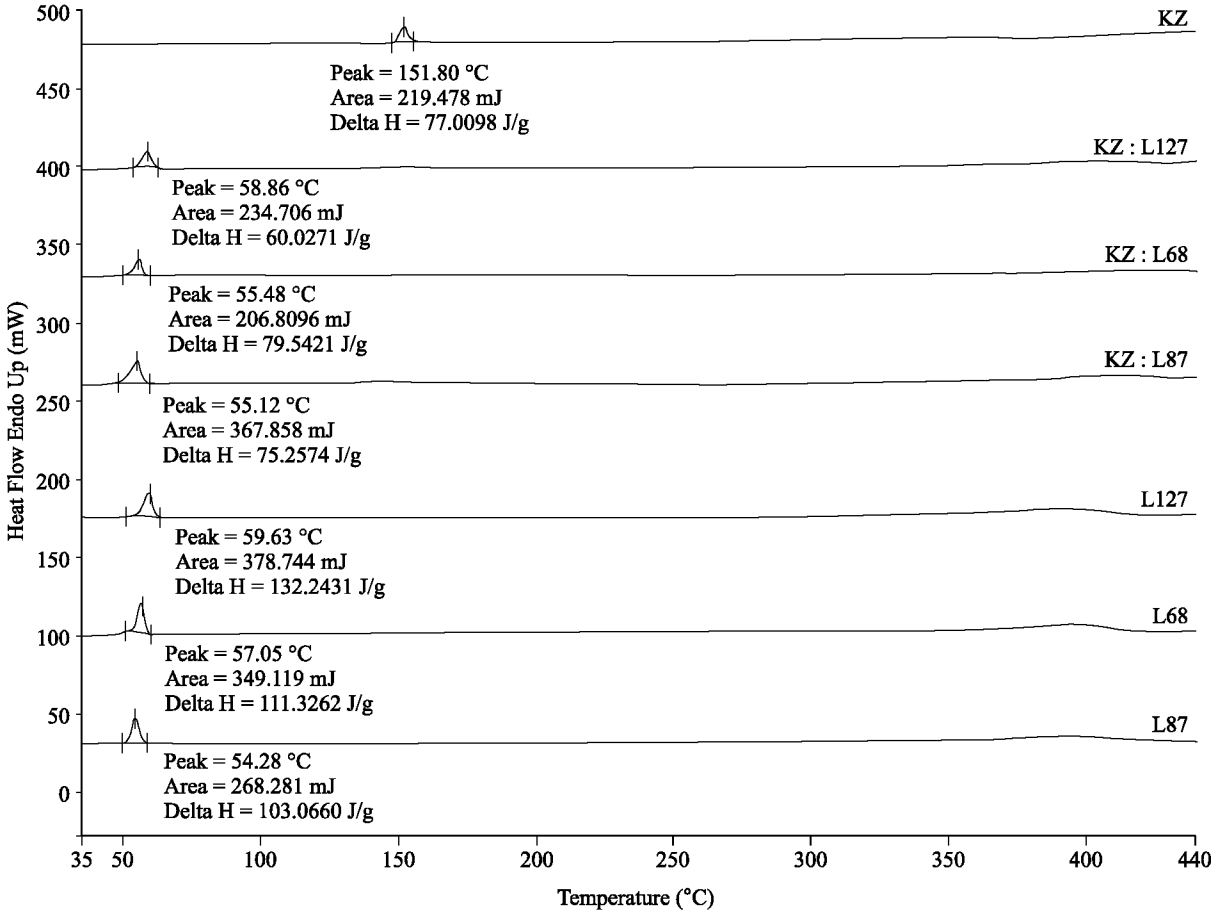


Figure 6.11 DSC thermograms for KZ, poloxamer and 1:1 mixtures of KZ and poloxamer generated at a heating rate of 10°C/min.

As expected for 1:1 mixtures of KZ and L127, L68 and L87 the melting point endotherm for the poloxamers shifted by ± 2 °C. The melting endotherm for KZ disappeared completely in all thermograms of mixtures that may be due to a change from a crystalline to amorphous form of KZ, as was observed when KZ and polysorbate 80 were mixed (**Figure 6.10**) or alternatively KZ may have dissolved in the molten poloxamer [7,41,455,456]. The use a smaller quantity of poloxamer in the binary mixture may have resulted in the presence of some crystalline KZ in the mixture, which in turn would be evident on the thermogram [41]. Landge et al. reported that the endotherm for pure KZ was due to the presence of the crystalline form of KZ [227].

The FTIR spectra for pure L127, L68, L87 and a combination of L127:L68:L87 is depicted in **Figure 6.12**. The individual spectra for L127, L68 and L87 alone are identical to that observed when the 1:1:1 m/m mixture was analysed. The poloxamers contain an antioxidant BHT that exhibits a C-H aromatic stretch at 2900 cm^{-1}, a C-C in-ring aromatic stretch between 1400 cm^{-1} and 1500 cm^{-1} and a C-H aromatic bend at 850 cm^{-1}. The bands representing the functional groups within the molecular structure of the poloxamer occur at 2900 cm^{-1} for medium C-H alkane stretching, between 1200 cm^{-1} and 1400 cm^{-1} for the C-H rock and C-H bend medium bands and the prominent band at 1100 cm^{-1} for the C-O alcohol and ether functional groups. The FTIR spectrum for KZ was compared to that of the combination of poloxamer rather than the spectra of the individual poloxamer.

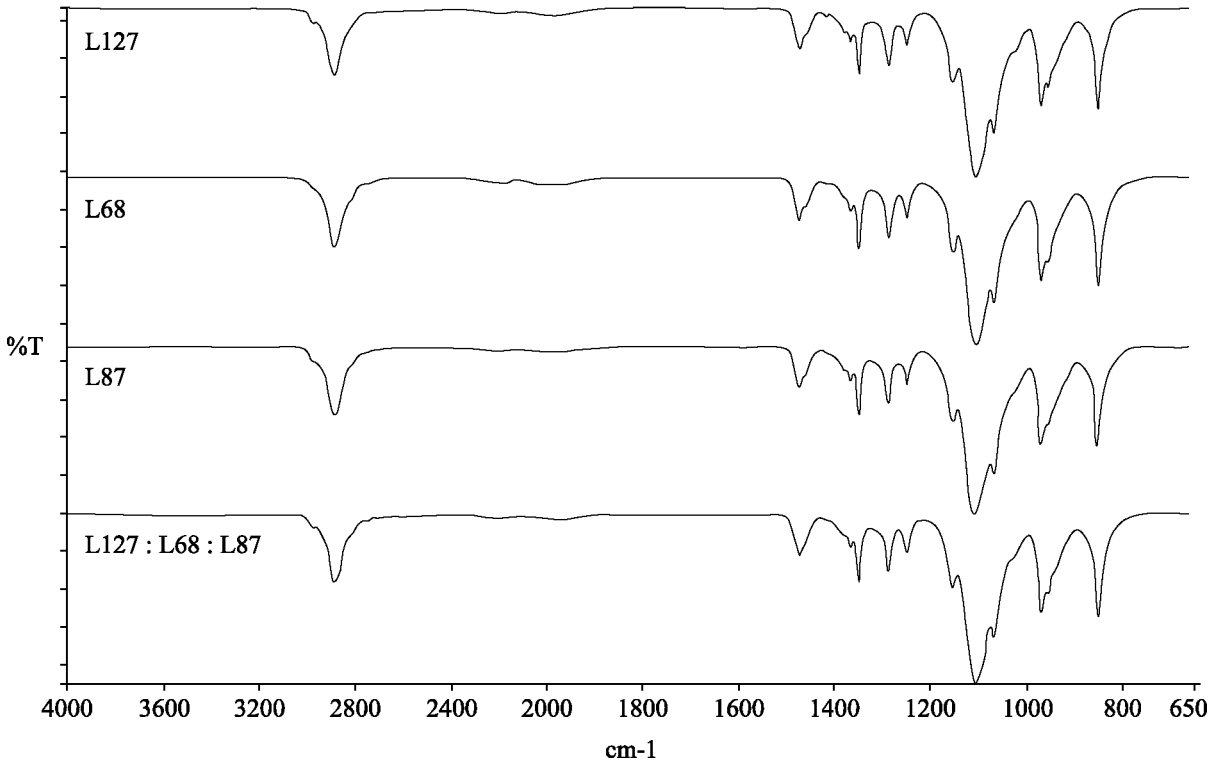


Figure 6.12 FTIR spectra for pure L127, L68, L87 and a ternary mixture of L127, L68 and L87.

The FTIR spectra for KZ, a mixture of poloxamers and a 1:1 m/m mixture of KZ and the poloxamers are depicted in **Figure 6.13**. The bands representing the major functional groups in the KZ structure are evident in the spectra for the KZ poloxamer mixtures. The aromatic C-H stretch between 2800 and 3200 cm^{-1} was shielded by the C-H alkane stretch in the poloxamer and no chemical shifts in the bands were observed. Therefore, a 1:1 physical mixture of KZ and the poloxamers does not appear to exhibit chemical incompatibility as has been reported [41].

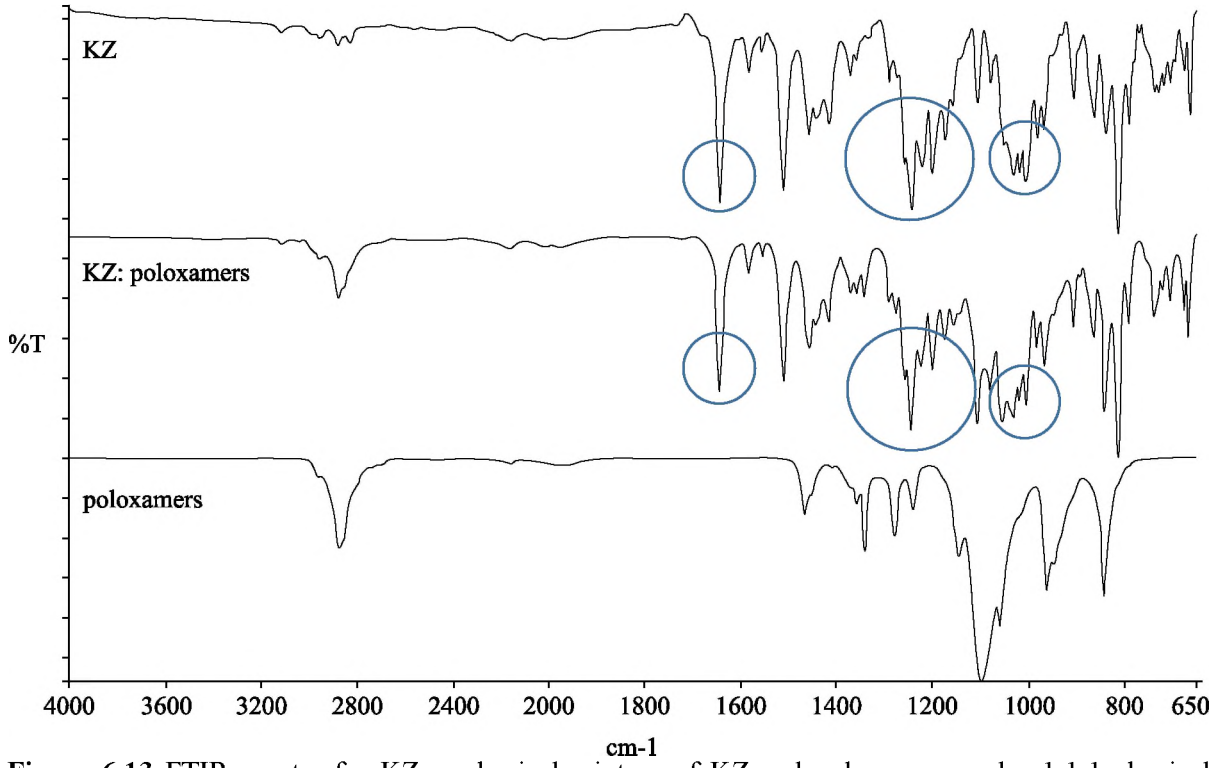


Figure 6.13 FTIR spectra for KZ, a physical mixture of KZ and poloxamers and a 1:1:1 physical mixture of only poloxamers.

6.2.2.2.5 Sodium metabisulphite

SM melts and decomposes at 150 °C [438] and this can be confirmed when evaluating the thermogram for the compound depicted in **Figure 6.14** in which the onset of the melting endotherm commences at approximately 150 °C. A 1:1 physical mixture of KZ and SM exhibits a melting point of approximately 151 °C for KZ albeit with an enthalpy one quarter of that for the pure compound. The melting point of SM increased from 176.54 to 208.23 °C with associated enthalpies reducing from 265.18 to 183.28 J/g. A small kink is observed on the broad endotherm that was not observed in the thermogram for pure SM that may be due to the formation of different polymorphs of SM that coexist and on heating overlap with the broad melting endotherm for SM with that for pure SM. The shift of the SM melting endotherm on addition of KZ resulted in an endotherm that was overlapped by the larger melting point endotherm that was of different intensity. KZ was considered to be compatible with SM as the melting point of KZ in the mixture was the same as for pure KZ. No peaks were observed at < 100 °C and it can be assumed that no degradation of KZ may occur during the manufacture of the vaginal gel and instability studies.

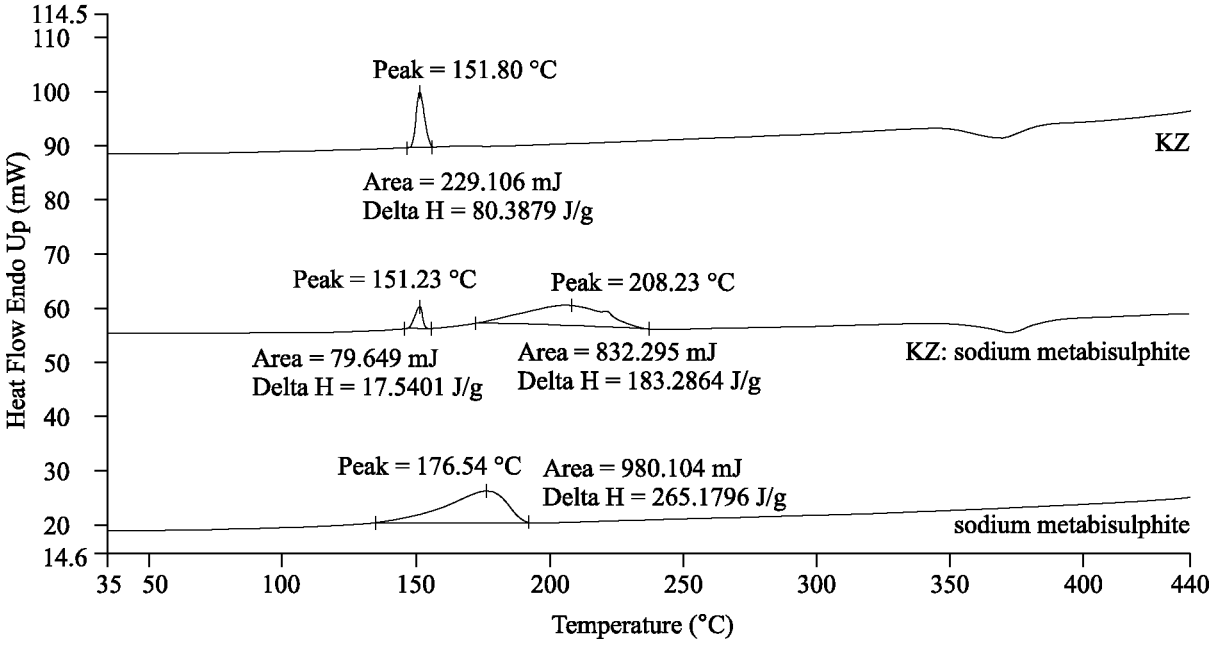


Figure 6.14 DSC thermogram for KZ, sodium metabisulphite and 1:1 mixture of KZ and sodium metabisulphite, generated at a heating rate of 10°C/min.

The FTIR spectra for KZ, a binary mixture of KZ and SM and pure SM, are depicted in **Figure 6.15**. The spectrum of pure sodium disulphite exhibits a very strong band for the symmetrical S=O stretch at 1150 cm^{-1}. The bands at 950 cm^{-1} and 1100 cm^{-1} may be due to sodium ions that are bound to different sulphur atoms and the disulphide (S-S) stretch is not visible in the IR range of the electromagnetic spectrum. The data generated correlates with the reported data [457]. The FTIR spectrum of a combination of KZ and SM revealed the presence of all signals for KZ and therefore suggests that there was no chemical interaction between KZ and SM.

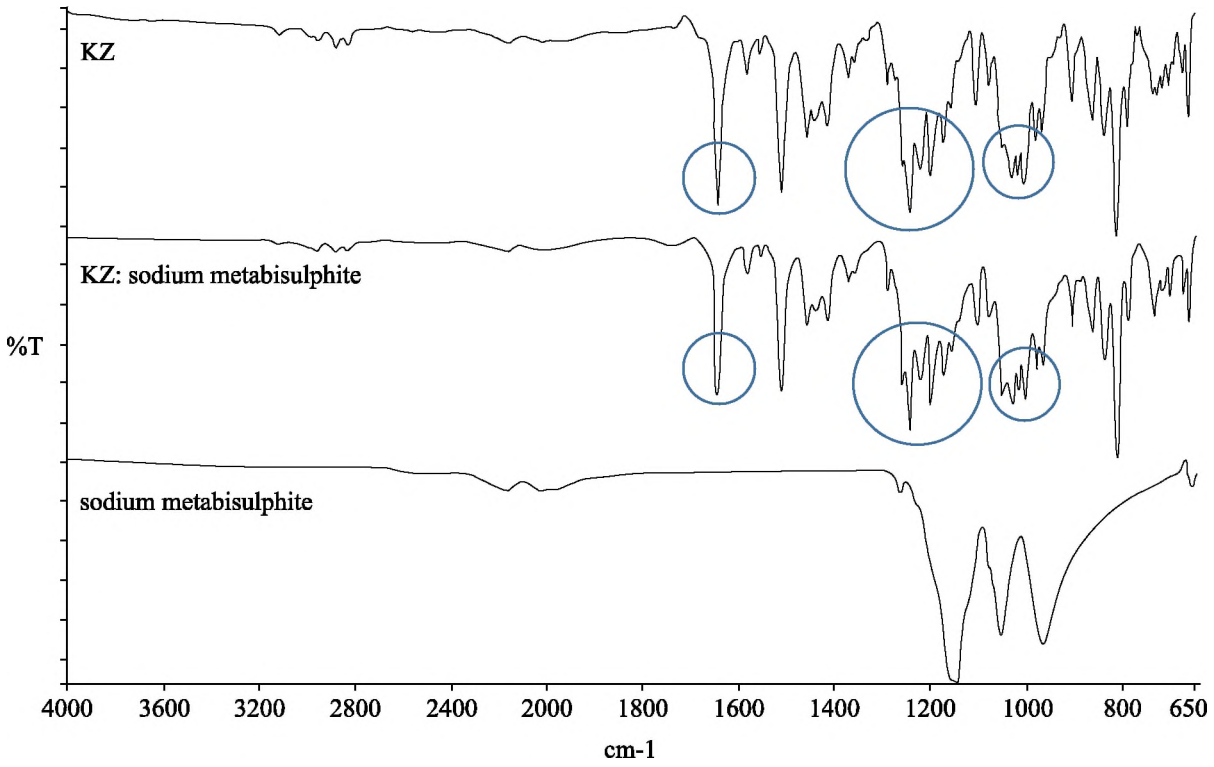


Figure 6.15 FTIR spectra for KZ, sodium metabisulphite and a 1:1 mixture of KZ and sodium metabisulphite.

6.2.3 Optimisation of the solvent system

The quaternary solvent system used was comprised of polysorbate 80, citric acid and ethanol made up to 50 g with a citrate-phosphate buffer of pH 5.0 that consisted of a 50:50% v/v mixture of 0.1 M citric acid and 0.2 M DHP. The 0.1 M citric acid solution was prepared by accurately weighing 21.00 g citric acid monohydrate and dissolving in 1000 mL HPLC grade water. Similarly, the 0.2 M DHP solution was manufactured by accurately weighing 28.40 g DHP and dissolving in 1000 mL HPLC grade water. The mixtures were sonicated until the powders had dissolved after which 500 mL 0.1 M citric acid and 500 mL 0.2 M DHP were mixed using a 1 L measuring cylinder and the pH adjusted to 5.0 using a 0.1 M NaOH solution. Polysorbate 80 was added to the solution in the amount indicated for the DoE and the mixture was stirred using a Labcon® Laboratory Magnetic Stirrer-hotplate. Ethanol was weighed and added shortly after the polysorbate 80 had been completely mixed. The pH of the solvent was then

measured. The solubility of KZ was quantitated by mixing an excess of KZ into 5 g of the quaternary solvent for one minute, sonicating the mixture for another minute and transferring the beaker to the magnetic stirrer for five minutes. Some KZ dissolved and excess KZ remained at the bottom of the beaker. The solution was then filtered using a 0.45 µm Millipore® Corporation HVLP Durapore membrane filter attached to a plastic 10 mL syringe and the filtrate analysed using a validated RP-HPLC method.

The % m/v amounts of polysorbate 80, citric acid and ethanol were identified with the aid of Design Expert® using a two-level factorial design with three centre points and eight factorial points. The amount of each of the components for the optimized solvent for use in the final formulation and the experimental amounts used are listed in **Table 6.2.** The aim of this optimisation study was to obtain a solvent system with the lowest possible concentration of each excipient with maximum solubility and minimal irritation potential of the dosage form.

Table 6.2 Experimental runs conducted to assess the impact of formulation of the quaternary solvent on KZ solubility.

SD	Run	A Polysorbate 80 % v/v	B Citric acid % m/v	C Ethanol % v/v
2	1	10	0	0
5	2	0	0	20
11	3	5	2.5	10
9	4	5	2.5	10
6	5	10	0	20
4	6	10	5	0
7	7	0	5	20
3	8	0	5	0
1	9	0	0	0
8	10	10	5	20
10	11	5	2.5	10

The amounts of polysorbate 80 (A), citric acid (B) and ethanol (C) used at a maximum or minimum % m/v or % v/v quantity are listed in **Table 6.3.** The independent input variables and ranges used were selected from data generated in preliminary studies and literature data [424].

Table 6.3 Summary of design factors.

Factor	Name	Units	Type	Subtype	Min	Max
A	Polysorbate 80	% v/v	Numeric	Continuous	0	10.00
B	Citric acid	% m/v	Numeric	Continuous	0	5.00
C	Ethanol	% v/v	Numeric	Continuous	0	20.00

The overall design summary consisted of linear and modified order models and all experiments were performed in a randomised manner to eliminate experimental bias. Mathematical models were generated for the three independent variables and optimisation of the factors was undertaken to establish

the combination of factors that resulted in the highest solubility of KZ whilst maintaining all input factor levels at low concentration. The interaction of factors was analysed for the impact on the solubility of KZ and pH, using perturbation plots, one-factor graphs, interaction plots, 2D contour plots and 3D response surface plots.

6.2.3.1 Model fitting

A linear order polynomial model was selected for the evaluation of the design space and the main model terms investigated included the intercept and three input variables. No aliases were identified for the linear model indicating that there are no unique design points or that an incorrect set of design points was selected for the model. A valid lack of fit test requires the df for the lack of fit test to be a minimum of three [404]. The df for the pure error test is equal to the number of centre point replications minus one. In this case, the lack of fit test had five df and pure error, two df. Fewer df may lead to the acceptance of a model that does not detect a lack of fit [403]. Other models that were assessed produced inadequate lack of fit t values and therefore a linear order polynomial model was used to interrogate the design space.

The standard error of fitting was 0.35 for all factors and the VIF and the multiple correlation coefficients were 1.00 and 0.00, respectively indicating that the design does not have any multi-linear constraints. The resolution of a two-level factorial design was calculated using a power tool, which resulted in a power at an α level of 5% to detect signal-noise ratios of 9.4% for 0.5 SD, 23.2% for one SD and 68.1% for two SDs. The average leverage calculated from all runs was 0.3636 which is < 1 and indicates that points within the design space do not need to be replicated. The condition number for the coefficient matrix was 1.00 indicating that no multi-collinearity between the model terms exists. The G efficiency is a measure of the average prediction variance as a percentage of the maximum prediction variance [458]. It is inversely proportional to maximum variance and was 78% in this case. As lack of fit increases, the value for G efficiency decreases [459] and the G-efficiency value of 78% was deemed acceptable and is indicative of a robust model fit [406-408].

6.2.3.2 Solubility of KZ in the solvent system

The solubility of KZ in the solvent system is an important consideration and is necessary to evaluate in order to determine the ratio of components that result in adequate solubility of KZ using the lowest possible concentration of each component of the system. The solubility of KZ was established by dissolving an excess of KZ in 5 g of the solvent mixture made using a specified amount of polysorbate 80, citric acid and ethanol made up to pH = 5.0 using the citrate-phosphate buffer. The mixtures were sonicated for 20 minutes and then placed in a Kimax® centrifuge tube and centrifuged for 5 minutes at 3000 rpm using a Model HN-SII centrifuge. The supernatant was filtered using a 0.45 µm Millipore®

Corporation HVLP Durapore membrane filter attached to a plastic 10 mL syringe filter. The filtrate was then analysed using a validated RP-HPLC method and the solubility reported in mg/mL.

6.2.3.2.1 Transformation of the solubility model

Box-Cox plots are used to evaluate the need for model transformation and the green line in **Figures 6.16** and **6.17** indicate the optimum lambda value for a specific power transform. The red lines in the plots indicate the boundaries of a 95% CI around the optimum λ value and the actual λ value, depicted by a blue line, should ideally fall within the boundaries of the 95% CI [458]. The ratio of the maximum and minimum λ value was 115.03 and as this value is > 10 a natural log transformation was performed as suggested for power transformations. The Box-Cox plot following log transformation depicted in **Figure 6.17** indicates that the blue line falls within the limits of the 95% CI indicating that the transformation was necessary and appropriate.

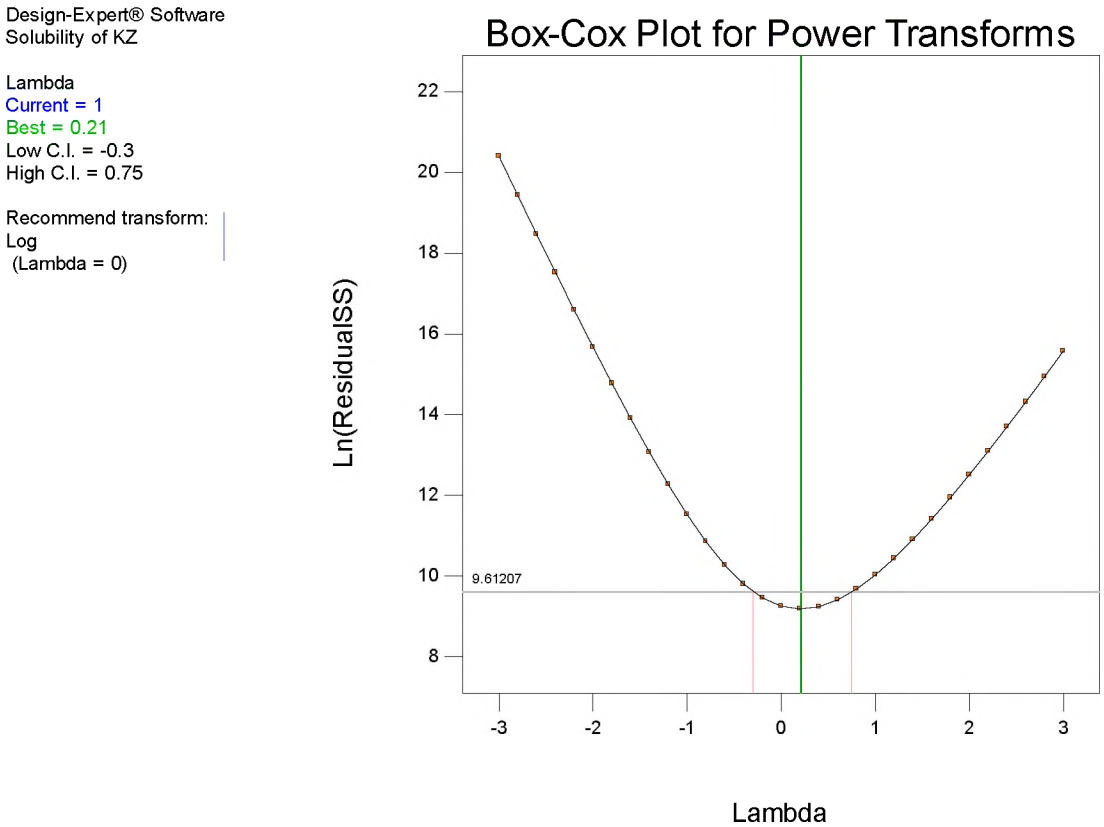


Figure 6.16 Box-Cox plot for the impact of solvent composition on the solubility of KZ prior to transformation.

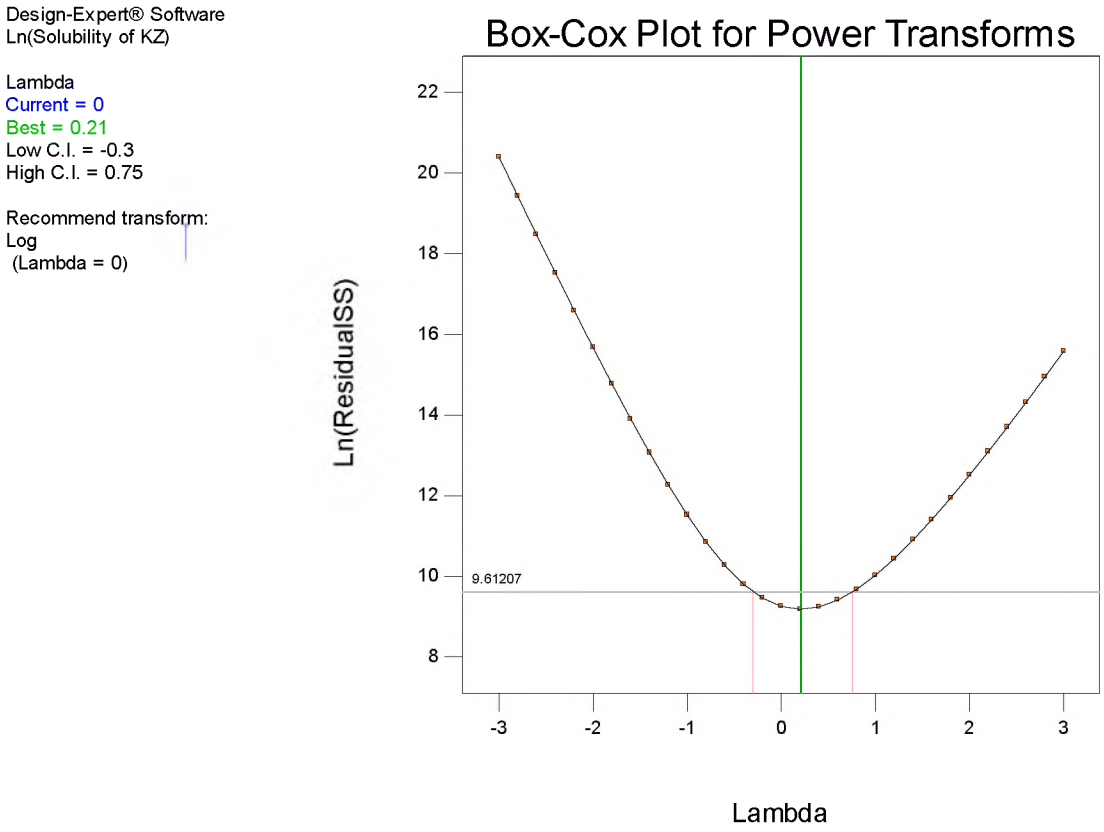


Figure 6.17 Box-Cox plot for the impact of solvent composition on the solubility of KZ following transformation.

6.2.3.2.2 Selection of significant factors affecting the solubility of KZ

Half normal, normal and Pareto chart plots are instruments used as selection tools for 2-level factorial designs. Half normal plots depict the standardised effects of the terms versus half-normal % probability and is used to temper the appropriate model by ignoring significant model terms such as citric acid in this case, as depicted in **Figure 6.18**, so as to reduce positive and negative effects and the error estimate points fall on a straight line plotted from zero (red line).

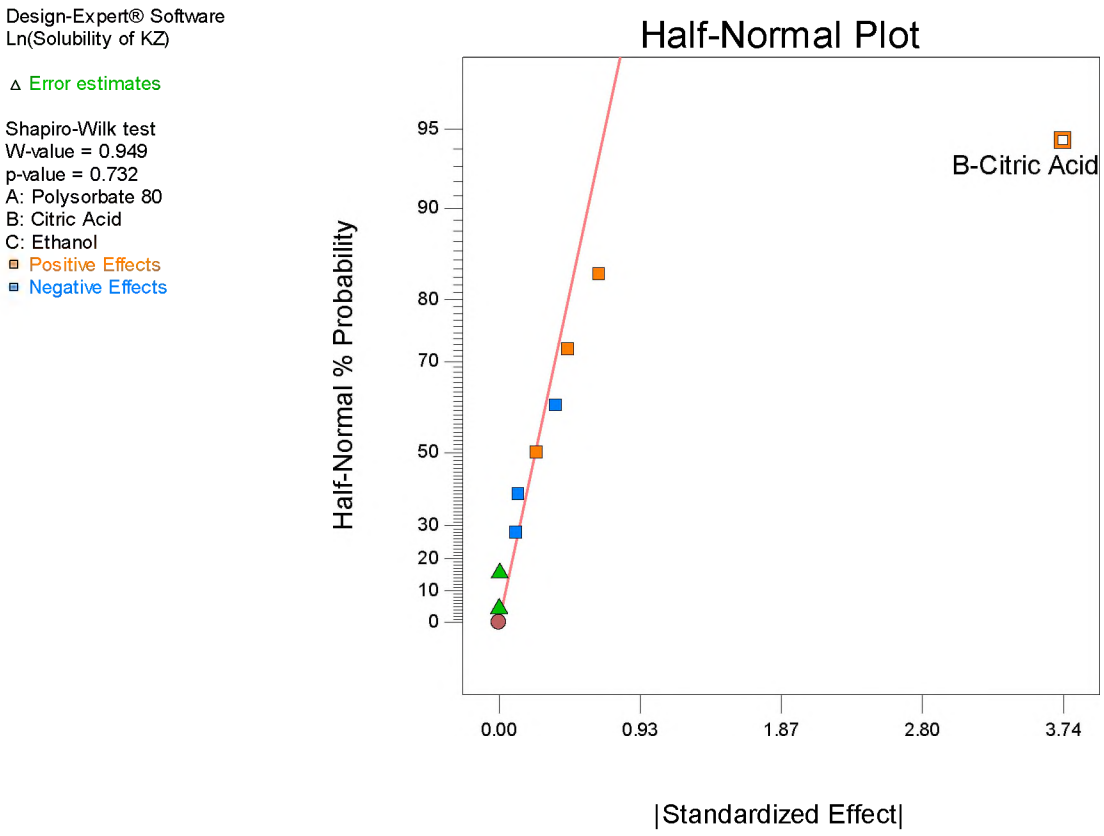


Figure 6.18 Half-normal plot for the impact of solvent composition on the solubility of KZ.

The Shapiro-Wilk test is a semi-parametric ANOVA test which is used to establish whether the significant terms deselected for the half-normal plot follow a normal distribution. If the p-value is > 0.1 then the term follows a normal distribution. The unselected terms reported exhibit a p-value = 0.732 and is normally distributed. The Shapiro-Wilk test produced a w-value = 0.695 (p = 0.003) prior to the deselection of citric acid from the half-normal plot and a w-value = 0.949 (p = 0.732) after deselection **(Figure 6.18)** indicating that the citric acid term (B) should be deselected as it exhibits a significant effect with a w–value closer to 1 and $p \geq 0.1$ following deselection from the half normal plot. No other statistically significant effects may be observed apart from citric acid and these data follow a normal distribution.

Normal plots are used to depict standardised effects versus a normal % probability and the same limits for the p- and w-values apply as for half-normal plots. Consequently, it is not necessary to analyse data using both approaches . Pareto charts as depicted in **Figure 6.19** can also be used to estimate significant model terms and facilitate removal of those terms from the selected model [390].

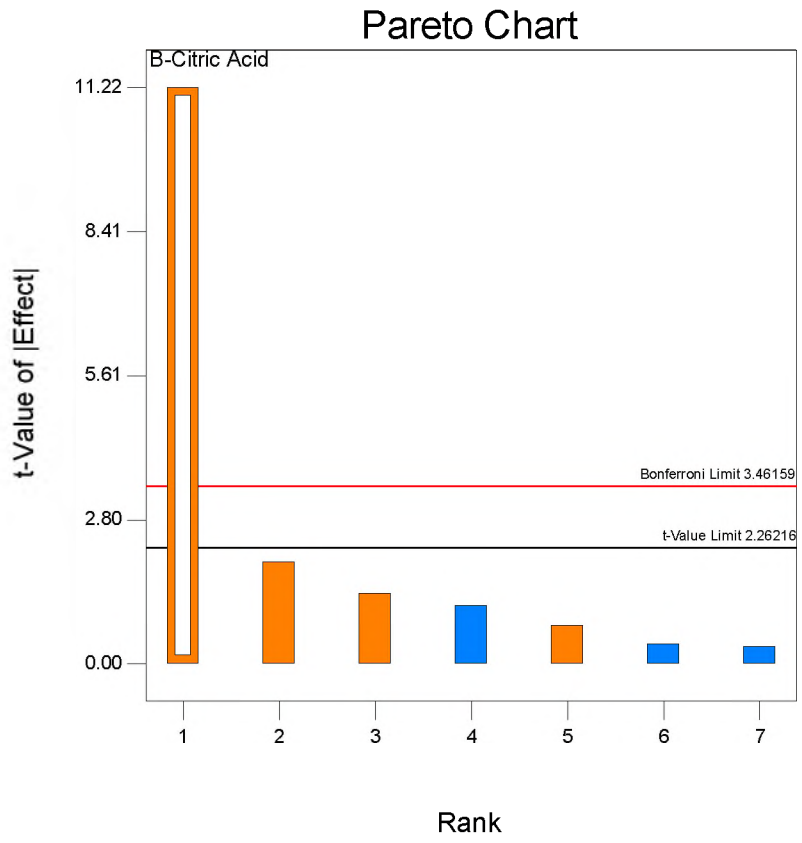


Figure 6.19 Pareto chart for impact of solvent composition on solubility of KZ.

The Pareto chart is used following evaluation of the half-normal plot to select the first significant model term. Effects above the Bonferroni limit of 3.58 are considered significant effects and effects between the Bonferroni and the limit for the t-value may be significant. Pareto charts are useful for the determination of the second largest significant effect in a set of data and in this case polysorbate 80 was identified as this term; however, the t-value limit is not reached and therefore polysorbate 80 remains an insignificant model term that may be included in the model to form a straight line in half-normal and normal plots. Of the three factors analysed, citric acid is the only input variable that has a significant effect on the solubility of KZ in this quaternary solvent system. The solubility of KZ can therefore be modulated by changing the citric acid content in the solvent system.

6.2.3.2.3 Analysis of variance

ANOVA was used to investigate the significance of the response model using Design Expert®. An ANOVA table summarizing the analysis of results for solubility of KZ exhibits a model F-value of 58.20, implying that the model is significant and indicates that there is a 0.01% ($p = 0.0001$) chance that the model F-value may have occurred due to noise (**Table 6.4**). The p-value for citric acid of < 0.0001 confirms the significance of the coefficient and facilitates an understanding of the CCD model.

Table 6.4 ANOVA data for the factorial model used to evaluate the impact of solvent composition on the solubility of KZ.

Source	Sum of squares	df	Mean square	F-value	p-value Prob > F	Significant difference
Model	27.95	1	27.95	58.20	< 0.0001	Yes
Citric acid	27.95	1	27.95	58.20	< 0.0001	Yes
Residual	4.32	9	0.48	-	-	Yes
Lack of fit	4.32	7	0.62	464.92	0.0021	Yes
Pure error	0.002655	2	0.001327	-	-	-
Total correction	32.27	10	-	-	-	-

The predicted R^2 of 0.8060 is in reasonable agreement with the adjusted R^2 of 0.8510 as summarized in **Table 6.5** [390]. Adequate precision, defined as the signal to noise ratio, should be > 4 and the value of 12.65 indicates that the model can be used to navigate the design space.

Table 6.5 Statistical measures of model adequacy.

Squared residuals and derivatives	Values
R^2	0.8660
Adjusted R^2	0.8510
Predicted R^2	0.8060
Adequate precision	12.65
SD	0.69
Mean	2.93
% Coefficient of variation	23.64
Predicted residual error sum of squares	6.26

The final equation for the solubility of KZ in actual terms is reported in **Equation 6.5** and provides an indication of the values for KZ solubility according to the significant factors. The polynomial expression contains the intercept and the first order main effect of citric acid which was the only significant term in the model. The sign and magnitude of the coefficient indicates the positive effect of citric acid on the solubility of KZ in the solvent system.

$$\ln(\text{KZ solubility}) = 1.06 + (0.75 \times \text{citric acid}) \qquad \textbf{Equation 6.5}$$

6.2.3.2.4 Evaluation of model fit for the solubility of KZ

Residual analysis and diagnostic plots are used to ensure that the assumptions made for ANOVA are met [458] and the internally studentized normal plot of residuals depicted in **Figure 6.20** appears adequate as all points fall almost on a straight line.

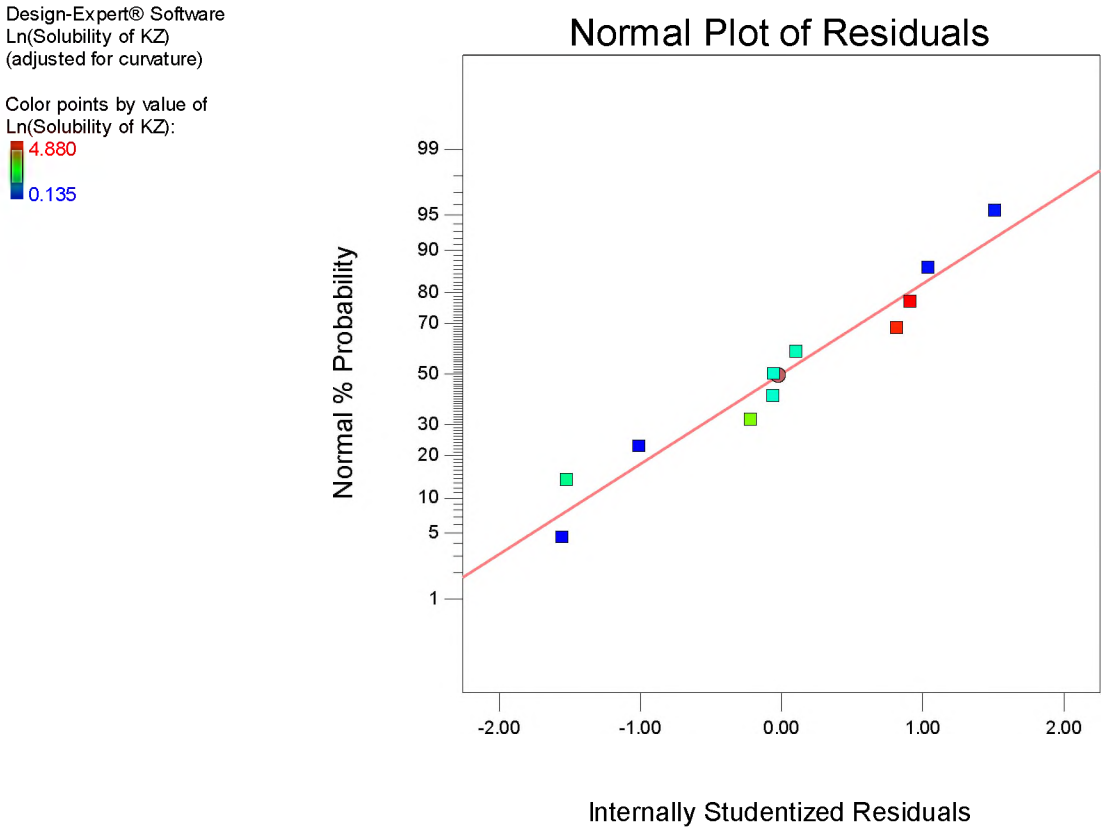


Figure 6.20 Internally studentized normal plot of residuals for the impact of solvent composition on the solubility of KZ.

The data points on the residuals versus run plot for internally studentized residuals depicted in **Figure 6.21** do not appear to follow a specific trend and this is an indication that not one input variable affected the solubility of KZ during experimentation and this is, in part, due to the randomized approach used. The red lines in **Figure 6.21** are the Pareto t-limits for this analysis.

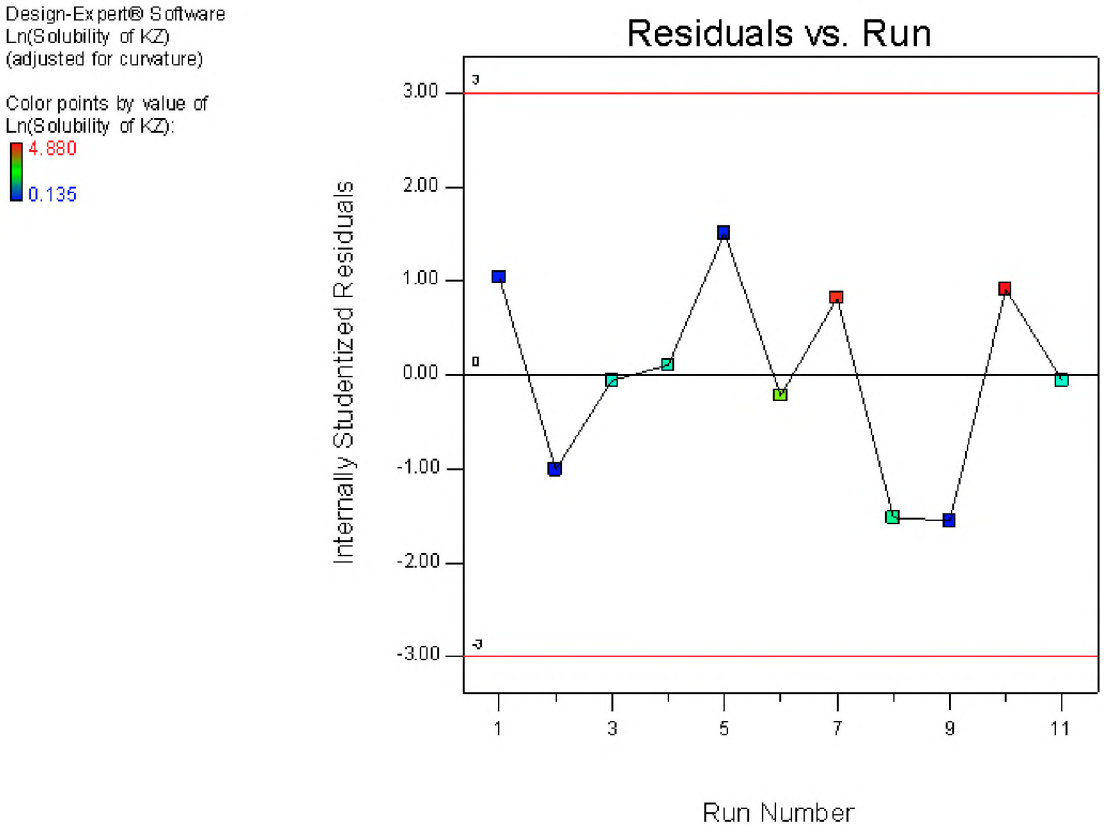


Figure 6.21 Internally studentized plot of residuals versus experimental run.

Externally studentized plots revealed the presence of no outliers thereby indicating that all data for all runs were adequately fitted to the model.

6.2.3.2.5 Graphical interpretation and evaluation of the model for KZ solubility

Perturbation plots demonstrate the sensitivity of a response to small changes in input factors and the plot for solubility of KZ when evaluating the impact of citric acid is depicted in **Figure 6.22**. As the amount of citric acid is increased the solubility of KZ in the solvent system increases and the plot suggests a high degree of sensitivity of the solubility to small changes in the amount of citric acid used in the solvent system.

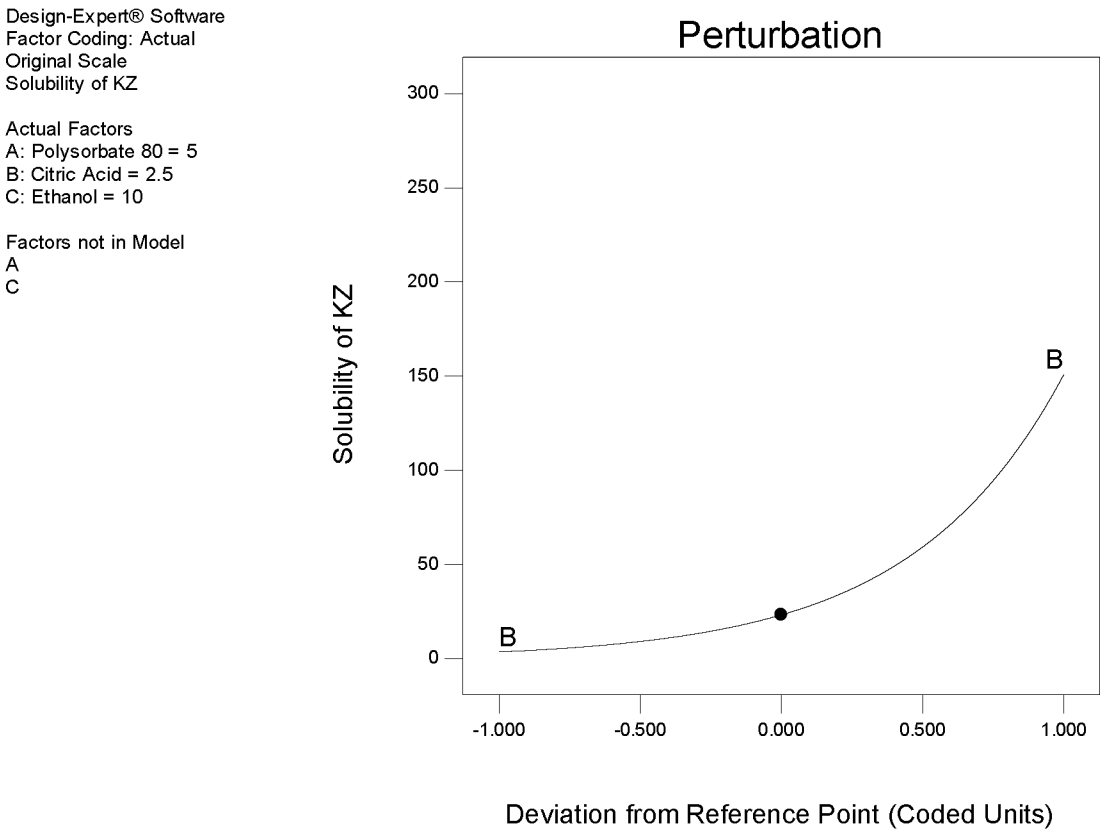


Figure 6.22 Perturbation plot demonstrating the impact of input variables on the solubility of KZ in the solvent system.

The solubility of KZ was sensitive to the amount of citric acid used in the solvent system as depicted in the one-factor graph depicted in **Figure 6.23**. The 'I beam' range is indicative of the least significant difference (LSD) calculation at a 95% confidence level and for a 5% m/v citric acid content the I beam had a wide range for the LSD whereas for 0% m/v citric acid a small LSD range was observed implying that as the amount of citric acid increases there is a decrease in the overall precision for the solubility of KZ [460]. One factor plots are not useful for evaluating systems in which the input factors interact, as the I beams may overlap resulting in a lack of precision for the model.

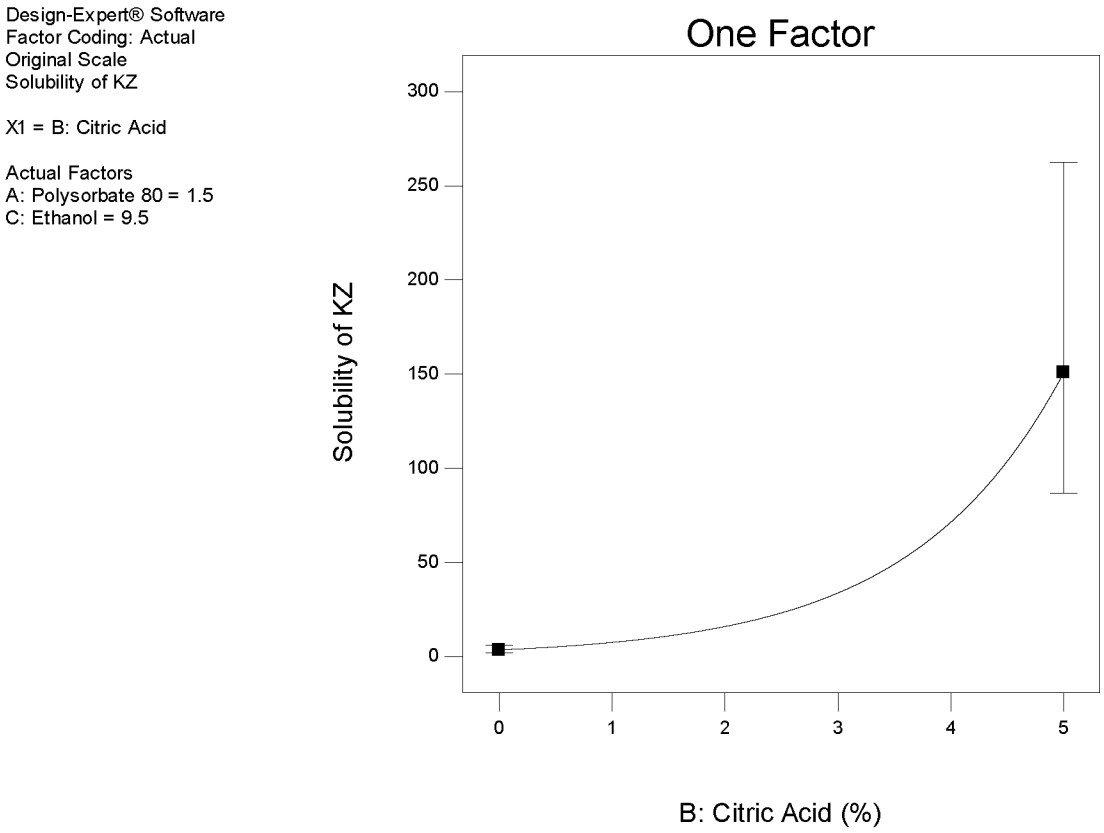


Figure 6.23 One factor plot depicting the impact of citric acid on the solubility of KZ.

There was no need to analyse an interaction plot as the only excipient that had a significant impact on the solubility of KZ was citric acid.

6.2.3.2.5.1 Polysorbate 80 and citric acid

Contour plots are 2D depictions of the impact of two variables on a response. A 2D contour plot depicting the impact polysorbate 80 and citric acid on the solubility of KZ is displayed in **Figure 6.24**. The solubility of KZ increases in a linear fashion as the concentration of citric acid is increased while maintaining the ethanol content at 9.5% v/v. As the concentration of polysorbate 80 is increased, the solubility of KZ in the solvent remains constant.

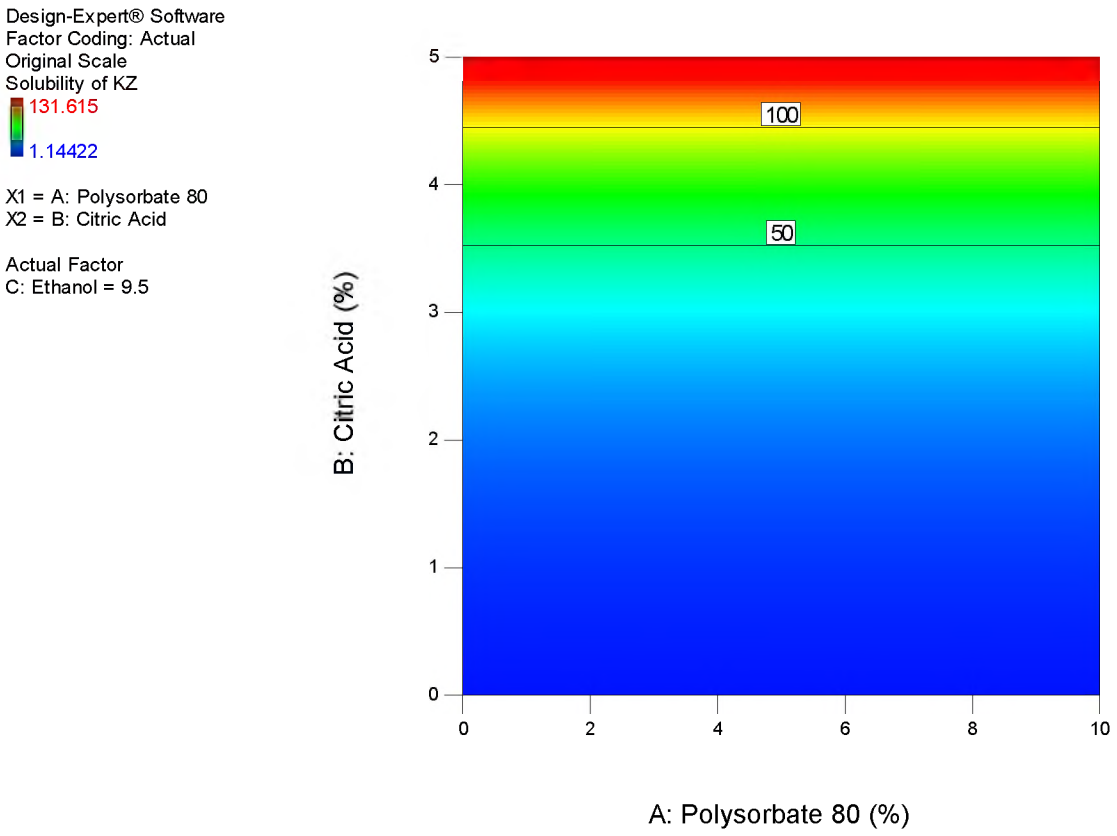


Figure 6.24 Contour plot depicting the impact of polysorbate 80 and citric acid on the solubility of KZ.

Three-dimensional (3D) response surface plots give shape to 2D contour plots and permit a better understanding of the influence of two factors in combination on the response under investigation. The 3D response surface plot depicting the impact of polysorbate 80 and citric acid on the solubility of KZ in the solvent system is depicted in **Figure 6.25**. It is clear from this plot that the influence of citric acid on KZ solubility is not linear. The shape of the contour plot curves slightly upwards from 0 to 5% m/v citric acid with constant ethanol content at 9.5% v/v. The amount of citric acid in the solvent system significantly influences the solubility of KZ. Since KZ is weakly basic it ionises in the presence of an acid and thus dissolves more readily in acidic media [461]. Therefore, the amount of citric acid can be used to modulate the solubility of KZ in the solvent. Polysorbate 80 does not appear to influence the solubility of KZ when the ethanol content is 9.5% v/v. A similar trend was observed for other amounts of ethanol used within the experimental range.

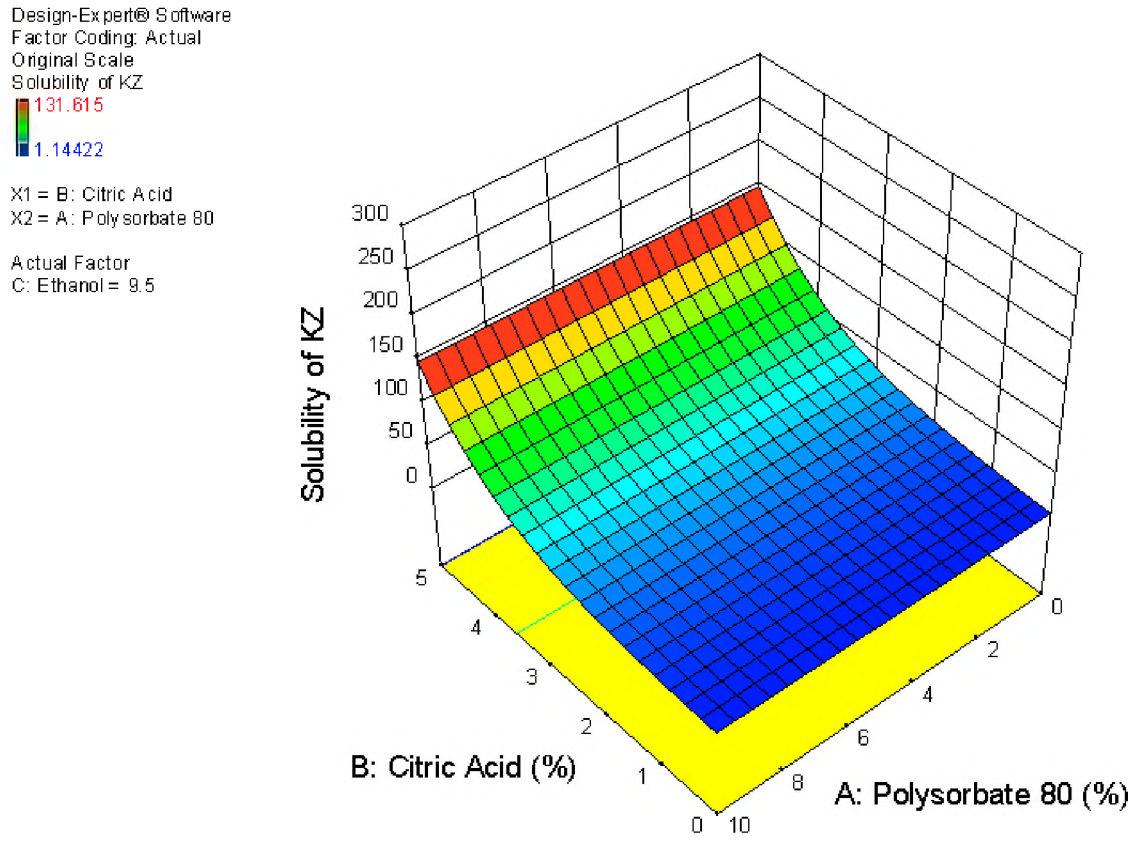


Figure 6.25 3D response surface plot depicting the impact of polysorbate 80 and citric acid on the solubility of KZ.

6.2.3.2.5.2 Citric acid and ethanol

A 2D contour plot depicting the impact of citric acid and ethanol content on the solubility of KZ in the solvent system is depicted in **Figure 6.26** and this confirms that citric acid is the only excipient that has a significant impact on the solubility of KZ. The plot reveals that as the citric acid levels increase the solubility of KZ increases with a polysorbate 80 content set at 1.5% v/v. The solubility of KZ remained constant as the ethanol concentration increased in the solvent system.

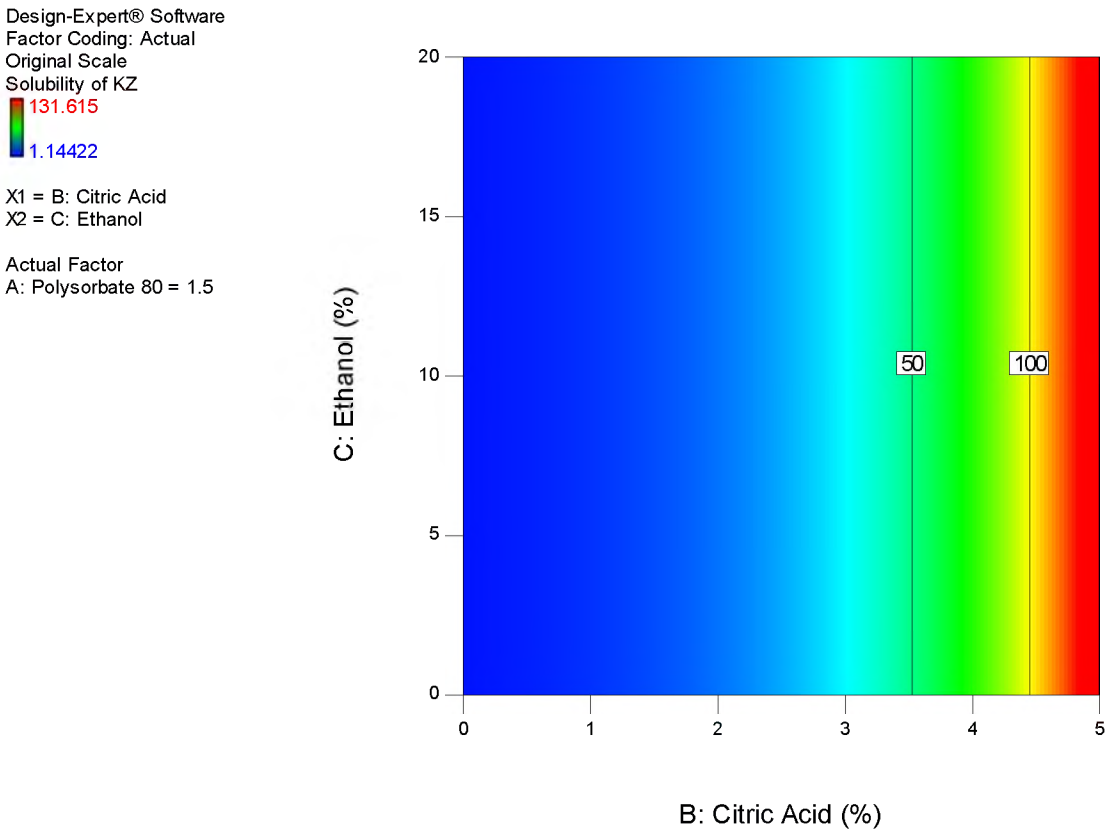


Figure 6.26 2D contour plot depicting the impact of citric acid and ethanol on the solubility of KZ.

The 3D response surface plot depicted in **Figure 6.27** complements **Figure 6.26** and reveals that an increase in the solubility of KZ is due to an increase in citric acid content. The plot exhibits a slight upward curve at high levels of citric acid for a specific ethanol concentration and the amount of KZ dissolved in the solvent system may be modulated using the citric acid content. Citric acid may, however, result in an overall decrease in pH causing irritation to the vaginal mucosa if the pH is < 3.5. Therefore, the solvent system must be able to dissolve an adequate amount of KZ, 2% m/v in this case, whilst using a minimum amount of citric acid in order to prevent irritation to the vaginal mucosa.

An increase in ethanol content does not appear to contribute to any changes in solubility of KZ within the 0 to 20% v/v limits of ethanol content evaluated when the polysorbate 80 content was set at 1.5% v/v.

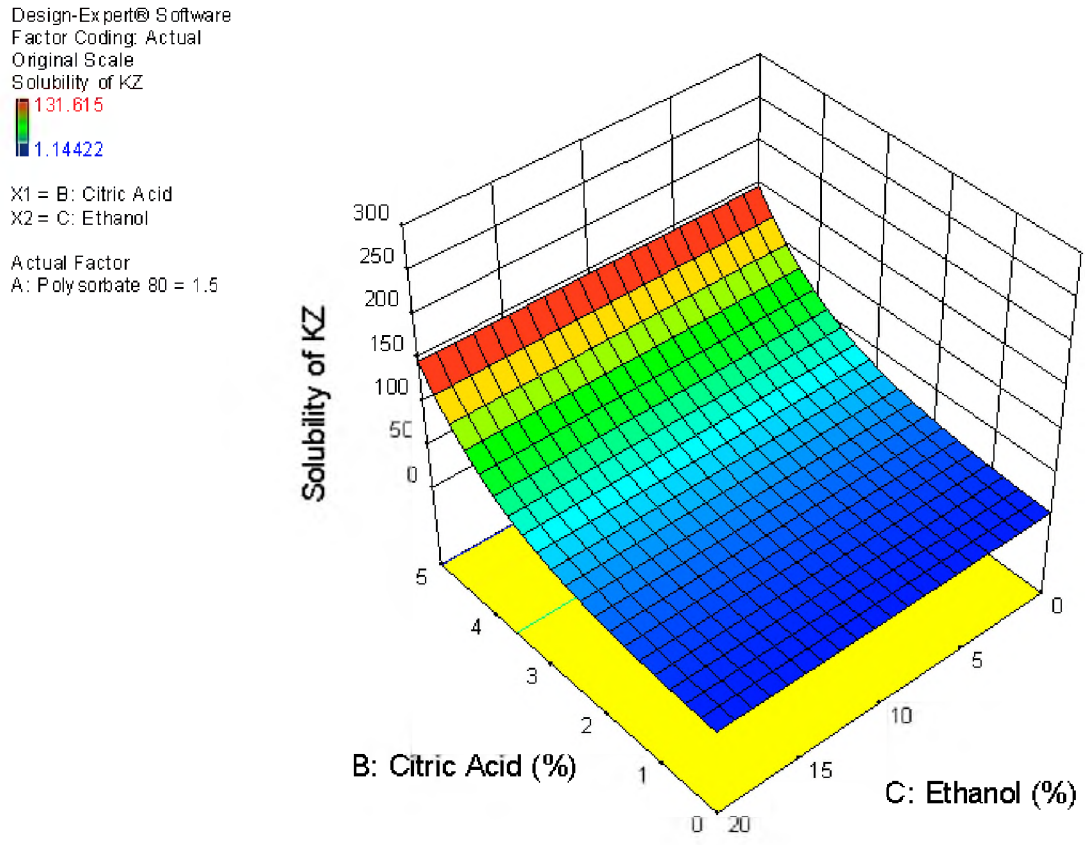


Figure 6.27 3D surface plot depicting the impact of citric acid and ethanol on the solubility of KZ.

6.2.3.2.5.3 Polysorbate 80 and ethanol

The response surface plot depicted in **Figure 6.28** suggests that ethanol and polysorbate 80 do not influence the solubility of KZ in any way when used within 0 to 10% v/v polysorbate 80 and 0 to 20% v/v ethanol limits when citric acid content is maintained at a 4% m/v level in the solvent system. The shape of the plot is flat and there are no lines evident on the yellow surface indicating there is no change in the solubility of KZ.

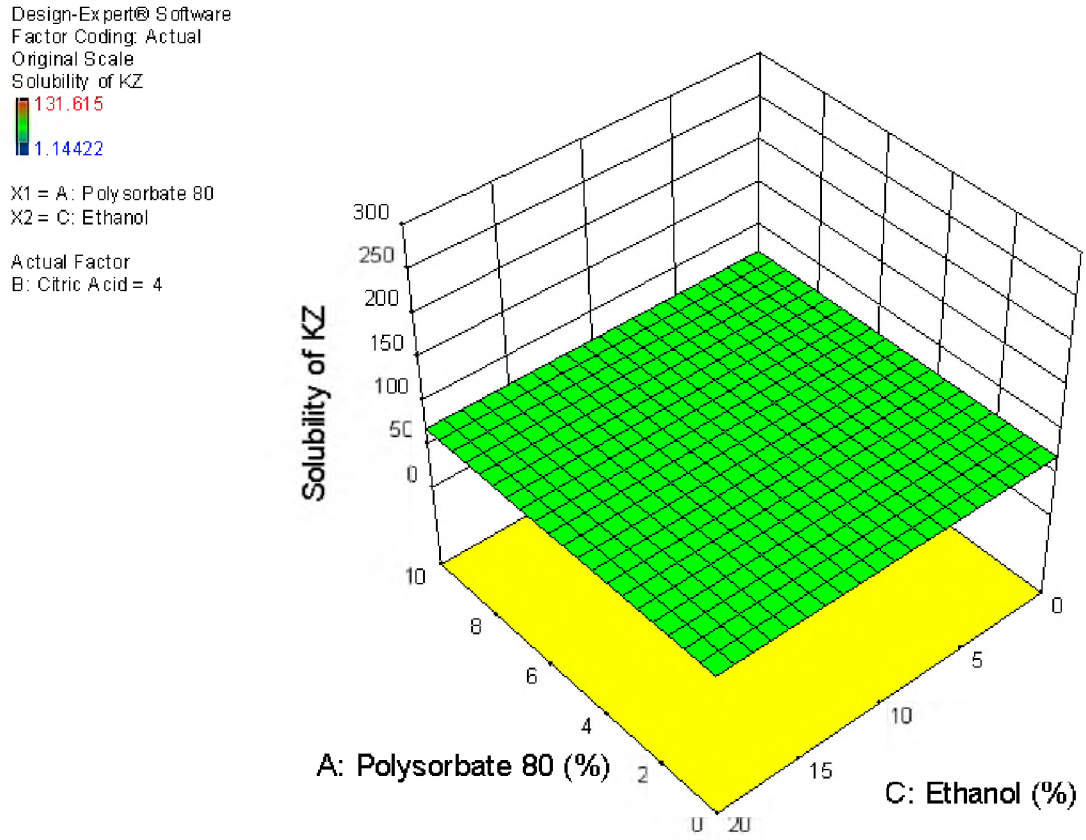


Figure 6.28 3D response surface plot depicting the impact of polysorbate 80 and ethanol on the solubility of KZ.

6.2.3.3 pH of the solvent

The solvent system was prepared using different ratios of the excipient levels as generated by the two-level factorial design and the mixtures were then made up to 50 g with a citrate-phosphate buffer of pH 5.0. The pH of the solvent is an important consideration as the solubility of KZ is affected by pH and the stability of the final formulation is likely to be dependent on the ultimate pH of the system.

6.2.3.3.1 Transformation of pH response model

The ratio of maximum to minimum λ-value for pH was 1.9 and as the value is < 10 no transformation for this response is necessary. The Box-Cox plot depicted in **Figure 6.29** reveals that λ = 1 and that a power transformation for this data is not required.

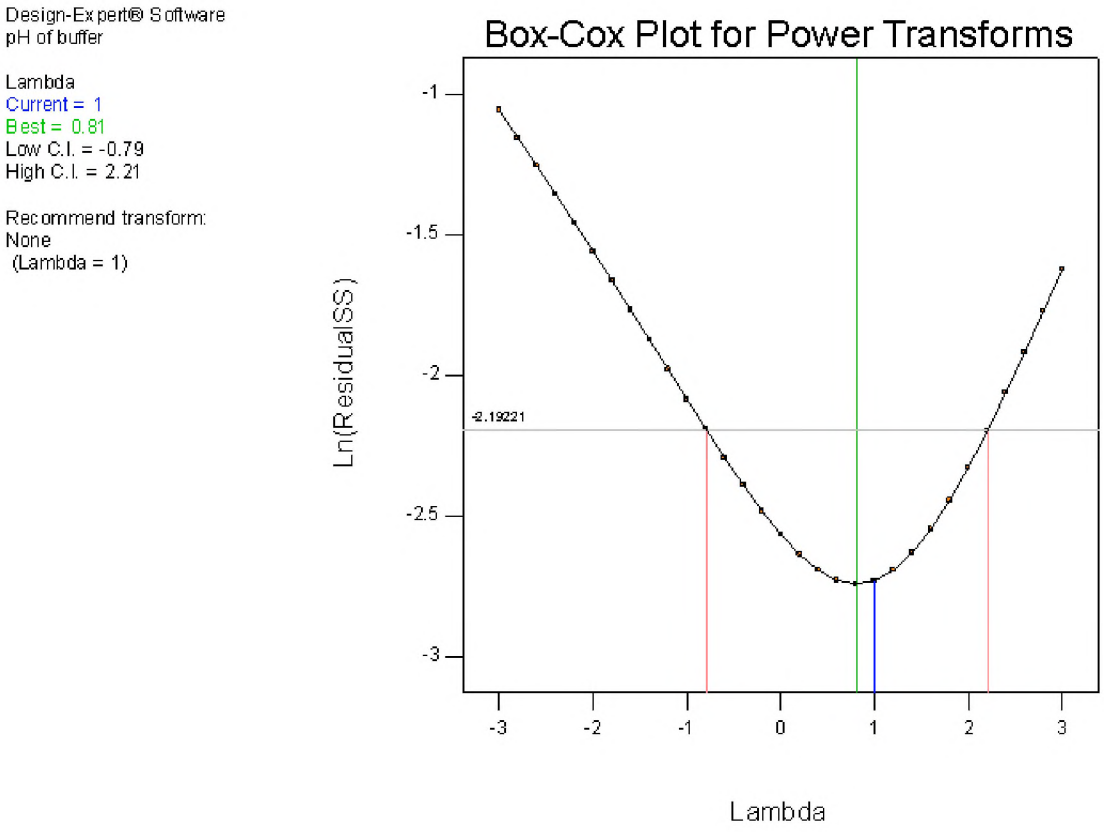


Figure 6.29 Box-Cox plot for the impact of solvent composition on the pH of the solvent system.

6.2.3.3.2 Selection of significant effects of pH

The half-normal plot in which citric acid and ethanol are significant factors in order for insignificant model terms and error estimates to fall in a straight line is depicted in **Figure 6.30**. The Shapiro-Wilk test produced a w-value = 0.924 and p-value = 0.559 ≥ 0.1 indicating that the significant model terms are normally distributed [462]. When no terms are selected, the Shapiro-Wilk's p-value was 0 indicating that there are significant terms in this model.

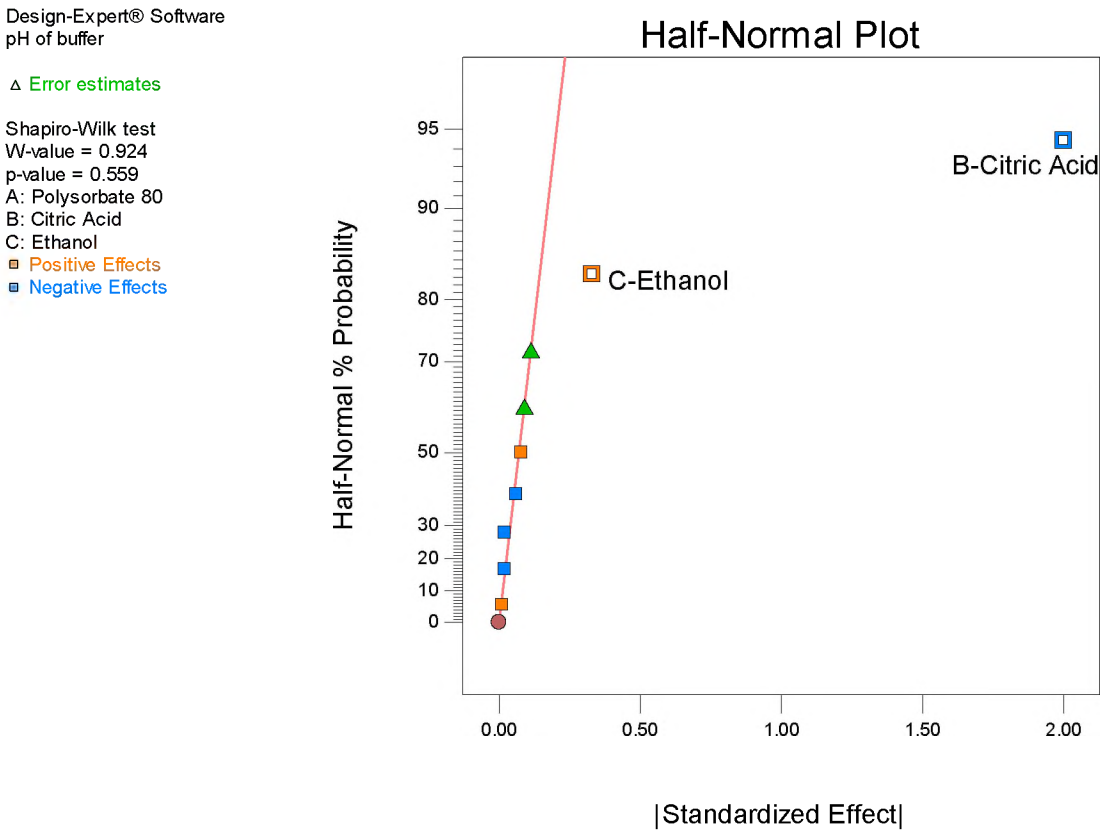


Figure 6.30 Half-normal plot for the impact of solvent composition on pH of the solvent system.

The normal plot is generally not required when a half-normal plot is used and it is more difficult to estimate significant model terms using a normal plot. In this case, the half-normal plot did not clearly indicate whether ethanol was a significant factor and a normal plot was required to confirm this finding. It was evident from the normal plot for pH that citric acid content is an outlier in this case **(Figure 6.31)** whereas ethanol is indeed a significant model term from the normal plot **(Figure 6.31)** and the Pareto chart depicted in **Figure 6.32**.

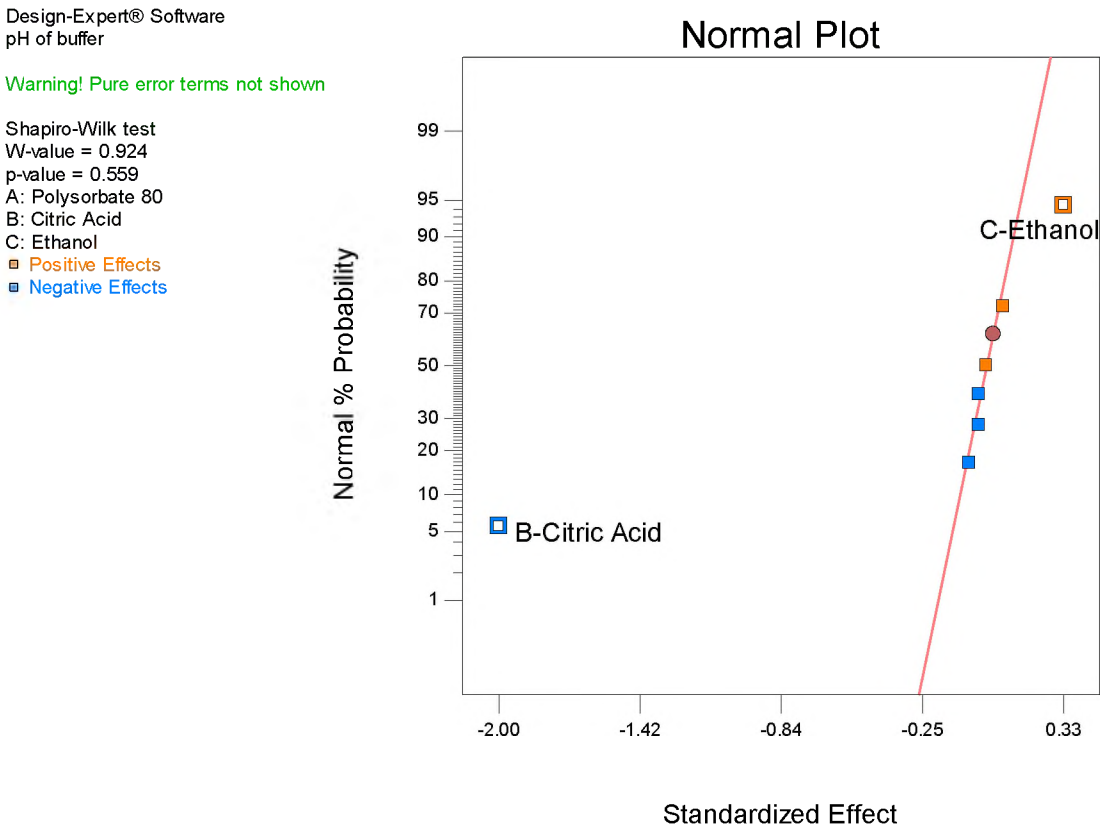


Figure 6.31 Normal plot for the impact of solvent composition on pH of the solvent system.

A Pareto chart reveals that citric acid and ethanol are indeed significant model terms as they fall above the Bonferroni limit of 3.58 and all insignificant model terms fall below the t-limit of 2.31. The data generated indicate that ethanol and citric acid content significantly affect the pH of the solvent system whereas the solubility of KZ in the solvent is solely affected by citric acid content. The solubility of KZ is dependent on the pH of the solvent in which it is dissolved.

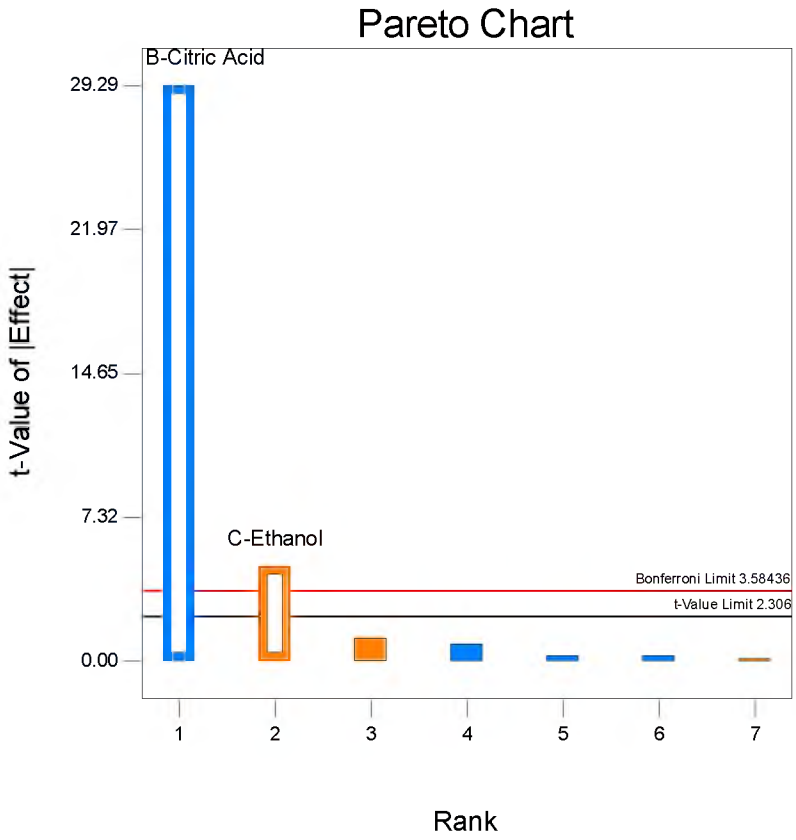


Figure 6.32 Pareto chart for impact of solvent composition on pH of the solvent system.

6.2.3.3.3 Analysis of variance for solvent pH

The ANOVA partial sum of squares data for solvent pH are summarized in **Table 6.6** and the model F-value of 27.24 and a p-value of 0.0003 indicate that the model is statistically significant. There is 0.03% chance that an F-value this large may be due to noise. Citric acid was a significant factor whereas ethanol was not significant, with $p < 0.0001$ and $p = 0.2639$ respectively. A lack of fit F-value of 8.92 and p-value of 0.1042 indicates that the model is not significant and there is a 10.42% chance of an F-value this large is due to noise. The insignificant lack of fit value is indicative that the data fits the model adequately.

Table 6.6 ANOVA data table for the factorial model for pH.

Source	Sum of squares	df	Mean square	F-value	p-value Prob > F	Significant difference
Model	8.22	2	4.11	27.24	0.0003	Yes
Citric acid	8.00	1	8.00	53.03	< 0.0001	Yes
Ethanol	0.22	1	0.22	1.44	0.2639	No
Residual	1.21	8	0.15	-	-	-
Lack of fit	1.16	6	0.19	8.92	0.1042	No
Pure error	0.043	2	0.022	-	-	-
Total correction	9.42	10	-	-	-	-

The R^2, adjusted (0.8400) and predicted (0.8070) R^2 values for the residuals were in close agreement [390]. An adequate precision of 11.49 indicates an adequate signal to noise ratio and suggests that the model can be used to navigate the design space.

Predictions of the pH when different levels of each of the significant model terms are used in the formulation can be made using **Equation 6.6**.

$$\text{pH of solvent} = 4.54 - (0.40 \times \text{citric acid}) + (0.017 \times \text{ethanol}) \qquad \textbf{Equation 6.6}$$

6.2.3.3.4 Evaluation of model fitting for pH of solvent

The normal plot of residuals for fitting of pH of solvent data is depicted in **Figure 6.33**. Almost all points are located in a straight line with moderate scattering of some points and as the data are not sigmoid or 'S' shaped it can be assumed that the residuals follow a normal distribution. As there is an autocorrelation between the residuals, the Shapiro-Wilk test for normality is invalidated and therefore it is not possible to calculate p- and w-values. The Shapiro-Wilk test depends on an assumption of independence, therefore visual assessment was adequate for the analysis of this plot.

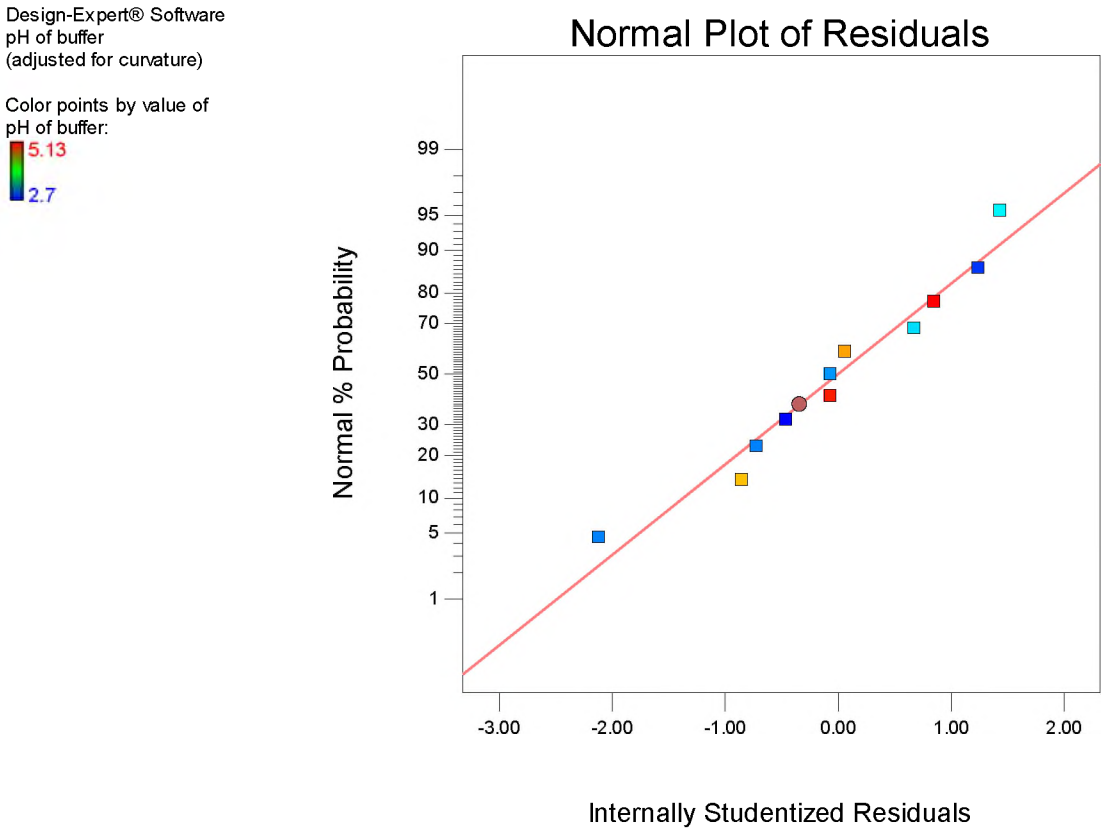


Figure 6.33 Internally studentized normal plot of residuals for the impact of input variables on pH.

The actual response versus predicted values plot is depicted in **Figure 6.34**. The points are located evenly with an equal number of points below and above the line indicating that the data fits the model and that a transformation might not be required.

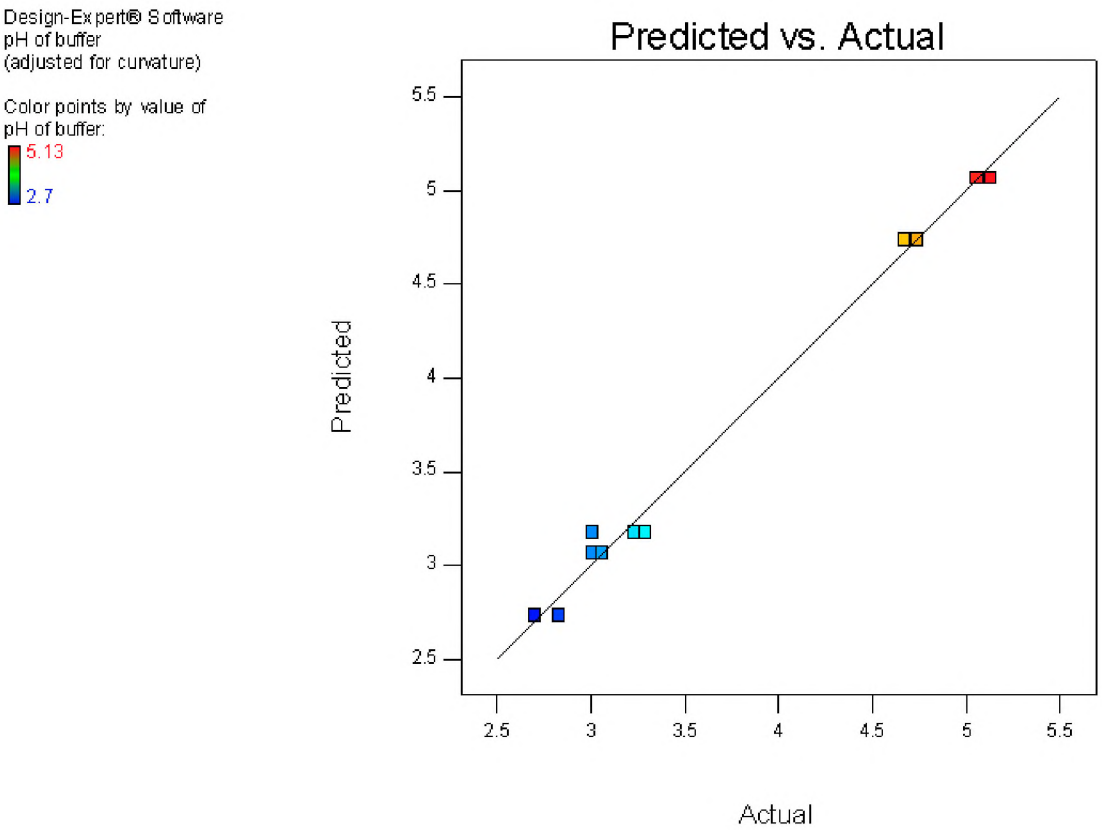


Figure 6.34 Observed versus predicted response plot for pH of the solvent.

6.2.3.3.5 Graphical interpretation and evaluation of response model for pH

The perturbation plot for pH depicted in **Figure 6.35** reveals the sensitivity of the solvent system to small changes in significant input factors. The perturbation plots for citric acid and ethanol content reveal that, as expected, as the concentration of citric acid increases, the pH of the solvent decreases. The steep slope observed for the citric acid plot indicates that pH is more sensitive to citric acid concentration than that of ethanol. As the amount of ethanol was increased in a 4% m/v citric acid and 1.5% v/v polysorbate 80 solution, the pH of the solvent increased only slightly. The slope of the ethanol plot is almost horizontal indicating that ethanol contributes only slightly to pH change when compared to citric acid levels. A low solvent pH contributes to an improvement in KZ solubility in aqueous media [265] and the amount of citric acid can be changed while keeping the amount of ethanol constant to control the pH of the solvent system.

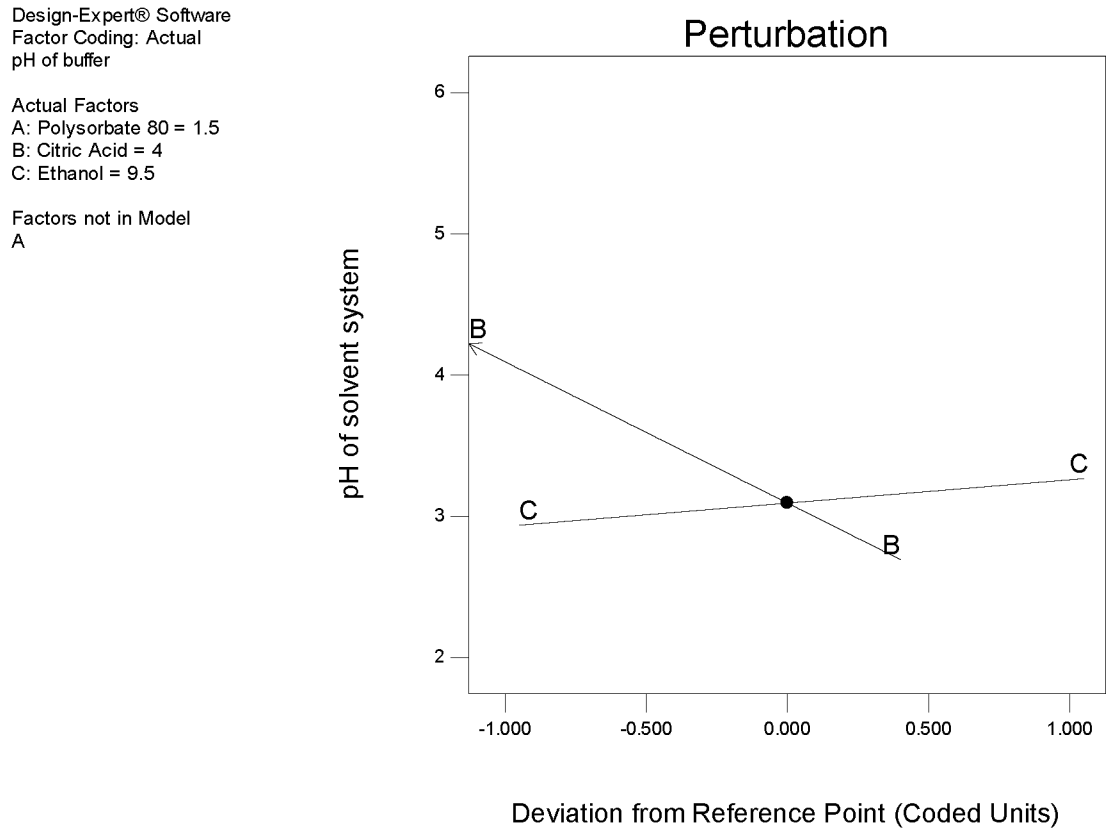


Figure 6.35 Perturbation plot demonstrating the impact of two input variables on the pH of the solvent system.

The effect of citric acid on the pH of the solvent system depicted as a one factor plot is depicted in **Figure 6.36**. Similar to the perturbation plot the slope for citric acid is relatively steep suggesting that citric acid has a significant impact on solvent pH. The LSD bars are relatively short indicating that pH can be precisely predicted and citric acid content from DoE studies can be used to change the pH of the solvent system with a low margin of error and these data are described in **§ 8.3** *vide infra.*

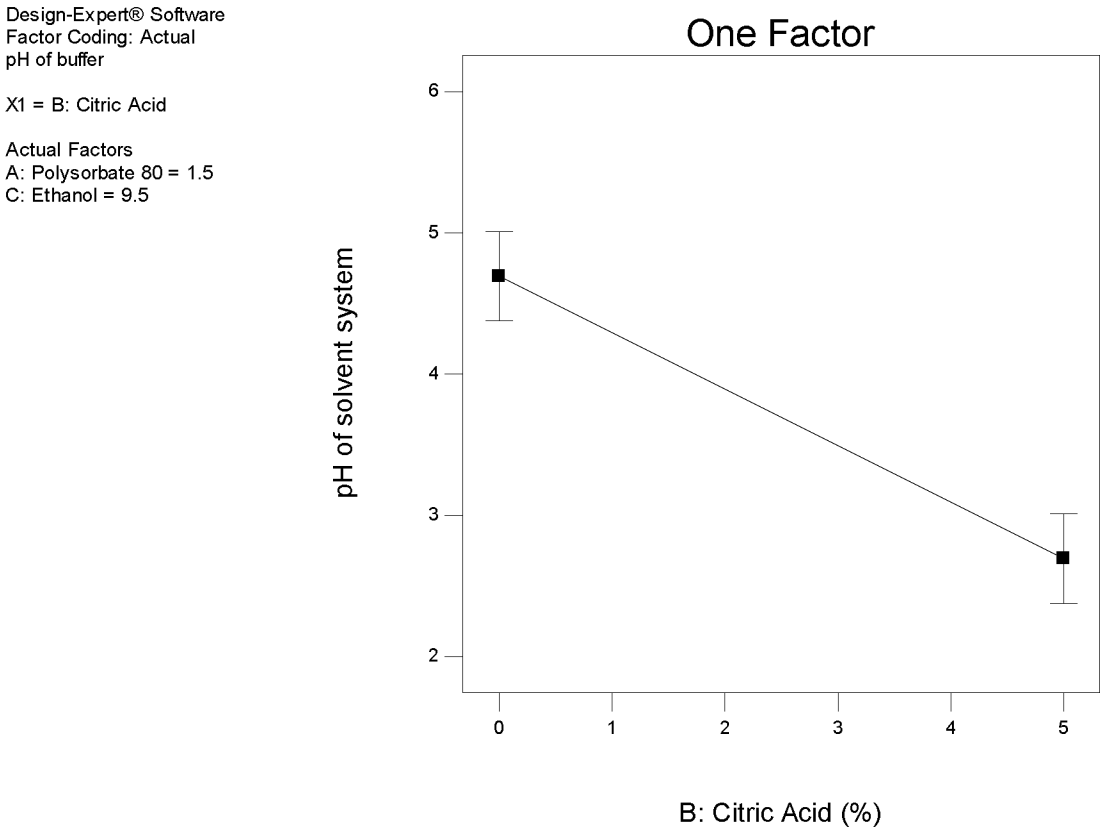


Figure 6.36 One factor plot depicting the impact of citric acid on solvent system pH.

The one factor plot depicting the impact of ethanol on pH of the solvent is displayed in **Figure 6.37.** Once again similar to the data observed in the perturbation plot, the one factor plot is a horizontal straight line further confirming that this factor does not have as significant an impact as citric acid content on pH.

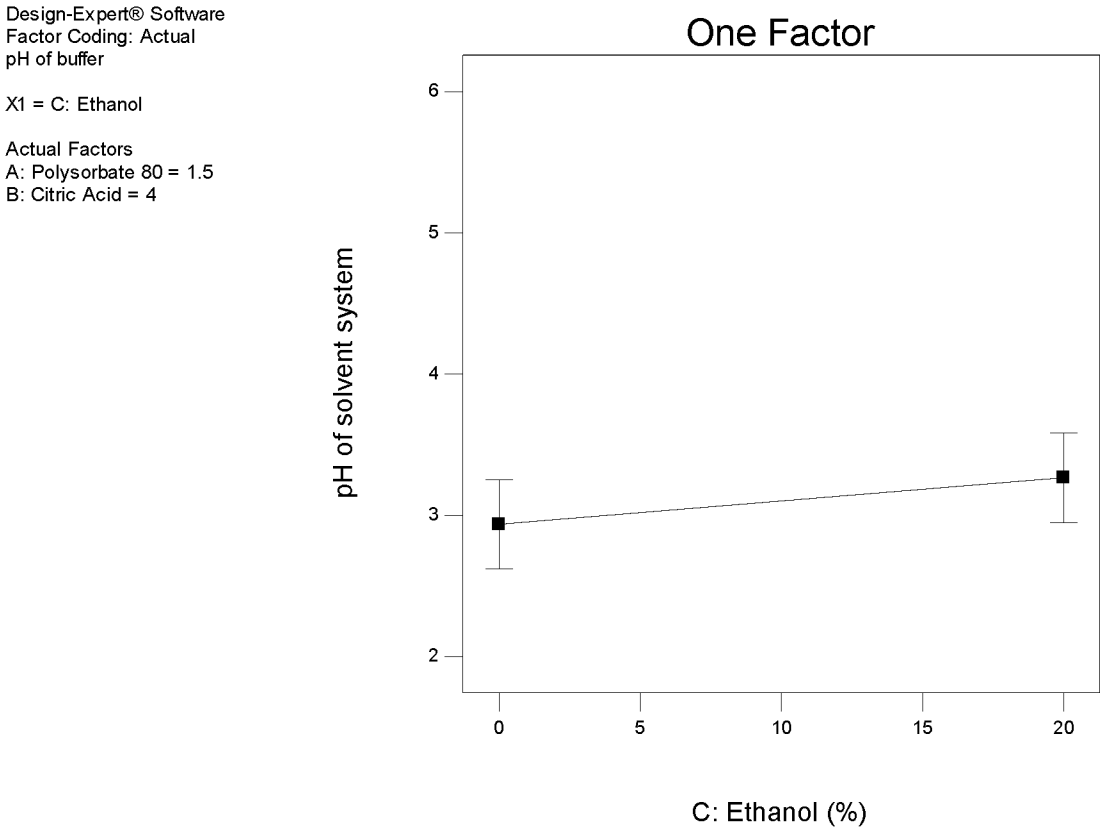


Figure 6.37 One factor plot depicting the impact of ethanol content on pH of the solvent system.

Interaction plots reveal the impact of two or more significant factors on each other when used at two different concentration levels and in this instance the data reveal parallel lines representing the levels of citric acid at 0 and 5% m/v as illustrated in **Figure 6.38**. The presence of parallel lines in the plot indicates that the effect of ethanol on solvent pH depends very slightly on the level at which citric acid is used. The parallel lines indicate values at the opposite sides of the range for citric acid content in the solvent system and demonstrate that changes in citric acid concentration within the range may result in a major change in the solvent pH system and that the effects of citric acid are not dependent on ethanol content. These data suggest that citric acid can be used to modulate solvent pH between approximately pH 2.0 – 5.0 and can be used advantageously during formulation development to modulate pH and solubility of KZ in solvent systems of a specific pH. The LSD bars do not overlap indicating a significant difference in the effect of citric acid on pH as was observed from data in **Figures 6.35** and **6.36**.

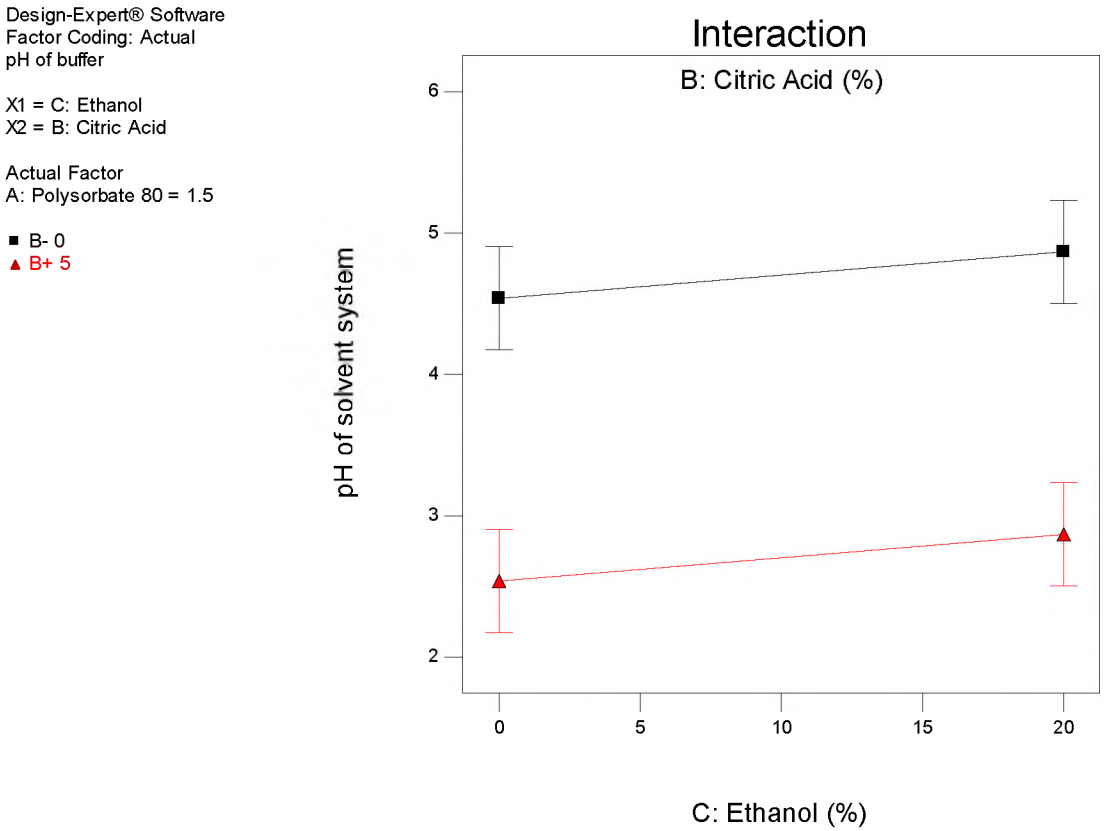


Figure 6.38 Interaction plot for citric acid at 0 and 5% and ethanol on pH of the solvent system.

The interaction between ethanol at 0 and 20% v/v and citric acid on the pH of the solvent is illustrated in **Figure 6.39**. The parallel lines imply that the effect of ethanol on pH is not dependent on the amount of citric acid used in the solvent. A change in ethanol concentration within the range of 0 to 20% v/v in the solvent system results in small changes of about 0.5 magnitude in pH. The LSD bars overlap indicating that the difference between the two levels is not significant, thereby confirming the observations depicted in **Figures 6.35** and **6.37**. From a formulation development perspective a change in ethanol content of the solvent may only slightly affect the solubility of KZ.

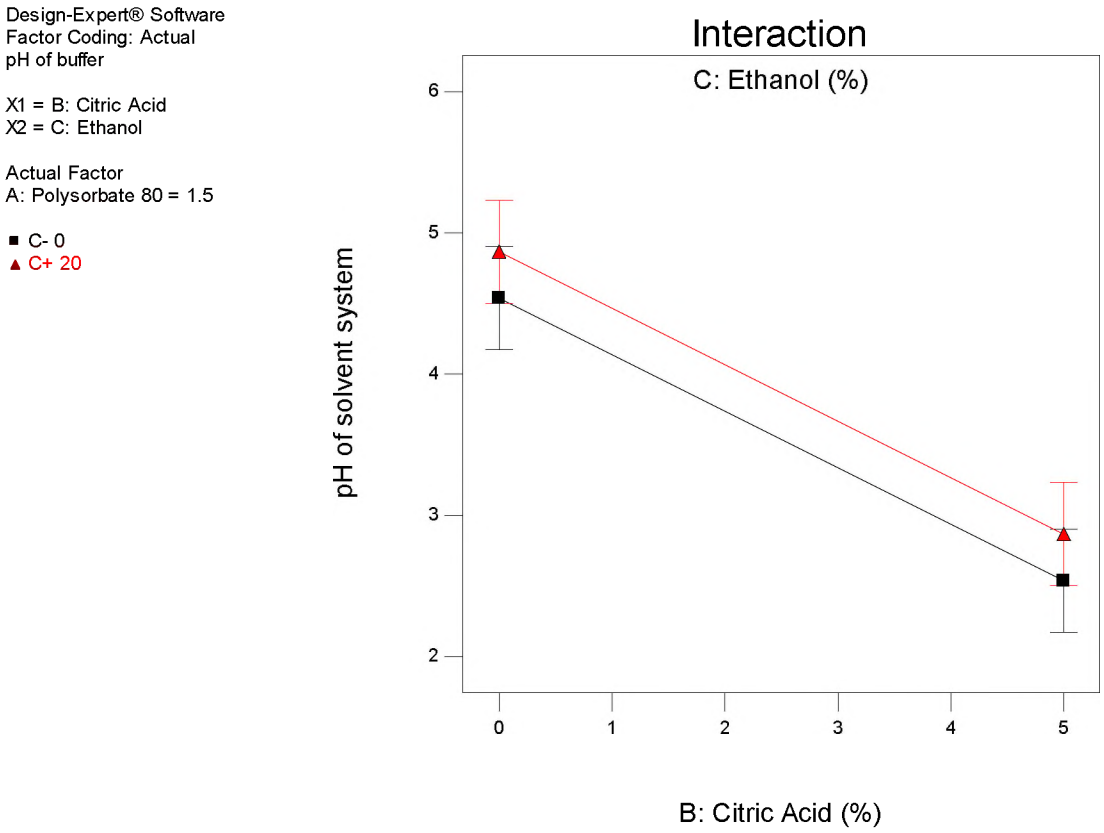


Figure 6.39 Interaction plot for ethanol at 0 and 20% and citric acid content on pH of the solvent.

6.2.3.3.5.1 Polysorbate 80 and citric acid

The 3D response surface plot depicting the impact of citric acid and polysorbate 80 content on pH of the solvent is shown in **Figure 6.40** from which it is clear that the pH of the solvent is dependent on citric acid content and the response is linear as the plot has a flat surface with the lowest pH evident when citric acid is used at the highest concentration of 5% m/v. Polysorbate 80 does not affect solvent pH within a 0 – 10% v/v range. Citric acid content is clearly the major determinant of solvent pH and as the citric acid concentration increases from 0 to 5% m/v the pH decreases from 4.5 to 2.5. This effect was expected as polysorbate 80 is a non-ionic compound and does not exert any effect on the pH of the solvent.

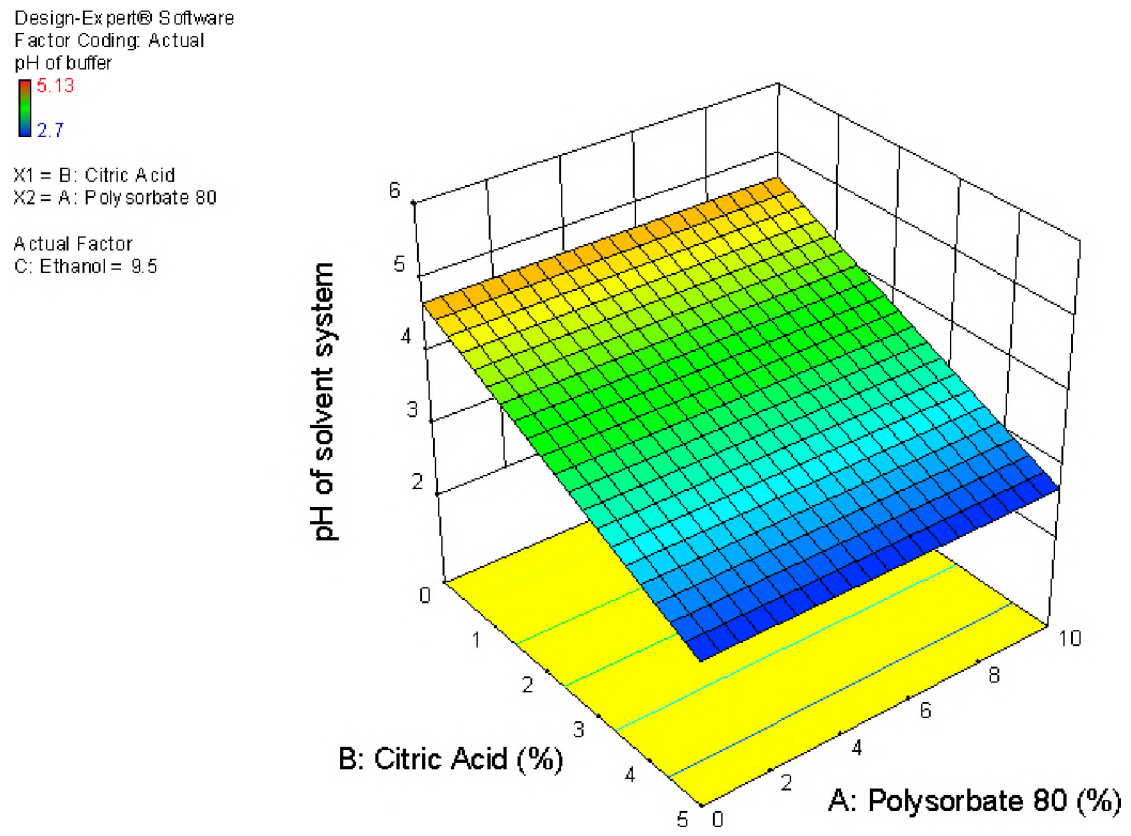


Figure 6.40 3D response surface plot depicting the impact of citric acid and polysorbate 80 content on pH of the solvent.

6.2.3.3.5.2 Citric acid and ethanol

A contour plot depicting the impact of ethanol and citric acid on solvent pH is displayed in **Figure 6.41**. From this plot it can be deduced that ethanol and citric acid have an antagonistic effect when modulating the pH of the quaternary solvent. As the citric acid concentration increases the pH of the solvent decreases whereas when the ethanol concentration increases the pH of the solvent increases slightly.

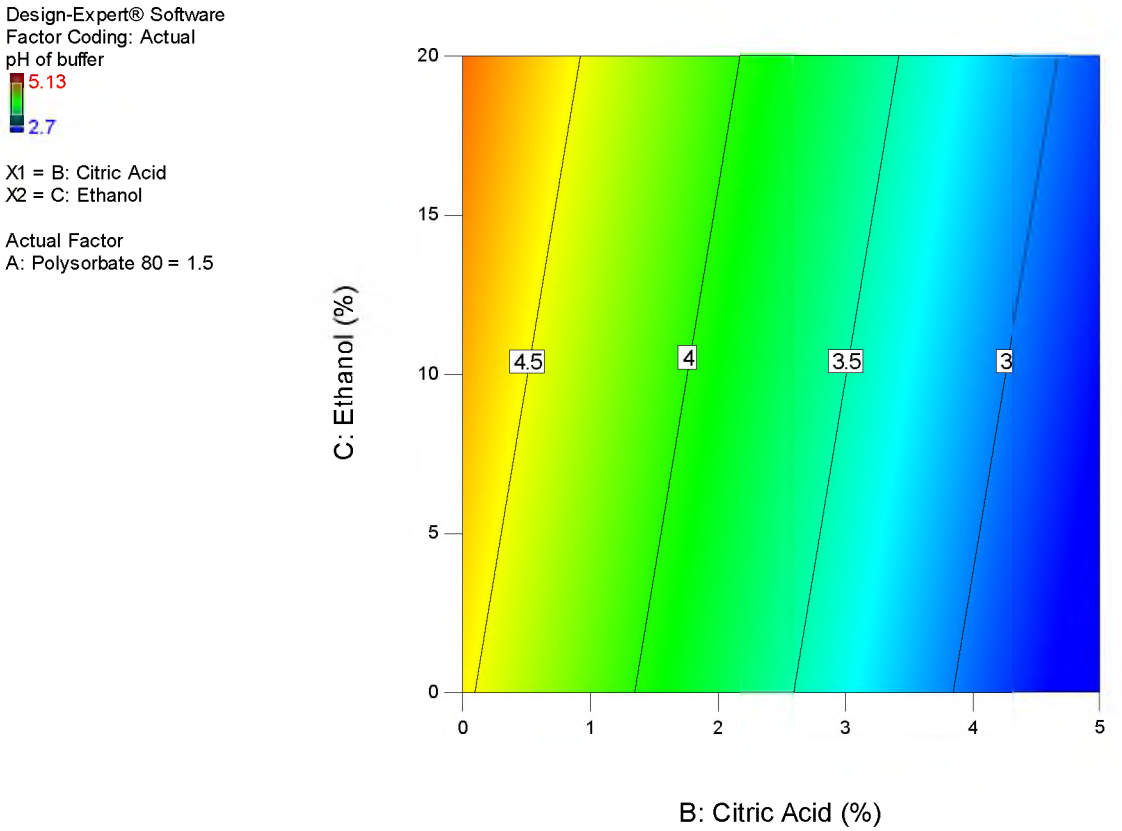


Figure 6.41 2D contour plot depicting the impact of ethanol and citric acid on pH of the solvent system.

The 3D response surface plot depicting the impact of ethanol and citric acid on pH of the solvent system is displayed in **Figure 6.42**. The surface of the plot is flat and is inclined with its lowest point at 0% v/v ethanol and 5% m/v citric acid when the concentration of polysorbate 80 is maintained at 1.5% v/v.

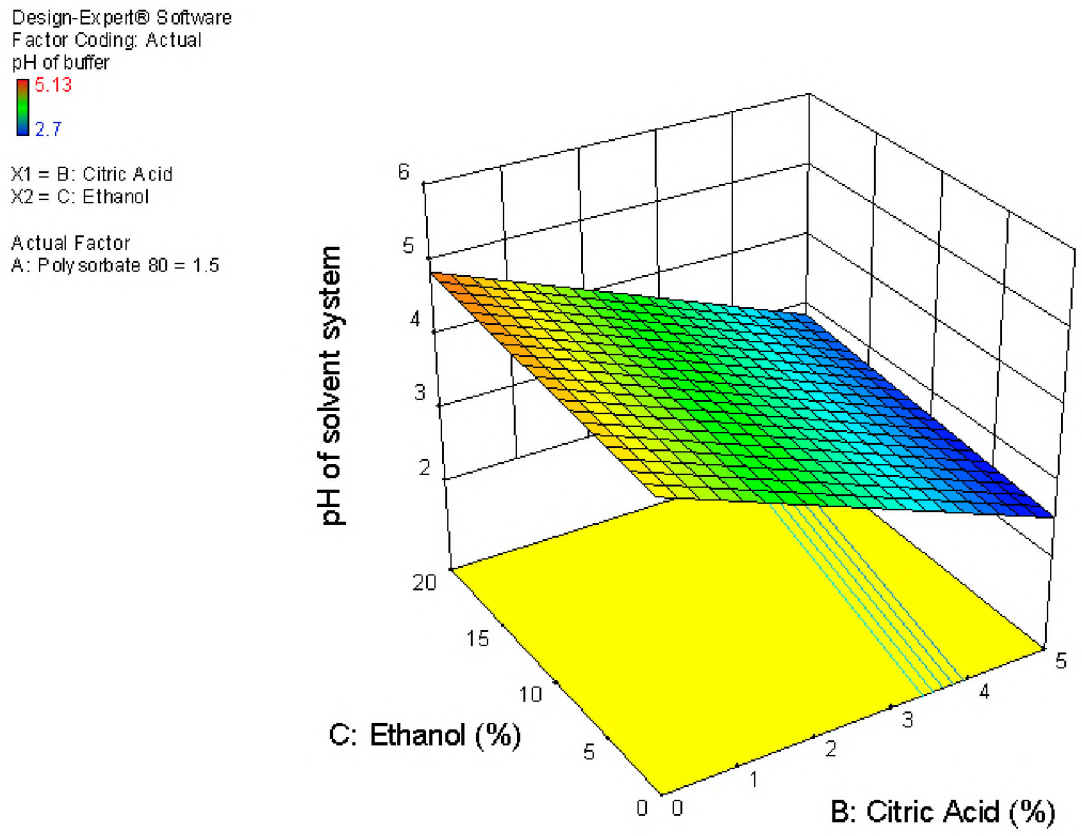


Figure 6.42 3D response surface plot depicting the impact of ethanol and citric acid content on pH of the solvent.

It can be deduced that ethanol (to a lesser extent) and citric acid (to a greater extent) change the pH of the solvent when the content of each factor is decreased or increased. This occurs due to the acidic properties of citric acid. The compound is a triacid and has pKa values of 3.13, 4.76 and 6.40 [190]. In contrast ethanol is slightly basic and exhibits a pKa of 16 [463] or 14 in water [464].

6.2.3.3.5.3 Polysorbate 80 and ethanol

The 3D response surface plot depicting the impact of ethanol and polysorbate 80 on the pH of the solvent system is depicted in **Figure 6.43** and reveals a slight incline in an otherwise flat surface to a low point when the polysorbate 80 and ethanol content is at 10% v/v and 0% v/v with citric acid content maintained at the 4% m/v level. This plot further confirms that ethanol has only a slight and polysorbate 80 no impact on the pH of the solvent system. The latter is likely a consequence of the neutral nature of the surfactant.

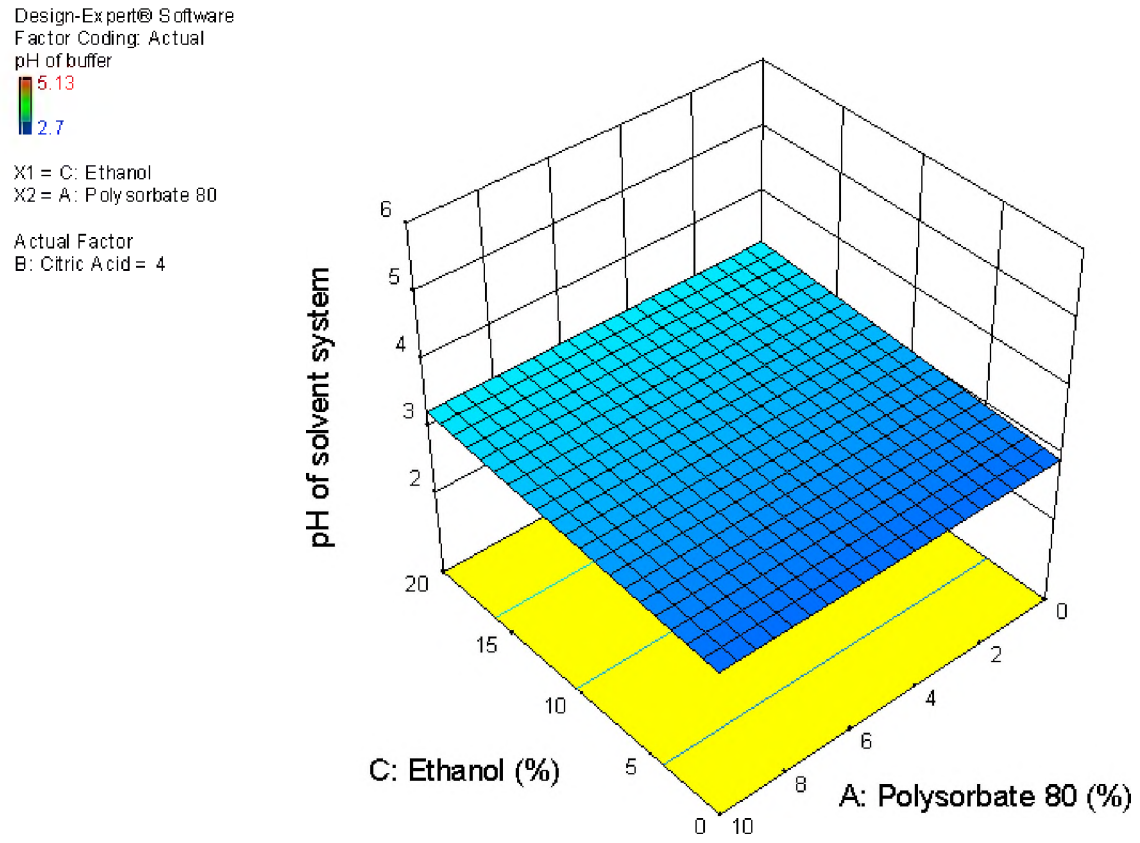


Figure 6.43 3D response surface plot depicting the impact of ethanol and polysorbate 80 on pH of the solvent system.

6.2.3.3.6 Selection of solvent composition

The optimised composition for the quaternary solvent system selected was comprised of 4% m/v citric acid, 1.5% v/v polysorbate 80 and 9.5% v/v ethanol made up to 50 g with citrate-phosphate buffer adjusted to pH 5.0. These components were selected based on the use of the lowest concentration of ethanol as aqueous solvents are desirable. In addition the highest pH possible for this solvent system was selected so as to establish maximum solubility and stability of KZ in the formulation. The pH selected for the solvent system was 3.5 and the solubility of KZ in this system was 71.41 mg/mL. This solvent system was identified as the optimum vehicle for the manufacture of a thermosetting KZ vaginal gel and that process is described in **Chapter 7** of this thesis.

6.2.4 Viscosity and pH of poloxamer solutions

Aqueous solutions with 10 to 20% m/v L127, L68 and L87 were manufactured by mixing the poloxamers in HPLC grade water and storing them in 50 g glass jars at ≤ 4°C for 48 and 72 hours with agitation every 24 hours. Clear poloxamer solutions were obtained and the viscosity of 10, 12, 14, 16, 18 and 20% m/v L127 and 2, 4, 6, 8, 10 and 12% m/v L68 and L87 solutions monitored. The temperature of the solutions was maintained at 22, 30, 37, 45 and 55°C using a Colora® Model NB-34980 Ultra-Thermostat water bath. A Model RVDVI+ Brookfield® DV-I+ viscometer was used to measure the viscosity (n = 3) of the solutions at the different temperatures. A model GLP 21 Crison® pH meter was used to monitor the pH of the different poloxamer solutions (n = 3). The accuracy and precision of the viscosity and pH assessment was such that the error bars are too small to be clearly observed in viscosity plots.

6.2.4.1 L127

The effect of 22 (ambient temperature), 30, 37 (body temperature), 45 and 55°C on the viscosity of the range of concentrations investigated is depicted in **Figure 6.44**. Concentrations of L127 of 10, 12 and 14% m/v demonstrated a similar viscosity when the temperature of the solution was increased from 22 to 55°C with viscosities < 10000 cP and liquid gels observed. The plot for the viscosity of different L127 concentrations are observed as a single line **(Figure 6.44)** as all exhibited a similar viscosity change. A gel manufactured using L127 at 16% m/v demonstrated an increase in viscosity as the temperature was increased and the viscosity observed was < 100000 cP. Solutions stored at 37, 45 and 55°C underwent a sol-gel transition but were liquid at 22°C. Solutions of 18 and 20% m/v L127 formed stiff gels with viscosities > 100000 cP at temperatures > 30°C and higher. Solutions of L127 maintained at temperatures > 55°C were not analysed as thermo-gelling behaviour at 37°C was critical to mimic biological conditions. The gels were also stored at 45 and 55°C to obtain the CMT of the poloxamers in solution which can vary between 15 and 40°C for L127 [465], 40°C and 80°C for L68 and L87 [334,466] and depending on their concentration in solution. Gels manufactured using L127 have been reported to exhibit sol-gel transitions at physiological temperature of 37°C from polymer concentrations of 16% m/v [336] and the viscosity observed in these studies **(Figure 6.44)** correlate with the reported data [334,336]. Therefore based on these data and information reported in **§ 5.3.4**, concentrations of L127 of 12 to 18% m/v were selected for use for the modulation of the thermosetting behaviour of the gels for the formulation optimisation process which is described in **Chapter 7**.

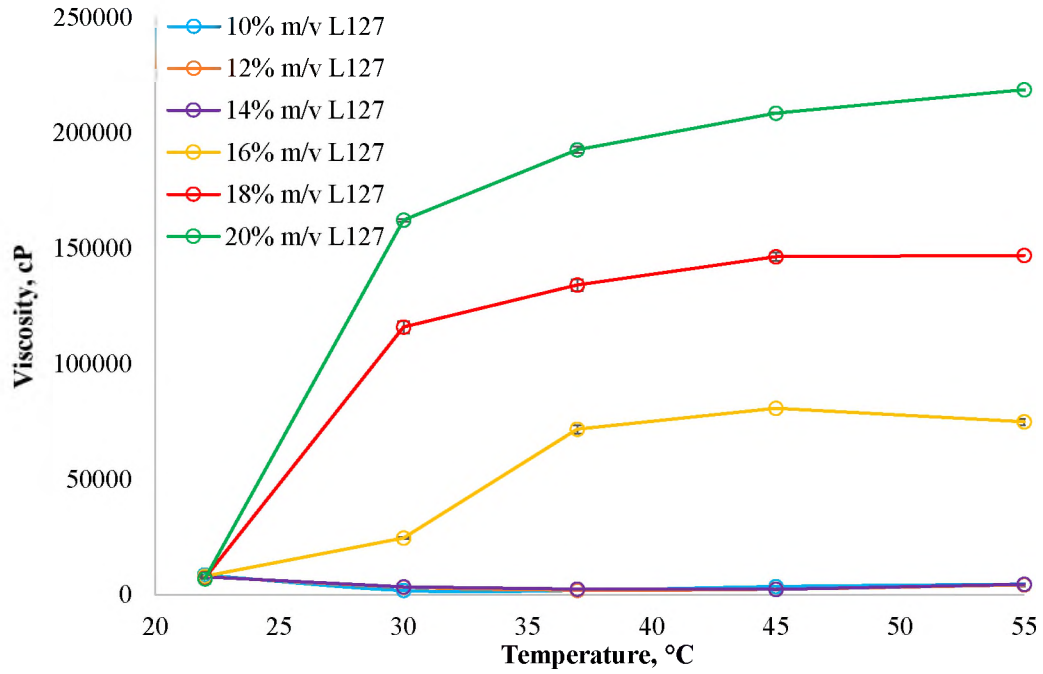


Figure 6.44 The effect of increasing temperature on the viscosity of gels manufactured using increasing concentrations of L127.

As pH is a critical parameter for vaginal formulations the pH of the gels manufactured using different concentrations of L127 was monitored and appeared to remain fairly constant at an almost neutral pH, as depicted in **Figure 6.45**. Caramella et al. reported that 15% m/v L127 exhibited a sol-gel transition at 30 °C [286] and the sol-gel transition of 20% m/v L127 was observed at 15 °C by Chaibva et al. [304]. Therefore the range of L127 was selected *viz.*, 10 to 20% m/v to ensure a sol-gel transition at approximately 35 °C as soon as the gel comes into contact with the vaginal mucosa. Sol-gel transitions at ≤ 35 °C may result in gelation of the dosage form during application making administration difficult. In addition, supplementary poloxamer inclusion that strengthen L127 micelles are an advantage due to their low molecular weight and the formation of smaller micelles that are located between larger L127 micelles resulting in a strong micellar structure.

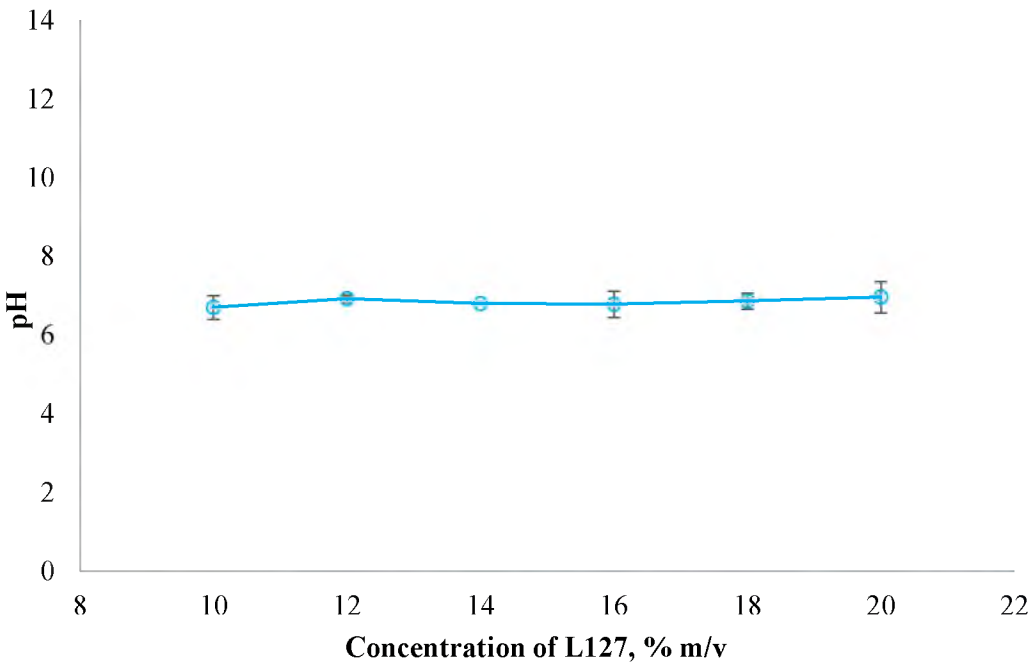


Figure 6.45 The effect of increasing L127 concentrations on pH of the gel.

6.2.4.2 L68

Increasing the amount of L68 used from two to 12% m/v in 2% m/v increments resulted in marginal viscosity changes when the temperature was increased from 22 to 55°C. The viscosity changes ranged between approximately 1000 and 7000 cP and all solutions remained in a liquid form over this temperature range. The four and 10% m/v L68 solutions exhibited a decrease in viscosity as the temperature was increased from 22 to 30°C after which a slight increase in viscosity was observed when the temperature increased from 30 to 37°C. A decrease in viscosity was again observed when the temperature was increased from 37 to 45°C after which an increase in viscosity to approximately 5000 cP at 55°C was observed. The viscosity change of the 2, 6, 8 and 12% m/v L68 solutions with increasing temperature followed a similar general profile **(Figure 6.46)**. The viscosities were slightly > 6000 cP at 22°C, decreased to between 2000 and 3000 cP at 30°C, increased to a maximum viscosity of approximately 7000 cP at 37°C and then decreased to between 2000 and 3000 cP at 45°C and eventually increased to between 4000 and 6000 cP at 55°C. No conclusive trend was observed for the viscosity changes as the temperature of the solutions was increased. L68 is reported to undergo sol-gel transition in solutions of 20% m/v at temperatures between 75 and 80°C [336]. These low viscosity values for L68 within the temperature range investigated was not critical as L68 was selected for inclusion to modulate the viscosity of solutions of multiple poloxamers of different molecular weight so as to achieve thermosetting from 35 °C and prevent gelling of the dosage form during application at temperatures < 37 °C. If this behaviour was achieved the formulation would set only when in contact with the vaginal mucosa.

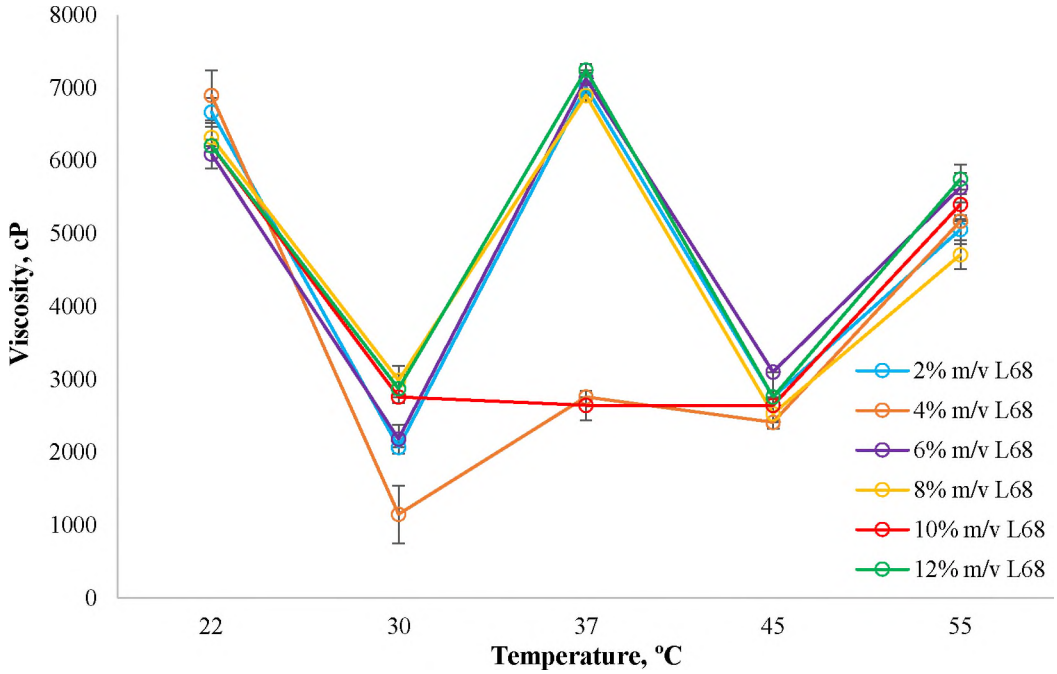


Figure 6.46 The effect of increasing temperature on the viscosity of solutions manufactured using increasing concentrations of L68.

Therefore L127 was used as it forms gels at temperatures close to physiological temperature without having to use excessive quantities in a formulation. If L68 alone were used to ensure gel formation at 37 °C concentrations of approximately 50% m/v would have to be used. This would be impractical and costly for the manufacture of formulations for low income countries. This erratic viscosity behaviour observed at low temperatures may be due to difficulty in accurately monitoring the viscosity of low viscosity solutions.

The pH of the L68 solutions of all concentration remained between a slightly acidic to neutral pH of between 5 to 7 **(Figure 6.47)**.

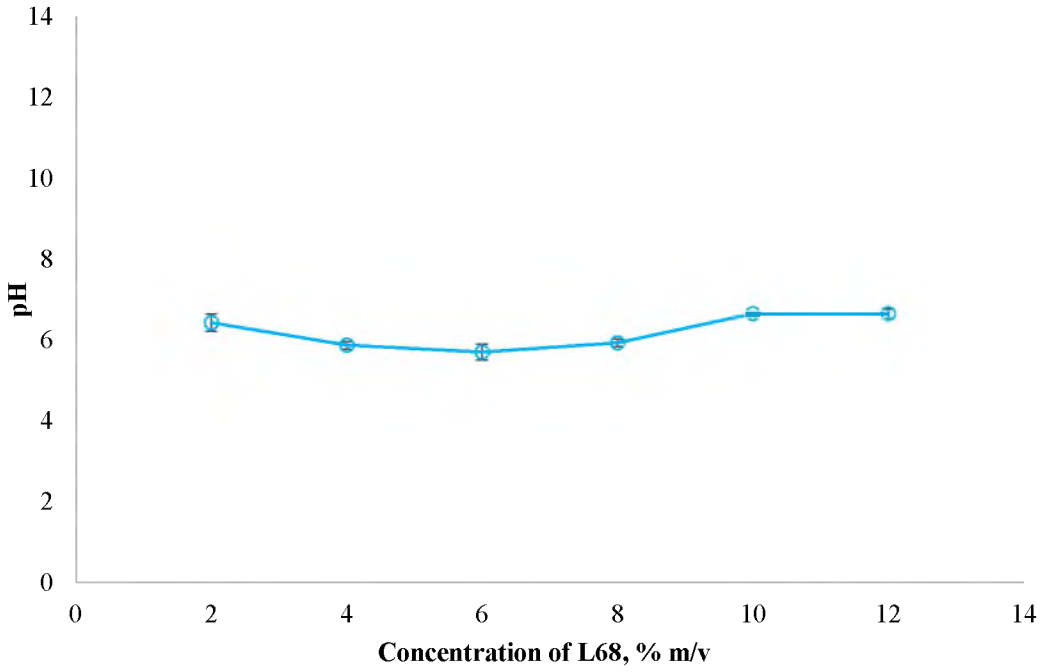


Figure 6.47 The effect of increasing L68 concentration on pH.

Although L68 did not gel within the concentration range used, concentrations of L68 between 7.50 to 12.50% m/v were selected for use during the formulation optimisation studies described in **Chapter 7** so as to modulate the viscosity of L127 to ensure thermosetting at 37°C.

6.2.4.3 L87

The viscosity of L87 solutions of different concentrations exhibited a decrease in viscosity from 5000 and 6100 cP as the temperature was increased from 22 to 30°C to approximately 2000 cP. As the temperature was increased further from 30 to 37°C, there was a slight reduction in viscosity to 1264 and 1724 cP for the 4 and 12% m/v L87 solutions respectively. The viscosity of 4 and 12% m/v solutions increased to 5172 cP with a temperature increase from 37 to 55°C (**Figure 6.48**). The viscosity of 2, 6, 8 and 10% m/v L87 solutions increased slightly to between 5000 and 6000 cP with a temperature increase from 30 to 37°C. From 37 to 45°C, a slight decrease in viscosity to between 1000 and 3000 cP was observed and from 45 to 55°C, an increase in viscosity to between 4000 to 6000 cP occurred. All solutions did not gel at elevated temperatures and L87 was to be included to modulate the thermosetting behaviour of the L127 gel in combination with L68. The L87 and L68 micelles or monomers are likely to be located between L127 micelles and/or monomers of the polymer thereby producing stiffer gel than when L127 is used alone.

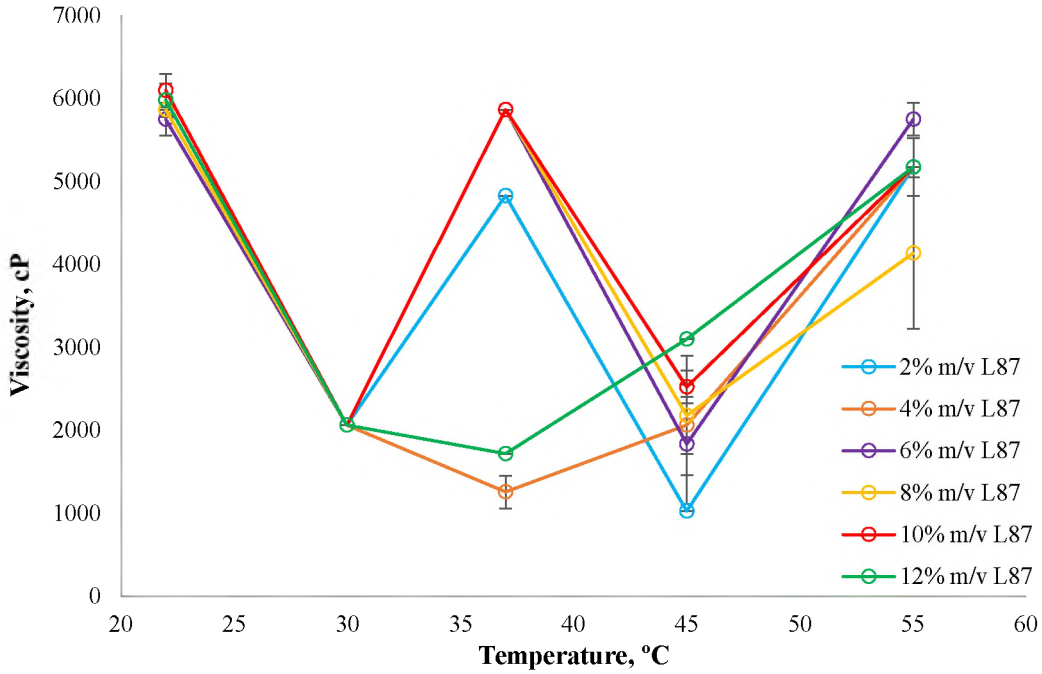


Figure 6.48 The effect of increasing temperature on the viscosity of solutions manufactured using increasing concentrations of L87.

The pH of 2 to 12% m/v L87 remained neutral as depicted in **Figure 6.49**.

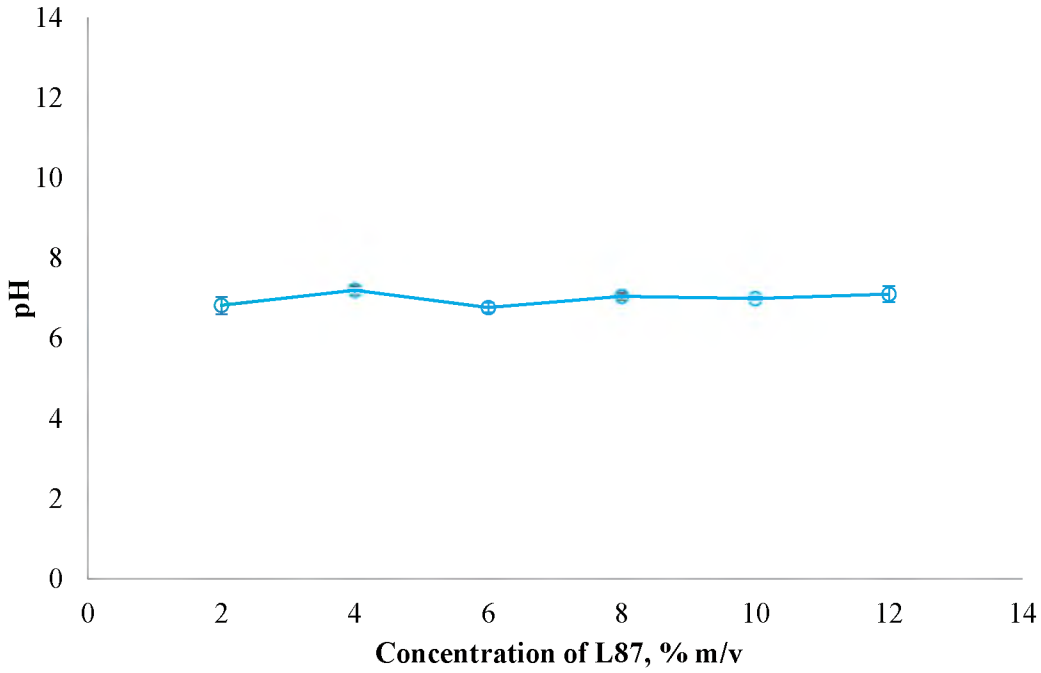


Figure 6.49 The effect of increasing L87 concentrations on pH.

A 2 to 6% m/v range of L87 was selected for evaluation during formulation optimisation to modulate the thermosetting behaviour of the gels at body temperature.

6.3 Conclusions

The preformulation studies conducted and reported were used to evaluate physical and thermal drug-excipient compatibility using FTIR and DSC. No obvious incompatibility was observed for all 1:1 KZ-excipient(s) mixtures. KZ was present in an amorphous form when in combination with polysorbate 80, L127, L68 and L87. The solvent system composition was optimised by investigating different concentrations of polysorbate 80, citric acid and ethanol with the ultimate target of maximizing solubility using the lowest amount of each of the excipients, whilst maintaining the pH of the system between three and four. The results of preformulation studies support the use of a cold manufacturing process to dissolve the poloxamer polymers in the solvent system to minimise the occurrence of any short term and avoid any long term interactions between the excipient and the solvent system. The range of poloxamers selected to modulate thermosetting behaviour of the gel was 12 to 18% m/v for L127, 7.50 to 12.50% m/v for L68 and 2 to 6% m/v for L87 based on the viscosity behaviour of the gels at different temperatures and the ultimate specifications and requirements for the gel formulation to be developed. The addition of L68 was required as gel stiffness or rigidity was reported to be affected by thermogelling polymers and although increasing L127 content can increase gel stiffness at high concentration it has a negative impact on drug release [467]. Therefore L68 was included to modulate

gel stiffness, sol-gel transition at 37 °C and KZ release [173]. Chen-Chow et al. reported that the rate of drug release from thermosetting gels depends on the micro-viscosity of the extra-micellar fluid, dimensions and number of the aqueous channels within the matrix and the equilibrium status of the drug between the micelles and the external aqueous phase [468]. The addition of small micelles formed by L68 and L87 may result in the formation of increasing numbers of aqueous channels within the matrix thereby enhancing dissolution. Thermosetting dosage forms in which L127 and L68 have been widely used have been summarized in **Table 5.2** [321,326-330].

L68 and L87 were included in the composition as they may increase the residence time of the gel in the vagina through the formation of a stiff gel following the sol to gel transition. Co-micellisation has not been widely studied and the reason for the formation of a stiff gel has not been elucidated. A hypothetical explanation may be a consequence of the low molecular weight poloxamers such as L68 and L87 forming tight crosslinks within the gel matrix through wedging between L127 molecules after sol-gel transitioning resulting in the formation of the stiffer gel containing hydrophobic moieties of different size. It is believed that the addition of a small quantity of L68 and/or L87 polymer may be necessary to maintain a solution of the gel formulation at ambient or storage temperatures < 37°C. The inclusion of L87 in gel formulation is not common but has been reported [183] as this low molecular weight material has a hydrophilic PEO/PPO composition. The use of this poloxamer was considered as it was believed that it would alter thermosetting behaviour and KZ release from thermosetting gel dosage forms. There was a need to monitor the impact of L87 on the gel when used in combination with L127 and L68 to modulate thermosetting and KZ release from the vaginal gel. A DoE described in **Chapter 7** was used to investigate the combined effects of L127, L68 and L87 on the specification of thermosetting KZ vaginal gel dosage forms. The pH of poloxamer solutions of all concentration in water exhibited a pH of between five and seven which is in agreement with reported values [469].

The complexity of using KZ in an aqueous gel for intra-vaginal use is a consequence of its low solubility in aqueous solution. The solubility in a solvent of high aqueous content, the lowest possible concentration of solvent components and a pH that is least irritating to the vaginal mucosa has been identified, manufactured and described.

CHAPTER SEVEN
FORMULATION AND CHARACTERISATION OF A THERMOSETTING VAGINAL GEL

7.1 Introduction

The formulation of *in situ* thermo-responsive gels is, in general, a simple process that requires the use of polymeric excipients such as poloxamers to modulate the thermosetting response at specific temperatures [180]. Poloxamers exhibit unique aggregation properties in aqueous media and form stable micellar systems that can include hydrophobic molecules such as KZ within the polymeric micellar core and thereby provide an attractive alternative for the delivery of water insoluble compounds [335]. Gels containing poloxamers are generally manufactured using hot and the cold processes [336,470-473] to yield gels that exhibit similar physico-chemical properties.

Hot processes involve the dissolution of poloxamers in aqueous solution at 70 °C. Water insoluble agents are dissolved in an organic solvent at the same temperature prior to mixing in the warm aqueous phase to produce a homogeneous solution. The solubilised drug is then added to the poloxamer solution at 70 °C and as the solution cools a gel forms [470,471]. The disadvantage of using the hot process is that the poloxamer clumps and is difficult to dissolve [471]. In addition the use of a high temperature may result in evaporation of the organic solvent if a lipophilic compound is used, which may exhibit thermal instability of the drug and/or other components in the formulation at elevated temperatures.

The use of the cold process was first described by Schmolka and this approach is the preferred and more widely used technique [474-481] for the formation of gels using poloxamers **(§ 5.3.4)** as it accommodates the inclusion of drugs that are heat labile and also limits the loss of solvent which may result in thixotropy and hamper manufacture [336,482]. In addition poloxamers are more soluble in cold aqueous media than in hot media [470,475,483,484] as a result of increased solvation and hydrogen bonding at lower temperatures [485]. At higher temperatures dehydration of the poloxamers occurs resulting in the formation of micelles that aggregate when the temperature is further increased, causing clumping. Gels prepared using a cold process require slow dispersion of the poloxamer in the aqueous phase at 4 to 5 °C while continuously agitating the mixture with or without the aid of refrigeration at that temperature for approximately five hours [470,471,475]. Lipophilic drugs are dissolved in an organic solvent at 4 - 5 °C prior to addition to the poloxamer dispersion [470,471].

The cold process approach was used to manufacture thermosetting gels containing KZ as the stability of KZ in aqueous media is dependent on temperature [43] and the compound was found to be unstable when subjected to heating at 60 °C for 4 hours **(§ 2.7.1)**. Consequently the use of a hot process may result in thermolytic degradation of KZ as the temperature used is ≥ 70 °C. KZ is a lipophilic molecule and therefore an organic solvent or a co-solvent system is necessary in order to ensure the molecule will

be incorporated into the gel in a solubilised form [27,37]. Consequently, the use of heat would cause evaporation of the organic solvent(s) eventually exhibiting an undesirable thixotropic effect during manufacture [336].

Following the optimization of solubilisation of KZ in a quaternary solvent (**§ 6.2.3**) it was then necessary to optimise the viscosity of the thermosetting gel in order to ensure that at 22 ºC or room temperature the product was liquid and when heated to 37 ºC formed a stiff gel. With this behaviour a dosage form with the potential to exhibit prolonged residence in the vagina would be produced. The L127, L68 and L87 grades of poloxamer were assessed and individual viscosity, poloxamer concentration and potential range of concentration were investigated and the results are described in **§ 6.2.4**.

Formulation development studies were undertaken using a DoE approach. Prior to the application of DoE for formulation development studies, the formulation variables and associated limits must be defined and understood within desired limits and attributes, after which it is vital to establish an appropriate experimental design in order to define the experimental matrix. The experiments defined in the matrix are then performed and the data generated for each response is collected and stored. The data are then mathematically and statistically evaluated and reduced to fit a mathematical relationship that is usually a polynomial equation. The fitness of the model is evaluated and the design space may then require reduction or be moved closer to an optimal region through data transformation to produce a better model fit. The optimal values for the factors analysed are then established using the information obtained from model fitting, data transformation and analysis of the plots generated using the data [344].

7.2 Materials

KZ and the poloxamers were donated by BASF (Ludwigshafen, Rhineland-Palatinate, Germany). The solvent system was manufactured according to the optimised formula reported in **§ 6.2.3.** All materials were used without further purification. Anhydrous glucose, yeast extract powder, nutrient agar and peptone powder were purchased from Merck® Laboratories (Wadeville, Gauteng, South Africa). *Candida albicans* was acquired from the UNESCO MIRCEN Yeast Culture Collection, Department of Microbial, Biochemical and Food Biotechnology, University of the Free State (Bloemfontein, Free State, South Africa) following completion of a materials transfer agreement for the period between 07/12/2014 to 31/12/2015. A copy of the agreement is included in **Appendix D**.

7.3 Method

All KZ dosage forms were manufactured in A-grade beakers and were protected from light using aluminium foil during production. The products were stored in amber glass bottles following manufacture in order to minimise exposure of KZ to light to prevent degradation. All products were manufactured on a weight for weight basis using the cold method of production [486].

The citrate-phosphate buffer (pH 5.0) was prepared as described in **§ 4.5.1.1** and the quaternary solvent system prepared by accurately weighing exactly 40 g citric acid into 850 g of the citrate-phosphate buffer pH 5.0 and sonicating the mixture for 30 minutes or until a clear solution had been formed, after which 15 g polysorbate 80 was added to the mixture and stirred using a magnetic stirrer until completely mixed. Exactly 95 g absolute ethanol was then added to the mixture that was stirred for a further 30 seconds. The pH of the solution was measured and established to be 3.5. A 1 kg batch of pH 3.5 solvent was manufactured for the manufacture of KZ gels.

Batches of 2% m/v KZ thermosetting vaginal gel (50 g) were manufactured with different amounts of poloxamer as defined in the CCD DoE. The amount of SM used was 1% m/v which is the maximum amount incorporated into topically applied formulations [190]. KZ (1 g) and SM (0.5 g) were dissolved in sufficient solvent in a 100 mL A-grade beaker covered with foil for the manufacture of 50 g of gel. The solution was mixed using a magnetic stirrer at 22 °C for 5 minutes and then sonicated for an additional five minutes prior to mixing for five minutes with the stirrer (**Figure 7.1**). The length of mixing and combined approach of using agitation and ultrasound ensured adequate mixing and dissolution of KZ and SM whilst protecting the integrity of KZ during dissolution through the activity of SM. The resultant product was a homogenous colourless to slightly yellow clear liquid.

Each batch of gel was prepared by weighing the exact amount of granular L127, L68 and/or L87 polymer into aluminium covered Consol® glass jars (Cape Town, Western Cape, South Africa). The granular polymeric materials were mixed prior to the addition of the KZ solution that was added with the aid of a glass rod so as to prevent splashing and loss of fluid. The bulk mixture was thoroughly mixed so as to ensure that the poloxamer molecules were adequately hydrated.

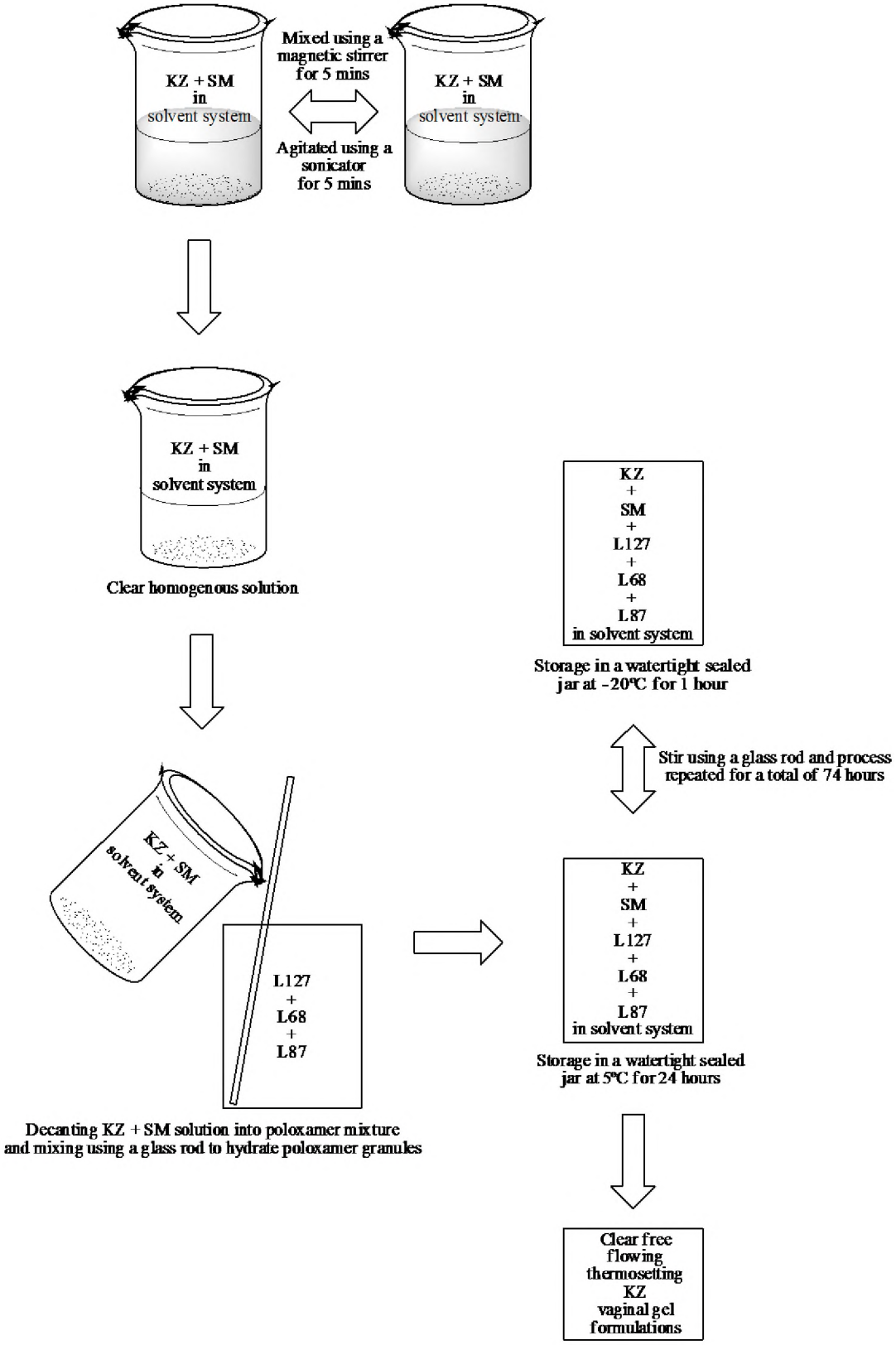


Figure 7.1 Schematic representation of the process used for the manufacture of thermosetting gels.

The product was packed into jars that were then sealed with the closure and over-sealed with Parafilm™ (Chicago, Illinois, USA) so as to prevent air ingress into the product . The jars were covered with

aluminium foil and placed in a Fuchsware® refrigerator (Midrand, Gauteng, South Africa) set at ≤ 4 °C for 24 hours in order to facilitate the cold production process and hydration of the poloxamers. The mixture was removed from the refrigerator after 24 hours, stirred using a glass rod and then transferred to a Model CF 355 KIC® Freezer maintained at -20 °C for 1 hour after which the product was again subjected to the process for a further 72 hours using a 24 hour cycle. The resultant gel was a clear free-flowing liquid that was assessed in respect of sol-gel transition time, viscosity at 22 °C and 37 °C, potency and KZ release at 24, 48 and 72 hours.

7.3.1 Sol-gel transition time

A number of simple and reliable 'bench-top' rheological tests have been used to assess the transition behavior of gel formulations and obviate the need for the acquisition of expensive rheometers and other such equipment [487,488]. A modified inverted test tube approach was used to test and monitor the sol-gel transition time of experimental formulations and was adapted from a variety of reported approaches [488-493]. Specifically, 1 mL of each test formulation was placed into a test tube of 9 mm diameter and 7.5 cm length and placed upright into a test-tube rack prior to placing the rack into a Labotec® oven (Midrand, Gauteng, South Africa) set to 37°C, for ten minutes. The tubes were then rotated through a 180° whilst still in the oven until the open side of the tube had been completely inverted. The time taken for the gel to flow to the opening of the tube was then monitored up to a maximum time of one minute. Formulations that did not flow during that period were considered suitable for further optimization studies as they exhibited low flow characteristics at body temperature.

7.3.2 Potency

A disc diffusion halo assay using agar plates inoculated with *Candida albicans* and discs of the formulation was used to establish the impact of poloxamer concentration and viscosity on the *ex vivo* potency of the formulations [494,495]. A yeast extract, peptone dextrose medium (YPD), was manufactured as a broth and was used in conjunction with agar extracts on 9 mm Sigma-Aldrich® disposable sterile petri dishes (St. Louis, Missouri, USA). The YPD was prepared by weighing 5 g yeast, 10 g peptone and 10 g glucose and adding the powders to 500 mL HPLC grade water. The YPD agar mixture was prepared by mixing 2.5 g yeast, 5 g peptone, 5 g glucose and 6.25 g agar in 250 mL HPLC grade water. The mixtures were then transferred to 500 mL Schott Duran® bottles and autoclaved with Brand® pipette tips (Wertheim, Baden-Württemberg, Germany), 4 mm Sigma-Aldrich® sterile discs (St. Louis, Missouri, USA) and McCartney bottles purchased from Lasec® (Port Elizabeth, Eastern Cape, South Africa). A model RAU-530D autoclave obtained from Rexall Industries (Qianzhen, Kaohsiung, Taiwan) used at a working pressure of 1.25 kg/cm^2 and temperature of 123 °C for 15 minutes was used to sterilize the materials [496].

In order to minimize contamination, an aseptic technique was used in conjunction with disinfection of hands and the use of sterile gloves worn at all times. Biological safety cabinet air flow was activated prior to use and the working surface disinfected with 70% v/v ethanol. The hot agar solution was allowed to cool to approximately 30 °C prior to pouring under laminar air flow into the petri dishes within a 40 cm radius of a naked Bunsen burner flame [496]. The petri dishes were filled with not more than half the height of the plates (15 mL) so as to avoid cross contamination [496]. Following the addition of the agar solution the plates were immediately covered so as to maintain sterility. The agar was then permitted to solidify at 22°C under laminar air-flow. The petri dishes were then sealed with Parafilm™ to reduce evaporation, after which they were stored in a refrigerator at 4°C and used within three days of pouring. Similar conditions were used for the nutrient broth that had been cooled to room temperature prior to storage.

The working surface of the laminar flow hood was swabbed using 70% v/v ethanol prior to inoculation of the agar plates with a vaginal strain of *Candida albicans*. The mouth of the container in which the YPD broth was stored was exposed to the open gas flame after which 10 mL of the broth was transferred using a sterile pipette tip into a sterile inoculation bottle. The cap of the inoculation bottle was immediately screwed onto the container and the bottle retained within range of the open flame. A wire inoculation loop was sterilized in a blue flame until the loop turned red, after which it was removed and allowed to cool within 20 cm of the flame. The Parafilm™ seal on the agar plate containing *Candida albicans* was carefully removed under laminar flow and the plate maintained in a horizontal position. The loop was then used to remove a single colony, representing single cell isolates from the surface of the agar, without cutting into the surface of the agar. The colony was then transferred to the inoculation bottle using the appropriate aseptic approach and the bottle loosely sealed to permit the *Candida albicans* colony to replicate aerobically in a constant environment chamber at an incubation temperature of 37 °C for 24 hours.

After 24 hours, approximately 1 mL of the YPD was removed and transferred aseptically into 5 mL YPD broth and the yeast incubated for a further 24 hours in order to produce fresh strains of *Candida albicans*.

The halo assay was performed by pipetting 10 µL of freshly cultured *Candida albicans* broth aseptically onto agar plates. The strain was evenly spread across the surface of the agar using an L-shaped glass rod that had been heated prior to spreading the inoculum. Four sterile filter disks were placed on each agar plate and 10 µL of each of the test formulations was placed onto one of the disks. Negative control plates with sterile filter discs and positive control plates with only the gel formulation without KZ were also used. All experiments were performed in triplicate on the same day and the plates were incubated for 48 hours to produce an adequate halo that could be accurately measured. The diameter of the halo

observed for KZ containing gels was measured and the inhibition of *Candida albicans* growth used for modelling and optimization of the formulation using Design Expert® software.

7.3.3 Viscosity of gel formulations

Viscosity is an important parameter that must be evaluated following the manufacture of thermosetting gels in general and specifically when targeting the vaginal route of delivery [286,318,497,498]. This category of gels should exhibit low viscosity at 22°C and high viscosity at 37°C. The targeted viscosity at 22°C was < 10000 cP and at 37°C was > 50000 cP. Approximately 25 g of each gel was placed into 50 mL beakers and the viscosity measured using a Brookfield® DVI+ Viscometer fitted with a helipath stand, operated at 5.8 rpm with spindle D (S94) over a 10 second period. The speed and spindle were selected so as to produce stable measurements for viscosity at the low and high specification limits using the same speed and spindle size, so as to obtain results that could be readily compared. Spindle D was selected so as to maintain a torque of between 10% and 90% read from the Brookfield® DVI+ Viscometer. The torque was used to calculate the viscosity of the sample. This range in torque must be respected so as to ensure the accuracy of the data that can be measured using a specific spindle at a specific speed. The gels were initially analysed in triplicate at 22°C after which they were placed in a Colora® Model NB-34980 Ultra-Thermostat water bath (Lorch, Hesse, Germany) set at 37°C and heated for ten minutes after which the viscosity was again measured (n = 3) whilst retaining the gels in the water bath.

7.3.4 KZ release at 24 hours, 48 hours and 72 hours

The *in vitro* release of KZ was assessed using an *in vitro* release method validated as described in **Chapter 4.** Approximately 1 g of test formulation was added to the donor compartment together with 1 mL SVF and KZ release assessed at 24, 48 and 72 hours so as to evaluate the extent of KZ release from the gel formulations. Samples of receptor fluid were analysed using a validated RP-HPLC method.

7.4 Results and discussion

7.4.1 Optimization of thermosetting behaviour using Central Composite Design

A CCD DoE was used to optimize the thermosetting behaviour of test formulations in which the amount of L127, L68 and L87 were varied and the sol-gel transition, potency, viscosity at 22 °C and 37 °C and release of KZ at 24, 48 and 72 hours monitored (dependent variables) for each experiment. The optimum amount of each of the poloxamers was established in order to obtain the best thermosetting response, *viz.*, sol-gel transition ≥ 60 seconds, zone of inhibition (potency) ≥ 15 mm, viscosity at 22°C ≤ 10000 cP and 37°C ≥ 10000 cP and ≥ 25% KZ released at 24 hours, 48 hours and 72 hours. The range of L127, L68 and L87 established and reported in **§ 6.2.4** was used with SM for this purpose. Optimisation of the formulation was achieved using a two-step process. Initially a CCD experimental design model (CCD I) was used to produce a formulation that demonstrated adequate gelling properties at 22°C and

37°C in order to define an optimised gel composition in terms of thermosetting characteristics. The second phase involved the use of CCD, *viz.*, CCD II to produce a gel with an optimal pH and antioxidant level in the defined solvent system such that a clear, colourless and stable gel was produced. Two separate CCD experiments were undertaken so as to distinguish between the optimisation of the gelling characteristics and stability of the formulation according to SM content, solvent pH, length of storage and storage temperature independently. Moreover, DoE can interpret each response with two input variables by means of contour plots and 3D response surface plots. Regression analysis permits a response and its input factors to be correlated using mathematical modelling; however, the relationship may be better understood using graphical methods of assessment. RSM is also not performed with > 6 input factors as the number of experiments increases as the number of input factors increase [387]. Therefore, two CCD studies were conducted instead of a single large experiment with many input factors.

A summary of CCD I in terms of initial design factors that included the amount of L127, L68 and L87 polymers used is listed in **Table 7.1**. The range of amount of L127, L68 and L87 investigated was 12 to 18% m/v, 7.5 to 12.5% m/v and 2 to 6% m/v respectively. The low and high values were used with Design Expert® software to generate a CCD model with six centre point replicates and 20 experiments were required (**Table 7.1**). The experiments were performed in the sequence generated by the software in order to minimise experimental bias and a copy of the batch production record for the formulations labelled as R1 to R20 obtained from CCD I is depicted in **Appendix E**. Batch summary records containing *in vitro* release profiles for R1 to R20 are reported in **Appendix F**.

Table 7.1 Randomised experimental runs conducted to assess the impact of polymer composition on gel properties.

SD	Run	A L127 % m/v	B L68 % m/v	C L87 % m/v
3	1	12.00	12.50	2.00
9	2	9.95	10.00	4.00
13	3	15.00	10.00	0.64
14	4	15.00	10.00	7.36
11	5	15.00	5.80	4.00
19	6	15.00	10.00	4.00
7	7	12.00	12.50	6.00
8	8	18.00	12.50	6.00
6	9	18.00	7.50	6.00
2	10	18.00	7.50	2.00
20	11	15.00	10.00	4.00
12	12	15.00	14.20	4.00
18	13	15.00	10.00	4.00
10	14	20.05	10.00	4.00
17	15	15.00	10.00	4.00
1	16	12.00	7.50	2.00
15	17	15.00	10.00	4.00
5	18	12.00	7.50	6.00
16	19	15.00	10.00	4.00
4	20	18.00	12.50	2.00

Mathematical models were used to assess the impact of amounts of polymer level (independent variables) on the seven responses monitored in order to identify the optimum composition for thermosetting behaviour, KZ release and potency of the formulations.

7.4.2 Model fitting

A quadratic order polynomial model was selected for the evaluation of the design space. All factors, *viz.,* L127, L68, L87, L127*L68, L68*L87, L127*L87 and the square of the factors, *viz.,* $L127^2$, $L68^2$ and $L87^2$ were model terms evaluated and analysed. No aliases were established in the quadratic order model and the lack of fit test was found to have 5 df with 5 df for pure error suggesting that the model is able to detect a lack of fit adequately.

The standard error within factors was 0.27, within binary combination of factors was 0.35 and within the square of factors was 0.26, suggesting no multi-linearity constraints existed in the model. The variance inflation factors (VIF) were all equal to one, indicating that well estimated coefficients had been identified. The multiple correlation coefficient was zero for all terms except the squared factor model terms, where the value of 0.0179 for the coefficient for this term is correlated to other terms. The average leverage calculated from all runs was 0.5 suggesting that the points in the design space do not require additional replication. The condition number of the coefficient matrix was < 100 with a reported value of 1.33 revealing that there is no multi-collinearity between the terms. A 74.7% G-efficiency value indicates that lack of fit was adequately low suggesting that the model fit is robust.

The use of a power tool is inefficient for RSM designs; therefore, a fraction of the design space (FDS) tool, which analyses the potential prediction performance of designs, was selected for use. A FDS plot was used to depict unscaled prediction variance versus a fraction of the paired design space [499]. The mean error type and one-sided t-test was used to calculate the FDS based on half-width of the CI as, illustrated in **Figure 7.2**.

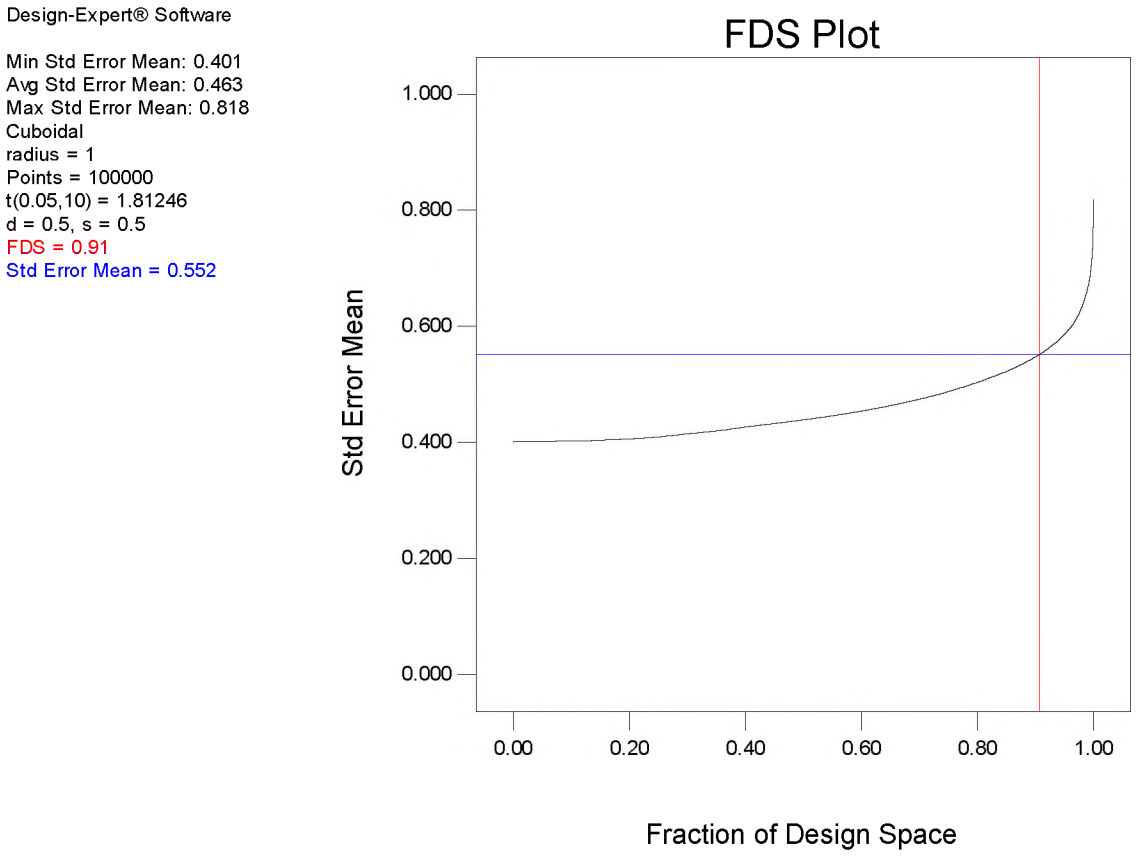


Figure 7.2 FDS plot depicting the fraction of the design space that can be navigated accurately.

The term 'd' corresponds to delta and is an indication of the minimum detectable change of a response, 's' corresponds to sigma and is an indication of the estimated SD whereas the term 'a' corresponds to alpha and is the level of significance used for statistical analyses, for example, 0.05 for a 5% Type I error risk at a 95% level of confidence. The FDS curve provides an indication of the percent or fraction of the design space that falls within a specified standard error of prediction [404]. In this case 91% of the design space falls within the 95% CI with a standard error of 0.552 for the mean (**Figure 7.2**). The value should be ≥ 80% for adequate power prediction and the value for 91% is an indication that the design space for the CCD is sufficiently robust to predict the outcome accurately for the responses monitored.

Overall the quadratic order polynomial model was found to be adequate for use to navigate the design space for these data.

7.4.3 Responses monitored

The impact of the amount of L127, L68 and L87 polymer used on sol-gel transition, potency, viscosity at 22°C and 37°C and KZ release at 24 hours, 48 hours and 72 hours was evaluated. The optimized formulation composition was selected in relation to the impact of input variables on the dependent parameters monitored. The model transformations recommended for these parameters are listed in **Table 7.2**. The response for potency and KZ release does not require model transformation for further analysis as the ratio of the maximum and minimum values for the response was < 10.00. The ratio of maximum to minimum values for sol-gel transition, viscosity at 22°C and viscosity at 37°C were established as 120.00, 63.37 and 328.43 and square root, inverse and natural log transformations were required so as to facilitate data analysis. Although coded values are recommended for use by Design Expert® the actual values are reported to facilitate an understanding of data presented in the plots.

Table 7.2 Responses for thermosetting gel formulations and respective model transformations.

Response	Parameter	Unit	Minimum observed	Maximum observed	Recommended transformation
R1	Viscosity at 37°C	cP	1034	339600	Natural log
R2	Viscosity at 22°C	cP	3103	196633	Inverse
R3	Drug release over 24 hours	$\mu g/cm^2$	1588.29	3603.58	None
R4	Drug release over 48 hours	$\mu g/cm^2$	1920.86	4307.07	None
R5	Drug release over 72 hours	$\mu g/cm^2$	2203.74	5023.59	None
R6	Sol-gel transition	s	0.50	60.00	Square root
R7	Potency Study	mm	8	34	None

The response coefficients, residuals and ANOVA are only reported for the viscosity of thermosetting gel at 37°C. The viscosity of the gels at 37°C is an important and critical response for this formulation and was therefore used as a basis for explanation of how data and response models were used in these studies using Design Expert® software, prior to further analysis of the contour and response surface plots. The transformed and untransformed model equations in actual terms are reported for all responses

monitored and the resultant equations may be used to establish the relationship and impact of the amount of L127, L68 and L87 on a response so as to simplify the identification of an optimized composition for the vaginal gel under development. 3D response surface plots for all other terms are reported in **Appendix G** and release profiles from six gels, *viz.*, one high, four moderate and one low KZ release per unit area, are reported and the balance of the profiles are depicted in batch summary records for formulations manufactured using CCD I (**Appendix F**).

7.4.3.1 Viscosity at 37°C

Statistical modeling was undertaken using a quadratic model with significant terms ($p < 0.05$) identified in order to define the final equations for the parameter. ANOVA data generated using Design Expert® are listed in **Table 7.3** and include sum of squares, df, mean square, F-value, p-value and significance of model terms. The model F-value of 24.86 implies that the model is significant and also indicates that there is a 0.01% ($p = 0.0001$) chance that the model F-value could be due to noise. A lack of fit F-value of 0.50 indicates that the lack of fit is insignificant relative to pure error and there is a 33.32% ($p = 0.3332$) chance that a lack of fit could occur due to noise. The lack of fit df = 5 and is within the acceptable range of 2 [401] and 5 [402] and is therefore a suitable number of df for model fit. The terms for L68, L87, L68*L87, L127*L87 and the square of all responses was found to be significant and these factors contribute to the viscosity of thermosetting gels at 37°C. The terms for L127 and L68*L87 were found to be the insignificant model terms with $p = 0.769$ and $p = 0.2592$ respectively.

Table 7.3 ANOVA data table for the CCD model for viscosity of thermosetting gels at 37°C.

Source	Sum of squares	df	Mean square	F-value	p-value Prob > F	Significant difference
Model	55.53	9	6.17	24.86	< 0.0001	Yes
A-L127	0.97	1	0.97	3.89	0.0769	No
B-L68	3.69	1	3.69	14.85	0.0032	Yes
C-L87	3.63	1	3.63	14.63	0.0033	Yes
L127*L68	2.94	1	2.94	11.85	0.0063	Yes
L127*L87	3.51	1	3.51	14.14	0.0037	Yes
L68*L87	0.36	1	0.36	1.43	0.2592	No
$L127^2$	4.58	1	4.58	18.46	0.0016	Yes
$L68^2$	1.40	1	1.40	5.65	0.0388	Yes
$L87^2$	1.73	1	1.73	6.96	0.0248	Yes
Residual	2.48	10	0.25	-	-	-
Lack of fit	1.49	5	0.30	1.50	0.3332	No
Pure error	0.99	5	0.20	-	-	-
Total correction	58.01	19	-	-	-	-

A high degree of correlation between the predicted and adjusted R^2 values was evident and R^2 value was close to 1. The predicted R^2 of 0.7731 is in reasonable agreement with the adjusted R^2 of 0.9187 (**Table 7.4**). An adequate precision, which is an indication of the signal to noise ratio, > 4 is recommended and the value of 17.42 is an indication that the model can be used to navigate the design space. The coefficient of variation (CV) measures the closeness of data to the mean and adequate precision and reliability of the model is evidenced by a low value for CV. The value for the CV was 5.30 indicating that the data generated, in these experiments, are reliable.

Table 7.4 Statistical measures of model adequacy.

Squared residuals and derivatives	Values
R^2	0.9572
Adjusted R^2	0.9187
Predicted R^2	0.7731
Adequate precision	17.42
SD	0.50
Mean	9.40
% Coefficient of variation	5.30
Predicted residual error sum of squares	13.16

The final equation for the viscosity of the gel monitored at 37°C reflected in actual terms is reported in **Equation 7.1** and can be used to make predictions in respect of this response for specific levels of input factors used. However, the equation should not be used to determine the relative impact of individual factors as the intercept for the relationship is not located at the centre of the design space and coefficients are scaled to accommodate units for the individual factors. Transformation of the data set will not allow for an accurate analysis of the equation as the terms are all calculated as a natural log value. Therefore the equation pertaining to the raw data set is also reported in **Equation 7.2** so that a suitable data analysis may be performed.

$$\ln(\text{viscosity at } 37\ ^{\circ}\text{C}) = 5.13 - (1.01 \times L127) + (2.19 \times L68) - (2.39 \times L87) - (0.08 \times L127 \times L68) + (0.11 \times L127 \times L87) + (0.040 \times L68 \times L87) - (0.060 \times L127^2) - (0.050 \times L68^2) + (0.090 \times L87^2) \quad \textbf{Equation 7.1}$$

The quadratic equation depicts the coefficient for the intercept, the first order main effects, interaction terms and higher order effects. The + or - operator sign and magnitude of the effect are used to quantitate the influence of each parameter on the natural log of viscosity of the gels at 37°C. The value for the coefficients reveal that the terms L168, L127*L87, L68*L87, $L87^2$ have a positive effect on the natural log of viscosity of the gel at 37 °C. The insignificant model terms, L127 and L127*L68, have a negative effect on the natural log of the viscosity at 37 °C response with the term L127*L68 exhibiting the least synergistic effect.

In terms of measurement of viscosity at 37 °C, the L127, L68 and L87 polymers exhibit a negative effect on the viscosity with L127 (199132 cP) and L87 (208643 cP) exhibiting effects of a similar magnitude and L68 (14552 cP) exhibiting an effect > 10 times lower than the other poloxamers as depicted in **Equation 7.2**.

$$\text{Viscosity at } 37\ ^{\circ}\text{C} = 1.72 - (199132.00 \times L127) - (14552.49 \times L68) - (208643.00 \times L87) + (84.30 \times L127 \times L68) + (12028.50 \times L127 \times L87) + (2028.80 \times L68 \times L87) + (5947.69 \times L127^2) + (311.63 \times L68^2) + (4291.56 \times L87^2)$$

Equation 7.2

The significant effects observed using untransformed data are revealed by terms for L127, L87, L127*L87 and $L127^2$ and these values are more reflective of the real time situation. Poloxamer L127 and L87 have similar ratios of PEO and PPO when compared to L68 and it is not surprising that they exhibit similar viscosities at 37 °C. However, L87 is seldom used for the manufacture of thermosetting gels and this information supports the fact that L87 can be used to modulate thermosetting behaviour in combination with L127. Poloxamer L68 exhibited an insignificant effect on the viscosity of the formulations at 37 °C. The relative impact of the factors can be better evaluated using a graphical representation of the model.

The contour and 3D response surface plots for viscosity at 37°C were generated for binary combinations of input factors and are depicted in **Figures 7.3 - 7.8**. On comparison of the contour and 3D response surface plots generated using transformed and untransformed data, similar responses were observed and the shape of the plot was enhanced following transformation. The shape of the plots reveals that moderate interactions exist between the variables under investigation.

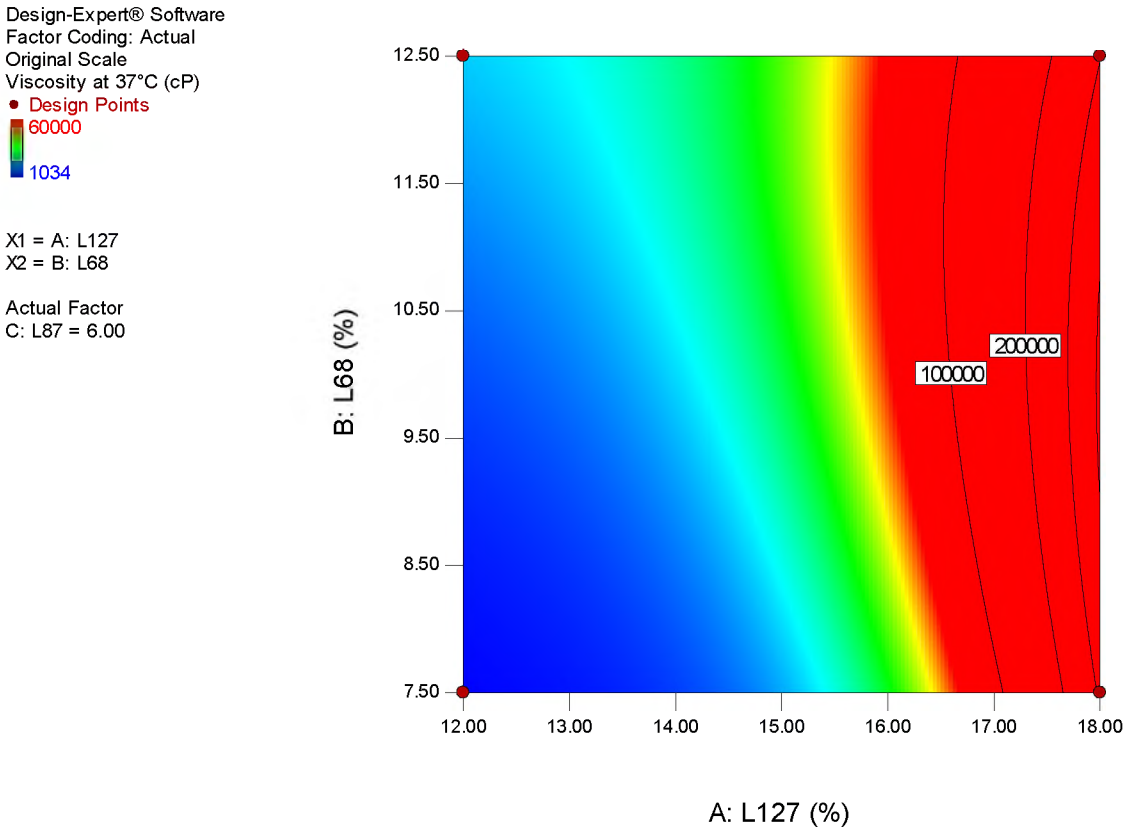


Figure 7.3 Contour plot depicting the impact of L68 and L127 content on viscosity at 37°C.

The interaction between the terms for L127 and L68 is depicted using contour (**Figure 7.3**) and 3D response surface (**Figure 7.4**) plots for the condition in which the amount of the L87 polymer is maintained at 6% m/v. The viscosity of thermosetting gels at 37°C is affected by the concentration of L127 and increases from 1034 to 60000 cP when the concentration of L127 increases from 12 to 18% m/v. The viscosity is most affected by the concentration of L68 when the concentration of L127 falls between 14 and 16% m/v L127 can therefore be used between these concentrations for viscosity modulation to obtain a better and accurate thermosetting behaviour.

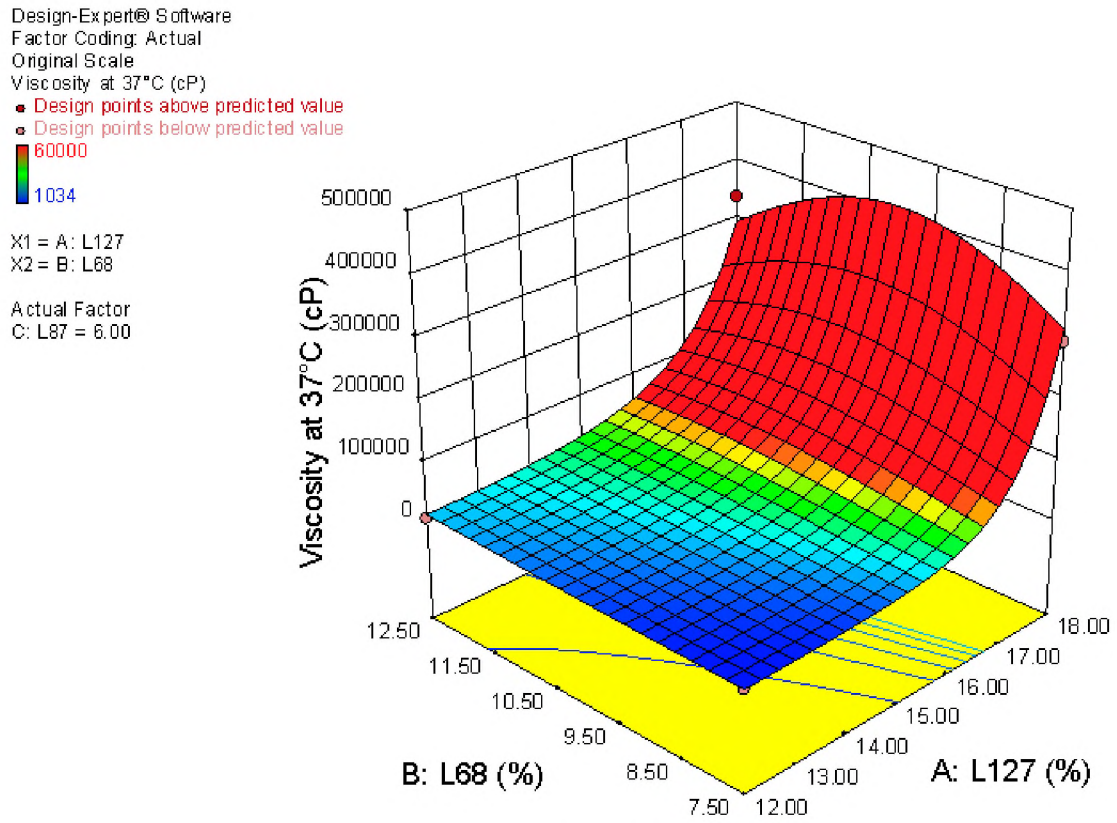


Figure 7.4 3D response surface plot depicting the impact of L68 and L127 content on viscosity at 37°C.

The 3D response surface plot (**Figure 7.4**) reveals a slight increase in the viscosity at 37°C when 18% m/v L127 and 9.5 - 10.5% m/v L68 are used and the lowest viscosity is observed when 12% m/v L127 and any concentration of the L68 polymer is used within this design space. The shape of the 3D response surface plot curves upwards from 12 to 18% m/v L127 content. When the amount of L127 is increased in the dosage form, the gels exhibit a dramatic increase in viscosity. The viscosity of the thermosetting formulation therefore is highly dependent on the amount of L127 used in the formulation. The amount of L68 does not appear to contribute to an increase in viscosity at 37°C to any great extent and the amount of L127 appears to be the primary contributing factor. Poloxamer L68 is generally added to thermosetting gels in combination with L127 in order to produce gels of low viscosity at room

temperature and for the modulation of sol-gel transitions to occur at specific temperatures or 37°C in this instance [328].

The interaction effect of poloxamer L127 and L87 is depicted in **Figures 7.5** and **7.6** that reveal that the viscosity of the formulation at 37°C increases linearly as the amount of L127 and L87 used is increased. The highest viscosity of > 300000 cP was observed when 18% m/v L127 and 6% m/v L87 were used whilst the content of L68 was maintained at 10% m/v. The combination of L127 and L68 has a positive effect on the viscosity of the formulation at 37°C.

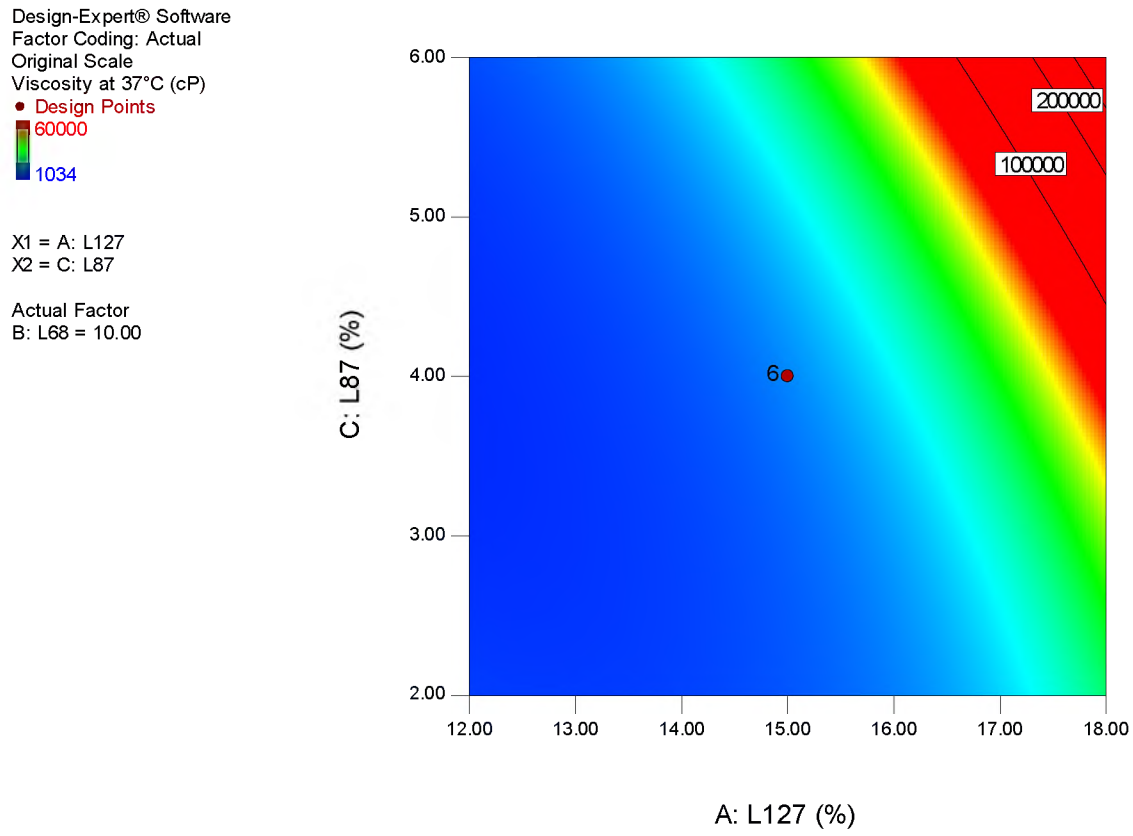


Figure 7.5 Contour plot depicting the impact of L87 and L127 content on viscosity at 37°C.

The shape of the 3D response surface plot is flat when low amounts of L127 and L87 are used and the surface elevates to produce a steep peak at one corner of the design space when the concentrations of both poloxamers are used at their upper limit. As the content of L127 and L87 increases there is a steep and dramatic increase in the viscosity of the formulation at 37°C. This positive increase in viscosity is desirable for a thermosetting formulation that is required to exhibit a low viscosity at room temperature and must solidify at 37°C when inserted into the vagina thereby preventing leakage of the formulation and increasing the residence time of KZ at the site of action.

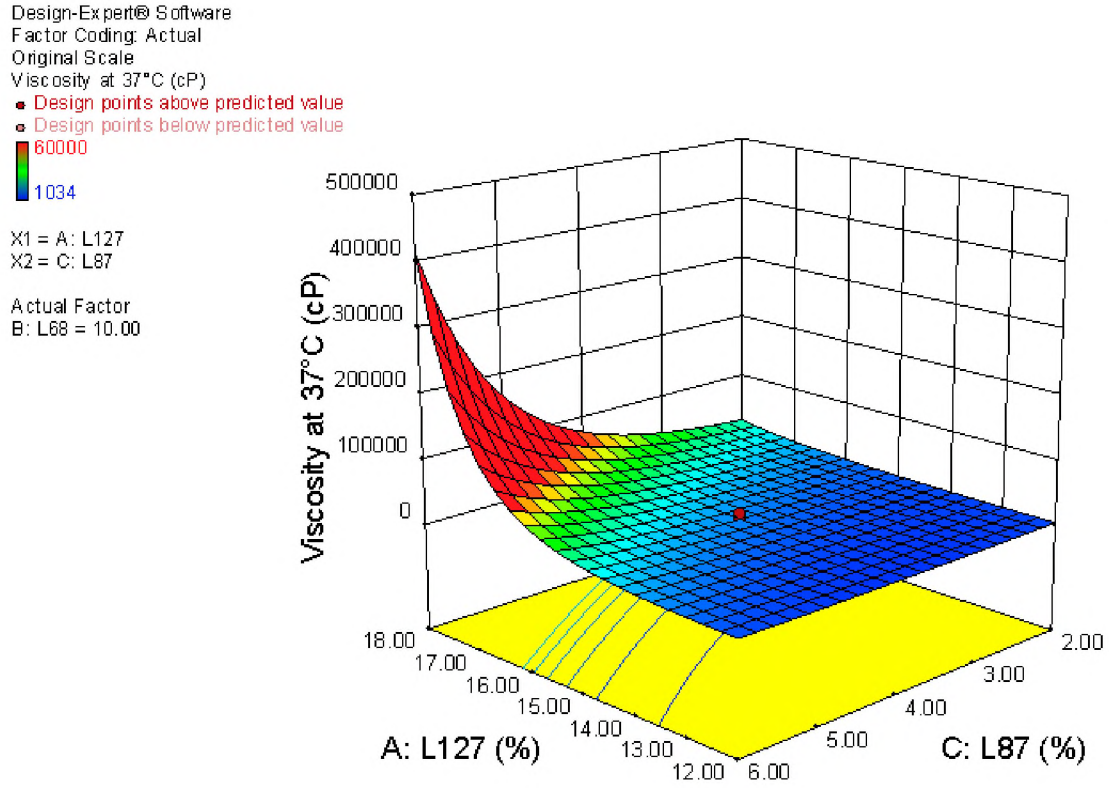


Figure 7.6 3D response surface plot depicting the impact of L87 and L127 content on viscosity at 37°C.

The contour and 3D response surface plots depicting the impact of the content of L68 and L87 on viscosity are shown in **Figures 7.7** and **7.8** for the condition when the content of L127 is maintained at the 16% m/v level. As the amount of L87 is increased the viscosity of the formulation increases from 1034 to 60000 cP. At a 3% m/v level of L87 an increase in L68 content resulted in an increase in viscosity until 10% m/v L68 was reached, after which the viscosity decreased slightly on further addition of the polymer. This phenomenon was significant when the amount of L87 used was between 5 to 6% m/v. When the amount of L68 is increased, the viscosity of the formulation increases to almost a plateau from 10.5% m/v which is evident from the shape of the contour lines on the plot. The shape of the 3D response surface plot is flat at low levels of L87 and forms a dome at high levels of L87, further confirming the observed relationship between L68 and L87.

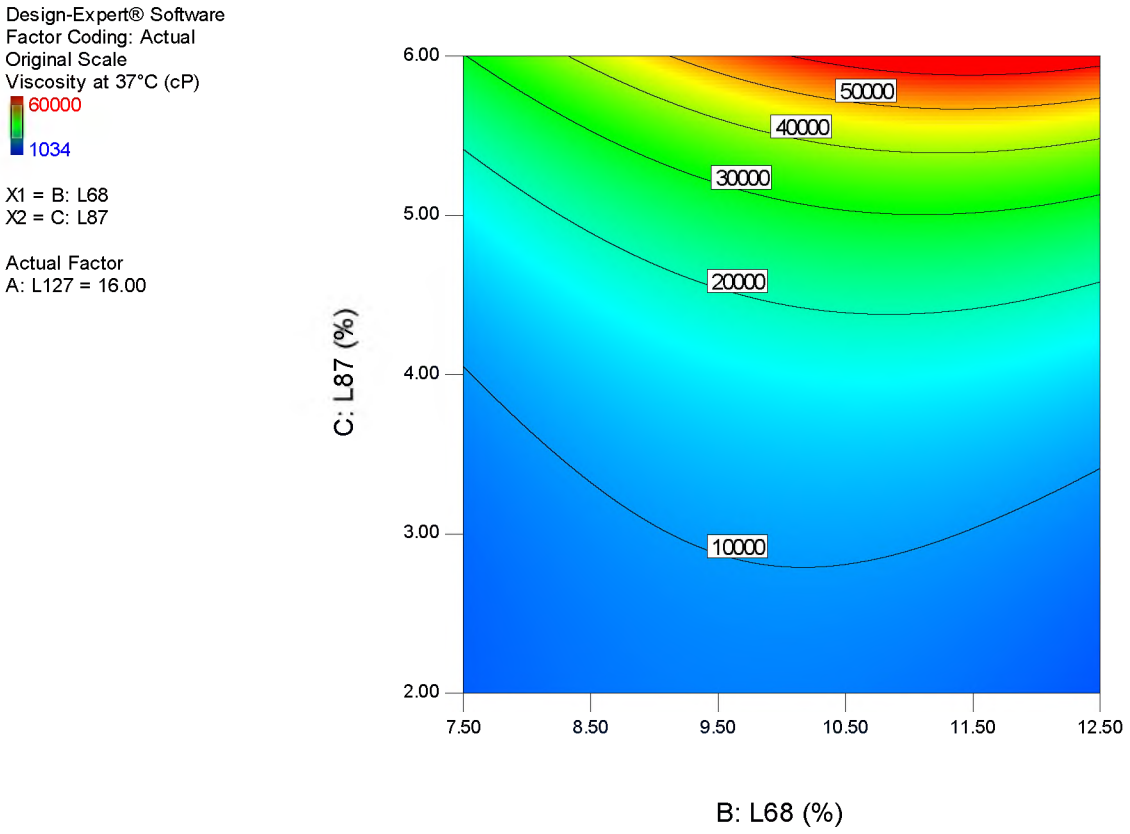


Figure 7.7 Contour plot depicting the impact of L87 and L68 levels on viscosity at 37°C.

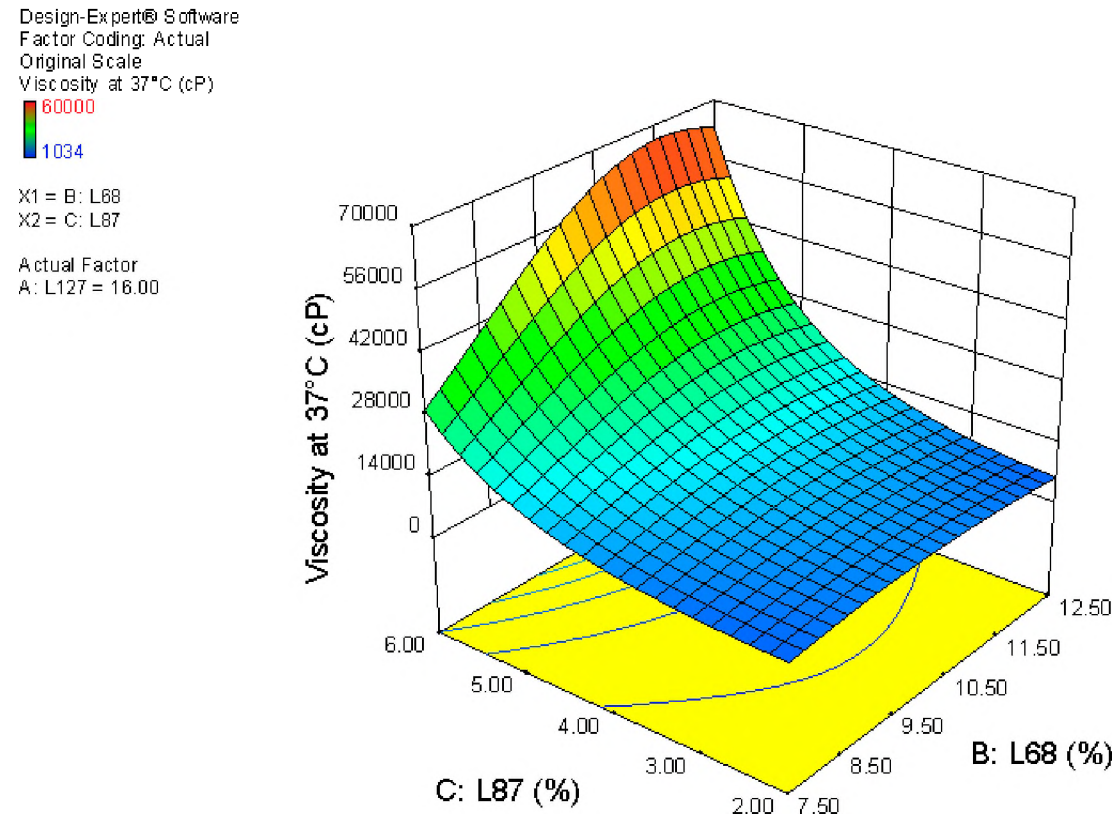


Figure 7.8 3D response surface plot depicting the impact of L87 and L68 levels on viscosity at 37°C.

The PEO chains of poloxamer are hydrophilic and interact with water molecules within the formulation via hydrogen bonding and Van Der Waals forces to produce low viscosity formulations whereas PPO chains are hydrophobic and tend to form clusters that result in a formulation of high viscosity [328,469]. The L127 grade of poloxamer is comprised of 64.3% PEO and 35.7% PPO, the L68 grade 74.8% PEO and 25.2% PPO and the L87 grade 63.4% PEO and 36.6% PPO [190], further providing evidence that by varying the proportion of different grades the ultimate viscosity of the dosage form can be modulated.

At very low poloxamer concentrations of between 0.0001 to 0.001% monomolecular micelles or pre-micellar forms occur in aqueous solution and, as the concentration is increased, multi-molecular micellar aggregates with a central hydrophobic core and peripheral chains that are hydrophilic and face towards the exterior of the micelle are formed [183,500]. When the poloxamer concentration in the formulation is further increased to the critical micelle concentration, micellar entanglement and packing occurs resulting in an increase in the viscosity of the dosage form. When the concentration of the poloxamer is used at a level above this critical value the micelles form a cubic lattice structure resulting in the conversion of the solution to a gel [184]. As the concentration of the poloxamers in the dosage form are further increased, the micelles rearrange to form cylindrical aggregates that eventually form a hexagonal phase [501] that are converted to form a lamellar phase at very high poloxamer concentrations. As the gel commences formation through the different phases from the cubic lattice

structure to a lamellar phase, it becomes increasingly more rigid and viscous as the micellar structure becomes more and more difficult to separate [328,502].

At low temperatures the poloxamers hydrate in aqueous solution and as the temperature of a solution increases the hydrogen bonds that form between the molecules of the aqueous solvent and hydrophilic PEO chains break [466,503-505] resulting in favourable conditions for hydrophobic interactions to occur, leading to the formation of micelles in which the PEO chains are oriented externally to the micelle [506,507]. At higher temperatures, depending on the concentration and molecular mass of the poloxamers present, greater chain friction, entanglement and micellar swelling occur [466,503-505,508] giving rise to micellar aggregation followed by the formation of a cubic lattice structure and eventually a lamellar phase resulting in the formation of a viscous gel [183,468,505,509,510].

The increase in viscosity observed at 37°C is due to swelling and the ultimate size of the micelles. The addition of a small amount of L68 in formulations containing L127 will initially result in a slight decrease in viscosity as the ratio of PEO in the gel increases [511,512], a phenomenon that was not observed for the design space described herein. The use of a high concentration of L87 (6% m/v) in the formulation resulted in micelle swelling at 37°C which manifested as an increase in viscosity whereas the addition of L68 increased the hydrophilicity of the formulation which resulted in a decrease in viscosity. However, the magnitude of the viscosity increase due to swelling of the L87 and L127 micelles was greater than the viscosity decrease, thereby resulting in an overall increase in the viscosity. When the amount of L127 used was maintained at 16% m/v with 3% m/v L87, the viscosity of the formulation increased slightly on addition of a small amount of the L68 polymer. This phenomenon may be due to the low amount of L87 present in the formulation which results in the formation of fewer micelles with a subsequent pronounced impact on the viscosity that may be due to the hydrophilicity induced by the presence of L68 than a likely increase in viscosity due to swelling of L127 micelles in the formulation. However, the addition of more L68 to the formulation may result in the formation of L68 micelles that contribute to an overall increase in the viscosity of the formulation. Low amounts of L87 did not affect the viscosity to any great extent as this grade of polymer has a similar PEO/PPO ratio to that of the L127 material.

The L127 and L87 grades of poloxamer have a positive and synergistic effect on the viscosity of the gels due to a similarity in their ratio of PEO to PPO. Therefore the addition of L87 to an aqueous solution containing L127 would not affect the PEO and PPO ratio and this would explain the observation of an apparent positive effect on viscosity [511,512]. The addition of L87 to the a solution of L127 has no impact on the hydrophobic-hydrophilic balance of micelles within a solution as the L87 polymer exhibits the same PEO to PPO ratio. Micelles aggregate to a greater extent when a more hydrophobic poloxamer with a high PPO number is added to a solution as the hydrophobicity of the

solution increases as the micelles rearrange and the hydrophobic cores come into contact with each other and hydrophilic chains interact with the aqueous vehicle. The larger number of aggregates formed results in an increase in the viscosity of the solution. Similarly, if more L127 is added to the solution, the viscosity would be expected to increase but not to the extent if a poloxamer of higher PPO content was used with the disruption of the hydrophobic-hydrophilic balance of the micellar solution. Alternatively, small L87 micelles may wedge between the L127 molecules resulting in the formation of a more compact micellar structure with increased viscosity.

The use of a combination of the L87 and L68 grades of poloxamer has a small but positive effect on the viscosity of the solution at 37°C and the maximum viscosity observed was < 70000 cP, primarily due to the low amount of each of the L87 and L68 polymers used. Both grades of poloxamer generally complement the effect of L127 which is the primary contributor to the thermosetting behaviour of the formulations as the PPO/PEO blocks are longer in chain length than for the other grades used. The addition of the L68 and L87 grades to the formulation ensures an accurate modulation of the thermosetting temperature as a consequence of the interaction between the long chains of L127 and disruption of the PPO and PEO ratio within the composition and these data are similar to those reported by Ibrahim et al. and Charrueau et al. [328,513].

7.4.3.2 Viscosity at 22°C

The final equation for the viscosity at 22°C is reported as **Equation 7.3**. The resultant equation is linear in which the L127, L68 and L87 grades are listed as main effects.

$$\frac{1}{\text{viscosity at } 22°C} = 0.00058 - (0.000018 \times L127) - (0.000015 \times L68) - (0.0000027 \times L87) \qquad \textbf{Equation 7.3}$$

The values for the coefficients and associated sign for the operator suggest that the main effects observed all have a negative impact on the inverse viscosity of the formulation at 22°C, with the L127 grade exhibiting the most pronounced effect. Evaluation of the response data observed for all experiments summarized in **Table 7.5** indicate that the amount of L87 has a minimal effect on the inverse of viscosity at 22°C and as the amount of L127 is increased the inverse of the viscosity at 22°C also increases; however, the impact is not as pronounced as that observed at 37°C. An increase in the amount of L68 used resulted in a slight increase in the inverse of viscosity at 22°C.

Table 7.5 Response data for each experiment.

SD	Run	Viscosity at 37°C cP	Viscosity at 22°C cP	Sol-gel transition s	Zone of inhibition mm	KZ released at 24 hours µg/cm²	KZ released at 48 hours µg/cm²	KZ released at 72 hours µg/cm²
3	**1**	5862	5517	30.00	12	2920.94	3185.50	3247.78
9	**2**	5517	5632	0.50	14	2779.98	3001.62	3299.39
13	**3**	5287	7931	0.50	11	3351.00	3540.77	3592.52
14	**4**	89997	8276	60.00	20	2984.24	3169.02	3219.63
11	**5**	1494	4138	0.50	8	3366.10	3542.55	3588.72
19	**6**	5747	7816	0.50	15	3603.58	3851.44	3914.00
7	7	6551	4597	15.00	12	2473.85	2666.71	2725.00
8	**8**	339600	196633	60.00	32	2670.07	3139.73	3264.45
6	**9**	291667	7931	60.00	34	2485.67	2861.32	2960.86
2	**10**	42870	7011	5.00	21	2923.03	3115.57	3168.93
20	**11**	6322	7471	0.50	19	3565.39	3748.36	3801.38
12	**12**	7701	8276	11.00	33	3167.34	3482.06	3567.79
18	**13**	5172	7586	5.00	22	3278.16	3552.57	3631.78
10	**14**	295467	12410	60.00	30	3559.48	3852.47	3929.02
17	**15**	17467	5632	0.50	19	3406.82	3902.93	4031.39
1	**16**	1034	3793	0.50	32	3293.74	3520.37	3573.61
15	**17**	8735	5747	5.00	27	3390.41	4307.07	5023.59
5	**18**	1034	3103	0.50	29	3422.16	3546.23	3583.61
16	**19**	9195	5862	0.50	23	2213.31	3066.24	3669.24
4	**20**	10340	6781	17.00	34	1588.29	1920.86	2203.74

The inverse of viscosity can be difficult to apply to actual situations and the equation depicting this response prior to transformation is illustrated in **Equation 7.4.** The untransformed data required the process order to be two-factor interaction (2FI); therefore, the first order terms as well as the interaction terms are displayed in the equation, unlike the equation generated following transformation which required a linear process order to be applied.

Viscosity at 22 °C = + 744562.00 – (41714.86 x L127) – (59365.40 x L68) – (100348.00 x L87) + (3087.57 x L127 x L68) + (4007.96 x L127 x L87) + (4717.55 x L68 x L87) **Equation 7.4**

L87 exhibits the highest negative impact on viscosity at 22 °C and L127 the lowest. A combination of poloxamers exhibits a positive effect on the viscosity of the formulation at 22 °C. This data reveals that the use of L87 helps to decrease the viscosity of the gel at lower temperatures which will ensure ease

of application of the gel into the vagina as a liquid. This occurs as the L87 grade is of lower molecular mass when compared to the other poloxamers used and thus forms small micelles at high temperatures due to the low degree of hydrophobic attraction between the PPO block copolymer in the molecule requiring high temperatures to facilitate sol-gel transitions. This phenomenon lowers the critical micelle temperature (CMT) of L127 and L68 resulting in the formation of a liquid gel at 22 °C and modulation of the CMT to 37 °C.

In general the low viscosity observed for the gels at 22°C was desirable as administration of the gel into the vagina would be facilitated and can be explained by the formation of stable monomeric poloxamer solution in aqueous vehicles at this temperature [328,514,515]. At higher concentrations of poloxamer the low number of poloxamer molecules contributing to the formation of aggregates within the formulation results in the low viscosity observed [502,516,517]. The target viscosity was < 10000 cP so as to ensure that the gel is free flowing and most combinations of poloxamer produced gels close to this viscosity.

7.4.3.3 Sol to gel transition

The equation for the sol to gel transition is reported in **Equation 7.5** that indicates the terms L127, L87, L127*L68 and L68*L87 have a negative effect on the function, square root of sol to gel transition and on the overall sol to gel transition response as depicted in **Equation 7.6.**

$\sqrt{\text{sol} - \text{gel transition}} = + 20.26 - (3.19 \times L127) + (1.07 \times L68) - (4.09 \times L87) - (0.10 \times L127 \times L68) + (0.22 \times L127 \times L87) - (0.087 \times L68 \times L87) + (0.13 \times L127^2) + (0.060 \times L68^2) + (0.29 \times L87^2)$ **Equation 7.5**

It is evident that the amount of L68 used has a pronounced and positive effect on the square root of the response as observed from the quadratic equation. As the concentration of L127 increases the time required for the gel to flow increases substantially **(Table 7.5)** whereas the level of L68 does not appear to exert a significant effect on the sol to gel transition. A synergistic effect is observed when the amount of L127 and L87 is increased and the transition of the phase increases up to a maximum of 60 seconds which was the limit for the response set for these experiments.

The equation for sol to gel transition prior to transformation is depicted in **Equation 7.6** and the terms are interpreted according to the untransformed equation as it is more suggestive of a real formulation effect.

$\text{Sol} - \text{gel} = + 228.82 - (32.73 \times L127) + (8.48 \times L68) - (41.91 \times L87) - (0.53 \times L127 \times L68) + (2.35 \times L127 \times L87) - (0.68 \times L68 \times L87) + (1.11 \times L127^2) + (0.22 \times L68^2) + (2.51 \times L87^2)$ **Equation 7.6**

Similar to observations for managing the transformed equation, L68 exhibits a positive effect on the sol to gel transition whereas L127 and L87 exhibit a negative effect with L87 exhibiting the most

pronounced effect suggesting that L68 improved the gel strength as a positive response. This result indicates that the addition of larger quantities of L68 facilitates more rapid gelation that form gels that retain this characteristic for a longer period of time. An increase in the content of L127 and L87 individually resulted in an apparent decrease in the strength of the gel over a period of time. However, the combination of L127 and L87 exhibited a positive response and suggests that these poloxamers can improve gel strength in combination. These data indicate that the sol to gel transition is complex and requires the analysis of individual and binary interactions in order to predict the actual response from a formulations composition. Therefore the CCD I approach will be used to elucidate an optimised poloxamer combination to produce a formulation composition that exhibits maximised gel strength.

An increase in the amount of L87 and L127 used and that have similar PEO/PPO ratios will result in the formation of stiff gels due to the organization of large L127 and small L87 micelles into a compact gel structure within the formulation. The use of L87 adds the requisite stiffness required to the formulation to ensure prolonged residence time in the vagina as evidence by the binary interaction effect of L127 and L87. The L68 individually seems to also contribute to gel stiffness. Charrueau et al. and Dumortier et al. reported the formation of stiff bio-adhesive gels when the amounts of L127 and L68 used in a formulation was increased [184,513]. Therefore, as the concentrations of each poloxamer increases, the resultant formulation will form into and remain a stiff gel for longer periods of time.

7.4.3.4 Potency studies

The final equation for potency is reported as **Equation 7.7**. The resultant quadratic equation for this response reveals that L127, L68, L87 and $L87^2$ exhibited a negative effect on the potency of the formulation.

$$\text{Potency} = +12.10 - (0.76 \times L127) - (1.07 \times L68) - (0.28 \times L87) + (0.067 \times L127 \times L68) + (0.042 \times L127 \times L87) + (2.91 \times 10^{-17} \times L68 \times L87) + (0.0037 \times L127^2) + 0.0053 \times L68^2) - (0.036 \times L87^2) \quad \textbf{Equation 7.7}$$

The highest positive synergistic effect was observed for the combination of L127 and L68. The data summarized in **Table 7.5** suggest that a high concentration of L127 and L68 when used together result in a formulation of higher potency as compared to when they are used in combinations where one is used at a maximum and the other at a minimum level. The lowest potency was observed when the amount of L87 and L68 were both used at the lowest level within the design space investigated. These data reveal that all three poloxamers may have an important role to play in the promotion of potency of the formulation for treating *Candida albicans* infections due to the fact that at 37°C the formulations remain solid and release KZ in a prolonged and sustained manner leading to an improved antifungal effect over 48 hours during which the yeast was cultured. In other words, the poloxamers in the formulations remain in a micellar form at 37°C and KZ released through a limited number of aqueous channels over a prolonged time period.

In addition, the poloxamers did not result in the formation of a halo on the agar plate when used in control experiments and therefore the thermosetting behaviour of the gel may have affected the antifungal effect of KZ by ensuring prolonged release of KZ. Moreover, KZ is a lipophilic molecule that is likely to remain within the hydrophobic core of the micelle and the destruction or rearrangement of micellar structures over time due to interaction with the components in the agar may slow the delivery of KZ in this agar system.

7.4.3.5 KZ release

The final equations for KZ release at 24, 48 and 72 hours are reported as **Equations 7.8 – 7.10** respectively.

KZ released at 24 hours = – 1867.18 + (568.07 x L127) + (365.32 x L68) + (328.40 x L87) + (14.66 x L127 x L68) + (3.76 x L127 x L87) + (13.67 x L68 x L87) – (24.97 x $L127^2$) – (36.12 x $L68^2$) – (67.51 x $L87^2$)

Equation 7.8

KZ released at 48 hours = – 5921.71 + (868.79 x L127) + (697.92 x L68) + (578.56 x L87) + (20.75 x L127 x L68) + (10.45 x L127 x L87) + (6.86 x L68 x L87) – (37.06 x $L127^2$) – (54.64 x $L68^2$) – (103.04 x $L87^2$)

Equation 7.9

KZ released at 72 hours = – 10270.19 + (1109.59 x L127) + (1091.37 x L68) + (1068.89 x L87) + (28.46 x L127 x L68) + (3.73 x L127 x L87) – (4.11 x L68 x L87) – (46.63 x $L127^2$) – (77.01 x $L68^2$) – (139.67 x $L87^2$)

Equation 7.10

The release of KZ over 24 hours was positively affected by the terms for L127, L68, L87, L127*L68, L127*L87 and L68*L87. KZ release over 48 hours was positively affected by the same terms and over 72 hours was affected by the same main effects in addition to the terms for L127*L68 and L127*L87. The most positive impact on cumulative amount of KZ released per unit area was observed for L127. The average amount of KZ released per unit area at 24, 48 and 72 hours was 3022.18 ± 525.91, 3348.67 ± 522.78, 3499.82 ± 564 μg/cm^2. Changes in poloxamer content in the formulation composition exerted small effects on KZ release. This occurred due to entrapment of KZ in micellar structures formed by L127, L68 and L87 and prolonging release of KZ. KZ remains trapped within the micelles in the donor compartment, while some of the unbound KZ permeates through the membrane and, over time, due to the destruction of the micelles, as some receptor medium may have permeated into the donor compartment resulting in dissolution of the gel matrix.

The overall trend observed within the design space suggests that the use of high concentrations of poloxamer results in a reduction in the amount of KZ released from the test formulations. KZ may be trapped within the hydrophobic interior of micelles resulting in a reduction in release and, as the poloxamer concentration increases, additional micelles are formed and entrap additional molecules of KZ within the formulation. In addition the release of KZ may be suppressed at 37 °C as the formulation

occurs as a gel in which the cubical micellar arrangement within the formulation limits the formation of aqueous channels between the micelles through which KZ diffuses [518]. An increase in poloxamer content reduces the quantity of solvent in which the KZ can dissolve. Consequently, KZ may be present in a crystalline form within micelles and the formulation that impacts the stability and reduces the release rate of KZ. It has been reported that lipophilic drugs partition preferentially into micelles within the gel structure resulting in a decrease in the amount of KZ released [467]. However, the slower rate of release may also be a consequence of membrane pores becoming blocked [275].

The release profiles of KZ from test batches R1, R7, R9, R11, R13, R18 and R20 are depicted in **Figure 7.9**. An increase in the L68 content appears to have a negative influence on KZ release when compared to release from formulations in which the L87 content is decreased as observed from the profiles for batches R9 and R20. When comparing release from batch R13 to the other release profiles a general increase in total poloxamer concentration appears to result in a decrease in the release of KZ. Batch R18 exhibits the highest amount of KZ released and contains 25.5% m/v of poloxamers whereas batch R20 exhibits the lowest KZ release and contains a total of 32.5% m/v of poloxamers. However, this trend does not appear to apply to all formulations as, if the total amount of poloxamers used was directly related to the extent of KZ release, batch R1 (26.5% m/v poloxamers) would have exhibited a higher extent of release when compared to batches R13 (29.0% m/v poloxamers) and R7 (30.5% m/v poloxamers) which in turn would have greater release of KZ than batch R9 (31.5% m/v).

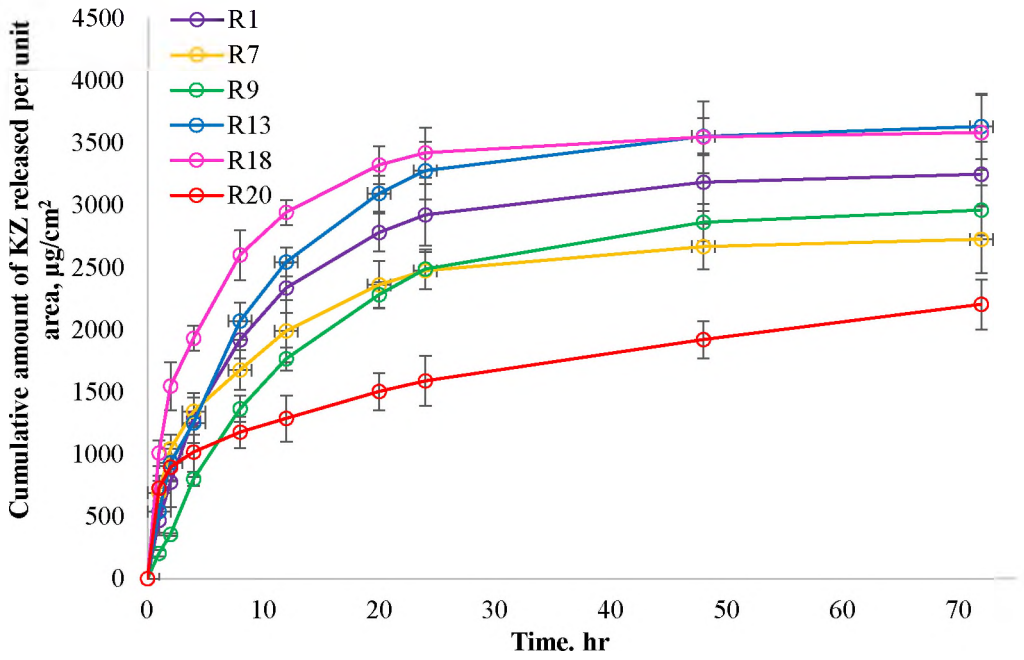


Figure 7.9 Cumulative amount of KZ released per unit area from formulations manufactured following CCD I.

Batch R20 exhibits the lowest rate and extent of KZ release per unit area and has a burst effect between 0 to 2 hours contributing to 40.66% of the total amount of KZ released over 72 hours. KZ release from batches R1, R7 and R9 are almost similar and a moderate amount of KZ was released overall. The greatest extent of KZ release was observed for batches R13 and R18 with batch R18 exhibiting the highest release from 0 to 24 hours and batch R13 plateauing from 48 hours.

The cause of the trends for KZ release in relation to the amount of L127, L68 and L87 is not clear from the release profiles suggesting that a more complex interaction between the factors exists. Therefore, quadratic equations were used **(Equations 7.8 – 7.10)** to examine the interactions and 3D surface plots depicted in **Appendix G**.

All *in vitro* release profiles are displayed within batch summary records and reported in **Appendix F**.

7.4.3.5.1 Model dependent analysis of *in vitro* release profiles

The *in vitro* release profiles generated for the gels were fitted to zero order, first order, Higuchi, Korsmeyer-Peppas and Hixson-Crowell models in order to elucidate the kinetics and mechanism of release of KZ from these gel. KZ release and time were plotted into the different models and values for R^2, the constant, K and the Korsmeyer-Peppas exponent, n values are listed in **Table 7.6**.

Table 7.6 Model dependent parameters for KZ release from thermosetting gels.

Gels	Zero Order		First Order		Higuchi		Korsmeyer- Peppas			Hixson-Crowell	
	R^2	K	R^2	K	R^2	K	R^2	K	n	R^2	K
R1	0.6229	0.17	0.4713	0.0080	0.8269	1.88	0.9160	0.55	0.43	0.6329	-0.0029
R2	0.7643	0.21	0.5665	0.010	0.8931	2.21	0.9548	0.22	0.58	0.7763	-0.0035
R3	0.5964	0.19	0.4757	0.0068	0.8058	2.13	0.9275	0.58	0.43	0.6072	-0.0033
R4	0.5906	0.18	0.4479	0.0075	0.8008	2.01	0.9114	0.45	0.49	0.6011	-0.0031
R5	0.5819	0.18	0.4627	0.0067	0.7937	2.00	0.9207	0.56	0.44	0.5918	-0.0031
R6	0.6446	0.21	0.5196	0.0073	0.8398	2.33	0.9250	0.57	0.46	0.6555	-0.0037
R7	0.6645	0.15	0.5382	0.15	0.8550	1.60	0.9319	0.50	0.40	0.6724	-0.0025
R8	0.7388	0.19	0.5269	0.011	0.9108	2.01	0.9454	0.048	0.67	0.7493	-0.0031
R9	0.7199	0.19	0.4983	0.012	0.8975	2.00	0.9345	-0.13	0.77	0.7296	-0.0031
R10	0.6439	0.17	0.5196	0.0075	0.8391	1.85	0.9259	0.44	0.46	0.6520	-0.0029
R11	0.5837	0.20	0.4644	0.0069	0.7952	2.21	0.9218	0.58	0.46	0.5945	-0.0034
R12	0.6531	0.21	0.4823	0.0091	0.8505	2.24	0.9276	0.30	0.58	0.665	-0.0035
R13	0.6542	0.20	0.5060	0.0087	0.8504	2.17	0.9369	0.34	0.48	0.6644	-0.0034
R14	0.6692	0.22	0.5454	0.0082	0.8570	2.34	0.9288	0.47	0.49	0.6795	-0.0037
R15	0.8915	0.34	0.6873	0.0092	0.8885	3.37	0.9742	0.57	0.47	0.6097	-0.0040
R16	0.6165	0.18	0.4790	0.0075	0.8223	1.96	0.9290	0.45	0.48	0.6264	-0.0030
R17	0.8055	0.28	0.5354	0.011	0.8439	2.90	0.9185	0.21	0.49	0.6220	-0.0048
R18	0.5621	0.15	0.4615	0.0054	0.7764	1.73	0.9188	0.68	0.36	0.5707	-0.0026
R19	0.8742	0.35	0.6632	0.010	0.8713	3.48	0.9808	0.43	0.46	0.6907	-0.0061
R20	0.8981	0.087	0.7904	0.0058	0.9861	0.87	0.9923	0.43	0.27	0.9020	-0.0014

Goodness of fit of one model is generally reported and chosen to describe drug release [257,519-521]. In these studies, due to the complexity of the formulations, models exhibiting R^2 value of ≥ 0.900 were considered unlike the value for R^2 of ≥ 0.9600 used as a limit for data presented in **Chapter 4**.

Batches R6, R11, R13, R15, R17 and R19 are the same formulation and repeated as part of the CCD design. The average of the R^2 values revealed that the data were best fitted to the Korsmeyer-Peppas model with a value for R^2 of 0.9429 and a corresponding value for n of 0.47. The release data for all other gels were also best fitted to the Korsmeyer-Peppas model with formulation R8 exhibiting $R^2 >$ 0.9000 for the Higuchi model and formulation R20 to both the Higuchi and Hixson-Crowell models. The exponent, n fell between 0.45 and 0.86 in most cases except for batches R1, R3, R5, R7, R18 and R20 gels.

The Korsmeyer-Peppas model generally describes drug release from a hydrophilic porous polymeric system [259]. The thermosetting gels, in this case, were manufactured using poloxamers that form a hydrophilic micellar system where the inter-micellar space containing the aqueous vehicle would form pores within the gel matrix. The limits for the release exponent, n represent Fickian diffusion at one end and case II relaxational transport at the other [522]. The mechanism of release when $n < 0.45$ cannot be described using this model but may be inferred according to the type of dosage form used. However, the correlation between time and drug release can be obtained from the R^2 values and kinetics of KZ release can be deduced according to the model the data fits. The values for n elucidated for batches R1, R3, R5, R7 and R11 were ≤ 0.45 with only a 0.05 unit deviation and may be described as technologies from which KZ release occurred by Fickian diffusion indicating that, in the main, KZ transport was diffusion controlled due to a concentration gradient that existed within the gel matrix that did not erode which was a phenomena also observed during IVRT. KZ release from batches R18 and R20 revealed low values for n and therefore release could be described only according to the model the data was best fitted to. The release profile from formulation R18 was best described by the Korsmeyer-Peppas model and is depicted in **Figure 7.9** in which it is clear KZ was released to a greater extent than from the other gels. The formulation for R18 was comprised of 12.00% m/v L127, 7.50% m/v L68 and 6.00% m/v L87 and has a relatively low total content of poloxamers when compared to the other gel formulations. Consequently, following hydration by the receptor fluid matrix dissolution may have occurred and therefore release driven predominantly by diffusion from a gel solution was likely to have occurred. Batch R20 revealed a value for n of 0.27 which is also the lowest value for this exponent recorded during these experiments. The release profile for KZ from this formulation also reveals the lowest amount of KZ released with an initial burst that could be due to the low amount of the L87 grade polymer in the formulation. The L87 grade of polymer forms small micelles that may lodge between larger micelles formed by the L127 and L68 grades of polymer resulting in a more compact gel structure as described in **§ 7.4.3.1**. As batch R20 formed a less rigid gel structure that collapses following

hydration by the receptor fluid, KZ release into the donor compartment is rapid and an initial burst effect is observed. The poloxamer content of 32.50% m/v in batch R20 is relatively high, however, and results in the formation of unimers of poloxamer that may block the pores of the membrane resulting in a low amount of KZ release at 72 hours. KZ release from batches R18 and R20 appeared to be membrane limited and therefore a low value for n was observed suggesting that the Korsmeyer-Peppas model was not suitable to describe KZ release. KZ release from batches R2, R4, R8, R9, R10, R12, R14 and R16 all exhibited values for n between 0.45 and 0.86 indicating that the mechanism of KZ release followed an anomalous, non-Fickian diffusion process indicating that drug release occurred by diffusion of KZ through the matrix as a result of a concentration gradient in addition to erosion of the matrix. Release data for batch R8 were adequately fitted to the Higuchi model indicating that KZ release is a function of diffusion through a porous matrix predominantly with little impact of erosion on the matrix. The formulation composition of batch R8 is comprised of the largest amount of each of the poloxamers with a total content of 36.50% m/v that forms a rigid matrix that can withstand hydration and erosion by the receptor medium.

The release kinetics of KZ from thermosetting gels tended to be best described using the Korsmeyer-Peppas model with release from some gels best fitted to other models. The differences observed are more than likely a consequence of large differences in poloxamer content of these gels for which the release mechanisms was difficult to identify as gels of different viscosity and rigidity were produced, some of which did not erode and others did .

7.4.4 Optimisation of poloxamer concentration in the gel formulation

The optimised formulation composition for the KZ vaginal gel was selected based on ranges for all responses monitored so as to identify the most desirable formulation composition following optimization with the aid of a CCD DoE and modelling of the data. The limits of viscosity for an ideal thermosetting vaginal gel require a maximum viscosity of > 50000 cP at 37 °C and minimum viscosity of < 10000 cP at 22 °C . The sol-gel transition response limit was set to 60 seconds for a stiff gel at 37 °C and the maximum potency of KZ against *Candida albicans* was essential. In addition KZ release at 24, 48 and 72 hours was required to be at the maximum for that period. A relatively low amount of KZ release was considered acceptable provided a maximum antifungal effect could be achieved in the vagina with minimal systemic absorption of the KZ. Furthermore, low amounts of KZ released from the formulation would suggest that KZ may diffuse slowly to the vaginal mucosa resulting in a sustained effect which may be suitable for the treatment of persistent yeast infections in the vagina. One formulation exhibited optimum desirability against the conditions set and the input variable levels were identified through modelling of the CCD data. The optimum poloxamer composition was established

as 16% m/v L127, 10% m/v L68 and 6% m/v L87 and was used as the basis for further optimisation processes.

7.5 Conclusions

The three grades of poloxamer tested play an important role in the manufacture of a high quality thermosetting KZ containing vaginal gel. The optimised levels of poloxamer to be used were identified using a CCD approach. The impact of L127, L68 and L87 content on viscosity at 37 °C was established and the maximum value for this response in addition to the viscosity of the gel at 22 °C, sol to gel transition time, potency and the % KZ released at 24, 48 and 72 hours were monitored. The polynomial equations generated for each response were used to explain the effect of input variables on the responses observed. The RSM approach used was deemed appropriate to determine the parameters for different models and also facilitated the success of model fitting. The successful development of a thermosetting gel requires careful consideration of a number of factors that can influence the performance of formulations and the use of DoE is a preferred approach that facilitates the design and manufacture of complex formulations. The CCD approach used permitted simultaneous investigation of the effects of three input variables in addition to the significance of their impact on the responses monitored. A maximum amount of information was generated using a small number of experiments to identify and establish possible interrelationships between the input variables and their impact on responses. The approach presented in this study is a useful methodology for the optimisation of thermosetting vaginal gels for delivering other hydrophobic drugs.

A low *in vitro* release rate of KZ was observed from test formulations although the release studies performed using a solution of KZ resulted in 100% release at 72 hours with the same *in vitro* test conditions. These data indicate that the release of KZ is retarded as a consequence of the formation of a gel matrix with the combination of poloxamers used resulting in sustained release of KZ [467]. KZ release from the gels was best fitted to the Korsmeyer-Peppas model and different release mechanisms were observed; however, the majority of the gels exhibited KZ release that followed an anomalous non-Fickian diffusive process. The sol to gel transition response may be used as an alternate test for viscosity as it is a simple and cheap experiment to undertake when expensive viscometers and rheometers are not available.

The sustained release observed for KZ in this study indicates that the gels manufactured using L127, L68 and L87 poloxamers have the potential to be administered as once daily doses. These gels can be self-administered as the formulations are liquid at 22 °C and would not exhibit embarrassing and uncomfortable leakage usually associated with the use of other semi-solid vaginal formulations. It is likely that the stiff colourless gel formed at 37 °C would remain in the vagina, unaffected by variable vaginal mucous discharge rates. Coitus and bowel movements are also unlikely to be affected by this

formulation as the soft semi-solid nature of the product is similar in consistency to vaginal mucous discharge and can be removed or moved without any feeling of discomfort. The thermosetting vaginal gel may also result in enhanced patient adherence due to the local treatment option for yeast infections and has the potential to eliminate RVVC. The product may be used to treat single isolated yeast infections successfully within a few days. This treatment option may also minimize the exposure of patients to the side effects associated with orally administered KZ and may be well tolerated and safe for use by pregnant women.

CHAPTER EIGHT
USE OF DOE TO MONITOR STABILITY OF THERMOSETTING GELS

8.1 Introduction

KZ is photo labile [156] and stability of the molecule in aqueous solution is dependent on pH and temperature [43]. The approach used in these studies, to enhance stability, was to shield KZ molecules within a hydrophobic PPO core through the formation of poloxamer micelles, as depicted in **Figure 8.1,** in which KZ is located in the core that is surrounded by hydrophilic PEO poloxamer chains and polysorbate 80. In this manner, the exposure of the KZ molecules to bulk aqueous and acidic vehicles in the proposed formulation is minimized [523]. The presence of non-ionic polysorbate 80 that is comprised of a lipophilic chain and a polar head strengthens the micellar structure of the poloxamer formulation and aids solubilisation of KZ [524].

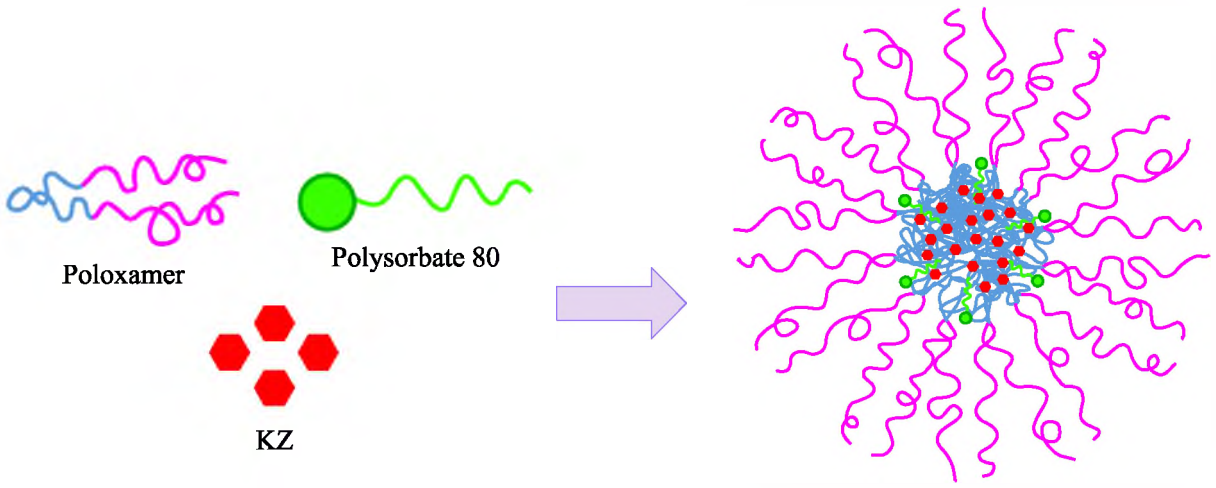


Figure 8.1 Schematic representation of micelle formation and KZ entrapment following an increase in temperature. Adapted from [525].

Novel pharmaceutical product development is often a long and tedious process that necessitates testing of many formulations and the attributes thereof, and in many cases requires retesting of small scale batches during early safety and clinical studies prior to scale up studies that then require validation and technology relocation [526]. The product that is developed is subjected to different conditions over accelerated, intermediate and long term time frames and assessed for product stability and/or changes of formulation at specific times [527,528]. Several potential products may be rejected following prolonged product development activities and stability testing that have involved the use of expensive human and other resources. Therefore, initial stability testing of pilot scale batch products should be performed in order to identify trends that may be exhibited by a product over time by exposure to different storage conditions prior to undertaking lengthy stability studies on large scale batches that are required for the purposes of product registration. Through the use of DoE it is possible to save time and

other resources and this approach was used for the purposes of assessing the stability of experimental thermosetting vaginal gels containing KZ.

A stable pharmaceutical formulation stored in specific packaging with an appropriate closure system must retain the original properties (within specified limits) and characteristics exhibited following initial manufacture [529-531]. Stability testing ensures the quality, safety and efficacy of marketed products by assessing the effect of environmental factors on a formulation, thereby permitting the prediction of an appropriate shelf-life and identification of the ideal storage conditions and labelling instructions for that product [530,531]. Stability testing is a requirement for regulatory approval of novel and generic formulations [532]. The European Medicines Agency Guidelines, ICH guidelines (Q1A (R2), Q1C and Q1E) and WHO technical reports are used to define conditions, that are specific for different regions, to be used for stability studies for pharmaceutical dosage forms [527,528,533-535].

The thermosetting KZ vaginal gels produced were comprised of a solvent system, poloxamer polymers and SM. The components of the solvent system included citric acid, polysorbate 80, ethanol and DHP and the composition of the solvent was optimised to achieve maximum solubility of KZ (**Chapter 6**). The concentration of poloxamer was optimised (**Chapter 7**) to ensure the optimum thermosetting behaviour for this dosage form. The stability of the gel, in terms of pH and SM content, is dependent on the amount of citric acid, ethanol and SM concentration used and is the focus of this chapter. The acidic vehicle is expected to affect the stability of KZ in the gel vehicle and SM was included so as to enhance the stability of the product. Stability studies were therefore conducted on formulations containing different amounts of SM and gels manufactured using solvents of pH 2.5 to 3.5 as this range is tolerated by human vaginal tissues. The results of these stability experiments form the basis of establishing the viability of formulations for further consideration for scale-up and product validation studies.

8.2 Method

All formulations were manufactured as described in **§ 7.3** and were stored in 50 g amber glass bottles sealed with a plastic lid and Parafilm™. Chromatograms developed following assessment of KZ indicated that the use of the plastic lid and/or Parafilm™ did not appear to result in any material leaching compounds that interfered with the analysis of KZ or the IS. A CCD face-centred experimental design was used to investigate the impact of three numeric and one categoric variable. Batches (50 g) of 2% m/v KZ gels were manufactured using the same amount of each of the poloxamers whilst adding different quantities of antioxidant and varying the pH according to a CCD DoE approach (CCD II) (**Table 8.1**). The variables investigated were the amount of SM, solvent pH, number of days stored (days) and storage temperature as summarised in **Table 8.1.** The numeric variables were investigated at three levels and the temperature at two levels. Stability was monitored in terms of KZ content, KZ released at 24, 48 and 72 hours, pH , viscosity at 22 ºC and 37 ºC.

Table 8.1 Summary of design factors for CCD II.

Factor	Name	Units	Type	Subtype	Min	Max
A	SM	% m/v	Numeric	Continuous	0.5	1.5
B	Solvent pH	-	Numeric	Continuous	2.5	3.5
C	Number of days stored	days	Numeric	Continuous	0	56
D	Storage temperature	ºC	Categoric	Nominal	25	40

The batches produced following the CCD II design, *viz.,* S1 to S9 were monitored to establish the appropriateness of the CCD approach for stability testing. The batch production records and batch summary records are reported in **Appendix H** and **Appendix I**. The formulation variables monitored during stability testing of batches S1 – S9 are listed in **Table 8.2.**

Table 8.2 Variable composition for formulations S1 to S9.

Formulation	SM, % m/v	Solvent pH
S1	0.50	3.00
S2	0.50	3.50
S3	1.00	3.00
S4	1.50	3.50
S5	1.50	3.00
S6	0.50	2.50
S7	1.50	2.50
S8	1.00	2.50
S9	1.00	3.50

The gels were placed in a Fuchsware® refrigerator set at 5 ± 3 °C, in a Gallenkamp® oven (Loughborough, Leicester, England) set at 25 ± 2 °C and in a Labotec® oven set at 40 ± 2 °C to simulate long term and accelerated storage conditions as described in the ICH Q1A (R2) guideline [535]. The products were stored at 5, 25 and 40 °C for a maximum of 56 days and samples were withdrawn for analysis and assessment at 7 (D7), 14 (D14), 28 (D28) and 56 (D56) days. The sample time points and responses monitored at the individual time points are listed in **Table 8.3**.

Table 8.3 Sample points and responses monitored during stability testing.

		KZ content %	KZ released 24 hours µg/cm²	KZ released 48 hours µg/cm²	KZ released 72 hours µg/cm²	Viscosity 22 °C cP	Viscosity 37 °C cP	pH
D0	-	x	x	x	x	x	x	x
D7	**5 °C**	x	-	-	-	-	-	-
	25 °C	x	x	x	x	x	x	x
	40 °C	x	x	x	x	x	x	x
D14	**5 °C**	x	-	-	-	-	-	-
	25 °C	x	x	x	x	x	x	x
	40 °C	x	x	x	x	x	x	x
D28	**5 °C**	x	-	-	-	-	-	-
	25 °C	x	x	x	x	x	x	x
	40 °C	x	x	x	x	x	x	x
D56	**5 °C**	x	-	-	-	-	-	-
	25 °C	x	x	x	x	x	x	x
	40 °C	x	x	x	x	x	x	x

Citrate-phosphate buffers of different pH were manufactured by changing the concentration of citric acid and maintaining the ethanol and polysorbate 80 concentrations at 9.5 and 1.5% v/v respectively. The pH was established using **Equation 6.6** and measured prior to incorporation in formulations.

Approximately 100 g of the solvent of each pH was manufactured by weighing the exact amount of citric acid identified using **Equation 6.6** and adding it to 85 g of a citrate-phosphate buffer of pH 5.0 before sonicating the mixture until a clear solution was produced. Exactly 1.5 g polysorbate 80 was added to the solution and stirred using a magnetic stirrer until the polysorbate 80 was completely mixed after which 9.5 g of absolute ethanol was added and the mixture was stirred for an additional 30 seconds using a magnetic stirrer. The pH of the solution was measured using a model GLP 21 Crison® pH meter. Thermosetting gels were manufactured using solvent systems of different pH as depicted in **Figure 7.1.**

The clear free flowing liquid gel formulations obtained were analysed for KZ content, KZ released at 24, 48 and 72 hours, viscosity at 22 °C and 37 °C and pH following each storage period.

8.2.1 KZ content

HPLC analysis was used to quantitate the amount of KZ in the gels following stability studies. The extraction of KZ from the gels was performed according to the extraction procedure described in **Chapter 3.** Approximately 1 g of the gel and 12 mg of IS were accurately weighed directly into a beaker containing 100 mL of a 50:50 v/v mixture of ACN and water and covered with Parafilm™ to prevent excessive solvent loss, was sonicated for 15 minutes prior to shaking in a Kimax® centrifuge tube with the aid of a Junior Orbit® shaker at 200 rpm for a further 15 minutes. The mixture was then centrifuged at 3000 rpm for 15 minutes using a Model HN-SII centrifuge 3000 rpm and the supernatant was filtered using a 0.45 µm HVLP Durapore syringe filter attached to a 10 mL plastic syringe. The compatibility of the syringe filters and plastic syringes when exposed to a mixture of ACN and water was established by monitoring the resulting chromatograms for extraneous peaks that could be attributed to leachable components of the plastic syringe and membrane filter and no extra peaks were present to interfere with the peaks for KZ and the IS. A 1.5 mL aliquot of the filtrate was transferred into a 2.0 mL Waters® screw top amber glass vial and analysed using the HPLC method developed and validated as described in **Chapter 2.**

8.2.2 KZ released at 24, 48 and 72 hours

The amount of KZ released was analysed using the validated *in vitro* release method described in **Chapter 4**. A 1 g aliquot of gel was added to the donor compartment of diffusion cells followed by the addition of 1 mL SVF. The amount of KZ released into the 50:50 v/v ACN and 0.05 M phosphate buffer (pH = 4.5) receptor medium at 24, 48 and 72 hours was determined and the data further reduced using Design Expert® software.

8.2.3 Gel pH

The pH of topical formulations should be monitored during long term storage to assess elements of incompatibility and avoid tissue irritation [536]. A Model GLP 21 Crison® Instruments pH meter was used to monitor pH changes of the gel following storage. Following calibration the electrode was immersed in the gel and the pH measured after 30 seconds exposure.

8.2.4 Viscosity at 22 °C and 37 °C

A Brookfield® viscometer fitted with spindle D (S94) and operated at 5.8 rpm with a 10 second helical path was used to establish the viscosity of the gels at 22 °C and 37 °C following storage . The viscosity of the gels was measured in triplicate at 22 °C heating to 37 °C for ten minutes. The gels were kept in a water bath during viscosity determination .

8.3 Results and discussion

8.3.1 Stability studies using Central Composite Design

Forty experiments were required for the CCD generated using Design Expert®, and included six centre point replicates (**Table 8.4**). The experiments were performed in the order generated by the software to minimise any potential experimental bias. This CCD was undertaken to monitor the impact of SM content, solvent pH, length of storage and storage temperature on the stability of the gels.

Table 8.4 Randomised experimental runs conducted to assess the impact of antioxidant concentration, solvent pH, length of storage and storage temperature on gel properties.

Formulation	SD	Run	A SM % m/v	B Solvent pH	C Days	D Storage temperature °C
S3	**35**	**1**	1.0	3.0	28	40
S2	**23**	**2**	0.5	3.5	0	40
S4	**28**	**3**	1.5	3.5	56	40
S3	**40**	**4**	1.0	3.0	28	40
S5	**30**	**5**	1.5	3.0	28	40
S3	**36**	**6**	1.0	3.0	28	40
S3	**38**	**7**	1.0	3.0	28	40
S3	**16**	**8**	1.0	3.0	28	25
S7	**26**	**9**	1.5	2.5	56	40
S9	**12**	**10**	1.0	3.5	28	25
S6	**1**	**11**	0.5	2.5	0	25
S3	**18**	**12**	1.0	3.0	28	25
S3	**37**	**13**	1.0	3.0	28	40
S2	**3**	**14**	0.5	3.5	0	25
S5	**10**	**15**	1.5	3.0	28	25
S6	**5**	**16**	0.5	2.5	56	25
S7	**2**	**17**	1.5	2.5	0	25
S7	**22**	**18**	1.5	2.5	0	40
S1	**29**	**19**	0.5	3.0	28	40
S2	**7**	**20**	0.5	3.5	56	25
S3	**15**	**21**	1.0	3.0	28	25
S8	**11**	**22**	1.0	2.5	28	25
S7	**6**	**23**	1.5	2.5	56	25
S3	**17**	**24**	1.0	3.0	28	25
S3	**14**	**25**	1.0	3.0	56	25
S3	**13**	**26**	1.0	3.0	0	25
S3	**33**	**27**	1.0	3.0	0	40
S1	**9**	**28**	0.5	3.0	28	25
S2	**27**	**29**	0.5	3.5	56	40
S4	**8**	**30**	1.5	3.5	56	25
S9	**32**	**31**	1.0	3.5	28	40
S6	**21**	**32**	0.5	2.5	0	40
S6	**25**	**33**	0.5	2.5	56	40
S3	**19**	**34**	1.0	3.0	28	25
S8	**31**	**35**	1.0	2.5	28	40
S4	**24**	**36**	1.5	3.5	0	40
S3	**39**	**37**	1.0	3.0	28	40
S4	**4**	**38**	1.5	3.5	0	25
S3	**20**	**39**	1.0	3.0	28	25
S3	**34**	**40**	1.0	3.0	56	40

Mathematical relationships were elucidated for four input variables and the seven response factors monitored, *viz.*, KZ content, KZ released at 24, 48 and 72 hours, pH, viscosity at 22 ºC and 37 ºC to identify the formulation that exhibited optimum stability and KZ release without an impact on the target viscosity of the gels at 22 ºC and 37 ºC or pH over the maximum stability test period investigated.

8.3.2 Model fitting

A 2FI polynomial model was identified and used for the evaluation of the proposed design space. All factors, *viz.*, SM content, solvent pH, number of days of storage and temperature individually in addition to binary combinations of these factors were model terms that were investigated. No aliases were identified and the lack of fit test produced 19 df for lack of fit and 10 df for pure error, suggesting that the correct set of design points had been selected and that the model was appropriate to detect any lack of fit for this design space.

The standard error of mean was found to be 0.22 for the parameters, SM, solvent pH, days, SM*storage temperature, solvent pH*storage temperature and days*storage temperature, 0.16 for days and 0.25 for solvent pH*days, SM*days and SM*solvent pH. The VIF and multiple correlation coefficient values were 1.00 and 0.00 respectively indicating that the design does not exhibit any multi-linearity constraints. The average leverage was 0.2750 suggesting that the points in the design space did not require additional replication. The condition number for the coefficient matrix was 1.00 suggesting that there was no multi-collinearity between the terms evaluated. The G-efficiency value was 61.2% indicating an adequately low lack of fit that is indicative that the model fitting process was robust. The mean error type and one-sided t-test was used to calculate the FDS based on the half-width of the CI (**Figure 8.2**). The values for d and s were set at 0.5 and a was set at 0.05 for a Type I error risk and 95% CI. Approximately 98% of the design space falls within the 95% CI with standard error of mean of 0.589. The FDS percentage was ≥ 80% indicating a robust design space had been identified and was able to accurately predict the outcome of responses. Overall the 2FI polynomial model was found to be adequate and was appropriate to use to navigate the design space.

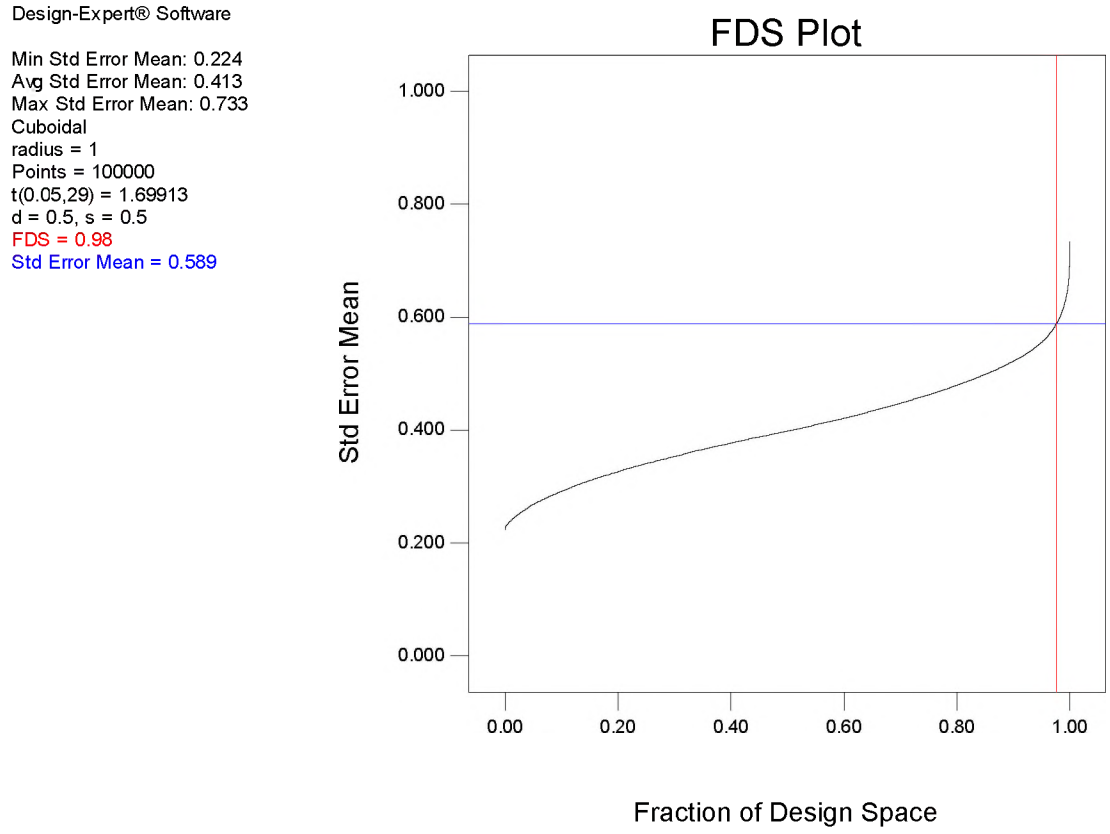


Figure 8.2 FDS plot depicting the percentage of the design space that can be accurately navigated.

8.3.3 Responses monitored

The impact of solvent pH, SM content, length of storage and temperature on KZ content, KZ release, viscosity and pH were evaluated in order to identify an optimised formulation composition. Any model transformations that were required are summarized in **Table 8.5.** The release of KZ at different times (R2 – R4) and viscosity at 22 °C (R6) were responses that exhibited a maximum to minimum ratio ≥ 10.00 and therefore natural log and inverse model transformations were performed to ensure robust analysis of the data could be achieved. The release of KZ resulted in lambda values = 0 and therefore natural log transformations were performed for these data. The difficulty in applying natural logarithms to KZ released or the inverse of viscosity to actual situations also required the analysis of equation prior to the transformation. In all cases the actual values were used in equations and response surface plots plotted in order to evaluate the experimental data, adequately.

Table 8.5 Responses obtained from thermosetting KZ vaginal gel formulations and respective model transformations required.

Response	Parameter	Unit	Minimum observed	Maximum observed	Recommended transformation
R1	KZ content	%	60.50	104.22	None
R2	KZ released at 24 hours	$\mu g/cm^2$	422.23	6579.61	Natural log
R3	KZ released at 48 hours	$\mu g/cm^2$	441.06	7247.19	Natural log
R4	KZ released at 72 hours	$\mu g/cm^2$	473.85	8766.49	Natural log
R5	Gel pH	-	2.95	4.08	None
R6	Viscosity at 22 °C	cP	3793	258367	Inverse
R7	Viscosity at 37 °C	cP	106875	400000	None

The data generated following analysis of KZ content and associated response coefficients, residuals and ANOVA are reported in detail, as the impact of different storage conditions on stability of the API is an important and critical response and is, therefore, used in this section as a basis for explaining the approach to model analysis. Detailed discussions of the data generated for the other responses are not reported, but the results of modelling and the final mathematical equations generated are reported for all responses as they give an indication of the relationship between the input factors and the response(s) monitored. The data obtained for all stability points and for all responses are listed in **Table 8.6** and the 3D response surface plots obtained for all responses are depicted in **Appendix J**.

Table 8.6 Stability response data obtained for formulations S1 to S9 at all sample times.

Storage conditions		Responses	S1	S2	S3	S4	S5	S6	S7	S8	S9
D0		KZ content, %	104.34	104.22	104.25	103.05	99.87	100.21	104.22	97.86	100.67
		KZ released at 24 hours, µg/cm²	4762.89	4321.64	4103.19	6579.61	6598.44	2708.09	2207.02	3655.65	3655.65
		KZ released at 48 hours, µg/cm²	5155.33	4527.51	4113.10	6606.59	6943.43	2866.18	2428.94	3877.72	3877.72
		KZ released at 72 hours, µg/cm²	5244.49	4561.64	4113.10	6622.45	6960.86	2903.40	2428.94	3921.29	3921.29
		pH	3.68	4.07	3.60	4.07	3.73	3.34	3.74	3.51	4.08
		Viscosity at 22 °C, cP	11493	10860	11146	3966	4138	8905	8102	7725	5297
		Viscosity at 37 °C, cP	201333	340450	186400	289450	333867	341550	306400	328968	264500
D7	5 °C	KZ content, %	104.16	103.13	103.51	103.02	98.91	99.34	103.95	97.05	99.97
	25 °C	KZ content, %	103.99	101.72	103.31	98.41	98.04	76.18	78.64	83.94	89.31
		KZ released at 24 hours, µg/cm²	4509.27	4423.90	4184.52	6449.30	4429.57	3493.53	2490.86	3991.10	2973.03
		KZ released at 48 hours, µg/cm²	4717.24	4898.75	4554.79	7133.16	4937.86	3581.59	2658.03	4245.41	3191.08
		KZ released at 72 hours, µg/cm²	4761.38	4996.48	4649.91	7299.91	4937.86	3593.71	2658.03	4297.11	3191.08
		pH	3.67	4.08	3.63	4.07	3.70	3.31	3.68	3.50	3.83
		Viscosity at 22 °C, cP	5630	8475	8893	6946	5309	8240	8060	7530	6874
		Viscosity at 37 °C, cP	267390	253500	220320	275220	335700	290970	306000	282040	251730
	40 °C	KZ content, %	101.28	103.74	101.66	100.74	98.67	89.34	81.20	79.54	83.47
		KZ released at 24 hours, µg/cm²	4845.88	5861.17	5288.80	5656.01	5873.86	2127.13	3863.26	3500.17	3921.55
		KZ released at 48 hours, µg/cm²	5284.20	5958.02	5288.80	6255.75	6588.66	2142.59	4048.09	3723.21	4128.09
		KZ released at 72 hours, µg/cm²	5310.37	5958.02	5288.80	6401.99	6771.83	2157.84	4095.01	3768.55	4188.99
		pH	3.64	4.05	3.61	4.05	3.68	3.27	3.70	3.35	3.89
		Viscosity at 22 °C, cP	4420	9210	9490	5360	5980	8905	7960	7840	6123
		Viscosity at 37 °C, cP	230400	325400	239833	278820	365720	282031	307196	279679	258566

Table 8.6 Stability response data obtained for formulations S1 to S9 at all sample times (continued).

Storage conditions		Responses	S1	S2	S3	S4	S5	S6	S7	S8	S9
D14	5 °C	KZ content, %	100.90	100.42	100.94	99.87	95.30	97.15	101.20	95.85	94.46
	25 °C	KZ content, %	101.15	101.04	99.36	92.45	94.92	69.62	56.82	65.56	72.99
		KZ released at 24 hours, µg/cm²	2469.27	2614.21	2266.22	1897.09	1705.36	2996.01	2508.22	2779.36	2199.68
		KZ released at 48 hours, µg/cm²	2752.91	2991.31	2603.98	1930.96	1878.89	3012.72	2535.58	2805.79	2357.93
		KZ released at 72 hours, µg/cm²	3108.48	3431.64	3186.83	2170.80	1878.89	3051.74	2599.34	2853.89	2380.15
		pH	3.67	4.05	3.44	3.90	3.55	3.25	3.53	3.53	3.61
		Viscosity at 22 °C, cP	4827	5172	5860	7241	6551	8030	7992	7675	8819
		Viscosity at 37 °C, cP	307500	178600	266500	202670	349971	285300	311733	236264	245620
	40 °C	KZ content, %	99.34	103.30	100.73	84.98	95.32	59.98	50.35	64.32	73.43
		KZ released at 24 hours, µg/cm²	2922.02	3761.43	2710.33	1663.74	2267.82	1941.74	2875.87	2124.53	3578.33
		KZ released at 48 hours, µg/cm²	3365.65	3805.68	2710.33	1693.44	2382.08	1967.4	2936.04	1980.61	3874.19
		KZ released at 72 hours, µg/cm²	3802.02	3805.68	2710.33	1903.79	2593.49	2113.2	2981.53	1980.61	3957.62
		pH	3.61	3.94	3.37	3.40	3.65	3.20	3.62	3.12	3.72
		Viscosity at 22 °C, cP	4482	5862	5517	6550	6896	6040	7683	7576	7537
		Viscosity at 37 °C, cP	292700	303100	293100	240150	383813	222560	311456	219943	233158
D28	5 °C	KZ content, %	92.75	89.36	87.11	85.99	83.86	79.48	82.67	86.34	82.32
	25 °C	KZ content, %	99.01	90.39	99.32	82.04	88.77	57.61	55.46	55.56	64.47
		KZ released at 24 hours, µg/cm²	3081.43	3868.91	2915.20	813.64	989.30	851.84	769.87	626.18	726.55
		KZ released at 48 hours, µg/cm²	3198.73	3993.24	3021.59	853.87	1037.82	851.84	816.97	632.26	793.45
		KZ released at 72 hours, µg/cm²	3427.57	4247.78	3157.51	861.09	1037.82	901.34	824.57	633.08	793.45
		pH	3.65	3.96	3.46	3.20	3.45	3.19	3.31	3.6	3.45
		Viscosity at 22 °C, cP	9310	9310	8270	7931	14019	7292	7360	7759	10685
		Viscosity at 37 °C, cP	267433	293100	252700	240300	400000	285300	317399	179100	228050
	40 °C	KZ content, %	94.17	102.93	87.38	73.85	80.71	54.98	49.14	45.54	56.77
		KZ released at 24 hours, µg/cm²	2800.10	3771.06	4013.04	713.56	935.33	965.39	617.37	762.2	1061.97
		KZ released at 48 hours, µg/cm²	2958.10	3922.25	4580.53	748.84	1006.50	965.39	636.13	826.04	1110.63
		KZ released at 72 hours, µg/cm²	3155.65	3922.25	5164.09	755.17	1018.22	969.95	641.35	841.12	1114.26
		pH	3.65	3.89	3.44	3.43	3.25	3.12	3.34	2.95	3.49
		Viscosity at 22 °C, cP	4597	9655	8620	6700	14924	5485	7506	7759	8965
		Viscosity at 37 °C, cP	285233	261700	158400	245668	400000	170489	313884	179100	260150

Table 8.6 Stability response data obtained for formulations S1 to S9 at all sample times (continued).

Storage conditions		Responses	S1	S2	S3	S4	S5	S6	S7	S8	S9
D56	5 °C	KZ content, %	85.93	76.15	72.57	75.72	73.36	67.25	71.49	69.13	73.24
	25 °C	KZ content, %	79.04	85.52	68.46	65.74	73.13	52.62	37.81	50.47	53.10
		KZ released at 24 hours, µg/cm^2	491.34	546.74	422.23	821.86	470.55	483.56	614.51	869.10	454.68
		KZ released at 48 hours, µg/cm^2	501.29	568.75	441.06	836.76	506.43	496.71	650.37	941.89	490.79
		KZ released at 72 hours, µg/cm^2	517.05	613.78	474.94	838.01	506.43	499.01	655.64	959.09	490.79
		pH	3.45	3.80	3.16	3.38	3.14	3.13	3.11	3.48	3.45
		Viscosity at 22 °C, cP	5333	9080	8810	7816	13133	7069	7126	7780	11030
		Viscosity at 37 °C, cP	307500	297900	214866	202133	400000	285000	317000	157520	254400
	40 °C	KZ content, %	85.73	93.76	71.64	60.50	67.31	49.85	47.88	35.54	51.97
		KZ released at 24 hours, µg/cm^2	666.67	730.50	686.06	720.77	531.93	451.14	494.12	549.16	649.09
		KZ released at 48 hours, µg/cm^2	668.15	731.28	739.19	733.83	555.96	472.57	495.95	554.49	649.5
		KZ released at 72 hours, µg/cm^2	668.15	731.28	743.28	734.93	561.76	473.85	495.95	555.21	649.5
		pH	3.43	3.82	3.18	3.38	3.17	3.01	3.11	2.95	3.49
		Viscosity at 22 °C, cP	4859	9540	6530	7471	13415	5170	7126	7586	8965
		Viscosity at 37 °C, cP	307500	274333	198600	236167	390780	106875	317000	179300	261700

8.3.3.1 KZ content

Statistical modelling was undertaken using a quadratic process order approach with significant terms ($p < 0.05$) selected to define final equations for the models. The ANOVA table generated using Design Expert® software (**Table 8.7**) reflects the sum of squares, df, mean square, F-value, p-value and statistical significance of the model terms. The model F-value of 19.44 implies that the model is significant and also indicates that there is a 0.01% ($p = 0.0001$) chance that the model F-value could occur due to noise. A lack of fit F-value of 2.33 indicates that the lack of fit is statistically insignificant relative to pure error and there is a 8.83% ($p = 0.0883$) chance that the lack of fit could occur due to noise. Significant model and insignificant lack of fit parameters are important, as these imply that the data is adequately fitted to the model. The terms for solvent pH, length of storage, a combination of anti-oxidant content and length of storage, a combination of pH and length of storage and the solvent pH and days were found to be significant factors that contributed to KZ content with p-values ≤ 0.05.

Table 8.7 ANOVA data table for the CCD for KZ content.

Source	Sum of squares	df	Mean square	F-value	p-value Prob > F	Significant difference
Model	13914.07	13	1070.31	19.44	< 0.0001	Yes
A-SM	176.70	1	176.70	3.21	0.0849	No
B-Solvent pH	2995.40	1	2995.40	54.40	< 0.0001	Yes
C-Days	1473.16	1	1473.16	26.75	< 0.0001	Yes
D-Storage temperature	2.54	1	2.54	0.046	0.8317	No
SM*solvent pH	135.84	1	135.84	2.47	0.1284	No
SM*days	356.27	1	356.27	6.47	0.0173	Yes
SM*storage temperature	0.74	1	0.74	0.014	0.9083	No
Solvent pH*days	779.53	1	779.53	14.16	0.0009	Yes
Solvent pH*storage temperature	0.20	1	0.20	0.0036	0.9529	No
Days*storage temperature	9.09	1	9.09	0.16	0.6879	No
SM^2	802.93	1	802.93	14.58	0.0007	Yes
Solvent pH^2	2908.87	1	2908.87	52.82	< 0.0001	Yes
$Days^2$	403.71	1	403.71	7.33	0.0118	Yes
Residual	1431.73	26	55.07	-	-	-
Lack of fit	1129.40	16	70.59	2.33	0.0883	No
Pure error	302.33	10	30.23	-	-	-
Total correction	15345.80	39	-	-	-	-

A high degree of correlation between the predicted and adjusted R^2 was observed with the R^2 value approaching 1 (**Table 8.8**). The predicted R^2 of 0.7694 is in reasonable agreement with the adjusted R^2 of 0.8601 as the difference is < 0.2000 [390]. An adequate precision of 17.39 is acceptable as it is > 4.00 indicates that the model can be used to navigate the design space.

Table 8.8 Statistical measures of model adequacy

Squared residuals and derivatives	Values
R^2	0.9067
Adjusted R^2	0.8601
Predicted R^2	0.7694
Adequate precision	17.39
SD	7.42
Mean	82.27
% Coefficient of variation	9.02
Predicted residual error sum of squares	3539.32

The final equation for KZ content using actual terms is reported in **Equation 8.1** and provides an indication of KZ content in relation to significant input variables.

KZ content = – 704.35 – (61.04 x SM) + (563.95 x solvent pH) – (2.48 x days) – (2.29 x storage temperature) – (11.66 x SM x solvent pH) – (0.34 x SM x days) – (0.39 x SM x storage temperature) + (0.50 x solvent pH x days) – (0.20 x solvent pH x storage temperature) + (0.024 x days x storage temperature) + (48.33 x SM^2) – (91.99 x solvent pH^2) + (0.011 x $days^2$) **Equation 8.1**

The values of the coefficients reveal that when the magnitude for solvent pH, solvent pH*days, days*storage temperature, SM^2 and $days^2$ increases the stability of KZ will also increase. The significant model term, solvent pH, exhibits the most pronounced effect on KZ content whereas the lowest impact was observed for $days^2$ with a coefficient of 0.011. A small change in the magnitude of the solvent pH will result in a large change in KZ content as compared to when there is a small change in the number of days used for the studies. In respect of the product and considering the sign of the coefficients when the solvent pH is increased the stability of KZ is enhanced within the 2.5 – 3.5 range for pH in this design space. On the other hand, when the formulation is stored for a longer period of time the formulation deteriorates to a small extent and the stability of KZ is reduced slightly.

3D response surface plots for content were generated for binary combinations of the numeric variables at both storage temperatures investigated and are depicted in **Figures 8.3 – 8.8.** The shapes of these plots reveal the presence of moderate interactions between the input factors investigated.

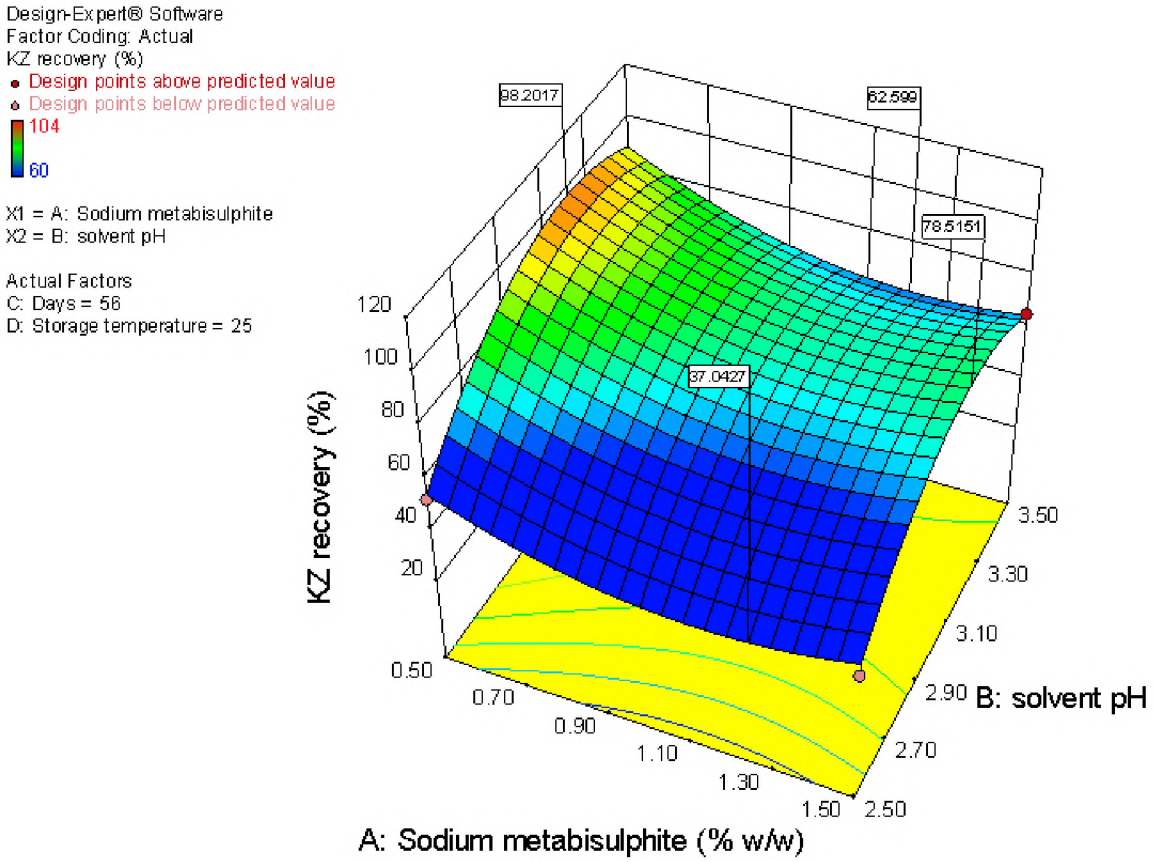


Figure 8.3 3D response surface plot depicting the impact of SM content and solvent pH on KZ content following storage for 56 days at 25 ºC.

The impact of the interaction between SM and solvent pH on KZ content of gels stored for 56 days at constant temperature are depicted in 3D surface plots at 25°C (**Figure 8.3**) and 40°C (**Figure 8.4**). The KZ content was at a maximum in vehicles of pH between 3.1 and 3.3 and a SM concentration of 0.5% m/v at 25 ºC. The highest KZ content of approximately 98.20% with 0.5% SM and approximately 78.51% with 1.5% m/v SM are easily identified by the flags depicted in **Figure 8.3**. A SM content of 0.5% m/v appears to be favourable in respect of maintaining the content of KZ at a relatively high level following storage at 25 ºC for 56 days using a solvent of pH 3.1 – 3.3. These data indicate that formulations with a solvent of pH 3.1 – 3.4 and that contain 0.50 – 0.55% m/v SM exhibit a KZ content ≥ 95% when stored at 25 ºC for 56 days, thereby complying with the ICH Q1A (R2) guideline [535].

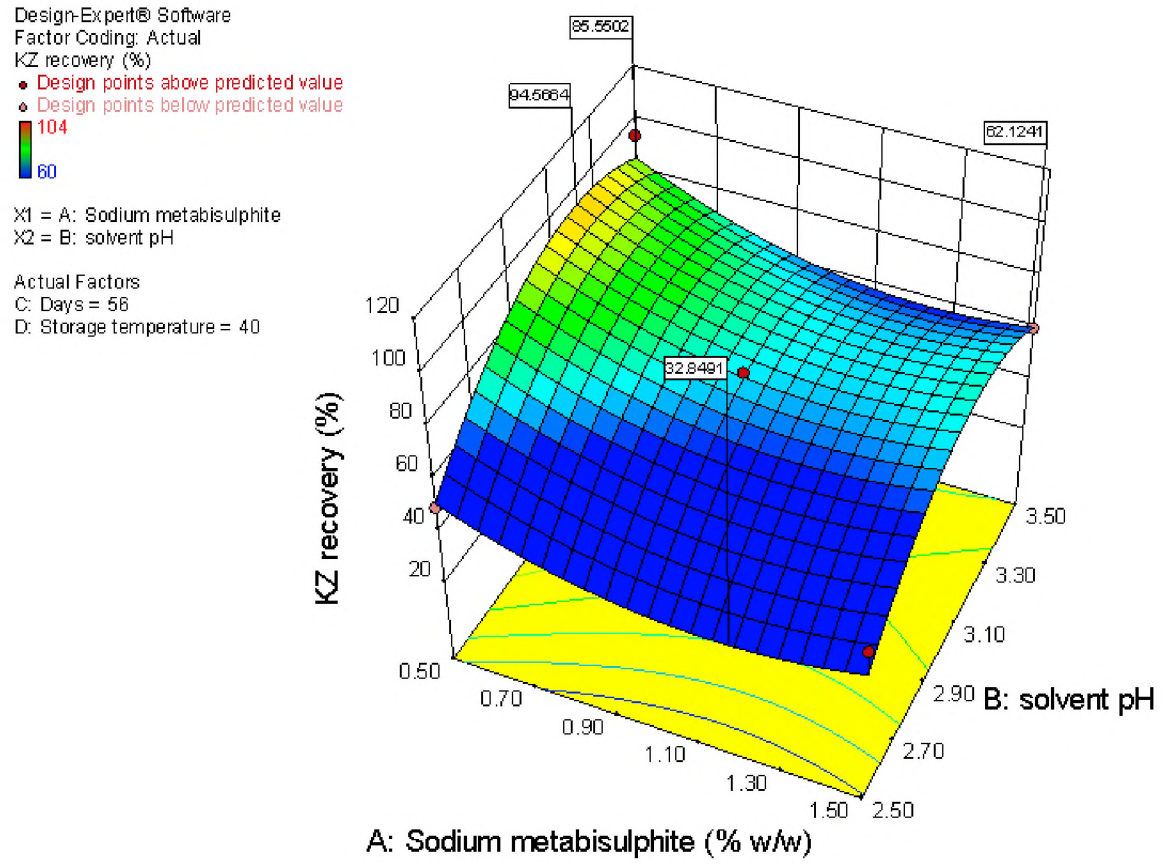


Figure 8.4 3D response surface plot depicting the impact of SM content and solvent pH on KZ content following storage for 56 days at 40 ºC.

A maximum KZ content of 94.57% was observed when 0.5% m/v SM and a solvent of pH between 3.1 and 3.3 were used in the composition (**Figure 8.4**). The recovery of KZ following storage at 40 ºC follows a similar trend to that observed at 25°C and the shape of the 3D response surface plots are similar. The lowest amount of KZ observed following storage at 40 ºC was 32.85% when a solvent of pH 2.5 and 1.20% m/v

SM were used. The same formulation exhibited a high KZ content of 85.55% when SM was included at a 0.5% m/v level and the pH of the vehicle was at the maximum level used in the design space, when compared to the same solvent pH, 1.50% m/v SM and storage at 40 ºC for which the KZ content was 62.12%. The difference in these values is approximately 20% and such a large difference cannot be attributed to variability likely to be observed within formulations. SM dissolves in water to form an acidic solution [26,537]; therefore, a large amount of SM may further lower the pH of the gels, resulting in the potential for increased hydrolysis of KZ. It is clearly evident that none of the dosage forms conformed to the specifications for KZ content after storage for 56 days at 40 ºC and therefore compliance with the ICH Q1A (R2) guideline was not achieved [535].

Following storage for 56 days, the lowest and highest content of KZ found was 62.36 and 104.96% when the amount of SM used was 1.2 and 0.5% m/v, respectively **(Figure 8.5)** at 25 ºC. A similar trend was observed following storage at 40 ºC **(Figure 8.6)** suggesting that the stability of KZ in the gel formulations is highly dependent on the amount of SM used and that low concentrations are preferable in formulation compositions. SM is an acidic compound that hydrolyses to form sodium bisulphite and eventually forms a weak sulphurous acid when mixed in acidic aqueous media [26,537]. As the amount of SM is increased the overall pH of the formulation decreases as observed from the data listed in **Table 8.6** possibly resulting in hydrolysis of KZ. Although SM is used as an anti-oxidant to minimise the oxidative degradation of KZ, it may, if used in relatively high concentrations, contribute to the hydrolysis of KZ in acidic solution. Therefore, a low concentration of SM of 0.5% m/v is preferred for this formulation as it has the least impact on the pH of the gel. At least 95% KZ was recovered following 14 days of storage at 25 ºC for a formulation that contained 0.5% m/v SM in a gel solvent of pH = 3.5. Alternate antioxidants were not investigated in these studies as preformulation studies suggested that the use SM would not result in interaction between KZ and SM. In addition SM is used as antioxidant in Ketazol® cream and is a known antimicrobial agent when used in acidic formulations, in addition to being an antioxidant [538]. As a consequence of these results there is clearly a need to investigate the use of alternate antioxidants for this formulation.

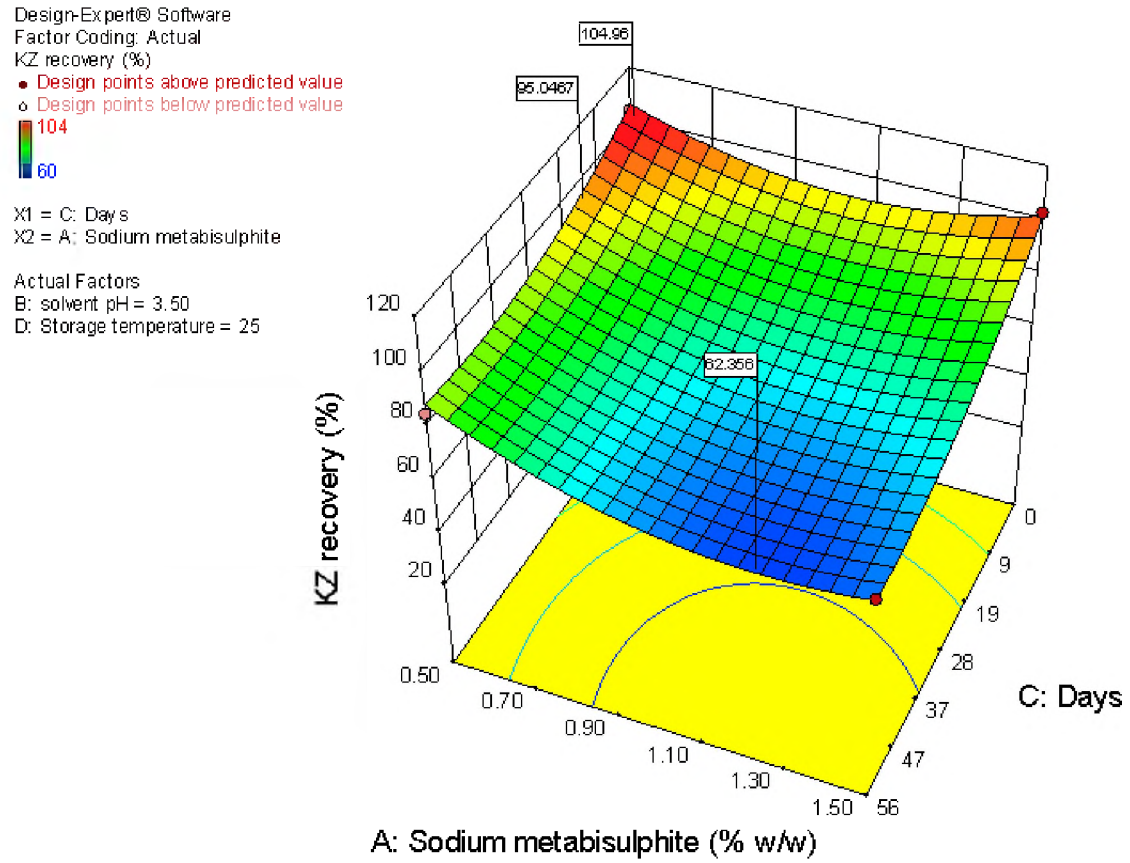


Figure 8.5 3D response surface plot depicting the impact of SM content and length of storage at 25 ºC on KZ content.

The lowest amount of KZ recovered of 58.10% was observed following storage for 56 days at 40 ºC for the gel in which 1.25% m/v SM was used **(Figure 8.6)**, whereas 95.26% KZ was recovered following storage for seven days at 40 ºC in a solvent of pH 3.5 when 0.5% m/v SM was included.

Several authors have reported that the inclusion of drugs within the hydrophobic core of micellar structures improves the stability of compound in aqueous formulations [333,335,539]. In addition rigid gel structures formed at temperatures ≥ 37 ºC reduce the rate of diffusion of drugs that may also be trapped within the hydrophobic core of micelles that form within the gel matrix thereby further reducing hydrolysis of that drug. In this case, the converse was observed as it appears as though the stability of KZ in this thermosetting gel formulation is enhanced at 25 ºC when compared to results observed for formulations stored at 40 ºC. Generally, at 25 ºC the formulation is in a liquid state in which case KZ is readily accessible to the vehicle and therefore more likely to undergo hydrolysis than when stored at 40 ºC. A simple explanation for this occurrence relates to the degradation of L68 at 40 ºC [540]. L68 degrades to form acidic compounds resulting in a decrease in pH of the gel which results in degradation of KZ. Degradation of the L68 polymer

would also result in a decrease in the viscosity of the formulation as the CMT increases with subsequent disruption of micelles and expulsion of KZ into the aqueous phase, leading to rapid hydrolysis during storage. At 25 ºC the L68 polymer is unlikely to degrade rapidly and would therefore provide some degree of protection for KZ over a longer period of time.

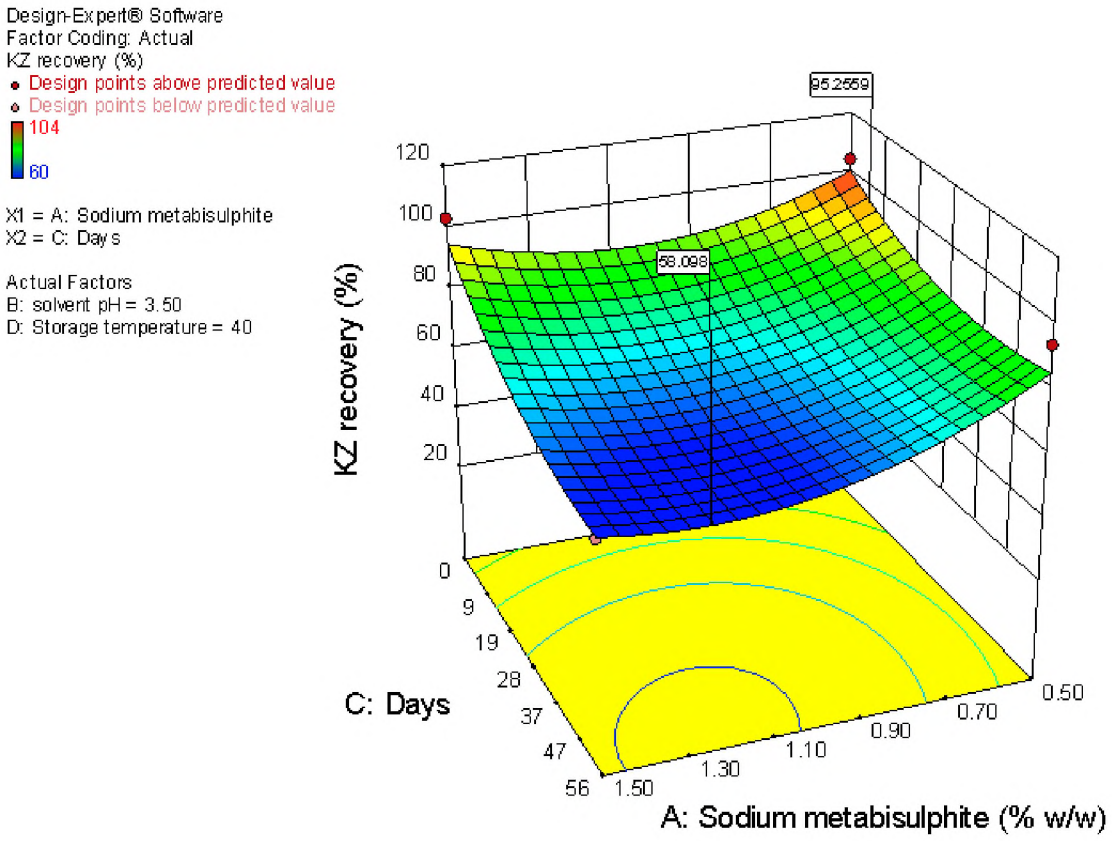


Figure 8.6 3D response surface plot depicting the impact of SM content and length of storage at 40 ºC on KZ content.

When a vehicle of pH 3.0 containing 0.5% m/v SM was used, 95.16% KZ was recovered following storage for 14 days at 25 ºC. The lowest amount of KZ of 69.92% was observed in a solvent of pH 2.5 following storage for 56 days (**Figure 8.7**). Higher solvent pH seems to be more favourable for ensuring the stability of KZ and may be due to hydrolysis of KZ in acidic media as described in § **2.7.2.1**.

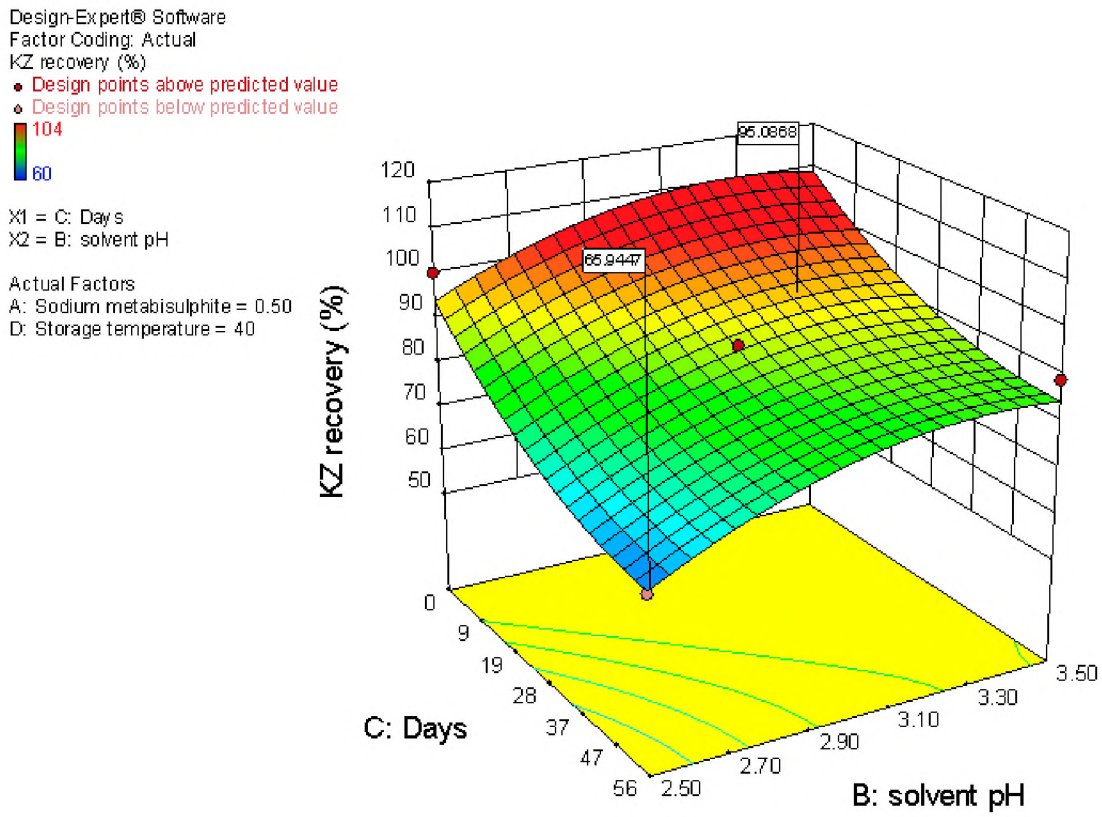


Figure 8.7 3D response surface plot depicting the impact of solvent pH and length of storage at 25 ºC on KZ content.

The lowest amount of KZ of 65.94% was observed following storage at 40 ºC for 56 days in a vehicle of pH 2.5 and 0.50% m/v SM **(Figure 8.8)**. It is evident that storage at 40 ºC has a detrimental effect on KZ stability at the lowest pH used within the design space. However, when a solvent of pH 3.2 was used in combination with 0.50% m/v SM, 95.09% KZ was recovered following storage for 21 days at 40 ºC.

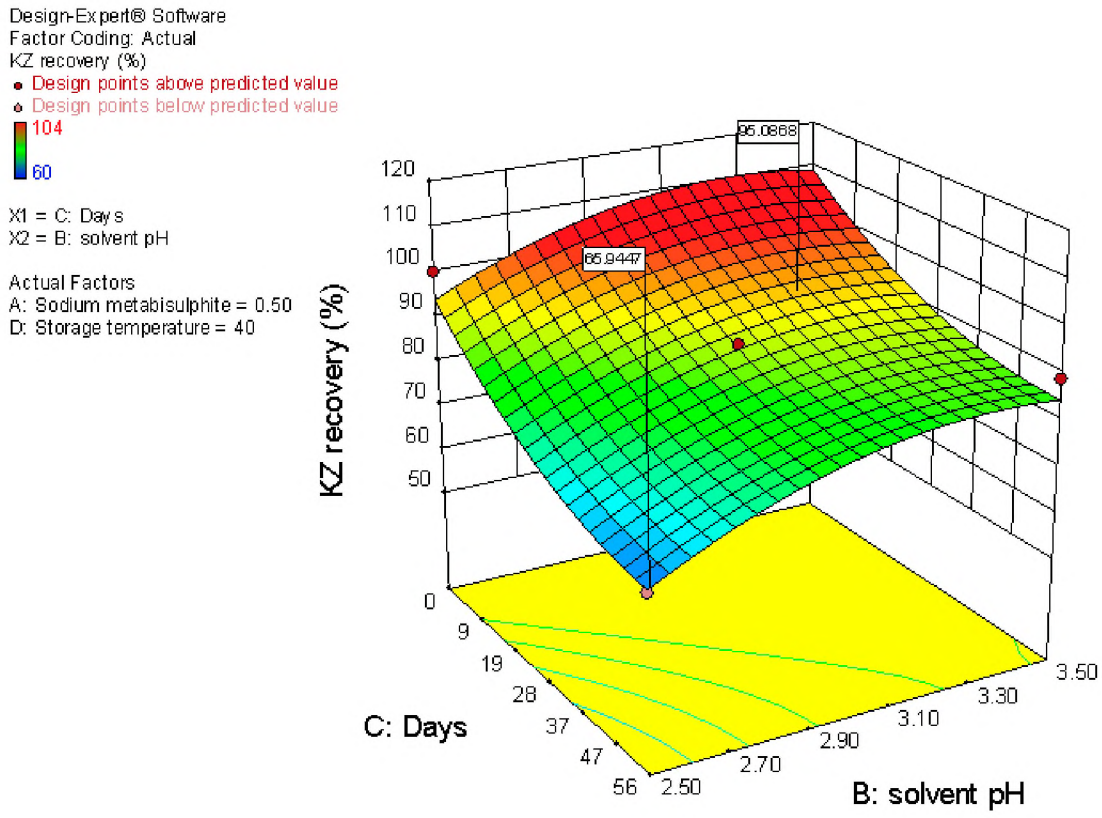


Figure 8.8 3D response surface plot depicting the impact of solvent pH and length of storage at 40°C on KZ content.

It is clear that the most stable formulation should include a solvent of pH between 3.1 and 3.3 and a SM content of 0.5% m/v when intended for storage at 25 ºC and 40 ºC. At least 95% KZ was recovered after 56 days of storage at 25 ºC; however, at 40 ºC and a higher solvent pH with 0.5% m/v SM, this was not the case. Therefore a number of formulations failed stability testing based on KZ content at all sample times investigated.

KZ content in addition to the other responses monitored for batches S1 to S9 at all sample points are reported in **Table 8.6.** The KZ content for batches S1 to S9 at 56 days when exposed to 25°C were 79.04, 85.52, 68.46, 65.74, 73.13, 52.62, 37.81, 50.47 and 53.10% with initial KZ contents of 104.34, 104.22,

104.25, 103.05, 99.87, 100.21, 104.22, 97.86 and 100.67%. During the first seven days, batch S7 exhibited the highest extent of KZ degradation of 25.58% and the formulation that exhibited the least degradation was batch S2 with a final KZ content of 82.08% calculated as a percentage of the initial KZ concentration at day 0. Batches S6 to S9 exhibited degradation of > 5% from the pull point, D7, when stored at 25°C and content of 99.66, 97.60, 99.10 and 98.17% KZ was recovered from batches S1, S2, S3 and S5 at the same sample point. Batch S3 was considered stable for up to 56 days at 25 °C when measured against the ICH Q1A (R2) guideline for stability testing of new drug substances and products with a 95.27% recovery of KZ [535] and batches S1, S2 and S5 were stable for a maximum of 14 days at 25 °C with KZ content of 96.94, 96.95 and 95.04% respectively. Batch S3 was considered the most stable formulation following storage at 25 °C for a maximum of 28 days.

The % KZ recovered from batches S1 to S9 stored at 40 °C for 56 days was 82.16, 89.96, 68.72, 58.71, 67.40, 49.75, 45.94, 36.32 and 51.62% as a percentage of the initial concentration. It is clear that > 5% KZ degraded after seven days of storage at 40 °C for batches S6, S7, S8 and S9. At least 97.07, 99.54, 97.52, 97.76 and 98.80% KZ was recovered from batches S1, S2, S3, S4 and S5 at the same sample point. Batch S4 was stable for at least seven days with 17.54% KZ degraded after 14 days storage at 40 °C. Batch S2 was found to be stable for over 28 days and only 10.04% KZ degraded when stored for 56 days at 40 °C. Batches S1, S3 and S5 were established as stable for over 14 days at 40 °C with ≤ 5% KZ degraded and only 9.75, 16.18 and 19.18% KZ degraded when stored at 40 °C for 28 days. Batch S2 was found to be the most stable formulation following storage at 40 °C for a maximum of 56 days.

Greater than 50% KZ was recovered from batches S1 through S9 following storage in the refrigerator for 56 days. All formulations except S9 were stable for over 14 days in the refrigerator with ≥ 95% KZ recovered and batch S9 was stable for over seven days with 6.17% KZ degraded following storage for 14 days in the refrigerator. None of the batches exhibited stability after 28 days with ≥ 5% KZ degradation at that time. Storage in the refrigerator appeared to enhance the stability of KZ over the 56 day period when compared to storage at 25 °C and 40 °C. However, all formulations stored in the refrigerator reached the limits specified in the ICH Q1A (R2) guideline prior to formulations stored at elevated temperatures [535]. None of the formulations could be stored in the refrigerator for longer than 14 days. Storage at low temperatures generally enhances stability, as the kinetic energy of the drug is low and therefore the activation energy for hydrolysis is difficult to achieve for the reaction to proceed [541]. In this case the viscosity of the thermosetting gel is lowest in the refrigerator and therefore KZ is readily available for reaction in the dosage form at low temperatures as the poloxamer molecules occur as unimers at low temperature. The formulation was expected to contain KZ molecules within the hydrophobic core of

micelles formed, thereby limiting exposure to the acidic vehicle, promoting enhanced stability of KZ. The stability of KZ at 40 °C was expected to be greater than that at 25 °C; however, as the L68 polymer degraded at 40 °C, the stability of the KZ in these dosage forms was almost similar at both temperatures, with the KZ content recovered following 28 days storage observed from formulations containing different SM and solvent pH falling within the specifications set.

The degradation profiles for all formulations stored at the three temperatures are depicted in **§ 8.3.4** *vide infra* and provide a better insight to the impact of storage conditions on product stability, in addition to the optimum formulation, developed to date using a DoE approach.

8.3.3.2 KZ released at 24, 72 and 48 hours

The final mathematical equations generated for KZ release at 24, 72 and 48 hours following modelling are listed as quadratic polynomial functions (**Equations 8.2 – 8.4**). There was a need to alter the model in order to fit the data adequately and therefore the natural log of KZ released was established for each time point. The equations prior to the transformation were also reported for better understanding of the data.

ln (KZ released at 24 hours) = + 16.80 – (1.77 x SM) + (16.92 x solvent pH) – (0.0065 x days) – (0.085 x storage temperature) + (0.33 x SM x solvent pH) + (0.0013 x SM x days) – (0.053 x SM x storage temperature) – (0.0082 x solvent pH x days) + (0.063 x solvent pH x storage temperature) + (0.00064 x days x storage temperature) + (0.32 x SM^2) – (2.76 x solvent pH^2) – (0.000055 x $days^2$) **Equation 8.2**

ln (KZ released at 48 hours) = + 16.92 – (1.60 x SM) + (17.02 x solvent pH) – (0.0065 x days) – (0.029 x storage temperature) + (0.29 x SM x solvent pH) + (0.0013 x SM x days) – (0.056 x SM x storage temperature) – (0.0076 x solvent pH x days) + (0.051 x solvent pH x storage temperature) + (0.00056 x days x storage temperature) + (0.29 x SM^2) – (2.77 x solvent pH^2) – (0.000092 x $days^2$) **Equation 8.3**

ln (KZ released at 72 hours) = – 18.19 – (1.54 x SM) + (17.88 x solvent pH) – (0.0055 x days) + (0.0018 x storage temperature) + (0.28 x SM x solvent pH) + (0.0011 x SM x days) – (0.047 x SM x storage temperature) – (0.0073 x solvent pH x days) + (0.043 x solvent pH x storage temperature) + (0.00029 x days x storage temperature) + (0.28 x SM^2) – (2.91 x solvent pH^2) – (0.00019 x $days^2$) **Equation 8.4**

The coefficients of the main effects suggest that the amount of SM used, length of storage and storage temperature have a negative effect on the natural log of KZ released at 24, 48 and 72 hours except for KZ released at 72 hours when storage temperature is considered and where a positive effect was observed. Solvent pH has a negative effect on the parameter. The most pronounced positive and negative effects were observed for solvent pH and SM content for all sample times.

The lowest overall main effect was observed for the length of storage. The lowest and highest amount of KZ released at 24, 48 and 72 hours was 422.23 to 6579.61 μg/cm^2, 441.06 to 7247.19 μg/cm^2 and 473.85 to 8766.49 μg/cm^2 respectively and these data are listed in **Table 8.9**.

Table 8.9 Response data generated for KZ release at different time points.

Formulation	SD	Run	KZ content %	KZ released at 24 hours μg/cm^2	KZ released at 48 hours μg/cm^2	KZ released at 72 hours μg/cm^2	Gel pH	Viscosity at 22 °C cP	Viscosity at 37 °C cP
S3	**35**	**1**	87.38	4013.04	4580.53	5164.09	3.44	8620	158400
S2	**23**	**2**	104.22	4321.64	4527.51	4561.64	4.07	10860	340450
S4	**28**	**3**	60.50	720.77	733.83	734.93	3.38	7471	236167
S3	**40**	**4**	82.01	3226.00	3660.94	3993.01	3.34	8161	400000
S5	**30**	**5**	80.71	935.33	1006.50	1018.22	3.25	14924	400000
S3	**36**	**6**	82.01	4091.32	4426.70	4808.94	3.34	7701	400000
S3	**38**	**7**	69.14	3431.44	3943.89	4319.85	3.21	8161	400000
S3	**16**	**8**	99.32	2915.20	3021.59	3157.51	3.46	8270	252700
S7	**26**	**9**	47.88	494.12	495.95	495.95	3.11	7126	317000
S9	**12**	**10**	64.47	726.55	793.45	793.45	3.45	10685	228050
S6	**1**	**11**	100.21	2708.09	2866.18	2903.40	3.34	8905	341550
S3	**18**	**12**	92.31	2713.42	2827.17	2934.23	3.54	7011	165700
S3	**37**	**13**	78.09	5968.95	7247.19	8766.49	3.20	8620	158400
S2	**3**	**14**	104.22	4321.64	4527.51	4561.64	4.07	10860	340450
S5	**10**	**15**	88.77	989.30	1037.82	1037.82	3.45	14019	400000
S6	**5**	**16**	52.62	483.56	496.71	499.01	3.13	7069	285000
S7	**2**	**17**	104.22	2207.02	2428.94	2428.94	3.74	8102	306400
S7	**22**	**18**	104.22	2207.02	2428.94	2428.94	3.74	8102	306400
S1	**29**	**19**	94.17	2800.10	2958.10	3155.65	3.65	4597	285233
S2	**7**	**20**	85.52	546.74	568.75	613.78	3.80	9080	297900
S3	**15**	**21**	96.88	2605.41	2698.71	2792.86	3.39	7011	165700
S8	**11**	**22**	55.56	626.18	632.26	633.08	3.60	7759	179100
S7	**6**	**23**	37.81	614.51	650.37	655.64	3.11	7126	317000
S3	**17**	**24**	96.88	3177.33	3269.62	3387.92	3.26	8276	203400
S3	**14**	**25**	68.46	422.23	441.06	474.94	3.16	8810	214866
S3	**13**	**26**	104.25	4103.19	4113.10	4113.10	3.60	11146	186400
S3	**33**	**27**	104.25	4103.19	4113.10	4113.10	3.60	11146	186400
S1	**9**	**28**	99.01	3081.43	3198.73	3427.57	3.67	9310	267433
S2	**27**	**29**	93.76	730.50	731.28	731.28	3.82	9540	274333
S4	**8**	**30**	65.74	821.86	836.76	838.01	3.38	7816	202133
S9	**32**	**31**	56.77	1061.97	1110.63	1114.26	3.49	8965	260150
S6	**21**	**32**	100.21	2708.09	2866.18	2903.40	3.34	8905	341550
S6	**25**	**33**	49.85	451.14	472.57	473.85	3.01	5170	106875
S3	**19**	**34**	92.31	3192.06	3368.28	3508.33	3.23	7011	165700
S8	**31**	**35**	45.54	762.20	826.04	841.12	2.95	7759	179100
S4	**24**	**36**	103.05	6579.61	6606.59	6622.45	4.07	3966	289450
S3	**39**	**37**	78.09	4013.04	4580.53	5164.09	3.55	8620	158400
S4	**4**	**38**	103.05	6579.61	6606.59	6622.45	4.07	3966	289450
S3	**20**	**39**	85.89	2887.78	2944.17	3164.22	3.32	7011	165700
S3	**34**	**40**	71.64	686.06	739.19	743.28	3.18	6530	198600

The equations for KZ released at 24, 48 and 72 hours prior to transformation are listed as **Equations 8.5 - 8.7** and offer a better overview of the real formulation effect on KZ release.

KZ released at 24 hours = – 42000.48 – (3630.09 x SM) + (29666.94 x solvent pH) + (77.98 x days) + (63.96 x storage temperature) + (1402.39 x SM x solvent pH) – (13.73 x SM x days) – (14.55 x SM x storage temperature) – (49.98 x solvent pH x days) + (43.49 x solvent pH x storage temperature) + (0.35 x days x storage temperature) – (96.57 x SM^2) – (4725.83 x solvent pH^2) + (0.45 x $days^2$) **Equation 8.5**

KZ released at 48 hours = – 44991.71 – (3093.34 x SM) + (31709.01 x solvent pH) + (78.96 x days) + (169.43 x storage temperature) + (1281.54 x SM x solvent pH) – (12.66 x SM x days) – (18.64 x SM x storage temperature) – (48.76 x solvent pH x days) + (36.16 x solvent pH x storage temperature) + (0.32 x days x storage temperature) – (217.45 x SM^2) – (5056.22 x solvent pH^2) + (0.32 x $days^2$) **Equation 8.6**

KZ released at 72 hours = – 49938.62 – (2876.66 x SM) + (35057.38 x solvent pH) + (87.95 x days) + (251.94 x storage temperature) + (1279.92 x SM x solvent pH) – (12.35 x SM x days) – (10.28 x SM x storage temperature) – (48.71 x solvent pH x days) + (31.20 x solvent pH x storage temperature) + (0.17 x days x storage temperature) – (356.07 x SM^2) – (5613.42 x solvent pH^2) + (0.14 x $days^2$) **Equation 8.7**

The coefficients of the main effects indicate that solvent pH, the number of days the gel is stored and storage temperature have a positive effect at 24, 48 and 72 hours, whereas SM content has a negative effect on the release of KZ. The magnitude of the effects exhibited by SM content and solvent pH are more pronounced than the effect of length and temperature of storage. The quadratic effects of binary combinations of input factors and the square of the functions have a relatively small impact on KZ release. Therefore, KZ release at 24, 48 and 72 hours is primarily dependent on SM content and solvent pH in the formulations. The storage conditions have a minimal impact on KZ release at the sample times assessed.

An increase in the SM content, in the gel formulation, results in a decrease in the amount of KZ released at 24, 48 and 72 hours. Conversely, an increase in solvent pH results in an increase in the amount of KZ released at all sample times investigated. The storage temperature had a small but positive impact on KZ release and an increase in the storage temperature from 25 °C to 40 °C resulted in a small increase in the amount of KZ released. The longer the gels were stored revealed that a slightly higher amount of KZ was released at 24, 48 and 72 hours and in order to achieve maximum amount of KZ released following long storage times would require the use of low amounts of SM and a high pH in the dosage form.

The increase in amount of KZ released, when the batches were stored at elevated temperatures for longer periods of time, may be due to the degradation of poloxamer L68 resulting in fewer molecules of the polymer in the dosage form, leading to an increase in the CMT [540]. Consequently less KZ would be incorporated within micelles and the dosage form would contain more unimers than micelles resulting in a less viscous formulation and more rapid KZ release from the dosage form. SM is an inorganic salt that may

have reduced the activity of water in the system by promoting hydrogen bonds between water molecules in the system thereby resulting in a salting out effect that dehydrates the hydrophilic corona of micelles [542]. Therefore inter-micellar attraction is induced and the formation of aggregates of micelles in the dosage form, resulting in an increase in viscosity at constant temperature and poloxamer concentrations when the amount of SM in the formulation is increased [543]. A low SM content would thus result in the production of a low viscosity formulation and KZ will be more rapidly released from these low viscosity dosage forms.

The release of KZ from the thermosetting gels increased significantly as solvent pH increased. It is evident from the information in **§ 8.3.3.1** that KZ undergoes hydrolytic degradation following storage of the dosage form at 25 ºC and 40 ºC. An increase in solvent pH resulted in a reduction of the degradation of KZ and may be the reason for the increase in KZ release when solvent pH increased. In addition as solvent pH was increased, less citric acid was used in the solvent system and the potential of salting out, similar to that observed when the SM content increased is minimised. A decrease in the amount of citric acid in the solvent system limited the potential for salting out to occur and resulted in less dehydration of the hydrophilic corona of micelles leading to a lower viscosity than when larger amounts of citric acid was present in the formulation and therefore KZ release from the gel increases.

8.3.3.3 pH

Following modelling of pH data, a quadratic polynomial best described the impact of input variables on gel pH and the final relationship is reported in **Equation 8.8**.

Gel pH = + 2.30 – (0.18 x SM) + (0.31 x solvent pH) – (0.0024 x days) – (0.26 x storage temperature) – (0.44 x SM x solvent pH) – (0.0071 x SM x days) – (0.008 x SM x storage temperature) – (0.00045 x solvent pH x days) + (0.083 x solvent pH x storage temperature) – (0.00014 x days x storage temperature) + (0.63 x SM^2) + (0.099 x solvent pH^2) + (0.000048 x $days^2$) **Equation 8.8**

The coefficients for the main effects suggest that solvent pH has a significant and positive impact on formulation pH with a positive coefficient of 0.31. SM content, length of storage and storage temperature have a low but negative impact on the pH of the gel. The low impact of SM content on the pH of the gel suggests that the use of a 0.05 M phosphate buffer system may counteract the acidity associated when increased amounts of SM are dissolved in water. The storage of the gels at 40°C over an extended period of time results in the gel becoming more acidic as observed from the data listed in **Table 8.6** and supports the observation reported by Wróblewska and Winnicka [544] where the stability of a KZ containing hydrogel was investigated. Formic and acetic acid are degradation products that are formed following storage of L68 at 40 ºC which may have resulted in the acidity observed in the formulations and that has been reported [540].

8.3.3.4 Viscosity at 22 °C and 37 °C

The final equations generated following modelling of viscosity data generated at 22 °C and 37 °C are reported as **Equations 8.9** and **8.10**. A 2FI process order was selected for model analysis and no transformation was required for viscosity at 22 °C **(Equation 8.9)**.

Viscosity at 22 °C = – 92.33 + (11218.95 x SM) + (3628.35 x solvent pH) – (238.82 x days) – (663.68 x storage temperature) – (4382.00 x SM x solvent pH) + (62.83 x SM x days) + (671.20 x SM x storage temperature) + (52.58 x solvent pH x days) + (29.40 x solvent pH x storage temperature) – (7.26 x days x storage temperature)

Equation 8.9

The coefficients of the main effects suggest that length of storage and storage temperature have a negative impact on viscosity of the formulation at 22 °C while increasing SM content and the solvent pH resulted in a positive effect. SM content exhibits the most significant effect on the viscosity of the formulation, whereas storage conditions have a minimal effect. It has been established that a decrease in the CMT is observed when the amount of inorganic salt in thermosetting formulations is increased [466,506,545]. An increase in the SM content may result in salting out effects, leading to an increase in viscosity when constant temperature and poloxamer concentration are used [542] as indicated on evaluation of **Equation 8.9.**

A quadratic process order with no further transformation was used to analyse the results for viscosity at 37 °C. Solvent pH and length of storage were established as the main effects and exhibited positive coefficients indicating that these input factors had a positive impact on the viscosity of the gel at this temperature and the mathematical expression of this data is reported in **Equation 8.10.**

Viscosity at 37 °C = – 0.000033 – (0.000057 x SM) + (0.000056 x solvent pH) + (41.07 x days) – (68247.10 x storage temperature) – (0.000010 x SM x solvent pH) + (1252.20 x SM x days) + (21792.60 x SM x storage temperature) + (92.67 x solvent pH x days) + (22069.20 x solvent pH x storage temperature) – (328.44 x days x storage temperature) + (0.000043 x SM^2) – (75720.00 x solvent pH^2) – (43.32 x $days^2$) **Equation 8.10**

The temperature at which the formulations were stored exhibited the highest numerical value for a coefficient but had a negative impact on the viscosity measured at 37 °C indicating that storage at 40 °C resulted in a general decrease in the viscosity at 37 °C. However, the longer the product was stored an increase in viscosity was observed. Storage of this formulation at 40 °C may have resulted in degradation of L68 [540] resulting in a lower number of poloxamer blocks in the gel and in a decrease in the viscosity of formulations at 37 °C. In addition extended storage times at elevated temperatures may have resulted in the further stabilisation of the micelles [333] as the micellar structures and aggregates are more prominent at temperatures ≥ 37 °C resulting in the increase in the viscosity at 37 °C, as predicted using **Equation 8.10**.

8.3.4 Degradation profiles for KZ in batches S1 to S9 following storage at 5 °C, 25 °C and 40 °C

The stability results obtained using the DoE approach for formulations stored at 25 °C and 40 °C revealed low KZ recovery. Consequently, the same formulations for batches S1 to S9 were manufactured and stored at 5 ± 3 °C in a refrigerator according to the ICH Q1A (R2) guideline [535]. Samples were harvested and analysed at 7, 14, 28 and 56 days. A semi log plot for KZ content is depicted in **Figure 8.9** and the degradation rate constant for KZ in each formulation was calculated.

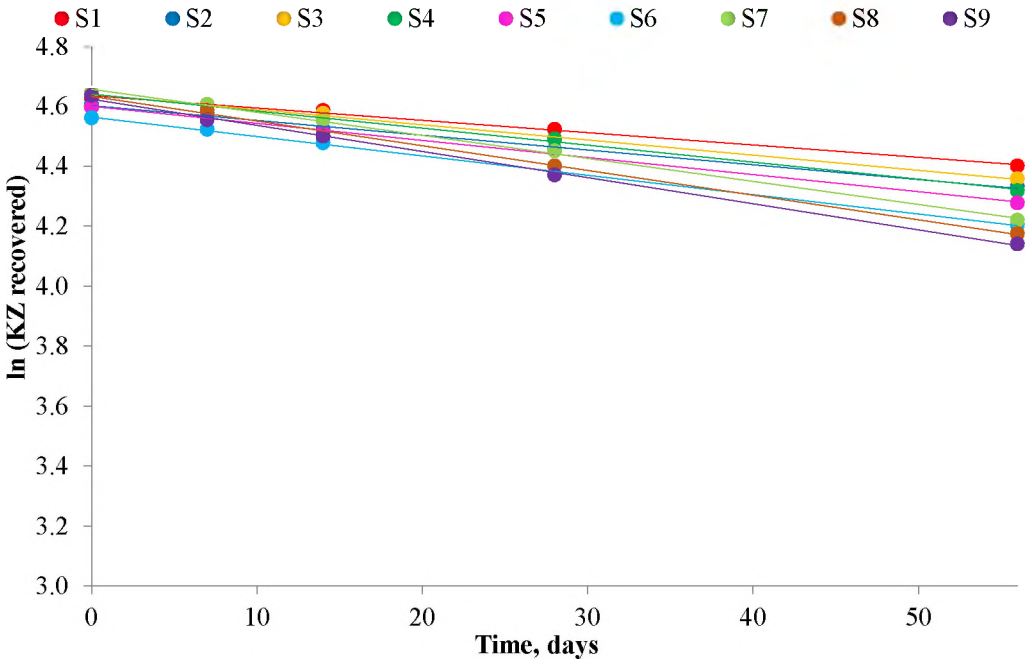


Figure 8.9 Semi-logarithmic plot of KZ content following storage of batches S1 to S9 at 5 ± 3 °C for 56 days.

In addition KZ content versus time was plotted as a semi-logarithmic plot for batches that had been stored at 25 ºC and 40 ºC and these data are depicted in **Figures 8.10 – 8.11** and the respective degradation rate constants are reported in **Table 8.10**. These data permit comparison of the degradation rates of KZ following storage at the three different temperatures.

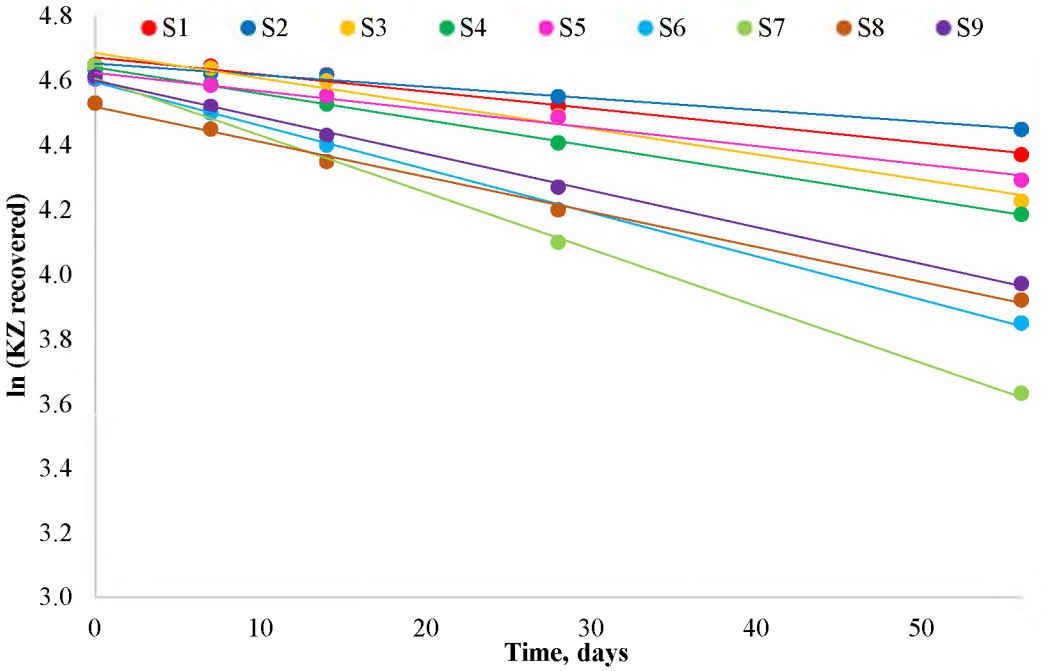


Figure 8.10 Semi-logarithmic plot of KZ content following storage of batches S1 to S9 at 25 ± 2 ºC for 56 days.

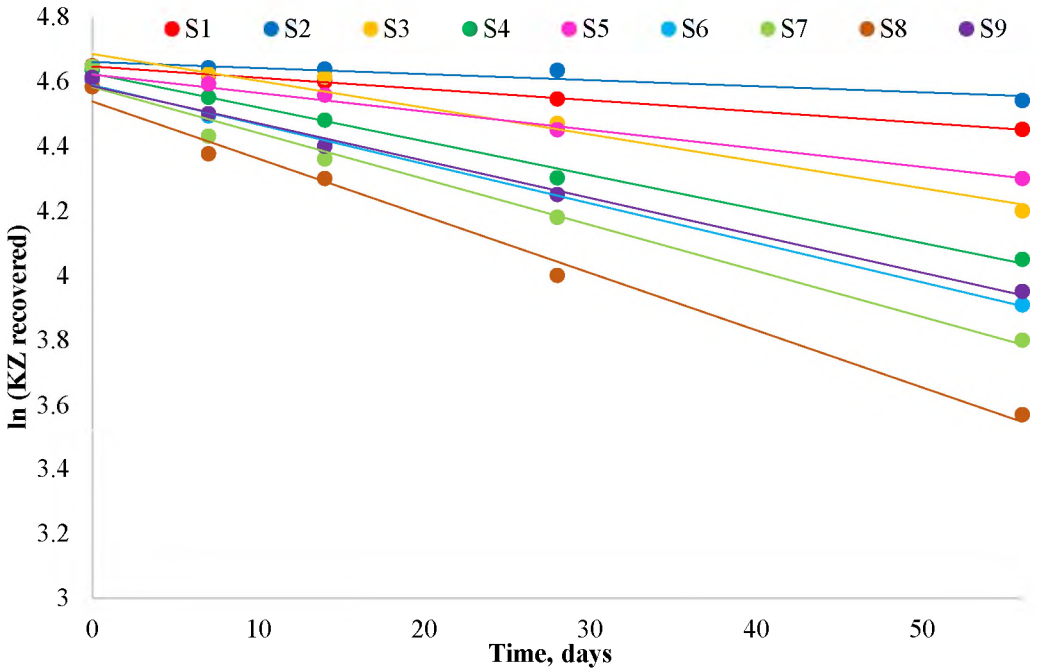


Figure 8.11 Semi-logarithmic plot of KZ content following storage of batches S1 to S9 at 40 ± 2 °C for 56 days.

Table 8.10 Degradation rate constants for KZ following storage of batches S1 to S9 at 5 °C, 25 °C and 40 °C.

Formulation	Degradation rate constant day^{-1}		
	5 °C	25 °C	40 °C
S1	-0.00423	-0.00501	-0.00706
S2	-0.00502	-0.00376	-0.00200
S3	-0.00532	-0.00745	-0.00830
S4	-0.00509	-0.00812	-0.01021
S5	-0.00505	-0.00567	-0.00578
S6	-0.00625	-0.01012	-0.01022
S7	-0.00667	-0.01620	-0.01154
S8	-0.00751	-0.01132	-0.01743
S9	-0.00684	-0.00991	-0.01101

The magnitude of the degradation rate constants increased as the storage temperature increased for batches S1, S3, S4, S5, S6, S8 and S9. The degradation rate constant decreased as storage temperature was increased for batch S2 and the highest degradation rate was observed following storage at 25 °C and the lowest following storage at 5 °C for formulation S7. The lowest degradation rates following storage at all temperatures was observed for formulation S2 that contained 0.5% m/v SM in a solvent of pH 3.5 which was also the optimum SM and solvent pH combination for the assurance of stability of KZ (**§ 8.3.3.1**). The degradation rates of the formulations did not appear to follow a specific trend, possibly due to the complex manner in which KZ may have degraded in these formulations. KZ degradation may have occurred in a

number of ways. Polymer L68 degradation at 40 °C to form acidic degradation products may have enhanced the hydrolysis of KZ. As a result of L68 degradation, a change in integrity of the gel matrix occurred increasing exposure of KZ to the acidic vehicle. Low poloxamer aggregation at low temperatures may also have resulted in an increase in exposure of KZ to the vehicle. Photolytic degradation of KZ may have commenced during the manufacturing process and wrapping containers in foil may not have provided sufficient protection from light. Gel manufacture in a facility with a safe light may be necessary. Storage of the gels in amber glass bottles provided some protection; however, the closure may not have been airtight and/or the humidity may have catalysed the degradation of KZ. Alternate container and/or closure system may have to be investigated. SM was not suitable for inclusion in this formulation as it forms acidic solutions thereby facilitating hydrolysis of KZ. In addition SM may be inadequate as antioxidant in this formulation and oxidation reactions could have occurred resulting in degradation.

8.4 Conclusions

KZ is a poorly water soluble compound that undergoes oxidation and hydrolysis in aqueous solution and some formulations [216,546]. The major challenge encountered in this study was the need to acidify the thermosetting gel formulation and solubilise KZ in an aqueous medium to produce a transparent gel. These formulation attributes of acidity of the gel and solubilisation of KZ in an aqueous solvent system contribute to the instability of KZ in this thermosetting vaginal gel formulation. Therefore a strategy was required to limit the instability of KZ in a simple formulation so as to produce a cost effective and reproducible treatment option for VVC. Solvent pH and the amount of SM were input variables investigated and predefined limits were set for this formulation type. KZ content was the main response monitored to establish the stability and potential shelf life of this formulation.

The CCD approach used to obtain stability data of the thermosetting KZ vaginal gels has demonstrated that DoE can be successfully used to design experiments to conduct studies to generate information relating to factors that impact the stability of thermosetting formulations. In addition, this approach permits simultaneous investigation of the effects of several formulation variables at any one time whilst establishing the significance of each input factor and response. The impact of input variables on KZ content was thoroughly investigated and the use of 3D response surface plots and associated mathematical relationships facilitated an understanding of the interactions between input factors and their impact on the stability and other attributes of the gel formulations. The polynomial equations generated were quadratic in nature as this model best describes the data generated. The quadratic model revealed single, binary combinations and squares of factors to describe responses such as amount of KZ released at 24, 48 and 72 hours, pH and viscosity at 22 °C and 37 °C. The use of a CCD enabled the relationship between the variables to be

established by conducting the lowest number of experiments to generate a maximum amount of information from the data.

Additional data obtained at each sample point were reported and the findings observed from stability studies assist in establishing strategies to improve the KZ gel formulations. The stability of KZ in the thermosetting gels following storage at 5 °C, 25 °C and 40 °C did not follow any specific trend as degradation of KZ was affected by SM content, solvent pH and storage conditions. Poloxamer molecules form unimers in solution at low temperature resulting in low viscosity gels. KZ was directly exposed to acidic vehicles in the dosage form following storage at 5 °C resulting in rapid hydrolysis of KZ. The data generated at each sample point confirm that a CCD approach may be adopted to produce an overall picture of the stability of formulations. However, a more thorough and accurate assessment of stability would be required for product registration. The approach to assessment of stability described in this chapter suggests that DoE may be used as a rapid and cost effective tool to provide an overall assessment of stability of topical formulations.

The major consequence of these studies is that there is a need for additional optimization studies to ensure the stability of the formulations so as to enhance the shelf-life of this product. The addition of SM in the gels resulted in significant changes in pH of the dosage forms which have a negative impact on the stability of KZ. This challenge can be obviated through the use of a low concentration of SM that should ensure sufficient antioxidant activity without compromising the overall pH of the gel, as the rate of hydrolysis of KZ is slower at higher pH. The analysis of other neutral antioxidants such as BHT used alone or in combination with SM should be investigated. BHT has been used as antioxidant for solution formulations of KZ [547].

The formation of stable micellar systems produced by dissolution of L127 have been reported [335,548,549]. The L68 polymer has been found to be unstable at 40 °C [540]. Consequently, an additional assessment of the stability of L68 is required to confirm whether L68 should be replaced by an alternate poloxamer or whether an alternate polymer that contributes to the strength and rigidity of the gel and that is stable at 40 °C should be used. Alternately, the stability of formulations at low temperatures ($\leq$ 25 °C) using different antioxidant(s) or in which KZ is protected from direct exposure to acidic vehicles can be undertaken. The co-solvency approach used in these studies to produce a vaginal gel containing solubilized KZ was adequate and the acidity of the formulation is a requirement to prevent irritation of the vaginal mucosa [550,551]. However, the pH of the gels may be increased to a maximum of 4.5 to further limit the hydrolysis of KZ; however, the compound does not dissolve completely at this pH. KZ molecules can be protected from hydrolysis in the formulation by incorporating KZ into solid lipid nanoparticles (SLN) [546],

nanostructured lipid carriers (NLC) and modified nano-vesicles [552] and stabilisation of nanoparticles in thermosetting gel formulations [553,554]. Solubilising KZ in polyamidoamine (PAMAM) dendrimers [555], encapsulated KZ loaded thermosetting gels [556-561], KZ/cyclodextrin complexes [562-568], solid dispersion of KZ in the hydrogel [7,31,563,569-572], dissolution of KZ in an emulsion prior to incorporation in a hydrogel base to form an emulgel [573], incorporating KZ in a self-emulsifying formulation [574-578] and nanoemulsions [579,580] are other strategies that could be used and attempted in future studies to limit exposure of the compound to the acidic thermosetting hydrogel formulated and optimised in this research.

CHAPTER NINE
CONCLUSIONS

Vulvovaginal candidiasis is a common fungal infection and an average of three infections occur annually in women suffering from repeated vulvovaginal candidiasis [1-3]. Uncomplicated vulvovaginal candidiasis can be treated within a few days using topical or oral treatment, whereas repeated vulvovaginal candidiasis requires extensive and prolonged treatment with potent azole therapeutic compounds [1]. *Candida albicans*, a yeast-like fungus, is the most common cause of local muco-cutaneous vulvovaginal candidiasis and invasive infections that affect other vital organs. Ketoconazole is an azole derivative that offers the possibility of cost effective treatment for vulvovaginal and/or repeated candidiasis when compared to other compounds in this class of molecules. Ketoconazole exhibits equivalent efficacy after administration using topical and systemic routes of administration, although systemic use is limited or has been discontinued in certain countries due to the high incidence of adverse effects, such as hepatoxicity, observed following systemic delivery. The topical administration of ketoconazole is considered safe with low systemic absorption. There exists a need for a vaginal formulation to treat vulvovaginal and/or repeated candidiasis to offer a safe treatment option for patients. In addition, alternate treatment options may then be selected based on the personal preference, social, economic and environmental considerations of the patient [284]. Ketoconazole has arguably, not yet been incorporated into any vaginal gel formulations to date and, therefore, considering the low efficacy following oral administration, may be an effective topical option for the treatment of vulvovaginal candidiasis.

The development and validation of a stability-indicating reversed-phase high performance liquid chromatography method with ultraviolet detection for the analysis of ketoconazole using the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use Guidelines was undertaken. A Phenomenex® Hyperclone 5 µm ODS C18 120 Å 150 x 4.6 mm i.d. column was used as the stationary phase and verapamil was used as the internal standard. Detection was achieved using a wavelength of 205 nm. The separation of ketoconazole and verapamil using a mobile phase composed of ACN: 0.05 M phosphate buffer (pH = 6) 50:50 v/v exhibited adequate resolution, peak asymmetry and tailing factors without the need for an additive to minimise peak tailing. The method was found to be linear over the concentration range 0.50 – 151.30 µg/mL and a correlation coefficient of 0.999 was established for the calibration curve. The precision of the method calculated at three levels *viz.*, intra-assay, intra- and inter-day precision. The standard deviation for responses was $\leq \pm 1.05$, % relative standard deviation $\leq 5\%$ and % recovery of $100 \pm 5\%$ in all cases and were within the target limits set in our laboratory. The method was established as accurate with a % Bias $\leq \pm 5\%$. The limits of quantitation and detection for ketoconazole

were 0.50 and 0.165 µg/mL. Ketoconazole was stable in solution to heating to a maximum temperature of 50°C for four hours. Ketoconazole was photolabile, susceptible to oxidation, acidic and alkali hydrolysis. The method was specific as all degradation peaks were resolved from the ketoconazole and verapamil responses observed for all analyses.

Commercially available topical formulations containing ketoconazole were assessed and characterised for viscosity, pH and content. Kez® shampoo, a liquid formulation was used in comparative testing to obtain information of the potential behavior of liquid KZ gel formulations. In addition, Fourier Transform-Infrared spectra and Differential Scanning Calorimeter thermograms were used to identify target characteristics for the development of a thermosetting vaginal gel formulation. The commercial formulations all exhibited a pH within the range that was least likely to cause irritation to human skin. Vaginal gels should be formulated at a pH that does not irritate and/or damage the vaginal mucosa and surrounding tissues. Analysis of viscosity data revealed that all formulations except the shampoo were semi-solid at 22 and at 37 °C. The shampoo became a liquid at the higher temperature. Extraction of ketoconazole from the commercial products was validated and a reversed-phase high performance liquid chromatographic method used to analyse content. All products were found to comply with their label claim following implementation of the extraction approach and this, together with the standard deviation, % relative standard deviation and % bias, indicate that the method was suitable for its intended purpose. Fourier Transform-Infrared Spectroscopy and Differential Scanning Calorimetry analysis revealed that ketoconazole was present in all commercial formulations in an amorphous form, which should, as far as possible, be reproduced in vaginal formulations for the purposes of maintaining stability and the therapeutic performance of ketoconazole.

The commercial products all contained 2% m/m ketoconazole and this amount of ketoconazole was assumed to be effective topically and was the starting strength used in formulation development activities. Xolegel® contains citric acid and Ketazol® cream contains sodium metabisulphite and these excipients were considered for inclusion to modulate pH and limit ketoconazole oxidation in the thermosetting gel. The colouring agent present in the gel and shampoo were not evaluated as these may stain undergarments and be uncomfortable for the patient. The effect of very hot climates on product integrity is important as liquid gels would solidify in the packaging and exhibit reduced flowability making administration difficult. The characterisation studies proved useful and generated valuable information about the commercial formulations that could facilitate formulation development activities. Sufficient information was generated during characterisation of the commercial products that permitted efficient preformulation and formulation studies to be undertaken for the development of a novel thermosetting vaginal gel containing ketoconazole.

In vitro release testing using Franz diffusion cells was undertaken to monitor ketoconazole release from novel vaginal formulations and commercial products. An *in vitro* release method was developed and validated to monitor ketoconazole release from commercially available formulations. The selection of a membrane for release studies was made following an assessment of the suitability of porcine vaginal mucosa and artificial membranes. A 0.2 µm Sartorius® cellulose acetate membrane was ultimately selected for use, as low binding and high permeability of ketoconazole using this membrane was observed. Receptor media of different composition were investigated and a medium of ethanol and 0.05 M phosphate buffer (pH = 4.5) in a 50:50 ratio was selected for these studies, as discrimination of ketoconazole release following testing of the different commercial products was observed. In addition, maximum ketoconazole release was achieved with the lowest quantity of ethanol possible whilst maintaining sink conditions for the release studies. The precision of the method was demonstrated through intra- and inter-day precision studies with a tolerance level for % relative standard deviation set at ≤ 10% [271]. Statistical comparison and mathematical modelling were performed on *in vitro* release profile data generated for the commercial formulations. Two-way analysis of variance and Tukey's multiple comparison testing proved to be insensitive, due to the low sample size used in these experiments and was therefore not able to discriminate effectively between the commercial formulations. Model dependent analysis demonstrated that the gel and shampoo and cream formulations exhibited different release kinetics and mechanism and the kinetics could not be attributed to one mathematical model as the data produced adequately fitted several models. The mechanism of release obtained from the n exponent of the Korsmeyer-Peppas model for all products was found to be mainly driven by an anomalous, non-Fickian diffusion. The model dependent analysis in combination with knowledge of formulation permitted the elucidation of release kinetics and mechanism from the formulations. Model independent analysis revealed that the release mechanism of ketoconazole from the gel and shampoo may be similar and that release from the cream was, as expected, different.

Vaginal drug delivery is an increasingly popular route of drug administration and has the potential for the delivery of ketoconazole to the vaginal mucosa without exposing patients to systemic side effects of the drug. The mucosa has a large surface area, in addition to extensive vascularisation, for the facilitation of ketoconazole transport and absorption [280]. Gel dosage forms that are similar to naturally occurring gels such as mucous are ideal for intra-vaginal use due to their high water and low excipient content, thereby minimising mucosal irritation and are relatively safe for application to inflamed and sensitive membranes [303]. The simplicity of gel formulations and their manufacturing processes make them a cost effective and attractive alternative to more complex technologies. A major disadvantage of vaginal formulations is the potential low residence time in the vagina as a result of physical and/or physiological expulsion that may result in unpredictable drug delivery and leakage of the formulation from the vagina causing discomfort,

which may be problematic for patient adherence to treatment. A temperature stimulus can be used to activate specialised hydrogels such as poloxamers, when formulated into *in situ* thermosetting gels that solidify at 37 °C. The use of this approach may result in increased residence times of the gel in the vagina, with an associated enhancement of therapeutic activity. Poloxamers occur as unimers at low temperature, form micelles at higher temperatures or micellar aggregates when the temperature increases further and are therefore suitable for the formulation of thermosetting gels. The novel thermosetting gels manufactured in these studies were designed to be liquid when administered into the vaginal lumen after which they would solidify to form a rigid gel that had the potential to remain in the vaginal cavity for an extended period of time for the successful treatment of vulvovaginal candidiasis. Poloxamers occur as compounds of different molecular weights containing different ratios of propylene oxide and polyethylene oxide. A combination of poloxamer grades 407, 188 and 237 was found suitable for the modulation of gelation temperature, in line with the requirements of this study, in which a gel that solidified rapidly following administration and that could withstand the physical and physiological environment of the vagina was formed.

Preformulation studies were performed to establish quality of materials and detect potential physical and/or chemical interactions between ketoconazole and excipients to be used for formulation development studies. Preformulation studies are undertaken to decrease the risk of chemical degradation, instability, changes in bioavailability, inefficacy and/or lack of safety during and after formulation development and commercialisation [419]. Differential Scanning Calorimetry thermograms and Fourier Transform-Infrared spectra were generated for individual compounds and 1:1 binary mixtures of ketoconazole and citric acid, disodium hydrogen phosphate, polysorbate 80, poloxamer 407, poloxamer 188, poloxamer 237 and sodium metabisulphite to evaluate physical and thermal ketoconazole-excipient compatibility. No obvious incompatibility was observed for all mixtures. Differential Scanning Calorimetry was useful to elucidate the form that ketoconazole existed in the mixture and it was established that an amorphous form occurred in combination with polysorbate 80 and all poloxamers. Ketoconazole is insoluble in water [13] and therefore a co-solvency approach was required to ensure complete dissolution of the drug in the formulation. Furthermore, the presence of the amorphous form of ketoconazole in the gel was likely to result in the formation of a clear gel, which from an aesthetic perspective would be of benefit during application to and removal from the vagina, as a suspended crystalline material may be confused with vaginal discharge and would not soil clothing.

Ketoconazole is a dibasic compound and is soluble in acidic vehicles. Consequently, it is suitable for inclusion in vaginal formulations required to treat local conditions [35]. Ketoconazole is also lipophilic and will therefore be sequestered within the hydrophobic core of any micelles that form in gels and would

therefore be shielded from the acidic vehicle. As ketoconazole is susceptible to acid catalysed hydrolysis and is poorly soluble, this phenomenon would enhance the solubility and stability of ketoconazole via micellar incorporation. The acidic vehicle in which ketoconazole was dissolved, prior to gel manufacture, was optimised for polysorbate 80, citric acid and ethanol content using a 2-level factorial design. The solubility of ketoconazole was maximised using the lowest amount of each excipient possible, whilst maintaining the pH of the vehicle to between 3 and 4. The poloxamer content was selected based on the viscosity data for gels formed from the poloxamers 407, 188 and 237 grades of polymer at 22 °C and 37 °C.

A cold process was used to manufacture the thermosetting gels to minimise interactions between the excipients and solvent system and to limit evaporation of ethanol, the absence of which, may have an undesirable and thixotropic effect during production [336]. The thermosetting behaviour of the gels was optimised using a Central Composite Design approach to identify a formulation comprising of poloxamers 407, 188 and 237 that exhibited prolonged release of ketoconazole. A total of 20 products were produced and the viscosity at 22 °C and 37 °C established. In addition, ketoconazole release at 24, 48 and 72 hours, sol-gel transition and potency against the vaginal strain of *Candida albicans* was evaluated. Contour and three-dimensional response surface plots were generated to evaluate the impact of binary combinations of input factors on gel viscosity at 37 °C and polynomial equations were elucidated for all responses to facilitate the explanation of the effects of the amount of poloxamer 407, 188 and 237 on product behaviour. Poloxamer 407 was found to be the primary contributor to the thermosetting behaviour of the gels, more than likely due to the presence of long propylene oxide/polyethylene oxide chains. The addition of poloxamer 188 and 237 was necessary to ensure accurate modulation of the gelation temperature. The effect of poloxamer 188 and 237 grades is more than likely due to the disruption of the ratio of propylene oxide to polyethylene oxide block copolymers that, in turn, affects the hydrophobic-hydrophilic balance of micelles within the dosage form. Furthermore, the interaction and wedging of poloxamer 188 and 237 between long chains of the poloxamer 407 grade may further impact polymer performance. A low viscosity at 22 °C was required to facilitate application of gel into the vaginal cavity. In this respect poloxamer 237 reduced the viscosity of the gels at 22°C, as it is of lower molecular mass compared to either poloxamer 407 and 188 and will form small micelles at temperatures higher than the critical micelle temperature of poloxamer 188 and 407 when used at similar concentrations. The addition of poloxamer 237 to a solution of poloxamers 407 and 188 lowers the critical micelle temperature of the solution, resulting in the formation of a liquid gel at 22 °C and modulation of the critical micelle temperature to 37 °C.

Ketoconazole release was monitored using a validated *in vitro* release method. Sustained release of ketoconazole from the thermosetting gels was observed, due to the formation of a gel matrix with the combination of poloxamers, suggesting that the thermosetting gels may be administered as once daily doses. The ketoconazole release kinetics from most gels following model dependent analysis tended to be best described by the Korsmeyer-Peppas model and the release mechanisms were difficult to identify. Gels of different rigidity and viscosity were produced due to large differences in poloxamer content forming gels which erodes while others did not. The sol-gel transition was assessed, using a simple approach, by monitoring the stiffness of the gel when subjected to the force of gravity. Sol-gel transition studies suggest that all three grades of poloxamer contribute to the stiffness of the gel to different degrees and the use of a combination of polymers results in the formation of a stiff gel that retains its form for longer than each of the individual materials alone. The sol-gel transition experiment provided useful information yet limited data relating to the viscosity and critical micelle temperature of the thermosetting behaviour of the gels was gained. The measurement of viscosity of the gels using viscometers and rheometers is important for the analysis of product performance and identification of the mechanisms behind the responses observed for the gels. Thermosetting gels should exhibit low viscosity at 22 ºC and high viscosity at 37 ºC with targeted viscosity at 22°C of < 10000 cP and at 37°C of > 50000 cP. A high viscosity at 37 ºC was highly dependent on the concentration of poloxamer 407 and on poloxamer 237 to a small extent. A combination of the three poloxamers modulated the viscosity to remain low at 22 ºC and high at 37 ºC. Potency studies revealed that an increase in the amount of each of the poloxamer grades in the formulation resulted in a decrease in potency against *Candida albicans* more than likely due to a delay in diffusion of ketoconazole from the gel into and through the agar used for the potency studies.

The optimised thermosetting gel can be self-administered as a liquid at 22 ºC and may coat the vaginal walls following administration prior to forming a stiff gel at 37 ºC. The formulation may remain in the vagina for an extended period of time despite conditions such vaginal mucous discharge or muscle contractions that would normally result in physical removal and physiological expulsion as occurs with other semi-solid dosage forms and is generally embarrassing and uncomfortable for patients. Patients are unlikely to experience discomfort and/or pain during coitus and bowel movement as the gel texture resembles vaginal mucous. This dosage form has the potential to be successfully used to treat yeast infections, eliminate side effects associated with systemic administration of ketoconazole and may be safe for use by pregnant women.

Ketoconazole is prone to acid and alkaline hydrolysis, thermal and photo-degradation and oxidises in aqueous solution. The gel pH was maintained in the 3.5 – 4.5 range to ensure that a dosage form was

produced that would not irritate the vaginal mucosa and that would have a minimal impact on vaginal flora. The low pH required is useful for the solubilisation of ketoconazole, yet is detrimental in respect of stability. The micelles that form in the gel shield ketoconazole molecules within a hydrophobic core and limits exposure of the compound to the acidic vehicle used in the formulation. In addition, a strategy to ensure long term stability of ketoconazole in the vehicle was required. The manufacturing process used was simple, cost effective and reproducible. The vehicle pH was modified using citric acid and stability enhancement with sodium metabisulphite. The amount of citric acid and sodium metabisulphite included was optimised to identify a formulation composition that exhibited the greatest stability. A Design of Experiments approach was once again used to evaluate the impact of input variables on formulation responses including ketoconazole content, amount of ketoconazole released per unit area at 24, 48 and 72 hours, gel pH and viscosity at 22 ºC and 37 ºC. The parameters were monitored to establish the stability and potential shelf life for this product. All 40 experiments were performed using 9 gel formulations *viz.*, batches S1 to S9 and were generated using the Central Composite Design. The dosage forms were assessed at three numeric levels for length of storage *viz.*, 0, 4 and 8 weeks and two categoric levels for temperature *viz.*, 25 ºC and 40 ºC. The container and closure system used for the gels were a 50 g amber glass bottle sealed with a plastic closure fitted with a liner and then over-sealed with Parafilm™. The gels were investigated at 0, 1, 2, 4 and 8 weeks following storage at 5 ºC, 25 ºC and 40 ºC for comparison to Central Composite Design generated stability data. Several factors affect the stability of ketoconazole in the gel and erratic degradation data that did not follow any specific trend were generated. The use of sodium metabisulphate added to the acidity of the formulation that in turn may have reduced the oxidation of ketoconazole but may enhance hydrolysis. While a more alkaline pH would slightly lower the rate and extent of degradation of ketoconazole when compared to the use of an acidic pH, a pH of 4.5 is acidic and therefore acid hydrolysis of ketoconazole may still occur. All formulations include poloxamers that form unimers at low temperatures. At these temperatures ketoconazole is present in an unprotected environment and in the acidic vehicle, resulting in the potential for rapid degradation of the molecule. The poloxamer 188 grade degrades at 40 ºC resulting in the formation of acidic degradation products that further reduce the stability of ketoconazole in gels stored at that temperature. This Central Composite Design approach was suitable for an overall assessment of stability using a minimum number of sample evaluations. This approach is simple, rapid and cost effective for conducting stability studies on experimental products and should be used in conjunction with comprehensive real time stability studies in which samples are pulled at appropriate times to augment product registration data. Although an optimised stable formulation for a thermosetting vaginal gel was not identified, the findings of these studies provide a starting point for establishing and implementing strategies to further develop and improve this novel vaginal gel with a composition that assures the stability of ketoconazole on long term storage. In addition, a number of

improvements can be made to this thermosetting gel in terms of formulation composition and the manufacturing process with a view to producing a stable thermosetting formulation. Compositions of ciprofloxacin hydrochloride *in situ* thermosetting formulation for ocular delivery containing poloxamer 407, chitosan and polyvinyl alcohol were stable at 40 ºC and 75% Relative Humidity for up to 45 days [581], a levofloxacin hemihydrate thermosetting ophthalmic hydrogel containing poloxamer 407, β-cyclodextrin and carbopol 940 was stable at 25 ºC for up to 4 weeks [582] and an *in situ* thermo-reversible mucoadhesive intranasal naratriptan hydrochloride gel containing poloxamer 407 and carbopol 934 was stable at 40 ºC and 75% Relative Humidity for up to three months [583].

A thermosetting gel has been successfully manufactured and exhibited adequate thermo-gelling properties at body temperature. However, the micellar structure of the system and gel matrix was not sufficiently optimised and degradation of ketoconazole occurred. The incorporation of ketoconazole into drug delivery systems such as inclusion complexes and nanoparticles has been reported, but the use of these approaches to stabilise ketoconazole may impact release and consequently therapeutic outcomes. Prolonged release of ketoconazole from a thermosetting hydrogel manufactured in these studies was achieved. Modulation of release may be enhanced through the addition of a penetration enhancer that is not irritable to the vaginal mucosa. Additional stability studies in which the poloxamer 188 grade is evaluated at 40 ºC should be undertaken to evaluate the stability of this compound in solution. Should degradation be evident, an alternate poloxamer or polymer must be considered in the composition. It would be useful to undertake stability studies on solutions of each polymer in aqueous solution as part of preformulation studies to provide information and permit optimisation of dosage forms using such materials. The use of sodium metabisulphite in the formulation did not enhance stability as expected and, as it is acidic, the use of an alternate neutral antioxidant such as butylated hydroxytoluene should be investigated.

Xolegel® is an amber coloured gel [168] containing FD&C yellow No 6 and No 10 dyes, whereas Kez® shampoo is a pink dosage form that contains the dye erythrosine C.I. 45430 [170]. The colour may be included to enhance the stability of ketoconazole and protect it from photolytic degradation rather than for aesthetic purposes alone. The inclusion of such agents in a vaginal gel may be considered if patient acceptance can be achieved. The use of Differential Scanning Calorimetry and Fourier Transform Infrared Spectroscopy for analysis of 1:1 binary mixtures of ketoconazole and excipients during preformulation studies may not be adequate for elucidating all stability considerations and as this is a semi-solid technology additional interaction studies in solution may be required. A combination of the vehicle, excipients and ketoconazole would require analysis using Differential Scanning Calorimetry, Fourier Transform-Infrared and Raman Spectroscopy and X-Ray Diffraction to obtain a more complete and thorough profile of potential

interactions. The container closure system used for storing the vaginal gel during stability studies may also not be ideal. The ICH Q8 (R2) guideline states the rationale for the selection of a container closure system and this should be further investigated to identify an appropriate container and closure system for this product [584].

This research has demonstrated that Response Surface Methodology in combination with Design of Experiments is suitable for the development of vaginal formulations. In addition, rapid and cost-effective studies for the assessment of stability of novel pharmaceutical products has been achieved. The application of the principles of Quality by Design and Quality Risk Management in combination with Response Surface Methodology would be applicable and useful for future studies to facilitate recognition of challenges, deficiencies and inconsistencies that might arise during the early stages of formulation development studies.

The results obtained in these studies demonstrate that the characterisation of commercial products, preformulation studies and the development of analytical, *in vitro* release and extraction methods play a vital role in formulation, production and product development studies for the manufacture of a novel thermosetting gel. The novelty described herein not only lies in the product but also the methods developed and used prior to and whilst formulating products and during stability studies. The use of surgically prepared porcine vaginal mucosa for *ex vivo* studies may be used as a means of obtaining data that can mimic *in vivo* release of the drug. Studies in which the *in situ* gelling and retention properties for the gel are measured would ensure a better understanding of the *in vi*vo behaviour of this technology. The characterisation of commercial products provided valuable information that proved useful during formulation development activities. The extraction of ketoconazole from commercial products was simple, reliable and is a new hither to unreported method of sample preparation. The use of a Design of Experiments for stability studies is arguably an original approach of obtaining stability data rapidly and cost effectively than real time studies and should be further investigated. Beyond the present work performed with a hydrophobic drug, this process should well be adapted to other insoluble compounds that are more stable than ketoconazole. In addition the use of water soluble active ingredients in this technology should not pose too many challenges for an experienced formulation scientist. Future studies should focus on stability enhancement, particularly studies shielding the ketoconazole molecule or any other antifungal agent from acidic vehicles that are necessary to ensure biocompatibility of intravaginal technologies.

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APPENDIX A
ETHICAL APPROVAL

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15 May 2014

Dear Ashmita Ramanah

RE: Ethical approval by the Faculty of Pharmacy's Ethics Committee (Tracking number PHARM 2014 - 9)

I am pleased to inform you that the Faculty of Pharmacy's Ethics Committee has decided that you do not require ethical approval for your research entitled:

Development, manufacture and assessment of ketoconazole (KZ) thermo-setting vaginal gels.

The reason why you do not require ethical approval is that you will be harvesting the vaginal membranes from pigs already slaughtered at the abattoir in Uitenhage.

Sincerely

C. Oltmann

Carmen Oltmann, PhD
Chairperson

APPENDIX B

TUKEY'S MULTIPLE COMPARISONS TEST RESULTS FOR SELECTION OF RECEPTOR MEDIUM

Table B1 Tukey's multiple comparison test for two-way ANOVA for KZ flux using a receptor fluid with 35% phosphate buffer of pH = 3.5.

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant	Summary	Adjusted P Value
0.5 hour					
Kez® shampoo vs. Xolegel®	333	-61.2 to 727	No	ns	0.1040
Ketazol® cream vs. Xolegel®	-82.1	-476 to 312	No	ns	0.8503
Ketazol® cream vs. Kez® shampoo	-415	-809 to -20.9	Yes	*	0.0386
1 hour					
Kez® shampoo vs. Xolegel®	22.5	-371 to 416	No	ns	0.9878
Ketazol® cream vs. Xolegel®	108	-286 to 502	No	ns	0.7587
Ketazol® cream vs. Kez® shampoo	85.2	-309 to 479	No	ns	0.8401
2 hours					
Kez® shampoo vs. Xolegel®	-7.79	-402 to 386	No	ns	0.9985
Ketazol® cream vs.Xolegel®	190	-204 to 584	No	ns	0.4384
Ketazol® cream vs. Kez® shampoo	198	-196 to 592	No	ns	0.4109
4 hours					
Kez® shampoo vs. Xolegel®	64.7	-329 to 459	No	ns	0.9037
Ketazol® cream vs. Xolegel®	-106	-500 to 288	No	ns	0.7652
Ketazol® cream vs. Kez® shampoo	-171	-565 to 223	No	ns	0.5099
8 hours					
Kez® shampoo vs. Xolegel®	212	-182 to 606	No	ns	0.3638
Ketazol® cream vs. Xolegel®	46.9	-347 to 441	No	ns	0.9480
Ketazol® cream vs. Kez® shampoo	-165	-559 to 229	No	ns	0.5321
12 hours					
Kez® shampoo vs. Xolegel®	73.3	-321 to 467	No	ns	0.8786
Ketazol® cream vs. Xolegel®	-46.9	-441 to 347	No	ns	0.9481
Ketazol® cream vs. Kez® shampoo	-120	-514 to 274	No	ns	0.7102
20 hours					
Kez® shampoo vs. Xolegel®	71.7	-322 to 466	No	ns	0.8834
Ketazol® cream vs. Xolegel®	196	-198 to 590	No	ns	0.4168
Ketazol® cream vs. Kez® shampoo	124	-270 to 518	No	ns	0.6935
24 hours					
Kez® shampoo vs. Xolegel®	2.39	-392 to 396	No	ns	0.9999
Ketazol® cream vs. Xolegel®	-63.8	-458 to 330	No	ns	0.9064
Ketazol® cream vs. Kez® shampoo	-66.2	-460 to 328	No	ns	0.8997

Table B2 Tukey's multiple comparison test for two-way ANOVA for KZ flux using a receptor fluid with 40% phosphate buffer of pH = 3.5.

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant	Summary	Adjusted P Value
0.5 hour					
Kez® shampoo vs. Xolegel®	376	-68.0 to 821	No	ns	0.1029
Ketazol® cream vs. Xolegel®	-159	-603 to 285	No	ns	0.6273
Ketazol® cream vs. Kez® shampoo	-535	-980 to -90.9	Yes	*	0.0181
1 hour					
Kez® shampoo vs. Xolegel®	-319	-764 to 125	No	ns	0.1807
Ketazol® cream vs. Xolegel®	-344	-788 to 101	No	ns	0.1429
Ketazol® cream vs. Kez® shampoo	-24.3	-469 to 420	No	ns	0.9887
2 hours					
Kez® shampoo vs. Xolegel®	-42.0	-486 to 402	No	ns	0.9669
Ketazol® cream vs.Xolegel®	-64.8	-509 to 380	No	ns	0.9232
Ketazol® cream vs. Kez® shampoo	-22.8	-467 to 422	No	ns	0.9901
4 hours					
Kez® shampoo vs. Xolegel®	-86.0	-530 to 358	No	ns	0.8694
Ketazol® cream vs. Xolegel®	-212	-657 to 232	No	ns	0.4444
Ketazol® cream vs. Kez® shampoo	-126	-571 to 318	No	ns	0.7418
8 hours					
Kez® shampoo vs. Xolegel®	-71.4	-516 to 373	No	ns	0.9077
Ketazol® cream vs. Xolegel®	-112	-556 to 332	No	ns	0.7901
Ketazol® cream vs. Kez® shampoo	-40.6	-485 to 404	No	ns	0.9691
12 hours					
Kez® shampoo vs. Xolegel®	-26.9	-471 to 417	No	ns	0.9862
Ketazol® cream vs. Xolegel®	-120	-565 to 324	No	ns	0.7631
Ketazol® cream vs. Kez® shampoo	-93.2	-538 to 351	No	ns	0.8486
20 hours					
Kez® shampoo vs. Xolegel®	-39.6	-484 to 405	No	ns	0.9705
Ketazol® cream vs. Xolegel®	-154	-598 to 290	No	ns	0.6447
Ketazol® cream vs. Kez® shampoo	-114	-559 to 330	No	ns	0.7820
24 hours					
Kez® shampoo vs. Xolegel®	-1.58	-446 to 443	No	ns	> 0.9999
Ketazol® cream vs. Xolegel®	92.5	-352 to 537	No	ns	0.8507
Ketazol® cream vs. Kez® shampoo	94.1	-350 to 539	No	ns	0.8460

Table B3 Tukey's multiple comparison test for two-way ANOVA for KZ flux using a receptor fluid with 50% phosphate buffer of pH = 3.5.

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant	Summary	Adjusted P Value
0.5 hour					
Kez® shampoo vs. Xolegel®	334	-45.0 to 713	No	ns	0.0878
Ketazol® cream vs. Xolegel®	-214	-594 to 165	No	ns	0.3297
Ketazol® cream vs. Kez® shampoo	-549	-928 to -169	Yes	**	0.0053
1 hour					
Kez® shampoo vs. Xolegel®	4.89	-374 to 384	No	ns	0.9994
Ketazol® cream vs. Xolegel®	-184	-563 to 195	No	ns	0.4334
Ketazol® cream vs. Kez® shampoo	-189	-568 to 190	No	ns	0.4155
2 hours					
Kez® shampoo vs. Xolegel®	359	-19.8 to 739	No	ns	0.0642
Ketazol® cream vs.Xolegel®	1.39	-378 to 381	No	ns	> 0.9999
Ketazol® cream vs. Kez® shampoo	-358	-737 to 21.2	No	ns	0.0654
4 hours					
Kez® shampoo vs. Xolegel®	136	-243 to 515	No	ns	0.6268
Ketazol® cream vs. Xolegel®	-56.4	-436 to 323	No	ns	0.9203
Ketazol® cream vs. Kez® shampoo	-192	-571 to 187	No	ns	0.4045
8 hours					
Kez® shampoo vs. Xolegel®	-63.1	-442 to 316	No	ns	0.9014
Ketazol® cream vs. Xolegel®	-260	-639 to 120	No	ns	0.2083
Ketazol® cream vs. Kez® shampoo	-196	-576 to 183	No	ns	0.3895
12 hours					
Kez® shampoo vs. Xolegel®	-7.64	-387 to 372	No	ns	0.9985
Ketazol® cream vs. Xolegel®	-240	-619 to 139	No	ns	0.2561
Ketazol® cream vs. Kez® shampoo	-232	-611 to 147	No	ns	0.2768
20 hours					
Kez® shampoo vs. Xolegel®	112	-267 to 491	No	ns	0.7262
Ketazol® cream vs. Xolegel®	-116	-495 to 263	No	ns	0.7090
Ketazol® cream vs. Kez® shampoo	-228	-607 to 152	No	ns	0.2899
24 hours					
Kez® shampoo vs. Xolegel®	-149	-529 to 230	No	ns	0.5700
Ketazol® cream vs. Xolegel®	-210	-589 to 169	No	ns	0.3428
Ketazol® cream vs. Kez® shampoo	-60.9	-440 to 318	No	ns	0.9079

Table B4 Tukey's multiple comparison test for two-way ANOVA for KZ flux using a receptor fluid with 60% phosphate buffer of pH = 3.5.

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant	Summary	Adjusted P Value
0.5 hour					
Kez® shampoo vs. Xolegel®	347	35.6 to 659	Yes	*	0.0285
Ketazol® cream vs. Xolegel®	-184	-496 to 128	No	ns	0.3011
Ketazol® cream vs. Kez® shampoo	-531	-843 to -220	Yes	**	0.0015
1 hour					
Kez® shampoo vs. Xolegel®	45.2	-266 to 357	No	ns	0.9241
Ketazol® cream vs. Xolegel®	-68.8	-381 to 243	No	ns	0.8339
Ketazol® cream vs. Kez® shampoo	-114	-426 to 198	No	ns	0.6143
2 hours					
Kez® shampoo vs. Xolegel®	-0.902	-313 to 311	No	ns	> 0.9999
Ketazol® cream vs.Xolegel®	-100	-412 to 211	No	ns	0.6840
Ketazol® cream vs. Kez® shampoo	-99.4	-411 to 212	No	ns	0.6886
4 hours					
Kez® shampoo vs. Xolegel®	9.24	-302 to 321	No	ns	0.9967
Ketazol® cream vs. Xolegel®	-202	-514 to 110	No	ns	0.2415
Ketazol® cream vs. Kez® shampoo	-211	-523 to 101	No	ns	0.2144
8 hours					
Kez® shampoo vs. Xolegel®	68.3	-243 to 380	No	ns	0.8361
Ketazol® cream vs. Xolegel®	-274	-586 to 37.8	No	ns	0.0889
Ketazol® cream vs. Kez® shampoo	-342	-654 to -30.5	Yes	*	0.0309
12 hours					
Kez® shampoo vs. Xolegel®	46.9	-265 to 359	No	ns	0.9187
Ketazol® cream vs. Xolegel®	-156	-468 to 156	No	ns	0.4128
Ketazol® cream vs. Kez® shampoo	-203	-515 to 109	No	ns	0.2383
20 hours					
Kez® shampoo vs. Xolegel®	-1.64	-313 to 310	No	ns	0.9999
Ketazol® cream vs. Xolegel®	-202	-514 to 110	No	ns	0.2408
Ketazol® cream vs. Kez® shampoo	-200	-512 to 111	No	ns	0.2458
24 hours					
Kez® shampoo vs. Xolegel®	4.55	-307 to 316	No	ns	0.9992
Ketazol® cream vs. Xolegel®	-58.9	-371 to 253	No	ns	0.8751
Ketazol® cream vs. Kez® shampoo	-63.4	-375 to 248	No	ns	0.8567

Table B5 Tukey's multiple comparison test for two-way ANOVA for KZ flux using a receptor fluid with 65% phosphate buffer of pH = 3.5.

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant	Summary	Adjusted P Value
0.5 hour					
Kez® shampoo vs. Xolegel®	225	-84.4 to 535	No	ns	0.1740
Ketazol® cream vs. Xolegel®	18.0	-292 to 328	No	ns	0.9874
Ketazol® cream vs. Kez® shampoo	-208	-517 to 102	No	ns	0.2211
1 hour					
Kez® shampoo vs. Xolegel®	-0.553	-310 to 309	No	ns	> 0.9999
Ketazol® cream vs. Xolegel®	-90.9	-401 to 219	No	ns	0.7282
Ketazol® cream vs. Kez® shampoo	-90.3	-400 to 220	No	ns	0.7310
2 hours					
Kez® shampoo vs. Xolegel®	-31.4	-341 to 279	No	ns	0.9622
Ketazol® cream vs.Xolegel®	-32.6	-342 to 277	No	ns	0.9592
Ketazol® cream vs. Kez® shampoo	-1.25	-311 to 309	No	ns	> 0.9999
4 hours					
Kez® shampoo vs. Xolegel®	129	-181 to 439	No	ns	0.5365
Ketazol® cream vs. Xolegel®	-8.71	-319 to 301	No	ns	0.9970
Ketazol® cream vs. Kez® shampoo	-138	-447 to 172	No	ns	0.4940
8 hours					
Kez® shampoo vs. Xolegel®	86.7	-223 to 397	No	ns	0.7487
Ketazol® cream vs. Xolegel®	-127	-437 to 183	No	ns	0.5469
Ketazol® cream vs. Kez® shampoo	-213	-523 to 96.4	No	ns	0.2045
12 hours					
Kez® shampoo vs. Xolegel®	-165	-475 to 145	No	ns	0.3704
Ketazol® cream vs. Xolegel®	-229	-539 to 80.8	No	ns	0.1657
Ketazol® cream vs. Kez® shampoo	-64.0	-374 to 246	No	ns	0.8529
20 hours					
Kez® shampoo vs. Xolegel®	-135	-444 to 175	No	ns	0.5084
Ketazol® cream vs. Xolegel®	-136	-446 to 174	No	ns	0.5000
Ketazol® cream vs. Kez® shampoo	-1.75	-312 to 308	No	ns	0.9999
24 hours					
Kez® shampoo vs. Xolegel®	-236	-546 to 73.8	No	ns	0.1502
Ketazol® cream vs. Xolegel®	-232	-542 to 77.5	No	ns	0.1582
Ketazol® cream vs. Kez® shampoo	3.71	-306 to 314	No	ns	0.9995

Table B6 Tukey's multiple comparison test for two-way ANOVA for KZ flux using a receptor fluid with 35% phosphate buffer of pH = 4.5.

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant	Summary	Adjusted P Value
0.5 hour					
Kez® shampoo vs. Xolegel®	508	-385 to 1401	No	ns	0.3259
Ketazol® cream vs. Xolegel®	-151	-1044 to 742	No	ns	0.8985
Ketazol® cream vs. Kez® shampoo	-659	-1552 to 234	No	ns	0.1667
1 hour					
Kez® shampoo vs. Xolegel®	573	-321 to 1466	No	ns	0.2479
Ketazol® cream vs. Xolegel®	74.2	-819 to 967	No	ns	0.9744
Ketazol® cream vs. Kez® shampoo	-498	-1392 to 395	No	ns	0.3387
2 hours					
Kez® shampoo vs. Xolegel®	292	-601 to 1186	No	ns	0.6754
Ketazol® cream vs.Xolegel®	136	-757 to 1030	No	ns	0.9163
Ketazol® cream vs. Kez® shampoo	-156	-1049 to 737	No	ns	0.8922
4 hours					
Kez® shampoo vs. Xolegel®	515	-379 to 1408	No	ns	0.3170
Ketazol® cream vs. Xolegel®	341	-552 to 1235	No	ns	0.5890
Ketazol® cream vs. Kez® shampoo	-174	-1067 to 720	No	ns	0.8684
8 hours					
Kez® shampoo vs. Xolegel®	811	-82.6 to 1704	No	ns	0.0777
Ketazol® cream vs. Xolegel®	242	-651 to 1135	No	ns	0.7620
Ketazol® cream vs. Kez® shampoo	-569	-1462 to 325	No	ns	0.2524
12 hours					
Kez® shampoo vs. Xolegel®	-185	-1078 to 709	No	ns	0.8527
Ketazol® cream vs. Xolegel®	72.2	-821 to 966	No	ns	0.9757
Ketazol® cream vs. Kez® shampoo	257	-637 to 1150	No	ns	0.7372
20 hours					
Kez® shampoo vs. Xolegel®	-176	-1070 to 717	No	ns	0.8645
Ketazol® cream vs. Xolegel®	135	-758 to 1029	No	ns	0.9174
Ketazol® cream vs. Kez® shampoo	312	-581 to 1205	No	ns	0.6409
24 hours					
Kez® shampoo vs. Xolegel®	-294	-1188 to 599	No	ns	0.6718
Ketazol® cream vs. Xolegel®	-185	-1078 to 709	No	ns	0.8526
Ketazol® cream vs. Kez® shampoo	110	-784 to 1003	No	ns	0.9449

Table B7 Tukey's multiple comparison test for two-way ANOVA for KZ flux using a receptor fluid with 40% phosphate buffer of pH = 4.5.

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant	Summary	Adjusted P Value
0.5 hour					
Kez® shampoo vs. Xolegel®	31.4	-118 to 181	No	ns	0.8491
Ketazol® cream vs. Xolegel®	-105	-254 to 45.2	No	ns	0.1970
Ketazol® cream vs. Kez® shampoo	-136	-286 to 13.9	No	ns	0.0778
1 hour					
Kez® shampoo vs. Xolegel®	57.3	-92.5 to 207	No	ns	0.5880
Ketazol® cream vs. Xolegel®	-67.8	-218 to 82.0	No	ns	0.4807
Ketazol® cream vs. Kez® shampoo	-125	-275 to 24.6	No	ns	0.1084
2 hours					
Kez® shampoo vs. Xolegel®	-13.2	-163 to 137	No	ns	0.9712
Ketazol® cream vs.Xolegel®	-31.8	-182 to 118	No	ns	0.8452
Ketazol® cream vs. Kez® shampoo	-18.6	-168 to 131	No	ns	0.9436
4 hours					
Kez® shampoo vs. Xolegel®	-9.85	-160 to 140	No	ns	0.9838
Ketazol® cream vs. Xolegel®	-177	-327 to -27.5	Yes	*	0.0202
Ketazol® cream vs. Kez® shampoo	-167	-317 to -17.6	Yes	*	0.0281
8 hours					
Kez® shampoo vs. Xolegel®	94.0	-55.8 to 244	No	ns	0.2612
Ketazol® cream vs. Xolegel®	5.75	-144 to 156	No	ns	0.9945
Ketazol® cream vs. Kez® shampoo	-88.3	-238 to 61.5	No	ns	0.3021
12 hours					
Kez® shampoo vs. Xolegel®	29.7	-120 to 180	No	ns	0.8634
Ketazol® cream vs. Xolegel®	-124	-274 to 26.0	No	ns	0.1129
Ketazol® cream vs. Kez® shampoo	-154	-303 to -3.71	Yes	*	0.0443
20 hours					
Kez® shampoo vs. Xolegel®	-12.0	-162 to 138	No	ns	0.9760
Ketazol® cream vs. Xolegel®	-191	-341 to -41.4	Yes	*	0.0126
Ketazol® cream vs. Kez® shampoo	-179	-329 to -29.4	Yes	*	0.0189
24 hours					
Kez® shampoo vs. Xolegel®	-17.3	-167 to 132	No	ns	0.9509
Ketazol® cream vs. Xolegel®	-59.4	-209 to 90.4	No	ns	0.5659
Ketazol® cream vs. Kez® shampoo	-42.1	-192 to 108	No	ns	0.7468

Table B8 Tukey's multiple comparison test for two-way ANOVA for KZ flux using a receptor fluid with 50% phosphate buffer of pH = 4.5.

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant	Summary	Adjusted P Value
0.5 hour					
Kez® shampoo vs. Xolegel®	482	54.0 to 911	Yes	*	0.0269
Ketazol® cream vs. Xolegel®	-128	-556 to 300	No	ns	0.7196
Ketazol® cream vs. Kez® shampoo	-610	-1038 to -182	Yes	**	0.0059
1 hour					
Kez® shampoo vs. Xolegel®	-25.8	-454 to 402	No	ns	0.9864
Ketazol® cream vs. Xolegel®	-50.3	-479 to 378	No	ns	0.9494
Ketazol® cream vs. Kez® shampoo	-24.5	-453 to 404	No	ns	0.9877
2 hours					
Kez® shampoo vs. Xolegel®	4.23	-424 to 432	No	ns	0.9996
Ketazol® cream vs.Xolegel®	-55.0	-483 to 373	No	ns	0.9400
Ketazol® cream vs. Kez® shampoo	-59.2	-487 to 369	No	ns	0.9308
4 hours					
Kez® shampoo vs. Xolegel®	-40.2	-468 to 388	No	ns	0.9673
Ketazol® cream vs. Xolegel®	-239	-667 to 189	No	ns	0.3391
Ketazol® cream vs. Kez® shampoo	-199	-627 to 230	No	ns	0.4648
8 hours					
Kez® shampoo vs. Xolegel®	-6.75	-435 to 421	No	ns	0.9991
Ketazol® cream vs. Xolegel®	-257	-685 to 172	No	ns	0.2909
Ketazol® cream vs. Kez® shampoo	-250	-678 to 178	No	ns	0.3085
12 hours					
Kez® shampoo vs. Xolegel®	-56.1	-484 to 372	No	ns	0.9376
Ketazol® cream vs. Xolegel®	-182	-610 to 246	No	ns	0.5222
Ketazol® cream vs. Kez® shampoo	-126	-554 to 302	No	ns	0.7268
20 hours					
Kez® shampoo vs. Xolegel®	-90.6	-519 to 338	No	ns	0.8462
Ketazol® cream vs. Xolegel®	-183	-611 to 245	No	ns	0.5187
Ketazol® cream vs. Kez® shampoo	-92.4	-521 to 336	No	ns	0.8406
24 hours					
Kez® shampoo vs. Xolegel®	-35.1	-463 to 393	No	ns	0.9750
Ketazol® cream vs. Xolegel®	-41.3	-470 to 387	No	ns	0.9656
Ketazol® cream vs. Kez® shampoo	-6.19	-434 to 422	No	ns	0.9992

Table B9 Tukey's multiple comparison test for two-way ANOVA for KZ flux using a receptor fluid with 60% phosphate buffer of pH = 4.5.

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant	Summary	Adjusted P Value
0.5 hour					
Kez® shampoo vs. Xolegel®	193	-45.1 to 431	No	ns	0.1213
Ketazol® cream vs. Xolegel®	-136	-374 to 102	No	ns	0.3245
Ketazol® cream vs. Kez® shampoo	-329	-567 to -90.6	Yes	**	0.0075
1 hour					
Kez® shampoo vs. Xolegel®	22.8	-215 to 261	No	ns	0.9660
Ketazol® cream vs. Xolegel®	-132	-370 to 106	No	ns	0.3435
Ketazol® cream vs. Kez® shampoo	-155	-393 to 83.4	No	ns	0.2393
2 hours					
Kez® shampoo vs. Xolegel®	23.9	-214 to 262	No	ns	0.9629
Ketazol® cream vs.Xolegel®	-66.1	-304 to 172	No	ns	0.7520
Ketazol® cream vs. Kez® shampoo	-90.0	-328 to 148	No	ns	0.5955
4 hours					
Kez® shampoo vs. Xolegel®	1.69	-236 to 240	No	ns	0.9998
Ketazol® cream vs. Xolegel®	-250	-488 to -12.1	Yes	*	0.0390
Ketazol® cream vs. Kez® shampoo	-252	-490 to -13.8	Yes	*	0.0377
8 hours					
Kez® shampoo vs. Xolegel®	24.5	-214 to 263	No	ns	0.9608
Ketazol® cream vs. Xolegel®	-327	-565 to -88.5	Yes	**	0.0078
Ketazol® cream vs. Kez® shampoo	-351	-589 to -113	Yes	**	0.0046
12 hours					
Kez® shampoo vs. Xolegel®	2.86	-235 to 241	No	ns	0.9995
Ketazol® cream vs. Xolegel®	-184	-422 to 53.9	No	ns	0.1427
Ketazol® cream vs. Kez® shampoo	-187	-425 to 51.0	No	ns	0.1354
20 hours					
Kez® shampoo vs. Xolegel®	-29.3	-267 to 209	No	ns	0.9448
Ketazol® cream vs. Xolegel®	-188	-427 to 49.7	No	ns	0.1322
Ketazol® cream vs. Kez® shampoo	-159	-397 to 79.0	No	ns	0.2224
24 hours					
Kez® shampoo vs. Xolegel®	-20.4	-259 to 218	No	ns	0.9726
Ketazol® cream vs. Xolegel®	-61.9	-300 to 176	No	ns	0.7784
Ketazol® cream vs. Kez® shampoo	-41.5	-280 to 197	No	ns	0.8926

Table B10 Tukey's multiple comparison test for two-way ANOVA for KZ flux using a receptor fluid with 65% phosphate buffer of pH = 4.5.

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant	Summary	Adjusted P Value
0.5 hour					
Kez® shampoo vs. Xolegel®	1005	234 to 1775	Yes	*	0.0110
Ketazol® cream vs. Xolegel®	-86.8	-857 to 684	No	ns	0.9534
Ketazol® cream vs. Kez® shampoo	-1091	-1862 to -321	Yes	**	0.0062
1 hour					
Kez® shampoo vs. Xolegel®	309	-461 to 1080	No	ns	0.5590
Ketazol® cream vs. Xolegel®	-346	-1116 to 424	No	ns	0.4861
Ketazol® cream vs. Kez® shampoo	-655	-1426 to 115	No	ns	0.1012
2 hours					
Kez® shampoo vs. Xolegel®	-27.8	-798 to 743	No	ns	0.9951
Ketazol® cream vs.Xolegel®	-220	-991 to 550	No	ns	0.7392
Ketazol® cream vs. Kez® shampoo	-193	-963 to 578	No	ns	0.7929
4 hours					
Kez® shampoo vs. Xolegel®	-91.7	-862 to 679	No	ns	0.9481
Ketazol® cream vs. Xolegel®	-271	-1041 to 500	No	ns	0.6374
Ketazol® cream vs. Kez® shampoo	-179	-949 to 591	No	ns	0.8182
8 hours					
Kez® shampoo vs. Xolegel®	-30.9	-801 to 740	No	ns	0.9940
Ketazol® cream vs. Xolegel®	-226	-997 to 544	No	ns	0.7276
Ketazol® cream vs. Kez® shampoo	-195	-966 to 575	No	ns	0.7877
12 hours					
Kez® shampoo vs. Xolegel®	105	-665 to 876	No	ns	0.9320
Ketazol® cream vs. Xolegel®	-151	-922 to 619	No	ns	0.8657
Ketazol® cream vs. Kez® shampoo	-257	-1027 to 514	No	ns	0.6657
20 hours					
Kez® shampoo vs. Xolegel®	1.97	-768 to 772	No	ns	> 0.9999
Ketazol® cream vs. Xolegel®	-245	-1015 to 525	No	ns	0.6897
Ketazol® cream vs. Kez® shampoo	-247	-1017 to 523	No	ns	0.6857
24 hours					
Kez® shampoo vs. Xolegel®	-62.5	-833 to 708	No	ns	0.9755
Ketazol® cream vs. Xolegel®	-124	-894 to 646	No	ns	0.9074
Ketazol® cream vs. Kez® shampoo	-61.5	-832 to 709	No	ns	0.9762

APPENDIX C

COMPARISON OF *IN VITRO* RELEASE PROFILES FOR KZ FROM XOLEGEL®, KEZ® SHAMPOO AND KETAZOL® CREAM USING DIFFERENT RECEPTOR MEDIA

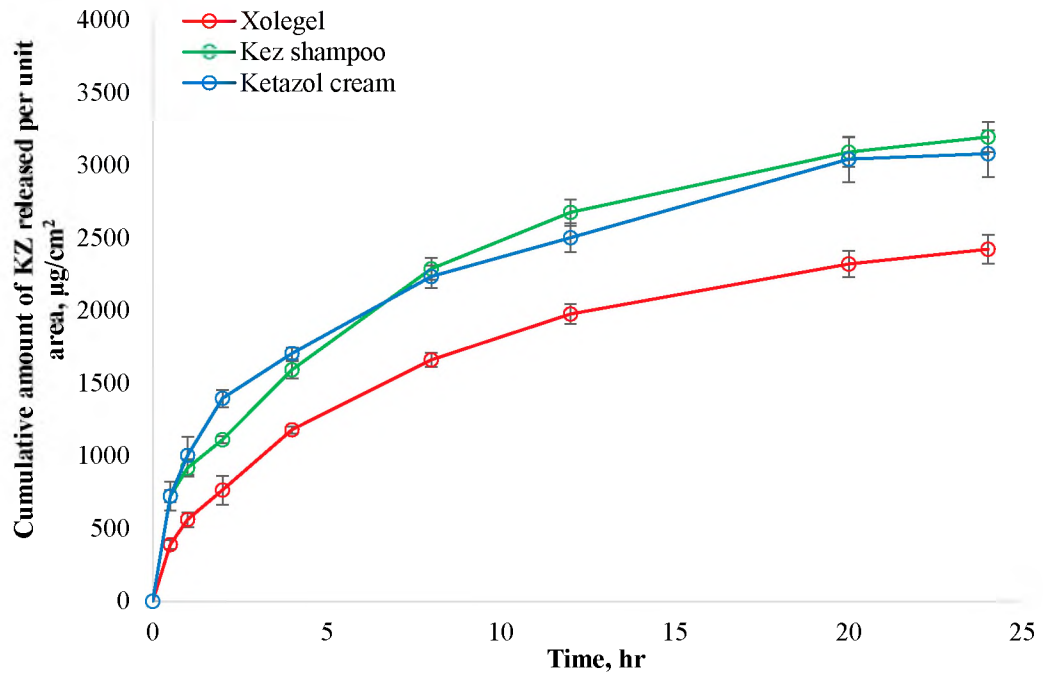


Figure C1 Cumulative amount of KZ released per unit area from Xolegel®, Kez® shampoo and Ketazol® cream using 35% phosphate buffer of pH = 3.5 in the receptor fluid.

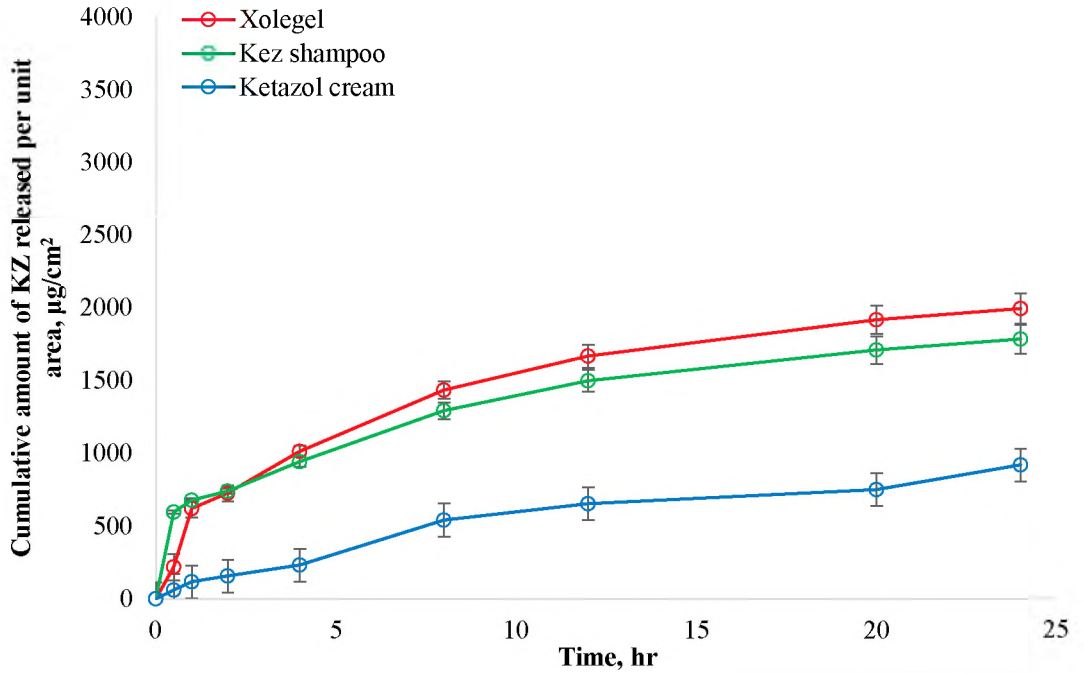


Figure C2 Cumulative amount of KZ released per unit area from Xolegel®, Kez® shampoo and Ketazol® cream using 40% phosphate buffer of pH = 3.5 in the receptor fluid.

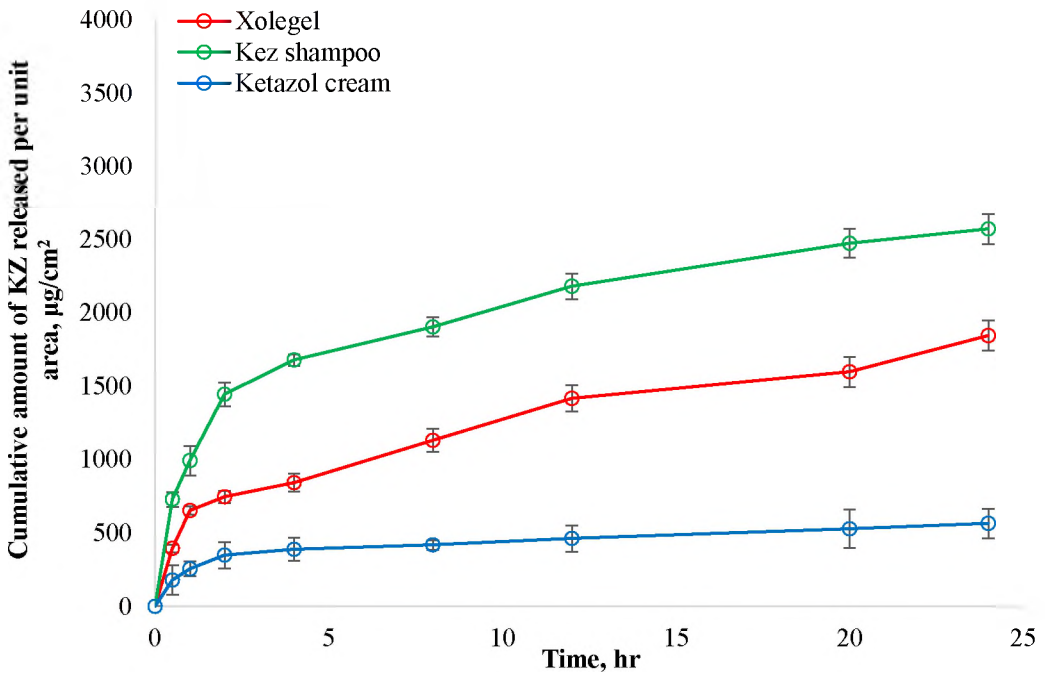


Figure C3 Cumulative amount of KZ released per unit area from Xolegel®, Kez® shampoo and Ketazol® cream using 50% phosphate buffer of pH = 3.5 in the receptor fluid.

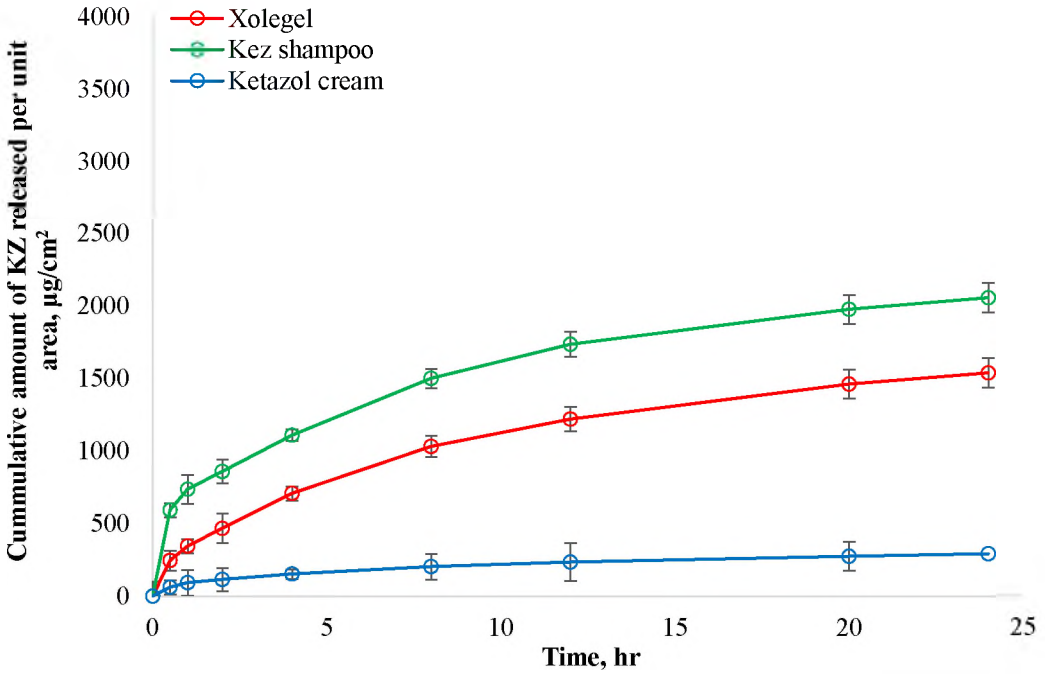


Figure C4 Cumulative amount of KZ released per unit area from Xolegel®, Kez® shampoo and Ketazol® cream using 60% phosphate buffer of pH = 3.5 in the receptor fluid.

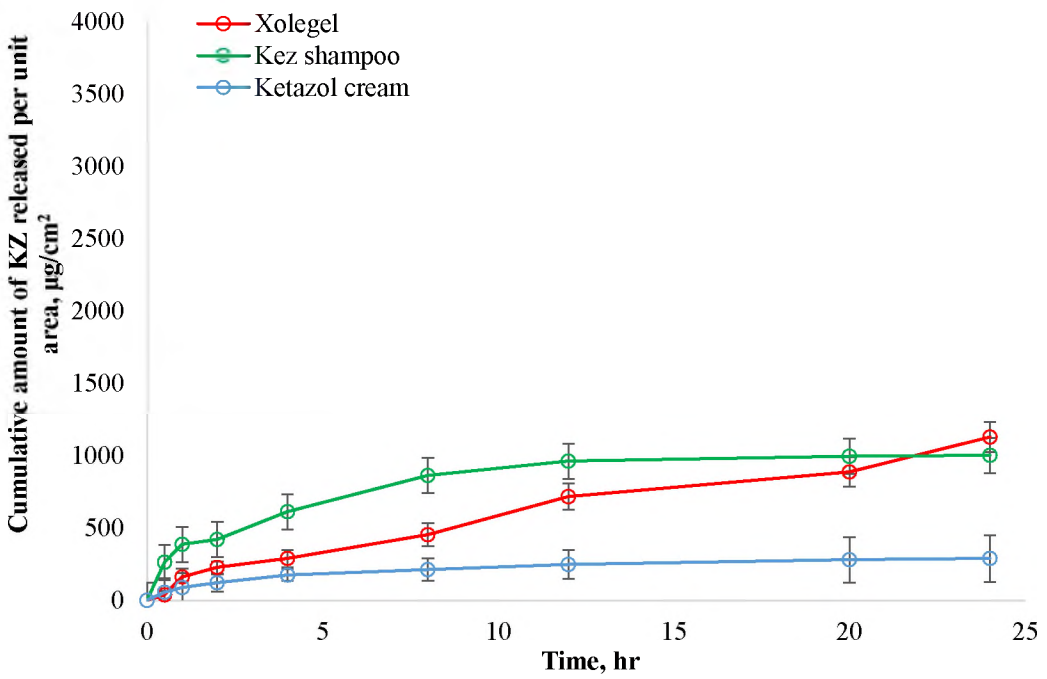


Figure C5 Cumulative amount of KZ released per unit area from Xolegel®, Kez® shampoo and Ketazol® cream using 65% phosphate buffer of pH = 3.5 in the receptor fluid.

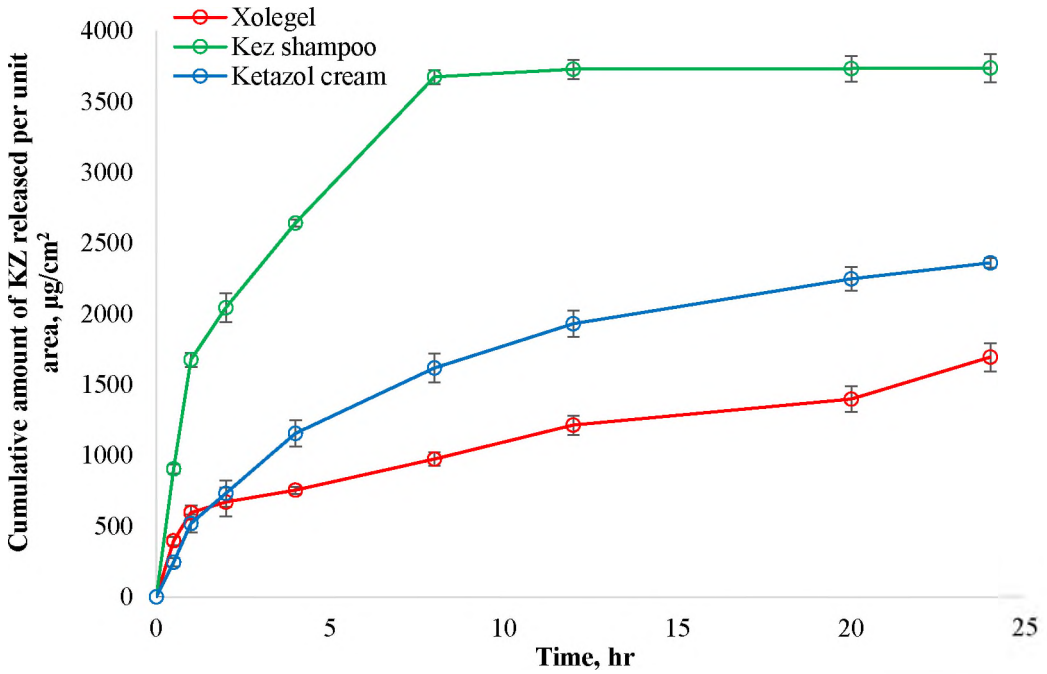


Figure C6 Cumulative amount of KZ released per unit area from Xolegel®, Kez® shampoo and Ketazol® cream using 35% phosphate buffer of pH = 4.5 in the receptor fluid.

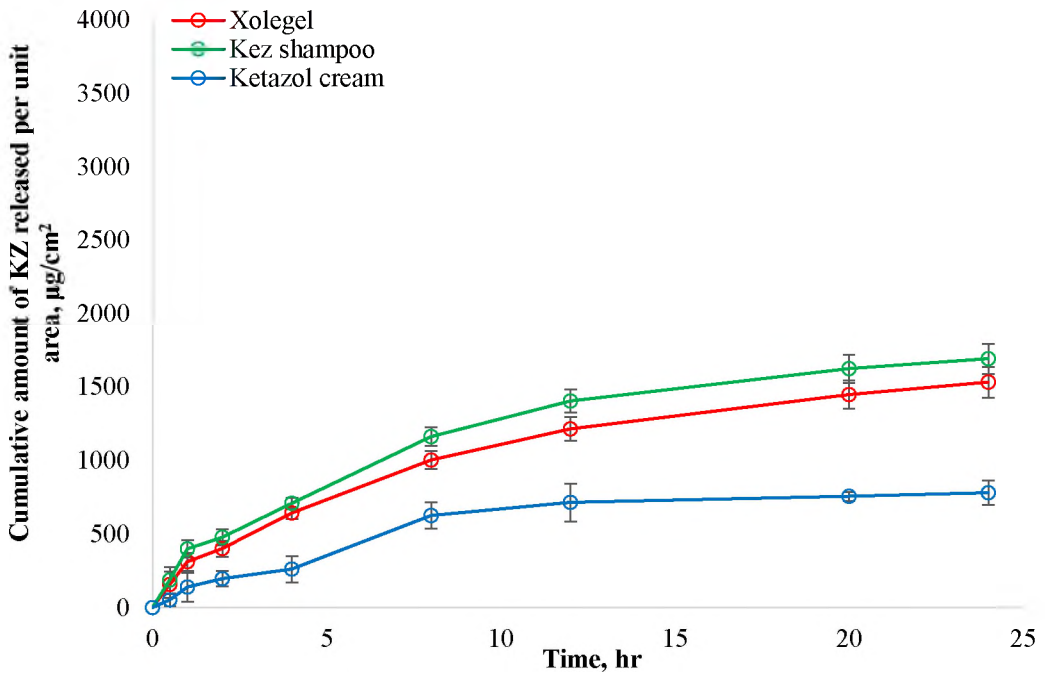


Figure C7 Cumulative amount of KZ released per unit area from Xolegel®, Kez® shampoo and Ketazol® cream using 40% phosphate buffer of pH = 4.5 in the receptor fluid.

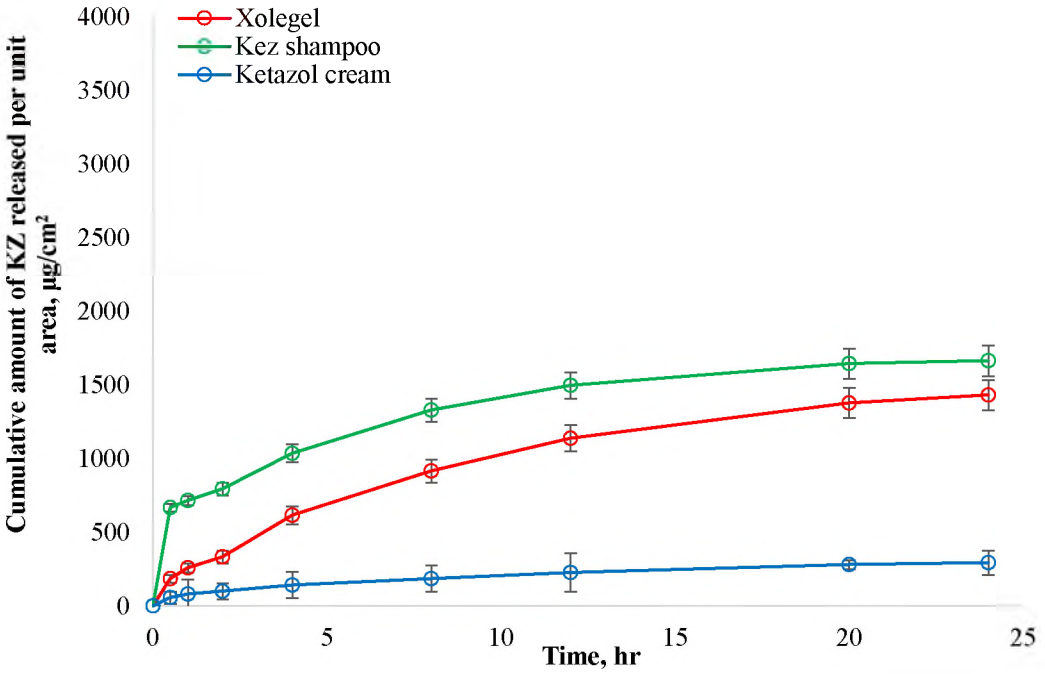


Figure C8 Cumulative amount of KZ released per unit area from Xolegel®, Kez® shampoo and Ketazol® cream using 50% phosphate buffer of pH = 4.5 in the receptor fluid.

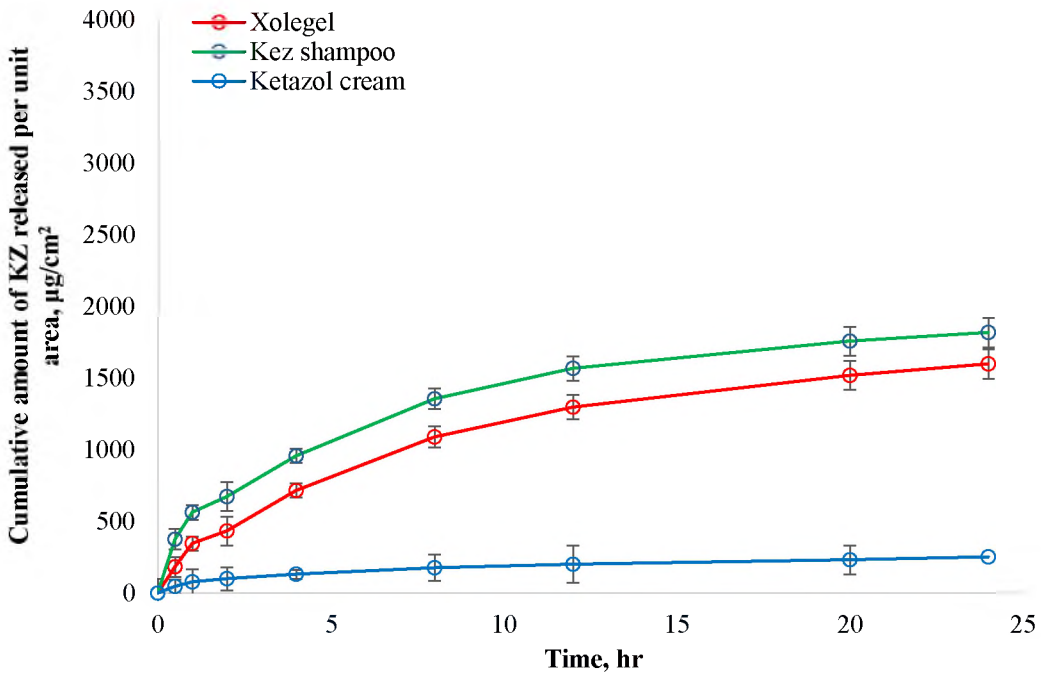


Figure C9 Cumulative amount of KZ released per unit area from Xolegel®, Kez® shampoo and Ketazol® cream using 60% phosphate buffer of pH = 4.5 in the receptor fluid.

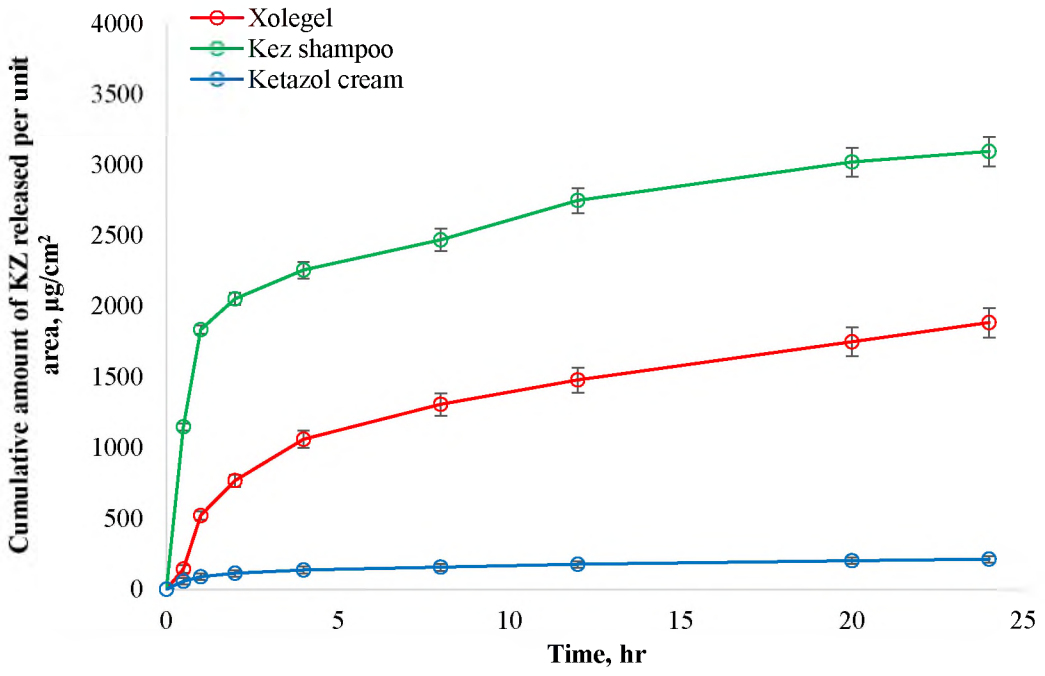


Figure C10 Cumulative amount of KZ released per unit area from Xolegel®, Kez® shampoo and Ketazol® cream using 65% phosphate buffer of pH = 4.5 in the receptor fluid.

APPENDIX D
MATERIALS TRANSFER AGREEMENT

UNESCO MIRCEN Yeast Culture Collection

Department of Microbial, Biochemical & Food Biotechnology
University of the Free State, P.O. Box 339, 9300 Bloemfontein, South Africa
Tel. (+27) 51-4019277
Fax: (+27) 51-4019376

ORDER FORM

Particulars of Recipient and Material requested:

Full Name ..... Ashmita Ramanah .....

Address: ..... Rhodes University .....

..... Po Box 94 .....

..... Grahamstown .....

..... 6140 .....

.....

Country ..... South Africa .....

Tel. No. ..... 0729253357 ..... Fax No. .....

E-mail address ..... ashmita.r@hotmail.com .....

Description of Material ("Material") ..... Candida albicans Ho 100

Period of Use: Indefinite ☐
Specified period ☑ Dates: 07 DEC 2014 – 31 DEC 2015

Handling Fee R200.00

..... RECIPIENT

..... (PROF R B WALKER) HEAD & DEAN
NAME AND DESIGNATION OF SIGNATORY WHO WARRANTS THAT HE/SHE HAS BEEN DULY AUTHORISED THERETO

Accepts herewith all the terms and conditions contained in the Materials Transfer Agreement attached hereto ("Agreement") to which the transfer of Material in terms hereof is subject to.

FACULTY OF PHARMACY
RHODES UNIVERSITY
GRAHAMSTOWN 6139
SOUTH AFRICA

MATERIALS TRANSFER AGREEMENT

MATERIALS TRANSFER AND SCOPE OF USE

1.1 The Recipient hereby agrees to accept transfer of the Material from the UNESCO MIRCEN, University of the Free State ("UFS") subject to the terms and conditions stated herein ("Agreement").

1.2.1 The Recipient shall only be entitled to make use of the Material, Replicates or Derivatives for *bona fide* research performed by the Recipient itself in the laboratories of the Recipient only, as well as related academic activities performed on the premises of the Recipient, provided that the aforesaid research and activities are not aimed, directly or indirectly, at achieving any profit or commercialisation purposes.

"Replicate" means any biological or chemical material that represents a substantially unmodified copy of the Material such as, but not limited to, material produced by growth of cells or microorganisms or amplification of Material. "Derivative" means material created from the Material that is substantially modified to have new properties.

1.3 The Recipient shall not, without the prior written consent of the UFS, distribute, sell, lend or in any manner deliver or hand over to any person any of the Material, Replicates and/or Derivatives or grant any rights to any person in respect thereof or the use thereof other than natural persons directly involved under the direct control and supervision of the Recipient and then only for purposes of the Recipient's own use.

1.6 The UFS shall not be restricted to perform any research or limited in any way to use any of the Material, Replicates and/or Derivatives for any purpose whatsoever, and no exclusivity of any nature is granted by the UFS to the Recipient.

1.7 The Recipient shall not be entitled to commercialise any results, discoveries, inventions or conclusions reached pursuant to the research performed by the Recipient in respect of or by using the Material, Replicates or Derivatives and other intellectual property derived from research carried out by the Recipient on or in respect of the Material, Replicates and/or Derivatives or allow any third party to do the same, without the prior written consent of the UFS.

1.9 The Recipient shall pay to the UFS a handling fee of the amount specified in this order in respect of the Material transferred by the UFS to the Recipient and the Recipient shall disburse all delivery costs incurred by the UFS to have the Material delivered to the Recipient, as well as all taxes, duties, tariffs and license fees payable to any relevant authority or otherwise, including import and export duties payable in transferring the Material to the Recipient. All amounts payable by the Recipient to the UFS in terms of this clause 1.9 shall be payable within 30 (THIRTY) days from date the Recipient receives an invoice from the UFS to that effect.

1.10 The Recipient shall at all times maintain the numbering and other identification methods employed and/or required by the UFS in respect of the Material, Replicates and/or Derivatives and the Recipient shall at all times ensure that the Material, Replicates and Derivatives can be identified in accordance with such identification systems.

1.13 The UFS shall at all times retain all rights, title and ownership of the Material and Derivatives to the extent that the same do not vest in any third party, and in the event that such rights vest in any third party, the UFS shall be entitled to exercise such rights on their behalf to the extent required by the UFS and/or such third parties to protect such rights.

TERM OF AGREEMENT

2.1 This Agreement shall apply with effect from the date any of the Material is supplied or delivered to the Recipient or the date this order is signed by the Recipient, whichever occurs first, and thereafter for as long as any of the Material, Replicates and/or Derivatives are in the possession of the Recipient, though it is recorded that the provisions of this Agreement shall remain to apply in respect of any breach of any provision hereof by the Recipient even if the Recipient is subsequent to such breach not in possession of any Material, Replicates and/or Derivatives any more.

2.2 The UFS shall at any time after expiry of the period referred to in this order be entitled to require the Material, Replicates and/or Derivatives to be either returned to the UFS or be destroyed by the Recipient.

LIMITATION OF LIABILITY AND INDEMNITY

3.1 The UFS shall not be liable for any claim or damages whatsoever resulting from the death of any living biological Material, or **resulting from the wrong or incomplete identification, coding, or naming of any Material.**

3.2 The UFS shall not be liable for any claim for damages, including any claim resulting from the death of or injury to any person, including legal costs incurred by any person, whether or not action is or was instituted, to the extent that such claims have resulted from any use, handling, receipt, storage,

transfer, disposal and any other activities relating to the Material, Replicates and/or Derivatives, and the Recipient accordingly hereby indemnify and hold harmless the UFS against any such claims.

3.3 The Material and technical information and assistance provided to the Recipient by the UFS as contemplated in this Agreement are provided as is without any warranty or implied warranty including without limitation, any warranty of merchantability, fitness for any particular purpose, typicality, safety, accuracy and non-infringement, save as expressly provided in this Agreement.

3.4 Without limiting the generality of the above, the UFS shall not be liable for any indirect, special, incidental or consequential damages (whether contractual, delictual or otherwise) of any kind, including loss of income or profits, in connection with or arising from this Agreement, the Material, Replicates and/or Derivatives.

INTELLECTUAL PROPERTY AND PUBLICATION

4.1 The Recipient undertakes to acknowledge the UFS and/or any other contributor indicated by the UFS as the source of the Material in all publications and patent applications that refer to the Material, Replicates and/or Derivatives.

4.2 If required by a third party contributor of the Material, the UFS may inform the contributor of the Recipient's identity.

4.3 The Recipient undertakes to notify the UFS of any acts taken by the Recipient to establish or secure any intellectual property rights relating to research carried out in respect of the Material, Replicates and/or Derivatives.

GOOD FAITH AND FRUSTRATION OF AGREEMENT

5.1 The Recipient undertakes not to frustrate the provisions and spirit of this Agreement or the rights of the UFS in respect of the Material by the interposing of juristic persons, trusts or other entities to perform acts falling outside the scope of use as contemplated in clause 1 hereof or which is otherwise contrary to the spirit of this Agreement.

5.2 The Recipient undertakes to act in good faith towards the UFS and not to do anything which may be prejudicial to the UFS for the duration of this Agreement as far as such acts or omissions relate to the Material.

MISCELLANEOUS

6.1 This Agreement shall be governed by and construed in accordance with the laws of South Africa.

6.2 This Agreement contains all the terms and conditions of the agreement between the parties concerning the subject-matter hereof and no terms, conditions, warranties or representations whatever apart from those contained in this Agreement have been made or agreed to by the parties.

6.3 No variation or consensual termination of this Agreement or any part thereof shall be of any force or effect unless in writing and signed by or on behalf of the Parties.

6.4 No relaxation or indulgence which the UFS may grant to the Recipient in regard to any of the Recipient's obligations in terms hereof shall constitute a waiver of or prejudice any of the UFS's rights in terms hereof.

APPENDIX E

BATCH SET PRODUCTION RECORDS AND SAMPLE BATCH PRODUCTION RECORD FOR DEVELOPMENT AND OPTIMISATION FORMULATIONS

Biopharmaceutics Research Laboratory
Department of Pharmaceutics, Faculty of Pharmacy
Rhodes University
Grahamstown 6140, South Africa

BATCH SET PRODUCTION RECORD 1/1

Product: Ketoconazole thermosetting vaginal Gel	**Date of Manufacture:** 05/11/2014 to 19/12/2014
Batch Set: Optimisation batches (R1 – R20)	**Batch Size:** 50g

Batch record issued by	**Date**
Batch record verified by	**Date**
Batch record verified by	**Date**
Batch record verified by	**Date**

SIGNATURE & INITIAL REFERENCE

Full name (Print)	Signature	Initials	Date

Biopharmaceutics Research Laboratory
Department of Pharmaceutics, Faculty of Pharmacy
Rhodes University
Grahamstown 6140, South Africa

BATCH PRODUCTION RECORD 1/2

Product: Ketoconazole thermosetting vaginal gel
Batch Number: R1
Date of Manufacture: 05/11/2014
Batch Size: 50 g

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	12.00	6000		
Poloxamer 188	RM000307	12.50	6250		
Poloxamer 237	RM000313	2.00	1000		
Sodium metabisulphite	RM1004933	1.00	500		
Ketoconazole	RM000242	2.00	1000		
Solvent system:	RM000242	70.50	35250		
Polysorbate 80	1043695	1.06	530		
Ethanol	-	6.70	3350		
Citric acid	241460 184	3.45	1725		
Disodium hydrogen phosphate	35716	0.85	425		
HPLC water	-	58.44	29220		

EQUIPMENT VERIFICATION

Description	Type/ Model	Verified by	Confirmed by
Top loading analytical balance	AG135 Mettler Toledo®		
Magnetic stirrer	Labcon®		
Sonicator	Branson® B12		
Freezer	Model CF 355 KIC®		
Refrigerator	Fuchsware®		

Biopharmaceutics Research Laboratory
Department of Pharmaceutics, Faculty of Pharmacy
Rhodes University
Grahamstown 6140, South Africa

BATCH PRODUCTION RECORD 2/2

Product: Ketoconazole thermosetting vaginal gel
Batch Number: R1
Date of Manufacture: 05/11/2014
Batch Size: 50 g

MANUFACTURING PROCEDURE

Steps	Procedure	Done by	Verified by
1	Citrate-phosphate buffer: Weigh citric acid and anhydrous disodium hydrogen phosphate and place into a Buckner bottle containing HPLC water. Sonicate until powders have dissolved. Adjust to pH 5.0 using 0.1 M sodium hydroxide.		
2	Weigh all poloxamers and place into labelled jars, wrapped in foil.		
3	Solvent system: Weigh citric acid, polysorbate 80 and ethanol. Place in a beaker, and then add citrate-phosphate buffer. Sonicate for 15 minutes. Use a magnetic stirrer to stir mixture for 15 minutes.		
4	Weigh 1000 mg ketoconazole and 500 mg sodium metabisulphite and add to solvent system. Cover the jar with foil and keep away from light. Sonicate for 5 minutes and use the magnetic stirrer to stir mixture for 5 minutes. Repeat sonication and stirring procedure until the solution is clear.		
5	Making sure that there is no residual powder in the beaker from step 4. Pour into the jar containing poloxamers, prepared in step 2.		
6	Using a glass rod, mix the contents of the jar from step 5, making sure the poloxamers are well hydrated.		
7	Seal jars using lid and Parafilm™. Freeze jars for 1 hour at -20°C, then move jars to thaw and chill in a refrigerator at 5°C for 24 hours. Stir gels using a glass rod and repeat freezing for 1 hour and chilling for 24 hours for a maximum of 74 hours.		

APPENDIX F

BATCH SUMMARY RECORDS FOR DEVELOPMENT AND OPTIMISATION FORMULATIONS

Biopharmaceutics Research Laboratory
Department of Pharmaceutics, Faculty of Pharmacy
Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

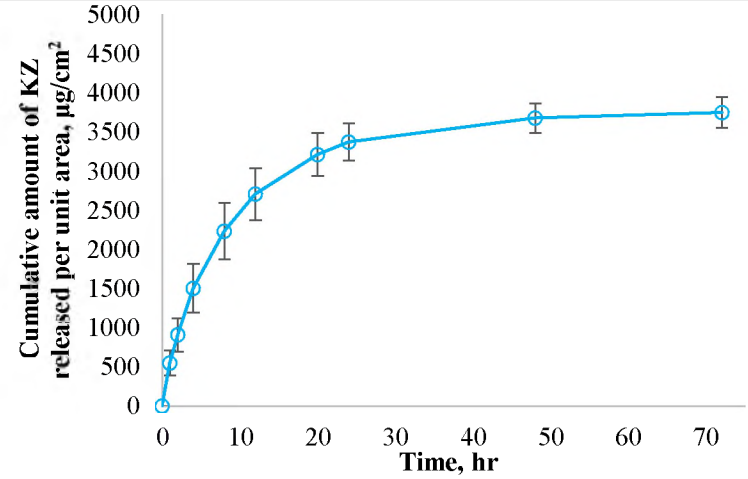
Product: Ketoconazole thermosetting vaginal gel
Batch size: 50 g
Batch Number: R1

Start of Manufacture: 05/11/2014 11:00
End of Manufacture: 08/11/2014 13:00

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	12.00	6000		
Poloxamer 188	RM000307	12.50	6250		
Poloxamer 237	RM000313	2.00	1000		
Sodium metabisulphite	RM1004933	1.00	500		
Ketoconazole	RM000242	2.00	1000		
Solvent system:	RM000242	70.50	35250		
Polysorbate 80	1043695	1.06	530		
Ethanol	-	6.70	3350		
Citric acid	241460 184	3.45	1725		
Disodium hydrogen phosphate	35716	0.85	425		
HPLC grade water	-	58.44	29220		

IN VITRO RELEASE PROFILE



GEL RESPONSES

Viscosity 22°C: 5517 cP

Viscosity 37°C: 5862 cP

Sol-gel transition: 30.00 s

Zone of inhibition: 12 mm

pH: 3.68

Appearance: Clear colourless liquid

Biopharmaceutics Research Laboratory
Department of Pharmaceutics, Faculty of Pharmacy
Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

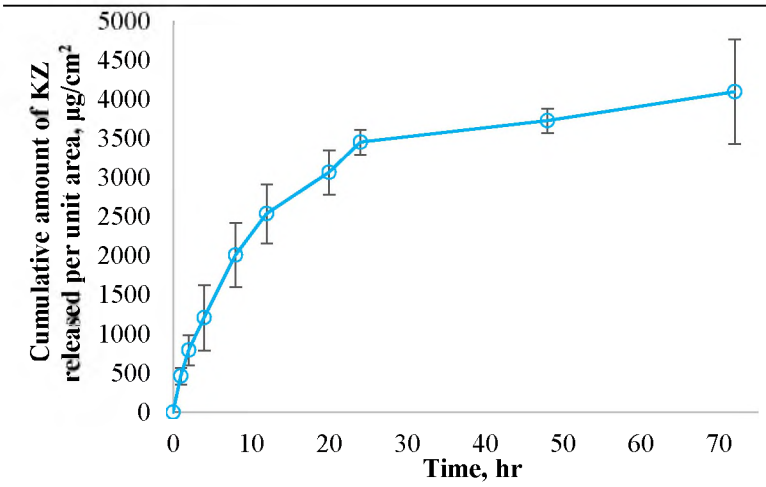
Product: Ketoconazole thermosetting vaginal gel
Batch size: 50 g
Batch Number: R2

Start of Manufacture: 05/11/2014 11:05
End of Manufacture: 08/11/2014 13:05

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	9.95	4975		
Poloxamer 188	RM000307	10.00	5000		
Poloxamer 237	RM000313	4.00	2000		
Sodium metabisulphite	RM1004933	1.00	500		
Ketoconazole	RM000242	2.00	1000		
Solvent system:	RM000242	73.05	36525		
Polysorbate 80	1043695	1.10	550		
Ethanol	-	6.94	3470		
Citric acid	241460 184	3.57	1785		
Disodium hydrogen phosphate	35716	0.88	440		
HPLC grade water	-	60.56	30280		

IN VITRO RELEASE PROFILE



GEL RESPONSES

Viscosity 22°C: 5632 cP

Viscosity 37°C: 5517 cP

Sol-gel transition: 0.50 s

Zone of inhibition: 14 mm

pH: 3.46

Appearance: Clear colourless liquid

Biopharmaceutics Research Laboratory
Department of Pharmaceutics, Faculty of Pharmacy
Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

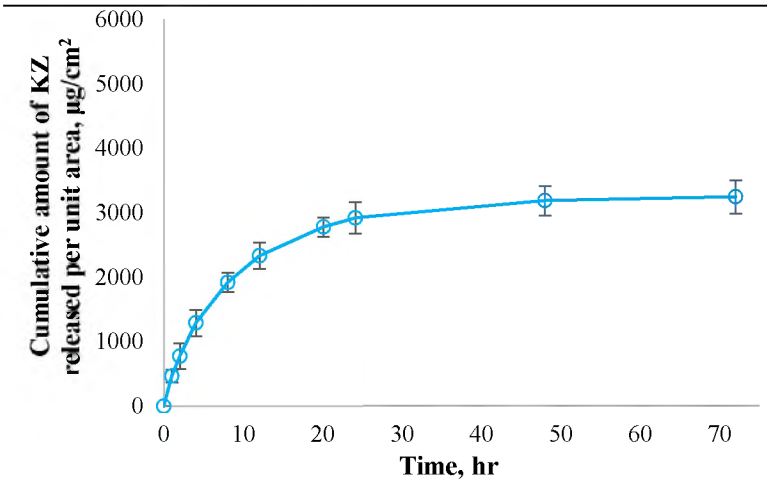
Product: Ketoconazole thermosetting vaginal gel
Batch size: 50 g
Batch Number: R3

Start of Manufacture: 05/11/2014 11:10
End of Manufacture: 08/11/2014 13:10

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	15.00	7500		
Poloxamer 188	RM000307	10.00	5000		
Poloxamer 237	RM000313	0.64	320		
Sodium metabisulphite	RM1004933	1.00	500		
Ketoconazole	RM000242	2.00	1000		
Solvent system:	RM000242	71.36	35680		
Polysorbate 80	1043695	1.07	535		
Ethanol	-	6.78	3390		
Citric acid	241460 184	3.49	1745		
Disodium hydrogen phosphate	35716	0.86	431		
HPLC grade water	-	59.16	29579		

IN VITRO RELEASE PROFILE



GEL RESPONSES

Viscosity 22°C: 7931 cP

Viscosity 37°C: 5287 cP

Sol-gel transition: 0.50 s

Zone of inhibition: 11 mm

pH: 3.63

Appearance: Clear colourless liquid

Biopharmaceutics Research Laboratory
Department of Pharmaceutics, Faculty of Pharmacy
Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

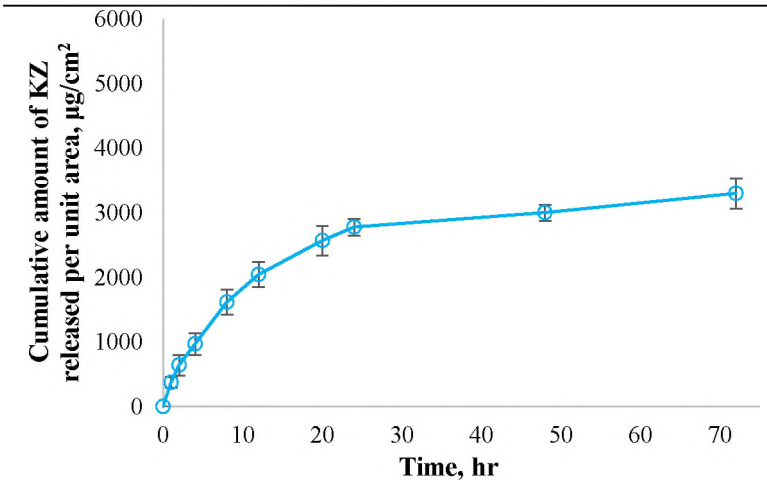
Product: Ketoconazole thermosetting vaginal gel
Batch size: 50 g
Batch Number: R4

Start of Manufacture: 11/11/2014 13:00
End of Manufacture: 14/11/2014 15:00

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	15.00	7500		
Poloxamer 188	RM000307	10.00	5000		
Poloxamer 237	RM000313	7.36	3680		
Sodium metabisulphite	RM1004933	1.00	500		
Ketoconazole	RM000242	2.00	1000		
Solvent system:	RM000242	64.64	32320		
Polysorbate 80	1043695	0.97	485		
Ethanol	-	6.14	3070		
Citric acid	241460 184	3.16	1580		
Disodium hydrogen phosphate	35716	0.78	390		
HPLC grade water	-	53.59	26795		

IN VITRO RELEASE PROFILE



GEL RESPONSES

Viscosity 22°C: 8276 cP

Viscosity 37°C: 89997 cP

Sol-gel transition: 60.00 s

Zone of inhibition: 20 mm

pH: 3.74

Appearance: Clear colourless liquid

Biopharmaceutics Research Laboratory
Department of Pharmaceutics, Faculty of Pharmacy
Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

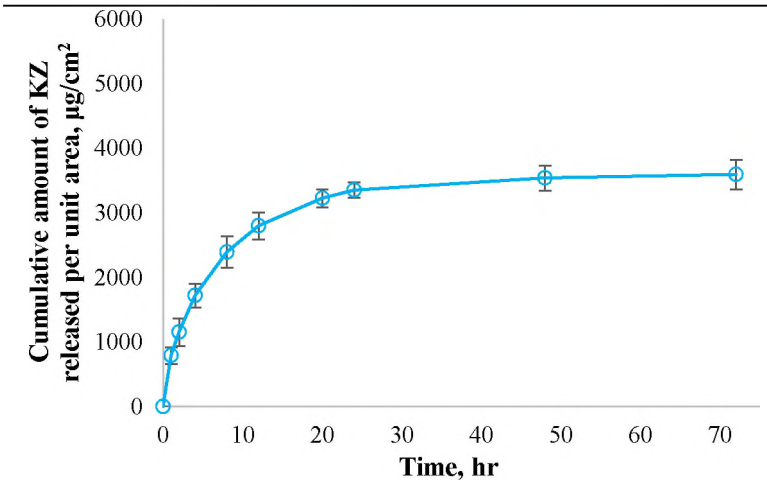
Product: Ketoconazole thermosetting vaginal gel
Batch size: 50 g
Batch Number: R5

Start of Manufacture: 11/11/2014 13:05
End of Manufacture: 14/11/2014 15:05

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	15.00	7500		
Poloxamer 188	RM000307	5.80	2900		
Poloxamer 237	RM000313	4.00	2000		
Sodium metabisulphite	RM1004933	1.00	500		
Ketoconazole	RM000242	2.00	1000		
Solvent system:	RM000242	72.20	36100		
Polysorbate 80	1043695	1.08	540		
Ethanol	-	6.86	3430		
Citric acid	241460 184	3.53	1765		
Disodium hydrogen phosphate	35716	0.87	435		
HPLC grade water	-	59.86	29930		

IN VITRO RELEASE PROFILE



GEL RESPONSES

Viscosity 22°C: 4138 cP

Viscosity 37°C: 1494 cP

Sol-gel transition: 0.50 s

Zone of inhibition: 8 mm

pH: 3.57

Appearance: Clear colourless liquid

Biopharmaceutics Research Laboratory
Department of Pharmaceutics, Faculty of Pharmacy
Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

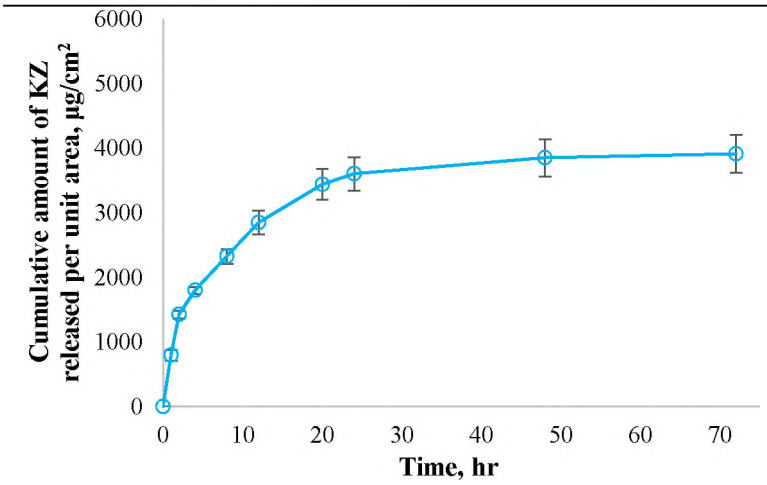
Product: Ketoconazole thermosetting vaginal gel
Batch size: 50 g
Batch Number: R6

Start of Manufacture: 11/11/2014 13:10
End of Manufacture: 14/11/2014 15:10

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	15.00	7500		
Poloxamer 188	RM000307	10.00	5000		
Poloxamer 237	RM000313	4.00	2000		
Sodium metabisulphite	RM1004933	1.00	500		
Ketoconazole	RM000242	2.00	1000		
Solvent system:	RM000242	68.00	34000		
Polysorbate 80	1043695	1.02	510		
Ethanol	-	6.46	3230		
Citric acid	241460 184	3.33	1665		
Disodium hydrogen phosphate	35716	0.82	410		
HPLC grade water	-	56.37	28185		

IN VITRO RELEASE PROFILE



GEL RESPONSES

Viscosity 22°C: 7816 cP

Viscosity 37°C: 5747 cP

Sol-gel transition: 0.50 s

Zone of inhibition: 15 mm

pH: 3.71

Appearance: Clear colourless liquid

Biopharmaceutics Research Laboratory
Department of Pharmaceutics, Faculty of Pharmacy
Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

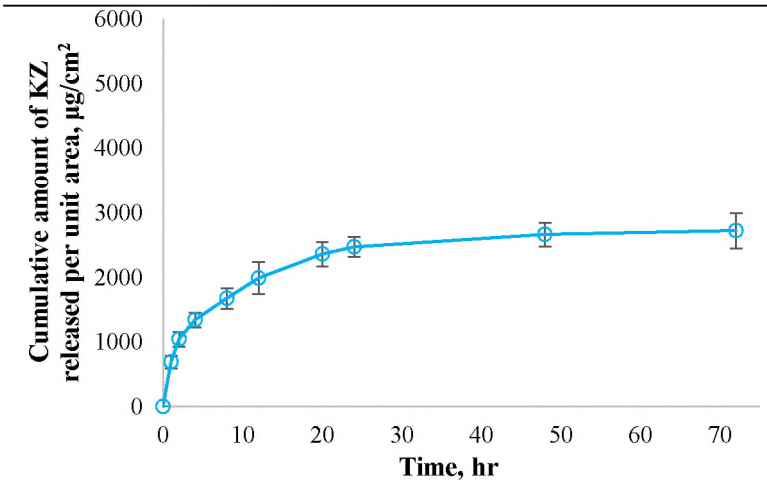
Product: Ketoconazole thermosetting vaginal gel
Batch size: 50 g
Batch Number: R7

Start of Manufacture: 11/11/2014 13:15
End of Manufacture: 14/11/2014 15:15

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	12.00	6000		
Poloxamer 188	RM000307	12.50	6250		
Poloxamer 237	RM000313	6.00	3000		
Sodium metabisulphite	RM1004933	1.00	500		
Ketoconazole	RM000242	2.00	1000		
Solvent system:	RM000242	66.50	33250		
Polysorbate 80	1043695	1.00	500		
Ethanol	-	6.32	3160		
Citric acid	241460 184	3.25	1625		
Disodium hydrogen phosphate	35716	0.80	400		
HPLC grade water	-	55.13	27565		

IN VITRO RELEASE PROFILE



GEL RESPONSES

Viscosity 22°C: 4597 cP

Viscosity 37°C: 6551 cP

Sol-gel transition: 15.00 s

Zone of inhibition: 12 mm

pH: 3.59

Appearance: Clear colourless liquid

Biopharmaceutics Research Laboratory
Department of Pharmaceutics, Faculty of Pharmacy
Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

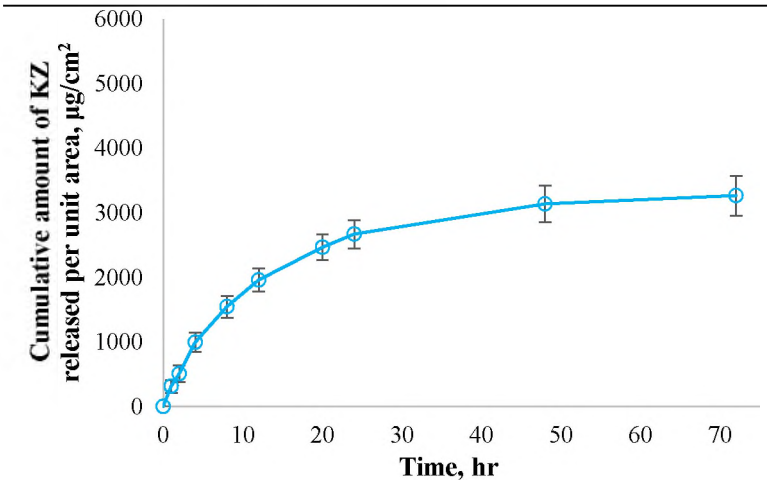
Product: Ketoconazole thermosetting vaginal gel
Batch size: 50 g
Batch Number: R8

Start of Manufacture: 02/12/2014 09:00
End of Manufacture: 05/12/2014 11:00

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	18.00	9000		
Poloxamer 188	RM000307	12.50	6250		
Poloxamer 237	RM000313	6.00	3000		
Sodium metabisulphite	RM1004933	1.00	500		
Ketoconazole	RM000242	2.00	1000		
Solvent system:	RM000242	60.50	30250		
Polysorbate 80	1043695	0.91	455		
Ethanol	-	5.75	2875		
Citric acid	241460 184	2.96	1480		
Disodium hydrogen phosphate	35716	0.73	365		
HPLC grade water	-	50.15	25075		

IN VITRO RELEASE PROFILE



GEL RESPONSES

Viscosity 22°C: 196633 cP

Viscosity 37°C: 339600 cP

Sol-gel transition: 60.00s

Zone of inhibition: 32 mm

pH: 3.73

Appearance: Clear colourless gel

Biopharmaceutics Research Laboratory
Department of Pharmaceutics, Faculty of Pharmacy
Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

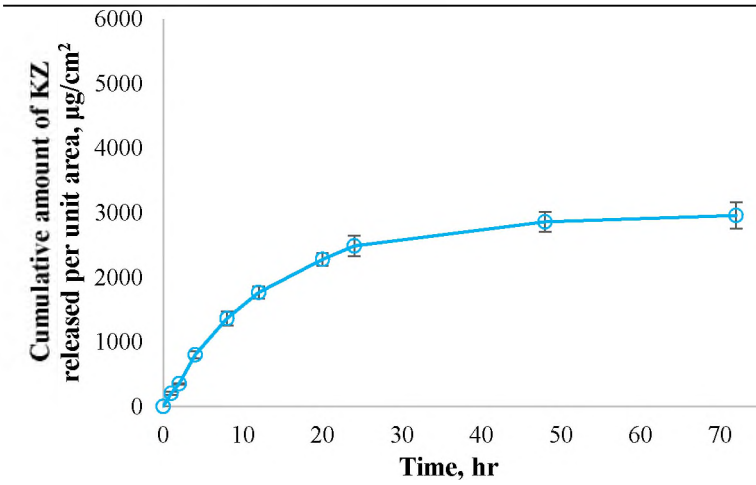
Product: Ketoconazole thermosetting vaginal gel
Batch size: 50 g
Batch Number: R9

Start of Manufacture: 02/12/2014 09:05
End of Manufacture: 05/12/2014 11:05

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	18.00	9000		
Poloxamer 188	RM000307	7.50	3750		
Poloxamer 237	RM000313	6.00	3000		
Sodium metabisulphite	RM1004933	1.00	500		
Ketoconazole	RM000242	2.00	1000		
Solvent system:	RM000242	65.50	32750		
Polysorbate 80	1043695	0.98	490		
Ethanol	-	6.22	3110		
Citric acid	241460 184	3.20	1600		
Disodium hydrogen phosphate	35716	0.79	395		
HPLC grade water	-	54.31	27155		

IN VITRO RELEASE PROFILE



GEL RESPONSES

Viscosity 22°C: 7931 cP

Viscosity 37°C: 291667 cP

Sol-gel transition: 60.00 s

Zone of inhibition: 34 mm

pH: 3.62

Appearance: Clear colourless liquid

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Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

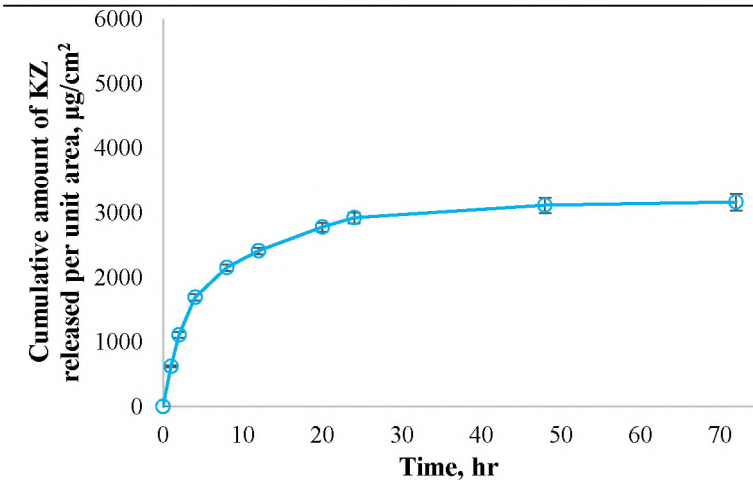
Product: Ketoconazole thermosetting vaginal gel
Batch size: 50 g
Batch Number: R10

Start of Manufacture: 02/12/2014 09:10
End of Manufacture: 05/12/2014 11:10

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	18.00	9000		
Poloxamer 188	RM000307	7.50	3750		
Poloxamer 237	RM000313	2.00	1000		
Sodium metabisulphite	RM1004933	1.00	500		
Ketoconazole	RM000242	2.00	1000		
Solvent system:	RM000242	69.50	34750		
Polysorbate 80	1043695	1.04	520		
Ethanol	-	6.60	3300		
Citric acid	241460 184	3.40	1700		
Disodium hydrogen phosphate	35716	0.84	420		
HPLC grade water	-	57.62	28810		

IN VITRO RELEASE PROFILE



GEL RESPONSES

Viscosity 22°C: 7011 cP

Viscosity 37°C: 42870 cP

Sol-gel transition: 5.00 s

Zone of inhibition: 21 mm

pH: 3.60

Appearance: Clear colourless liquid

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Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

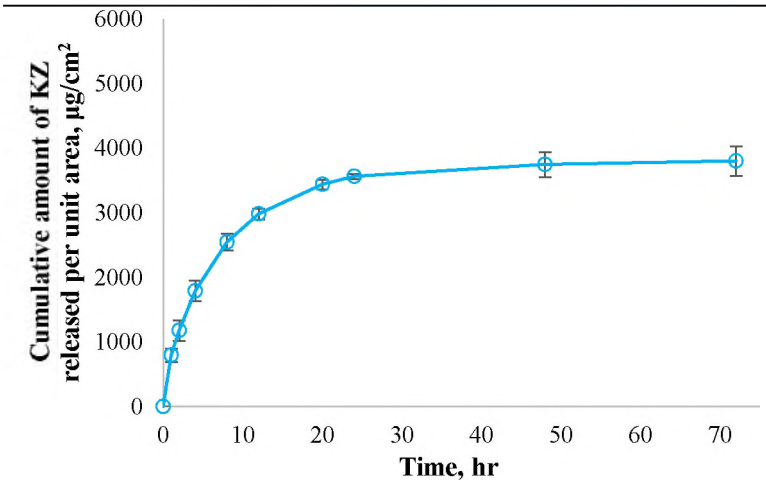
Product: Ketoconazole thermosetting vaginal gel
Batch size: 50 g
Batch Number: R11

Start of Manufacture: 08/12/2014 10:00
End of Manufacture: 11/12/2014 12:00

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	15.00	7500		
Poloxamer 188	RM000307	10.00	5000		
Poloxamer 237	RM000313	4.00	2000		
Sodium metabisulphite	RM1004933	1.00	500		
Ketoconazole	RM000242	2.00	1000		
Solvent system:	RM000242	68.00	34000		
Polysorbate 80	1043695	1.02	510		
Ethanol	-	6.46	3230		
Citric acid	241460 184	3.33	1665		
Disodium hydrogen phosphate	35716	0.82	410		
HPLC grade water	-	56.37	28185		

IN VITRO RELEASE PROFILE



GEL RESPONSES

Viscosity 22°C: 7471 cP

Viscosity 37°C: 6322 cP

Sol-gel transition: 0.50 s

Zone of inhibition: 19 mm

pH: 3.70

Appearance: Clear colourless liquid

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Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

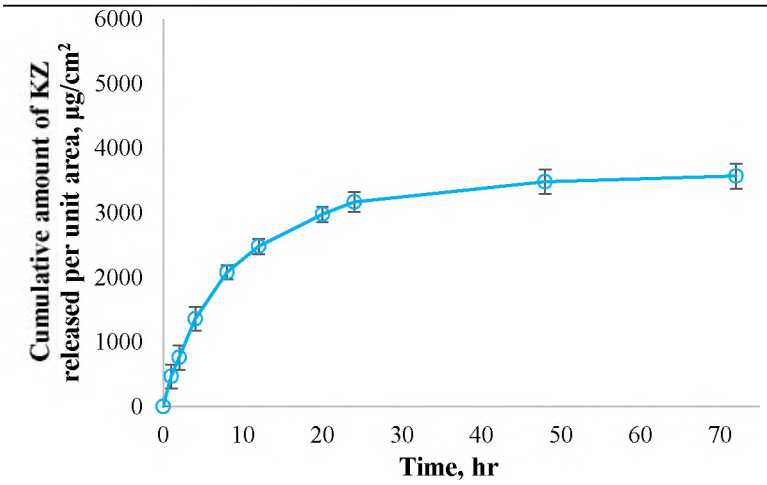
Product: Ketoconazole thermosetting vaginal gel
Batch size: 50 g
Batch Number: R12

Start of Manufacture: 08/12/2014 10:05
End of Manufacture: 11/12/2014 12:05

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	15.00	7500		
Poloxamer 188	RM000307	14.20	7100		
Poloxamer 237	RM000313	4.00	2000		
Sodium metabisulphite	RM1004933	1.00	500		
Ketoconazole	RM000242	2.00	1000		
Solvent system:	RM000242	63.80	31900		
Polysorbate 80	1043695	0.96	480		
Ethanol	-	6.06	3030		
Citric acid	241460 184	3.12	1560		
Disodium hydrogen phosphate	35716	0.77	385		
HPLC grade water	-	52.89	26445		

IN VITRO RELEASE PROFILE



GEL RESPONSES

Viscosity 22°C: 8276 cP

Viscosity 37°C: 7701 cP

Sol-gel transition: 11.00 s

Zone of inhibition: 33 mm

pH: 3.66

Appearance: Clear colourless liquid

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Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

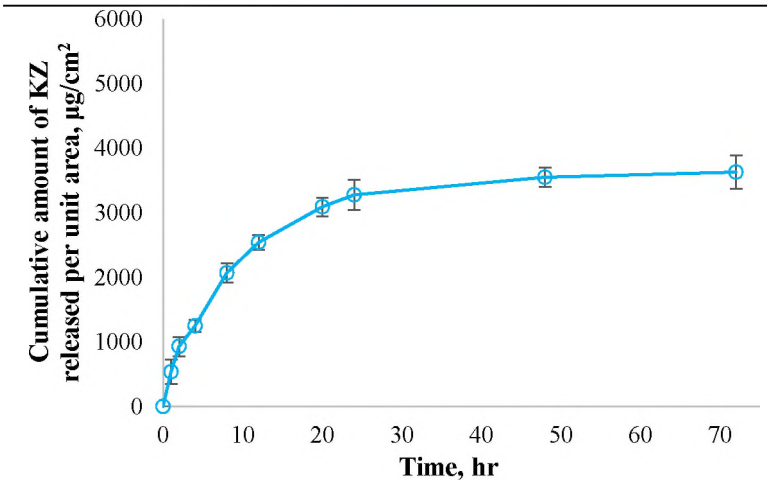
Product: Ketoconazole thermosetting vaginal gel
Batch size: 50 g
Batch Number: R13

Start of Manufacture: 08/12/2014 10:10
End of Manufacture: 11/12/2014 12:10

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	15.00	7500		
Poloxamer 188	RM000307	10.00	5000		
Poloxamer 237	RM000313	4.00	2000		
Sodium metabisulphite	RM1004933	1.00	500		
Ketoconazole	RM000242	2.00	1000		
Solvent system:	RM000242	68.00	34000		
Polysorbate 80	1043695	1.02	510		
Ethanol	-	6.46	3230		
Citric acid	241460 184	3.33	1665		
Disodium hydrogen phosphate	35716	0.82	410		
HPLC grade water	-	56.37	28185		

IN VITRO RELEASE PROFILE



GEL RESPONSES

Viscosity 22°C: 7586 cP

Viscosity 37°C: 5172 cP

Sol-gel transition: 5.00 s

Zone of inhibition: 22 mm

pH: 3.69

Appearance: Clear colourless liquid

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Department of Pharmaceutics, Faculty of Pharmacy
Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

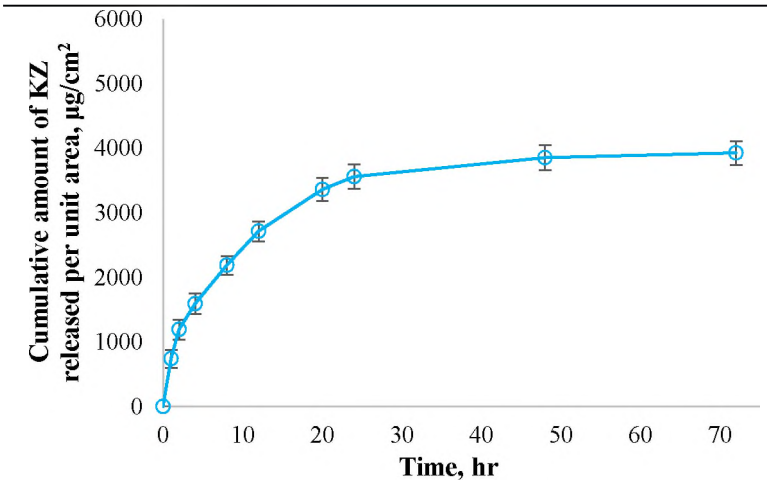
Product: Ketoconazole thermosetting vaginal gel
Batch size: 50 g
Batch Number: R14

Start of Manufacture: 08/12/2014 10:15
End of Manufacture: 11/12/2014 12:15

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	20.05	10025		
Poloxamer 188	RM000307	10.00	5000		
Poloxamer 237	RM000313	4.00	2000		
Sodium metabisulphite	RM1004933	1.00	500		
Ketoconazole	RM000242	2.00	1000		
Solvent system:	RM000242	62.95	31475		
Polysorbate 80	1043695	0.94	470		
Ethanol	-	5.98	2990		
Citric acid	241460 184	3.08	1540		
Disodium hydrogen phosphate	35716	0.76	380		
HPLC grade water	-	52.19	26095		

IN VITRO RELEASE PROFILE



GEL RESPONSES

Viscosity 22°C: 12410 cP

Viscosity 37°C: 295467 cP

Sol-gel transition: 60.00 s

Zone of inhibition: 30 mm

pH: 3.73

Appearance: Clear colourless gel

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Department of Pharmaceutics, Faculty of Pharmacy
Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

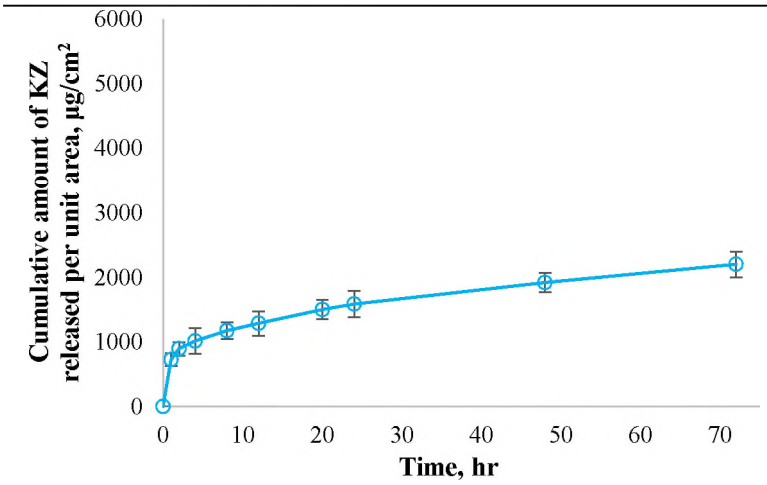
Product: Ketoconazole thermosetting vaginal gel
Batch size: 50 g
Batch Number: R15

Start of Manufacture: 15/12/2014 10:30
End of Manufacture: 18/12/2014 12:30

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	15.00	7500		
Poloxamer 188	RM000307	10.00	5000		
Poloxamer 237	RM000313	4.00	2000		
Sodium metabisulphite	RM1004933	1.00	500		
Ketoconazole	RM000242	2.00	1000		
Solvent system:	RM000242	68.00	34000		
Polysorbate 80	1043695	1.02	510		
Ethanol	-	6.46	3230		
Citric acid	241460 184	3.33	1665		
Disodium hydrogen phosphate	35716	0.82	410		
HPLC grade water	-	56.37	28185		

IN VITRO RELEASE PROFILE



GEL RESPONSES

Viscosity 22°C: 5632 cP

Viscosity 37°C: 17467 cP

Sol-gel transition: 0.50 s

Zone of inhibition: 19 mm

pH: 3.70

Appearance: Clear colourless liquid

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Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

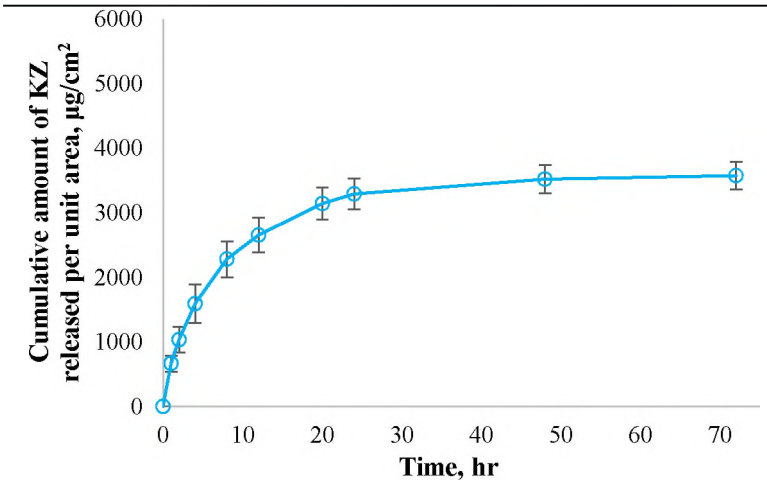
Product: Ketoconazole thermosetting vaginal gel
Batch size: 50 g
Batch Number: R16

Start of Manufacture: 15/12/2014 10:35
End of Manufacture: 18/12/2014 12:35

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	12.00	6000		
Poloxamer 188	RM000307	7.50	3750		
Poloxamer 237	RM000313	2.00	1000		
Sodium metabisulphite	RM1004933	1.00	500		
Ketoconazole	RM000242	2.00	1000		
Solvent system:	RM000242	75.50	37750		
Polysorbate 80	1043695	1.13	565		
Ethanol	-	7.17	3585		
Citric acid	241460 184	3.69	1845		
Disodium hydrogen phosphate	35716	0.91	455		
HPLC grade water	-	62.59	31295		

IN VITRO RELEASE PROFILE



GEL RESPONSES

Viscosity 22°C: 3793 cP

Viscosity 37°C: 1034 cP

Sol-gel transition: 0.50 s

Zone of inhibition: 32 mm

pH: 3.56

Appearance: Clear colourless liquid

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Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

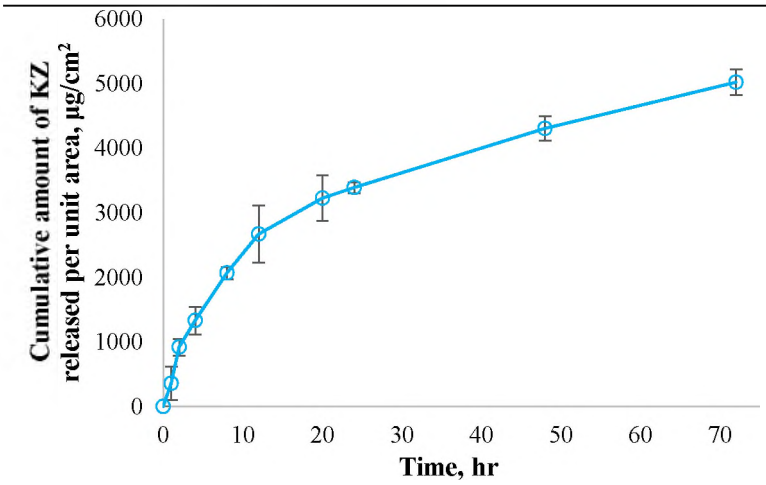
Product: Ketoconazole thermosetting vaginal gel
Batch size: 50 g
Batch Number: R17

Start of Manufacture: 15/12/2014 10:40
End of Manufacture: 18/12/2014 11:40

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	15.00	7500		
Poloxamer 188	RM000307	10.00	5000		
Poloxamer 237	RM000313	4.00	2000		
Sodium metabisulphite	RM1004933	1.00	500		
Ketoconazole	RM000242	2.00	1000		
Solvent system:	RM000242	68.00	34000		
Polysorbate 80	1043695	1.02	510		
Ethanol	-	6.46	3230		
Citric acid	241460 184	3.33	1665		
Disodium hydrogen phosphate	35716	0.82	410		
HPLC grade water	-	56.37	28185		

IN VITRO RELEASE PROFILE



GEL RESPONSES

Viscosity 22°C: 5747 cP

Viscosity 37°C: 8735 cP

Sol-gel transition: 5.00 s

Zone of inhibition: 27 mm

pH: 3.68

Appearance: Clear colourless liquid

Biopharmaceutics Research Laboratory
Department of Pharmaceutics, Faculty of Pharmacy
Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

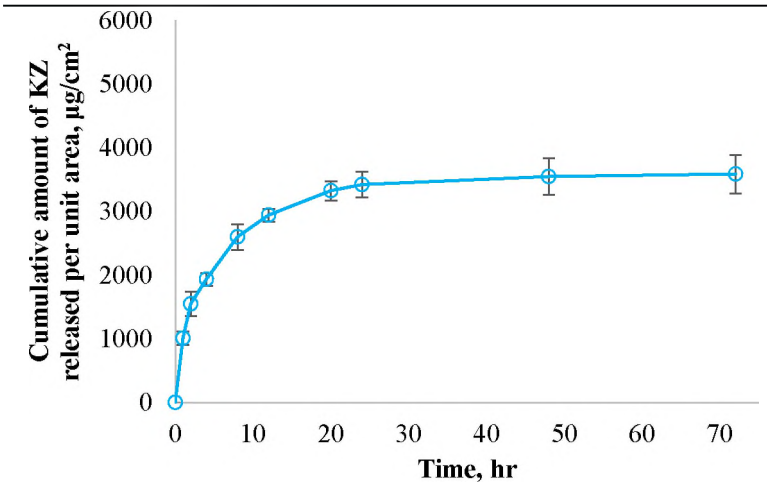
Product: Ketoconazole thermosetting vaginal gel
Batch size: 50 g
Batch Number: R18

Start of Manufacture: 15/12/2014 15:00
End of Manufacture: 18/12/2014 17:00

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	12.00	6000		
Poloxamer 188	RM000307	7.50	3750		
Poloxamer 237	RM000313	6.00	3000		
Sodium metabisulphite	RM1004933	1.00	500		
Ketoconazole	RM000242	2.00	1000		
Solvent system:	RM000242	71.50	35750		
Polysorbate 80	1043695	1.07	535		
Ethanol	-	6.79	3395		
Citric acid	241460 184	3.50	1750		
Disodium hydrogen phosphate	35716	0.86	430		
HPLC grade water	-	59.28	29635		

IN VITRO RELEASE PROFILE



GEL RESPONSES

Viscosity 22°C: 3103 cP

Viscosity 37°C: 1034 cP

Sol-gel transition: 0.50 s

Zone of inhibition: 29 mm

pH: 3.75

Appearance: Clear colourless liquid

Biopharmaceutics Research Laboratory
Department of Pharmaceutics, Faculty of Pharmacy
Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

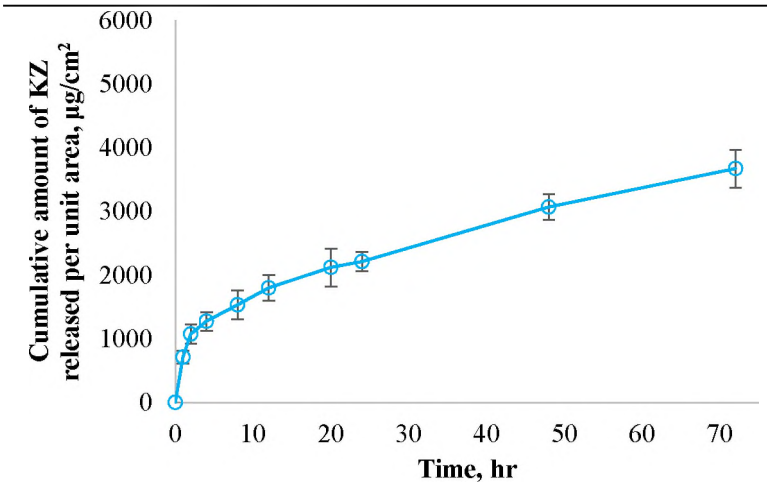
Product: Ketoconazole thermosetting vaginal gel
Batch size: 50 g
Batch Number: R19

Start of Manufacture: 15/12/2014 15:10
End of Manufacture: 18/12/2014 17:10

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	15.00	7500		
Poloxamer 188	RM000307	10.00	5000		
Poloxamer 237	RM000313	4.00	2000		
Sodium metabisulphite	RM1004933	1.00	500		
Ketoconazole	RM000242	2.00	1000		
Solvent system:	RM000242	68.00	34000		
Polysorbate 80	1043695	1.02	510		
Ethanol	-	6.46	3230		
Citric acid	241460 184	3.33	1665		
Disodium hydrogen phosphate	35716	0.82	410		
HPLC grade water	-	56.37	28185		

IN VITRO RELEASE PROFILE



GEL RESPONSES

Viscosity 22°C: 5862 cP

Viscosity 37°C: 9195 cP

Sol-gel transition: 0.50 s

Zone of inhibition: 23 mm

pH: 3.70

Appearance: Clear colourless liquid

Biopharmaceutics Research Laboratory
Department of Pharmaceutics, Faculty of Pharmacy
Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

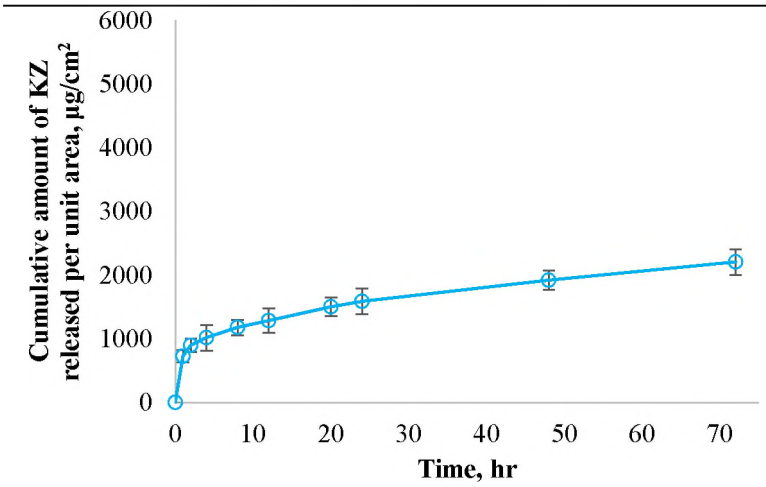
Product: Ketoconazole thermosetting vaginal gel
Batch size: 50 g
Batch Number: R20

Start of Manufacture: 15/12/2014 15:15
End of Manufacture: 18/12/2014 17:15

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	18.00	9000		
Poloxamer 188	RM000307	12.50	6250		
Poloxamer 237	RM000313	2.00	1000		
Sodium metabisulphite	RM1004933	1.00	500		
Ketoconazole	RM000242	2.00	1000		
Solvent system:	RM000242	64.50	32250		
Polysorbate 80	1043695	0.97	485		
Ethanol	-	6.13	3065		
Citric acid	241460 184	3.16	1580		
Disodium hydrogen phosphate	35716	0.78	390		
HPLC grade water	-	53.46	26735		

IN VITRO RELEASE PROFILE



GEL RESPONSES

Viscosity 22°C: 6781 cP

Viscosity 37°C: 10340 cP

Sol-gel transition: 17.00 s

Zone of inhibition: 34 mm

pH: 3.74

Appearance: Clear colourless liquid

APPENDIX G

3D SURFACE PLOTS FROM DEVELOPMENT AND OPTIMISATION STUDIES

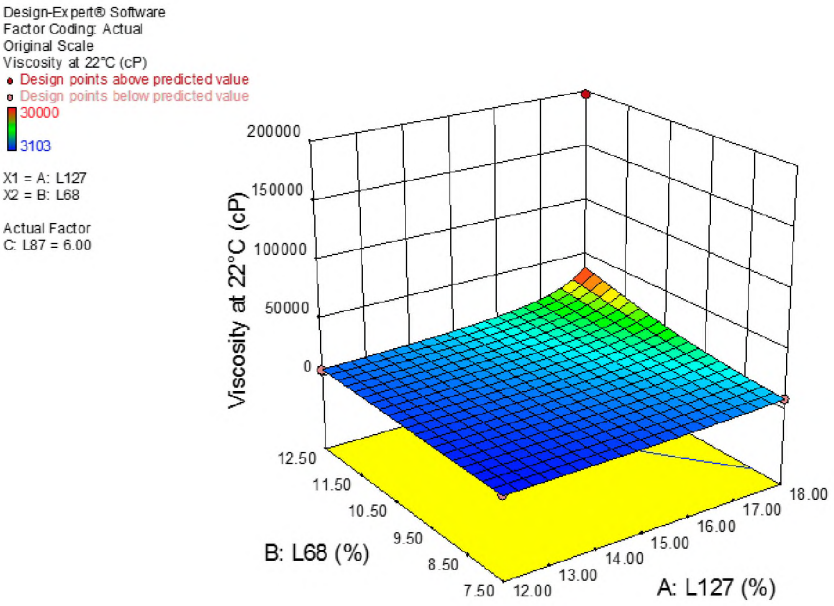


Figure G1 3D response surface plot depicting the impact of L127 and L68 on viscosity at 22 ºC.

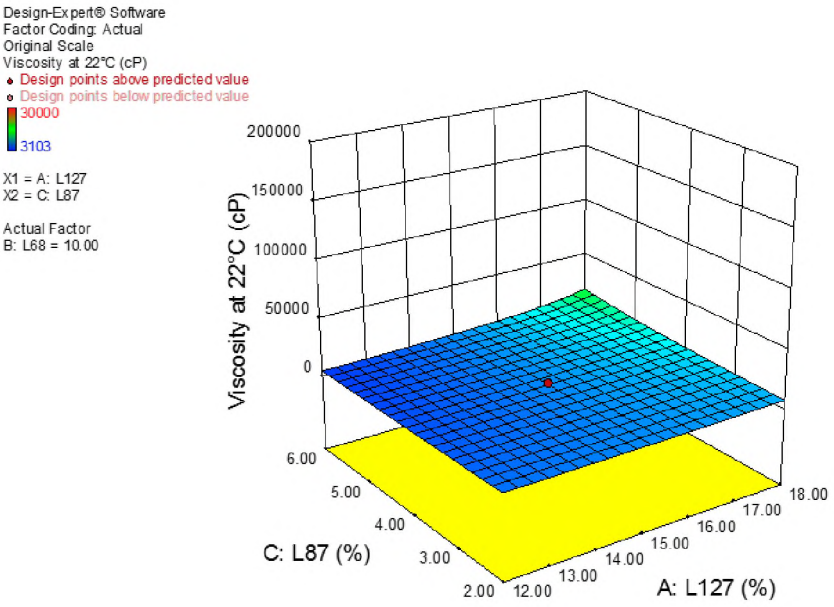


Figure G2 3D response surface plot depicting the impact of L127 and L87 on viscosity at 22 ºC.

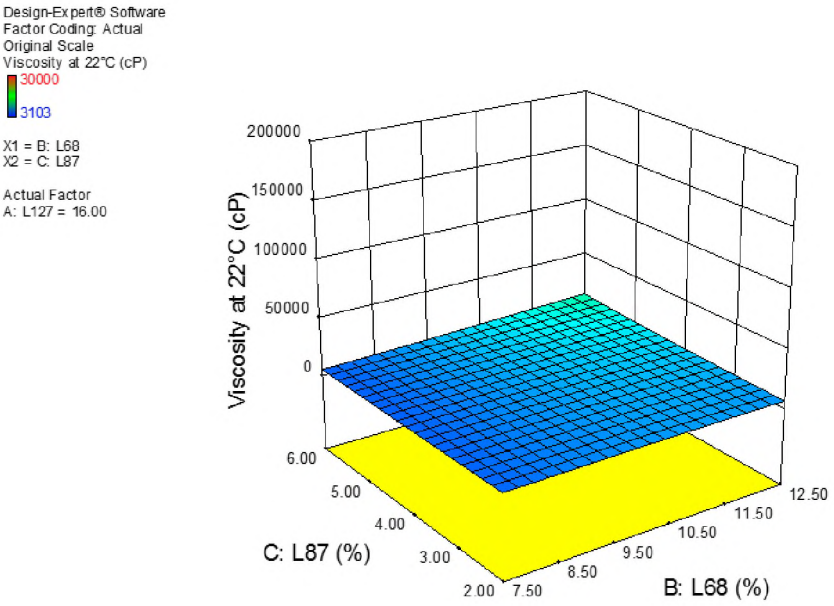


Figure G3 3D response surface plot depicting the impact of L68 and L87 on viscosity at 22 ºC.

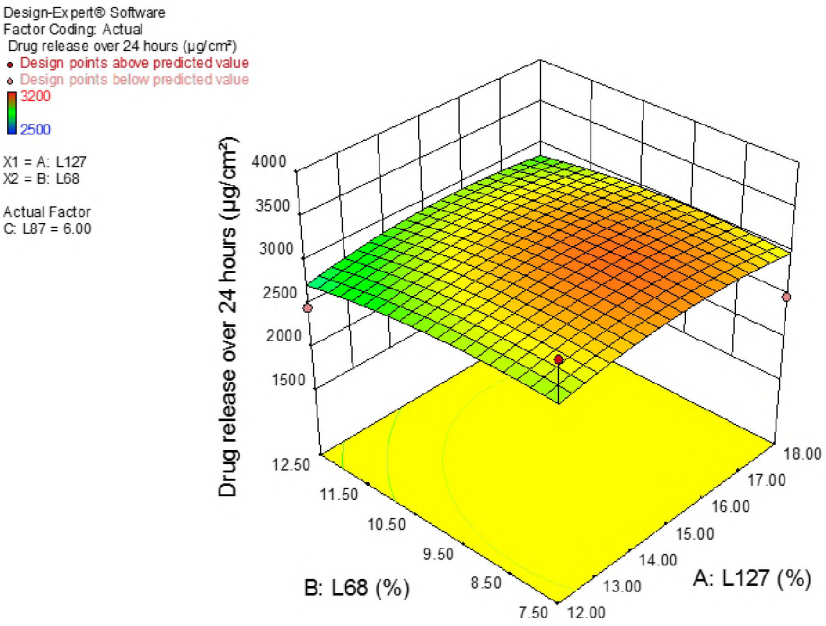


Figure G4 3D response surface plot depicting the impact of L127 and L68 on KZ release over 24 hours.

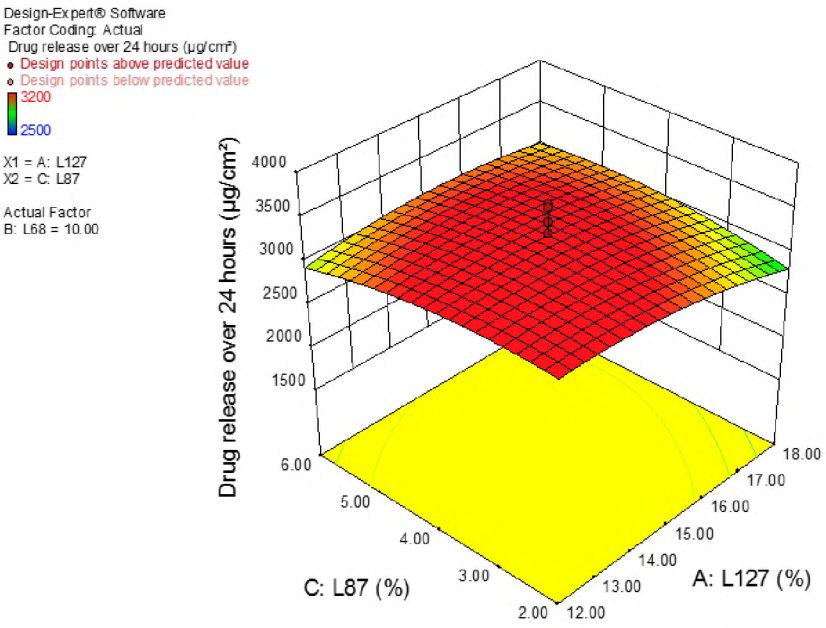


Figure G5 3D response surface plot depicting the impact of L127 and L87 on KZ release over 24 hours.

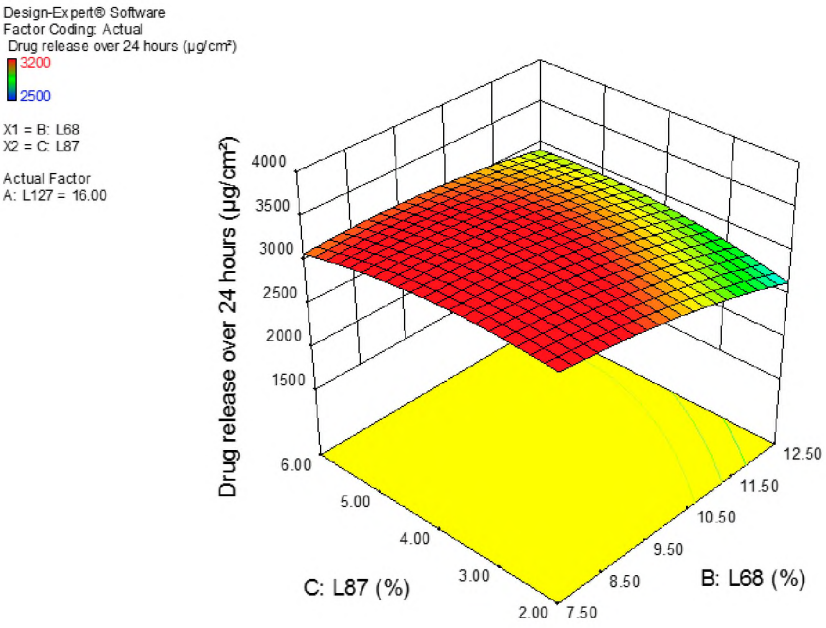


Figure G6 3D response surface plot depicting the impact of L68 and L87 on KZ release over 24 hours.

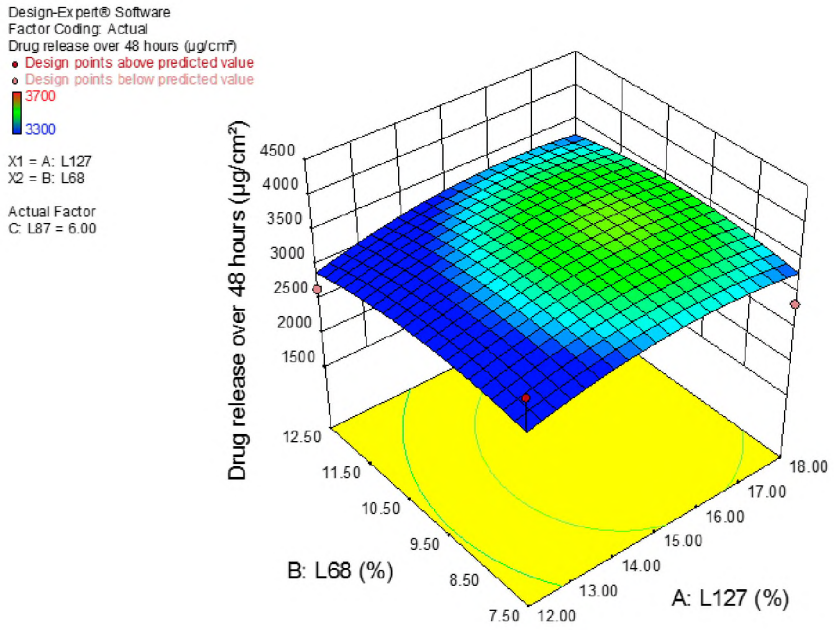


Figure G7 3D response surface plot depicting the impact of L127 and L68 on KZ release over 48 hours.

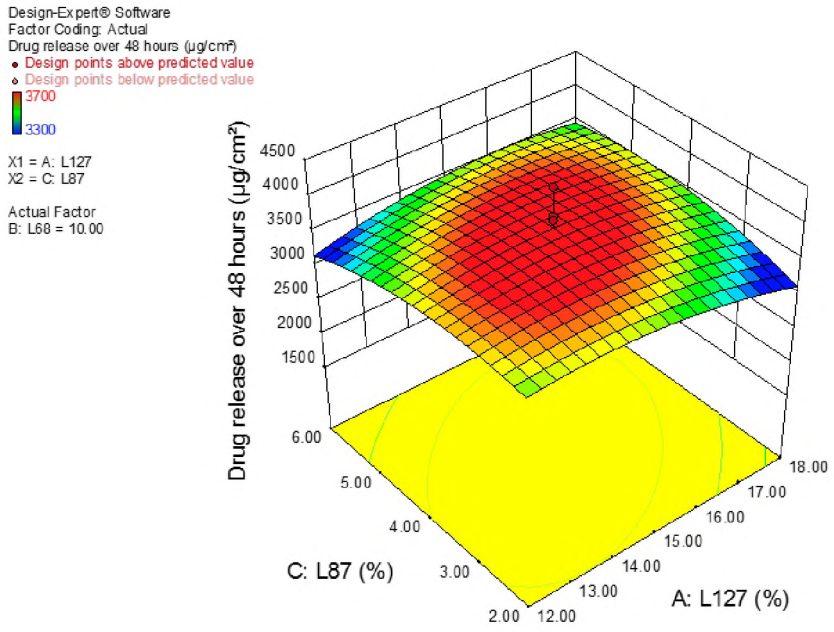


Figure G8 3D response surface plot depicting the impact of L127 and L87 on KZ release over 48 hours.

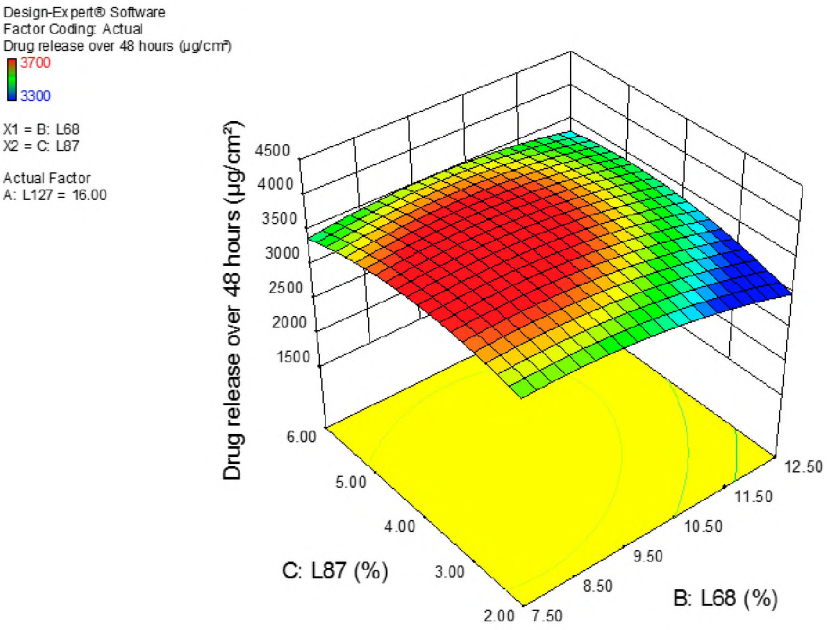


Figure G9 3D response surface plot depicting the impact of L68 and L87 on KZ release over 48 hours.

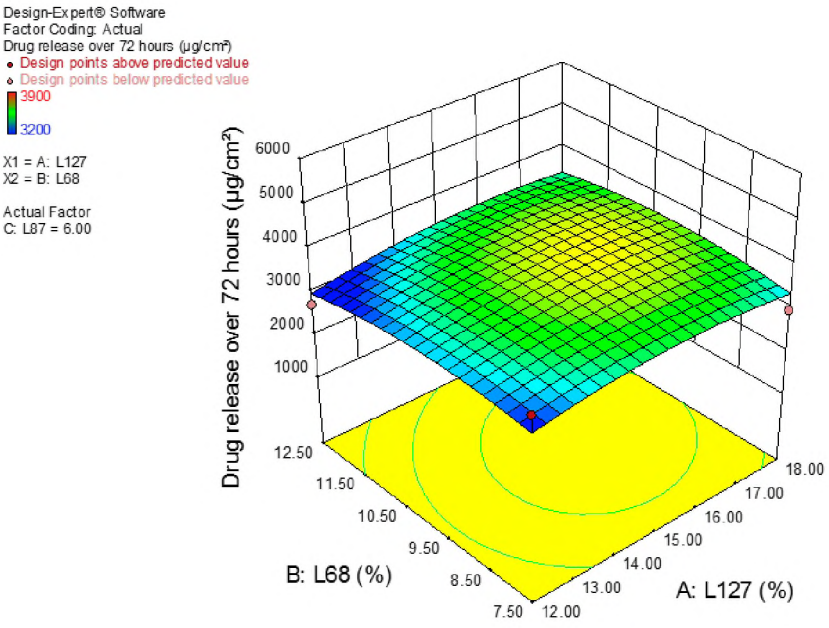


Figure G10 3D response surface plot depicting the impact of L127 and L68 on KZ release over 72 hours.

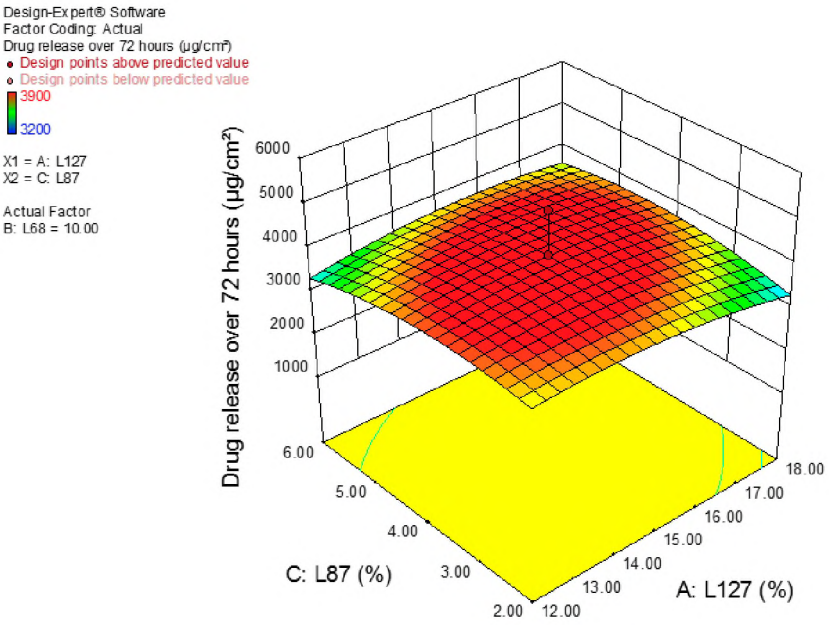


Figure G11 3D response surface plot depicting the impact of L127 and L87 on KZ release over 72 hours.

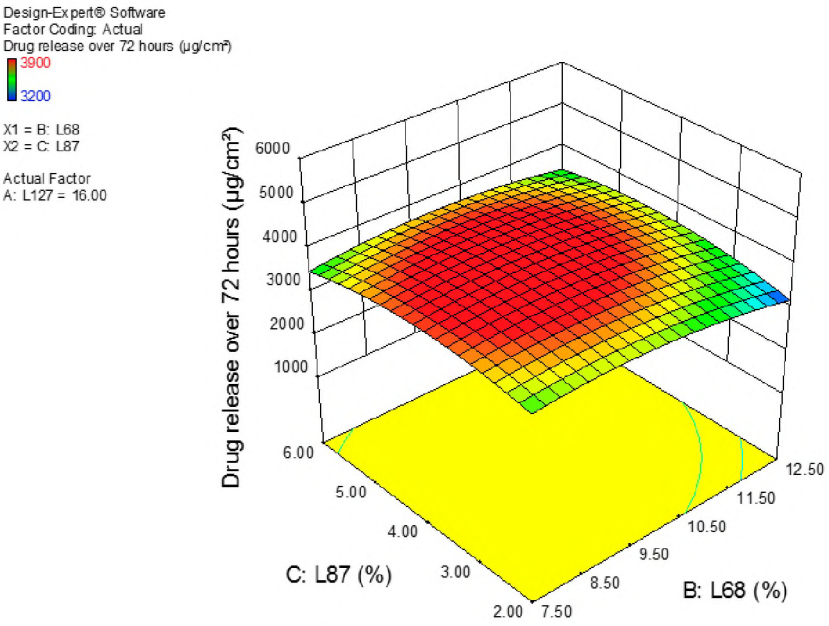


Figure G12 3D response surface plot depicting the impact of L168 and L87 on KZ release over 72 hours.

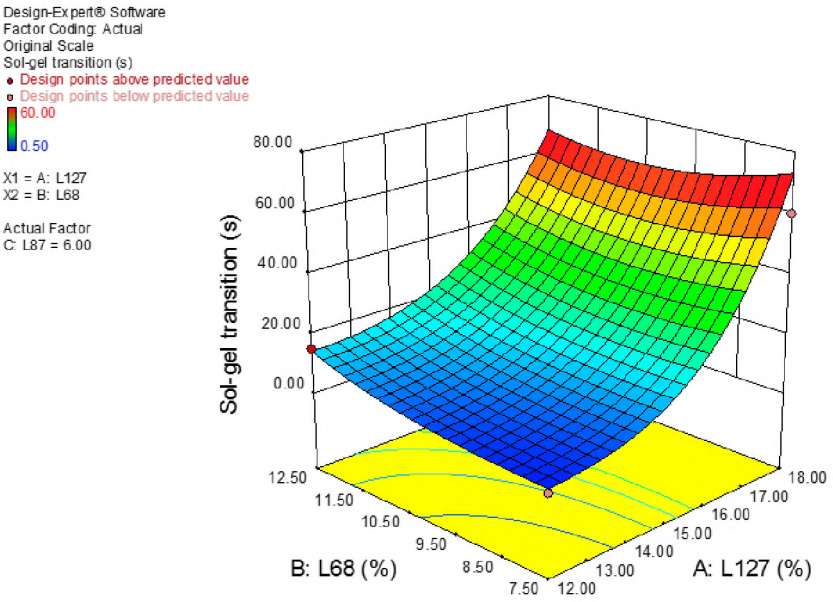


Figure G13 3D response surface plot depicting the impact of L127 and L68 on sol-gel transition.

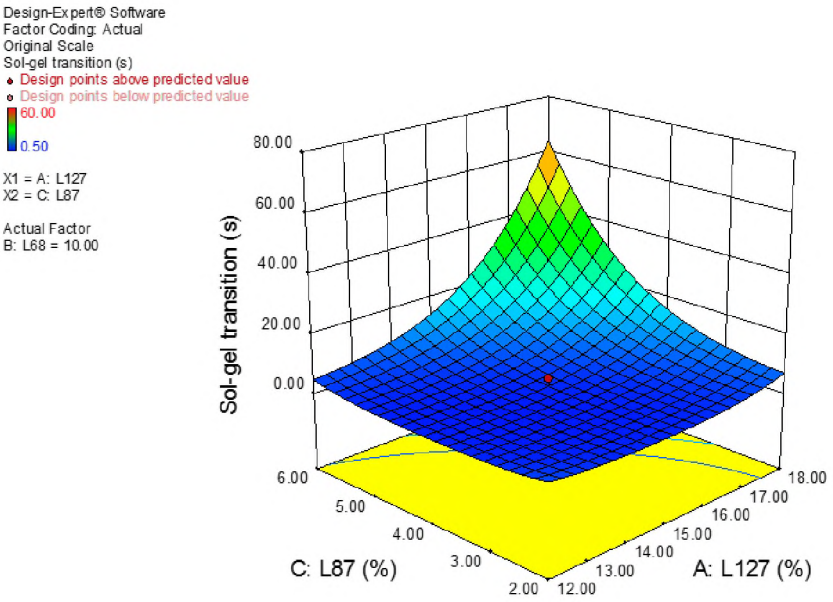


Figure G14 3D response surface plot depicting the impact of L127 and L87 on sol-gel transition.

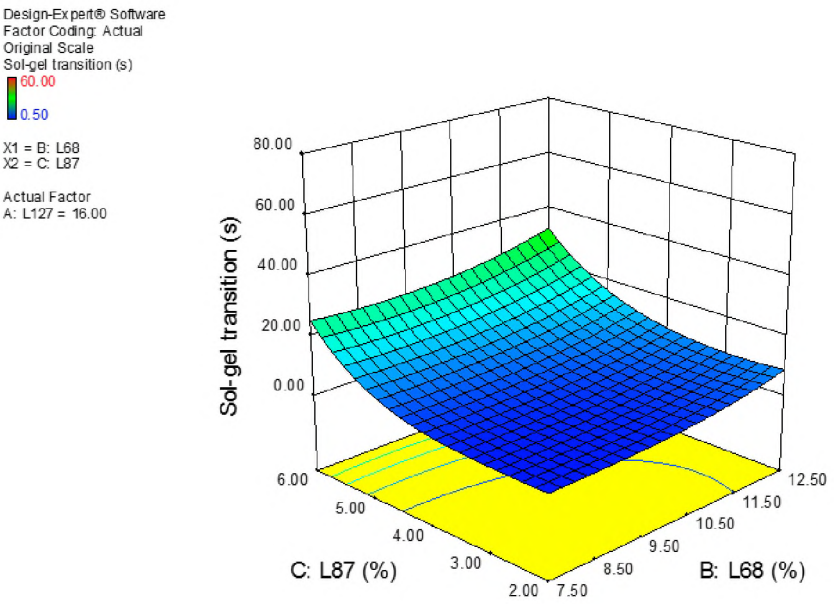


Figure G15 3D response surface plot depicting the impact of L68 and L87 on sol-gel transition.

APPENDIX H

BATCH SET PRODUCTION RECORDS AND SAMPLE BATCH PRODUCTION RECORD FOR STABILITY FORMULATIONS

Biopharmaceutics Research Laboratory
Department of Pharmaceutics, Faculty of Pharmacy
Rhodes University
Grahamstown 6140, South Africa

BATCH SET PRODUCTION RECORD 1/1

Product: Ketoconazole thermosetting vaginal gel

Date of Manufacture: 18/06/2015 to 11/08/2015

Batch Set: Stability batches (S1 – S9)

Batch Size: 50 g

Batch record issued by	**Date**
Batch record verified by	**Date**
Batch record verified by	**Date**
Batch record verified by	**Date**

SIGNATURE & INITIAL REFERENCE

Full name (Print):	Signature	Initials	Date

Biopharmaceutics Research Laboratory
Department of Pharmaceutics, Faculty of Pharmacy
Rhodes University
Grahamstown 6140, South Africa

BATCH PRODUCTION RECORD 1/2

Product: Ketoconazole thermosetting vaginal gel **Date of Manufacture:** 18/06/2015

Batch Number: S1 **Batch Size:** 50 g

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	17.00	8500		
Poloxamer 188	RM000307	7.36	3680		
Poloxamer 237	RM000313	6.38	3190		
Sodium metabisulphite	RM1004933	0.5	250		
Ketoconazole	RM000242	2.00	1000		
Solvent system (pH 3):		66.76	33380		
Polysorbate 80	1043695	1.00	500		
Ethanol	-	6.34	3170		
Citric acid	241460 184	3.27	1635		
Disodium hydrogen phosphate	35716	0.81	405		
HPLC water	-	55.34	27670		

EQUIPMENT VERIFICATION

Description	Type/ Model	Verified by	Confirmed by
Top loading analytical balance	AG135 Mettler Toledo®		
Magnetic stirrer	Labcon®		
Sonicator	Branson® B12		
Freezer	Model CF 355 KIC®		
Refrigerator	Fuchsware®		

Biopharmaceutics Research Laboratory
Department of Pharmaceutics, Faculty of Pharmacy
Rhodes University
Grahamstown 6140, South Africa

BATCH PRODUCTION RECORD 2/2

Product: Ketoconazole thermosetting vaginal gel **Date of Manufacture:** 18/06/2015

Batch Number: S1 **Batch Size:** 50 g

MANUFACTURING PROCEDURE

Steps	Procedure	Done by	Verified by
1	Citrate-phosphate buffer: Weigh citric acid and anhydrous disodium hydrogen phosphate and place into a Buckner bottle containing HPLC water. Sonicate until powders have dissolved. Adjust to pH 5.0 using 0.1 M sodium hydroxide.		
2	Weigh all poloxamers and place into labelled jars, wrapped in foil.		
3	Solvent system: Weigh citric acid, polysorbate 80 and ethanol. Place in a beaker, and then add citrate-phosphate buffer. Sonicate for 15 minutes. Use a magnetic stirrer to stir mixture for 15 minutes.		
4	Weigh 1000 mg ketoconazole and sodium metabisulphite and add to solvent system. Cover the jar with foil and keep away from light. Sonicate for 5 minutes and use the magnetic stirrer to stir mixture for 5 minutes. Repeat sonication and stirring procedure until the solution is clear.		
5	Making sure that there is no residual powder in the beaker from step 4. Pour into the jar containing poloxamers, prepared in step 2.		
6	Using a glass rod, mix the contents of the jar from step 5, making sure the poloxamers are well hydrated.		
7	Seal jars using lid and parafilm™. Freeze jars for 1 hour at -20°C, then move jars to thaw and chill in a refrigerator at 5°C for 24 hours. Stir gels using a glass rod and repeat freezing for 1 hour and chilling for 24 hours for a maximum of 74 hours.		

APPENDIX I

BATCH SUMMARY RECORDS FOR STABILITY FORMULATIONS

Biopharmaceutics Research Laboratory
Department of Pharmaceutics, Faculty of Pharmacy
Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

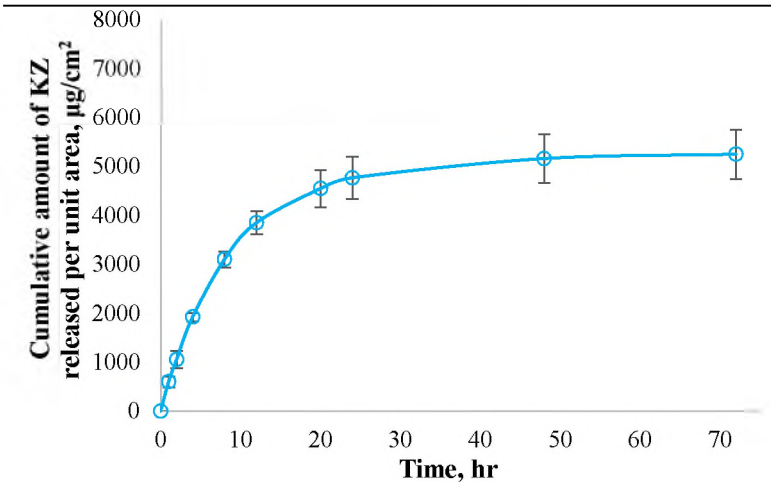
Product: Ketoconazole thermosetting vaginal gel
Batch Size: 50 g
Batch Number: S1

Start of Manufacture: 18/06/2015 13:00
End of Manufacture: 21/06/2015 15:00

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	17.00	8500		
Poloxamer 188	RM000307	7.36	3680		
Poloxamer 237	RM000313	6.38	3190		
Sodium metabisulphite	RM1004933	0.50	250		
Ketoconazole	RM000242	2.00	1000		
Solvent system (pH 3.00):		66.76	33380		
Polysorbate 80	1043695	1.00	500		
Ethanol	-	6.34	3170		
Citric acid	241460 184	2.84	1420		
Disodium hydrogen phosphate	35716	0.81	405		
HPLC grade water	-	55.77	27885		

IN VITRO RELEASE PROFILE



GEL RESPONSES

KZ content: 104.34%

Viscosity 22°C: 11493 cP

Viscosity 37°C: 201333 cP

pH: 3.68

Biopharmaceutics Research Laboratory
Department of Pharmaceutics, Faculty of Pharmacy
Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

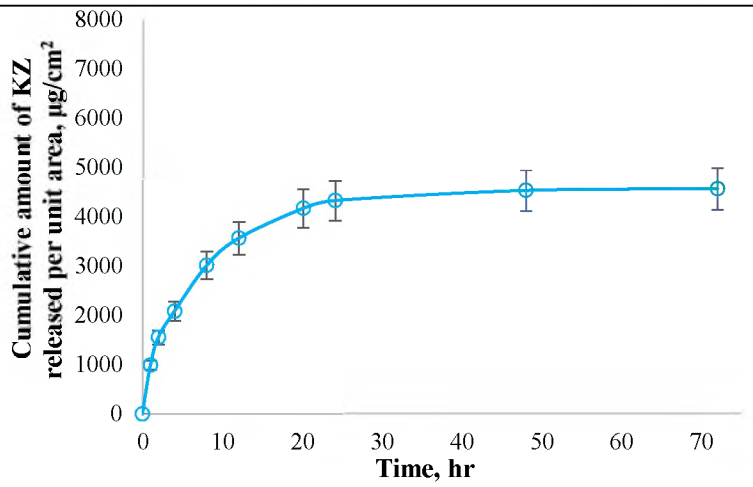
Product: Ketoconazole thermosetting vaginal gel
Batch Size: 50 g
Batch Number: S2

Start of Manufacture: 18/06/2015 13:10
End of Manufacture: 21/06/2015 15:10

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	17.00	8500		
Poloxamer 188	RM000307	7.36	3680		
Poloxamer 237	RM000313	6.38	3190		
Sodium metabisulphite	RM1004933	0.50	250		
Ketoconazole	RM000242	2.00	1000		
Solvent system (pH 3.50):		66.76	33380		
Polysorbate 80	1043695	1.00	500		
Ethanol	-	6.34	3170		
Citric acid	241460 184	2.01	1005		
Disodium hydrogen phosphate	35716	0.81	405		
HPLC grade water	-	56.60	28300		

IN VITRO RELEASE PROFILE



GEL RESPONSES

KZ content: 104.22%

Viscosity 22°C: 10860 cP

Viscosity 37°C: 340450 cP

pH: 4.07

Biopharmaceutics Research Laboratory
Department of Pharmaceutics, Faculty of Pharmacy
Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

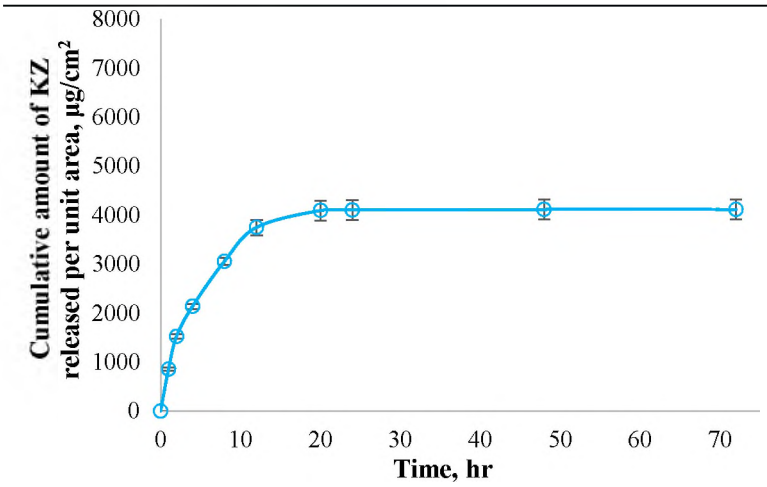
Product: Ketoconazole thermosetting vaginal gel
Batch Size: 50 g
Batch Number: S3

Start of Manufacture: 18/06/2015 13:20
End of Manufacture: 21/06/2015 15:20

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	17.00	8500		
Poloxamer 188	RM000307	7.36	3680		
Poloxamer 237	RM000313	6.38	3190		
Sodium metabisulphite	RM1004933	1.00	500		
Ketoconazole	RM000242	2.00	1000		
Solvent system (pH 3.00):		66.26	33130		
Polysorbate 80	1043695	0.99	495		
Ethanol	-	6.29	3145		
Citric acid	241460 184	2.82	1410		
Disodium hydrogen phosphate	35716	0.80	400		
HPLC grade water	-	55.36	27680		

IN VITRO RELEASE PROFILE



GEL RESPONSES

KZ content: 104.25%

Viscosity 22°C: 11146 cP

Viscosity 37°C: 186400 cP

pH: 3.60

Biopharmaceutics Research Laboratory
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Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

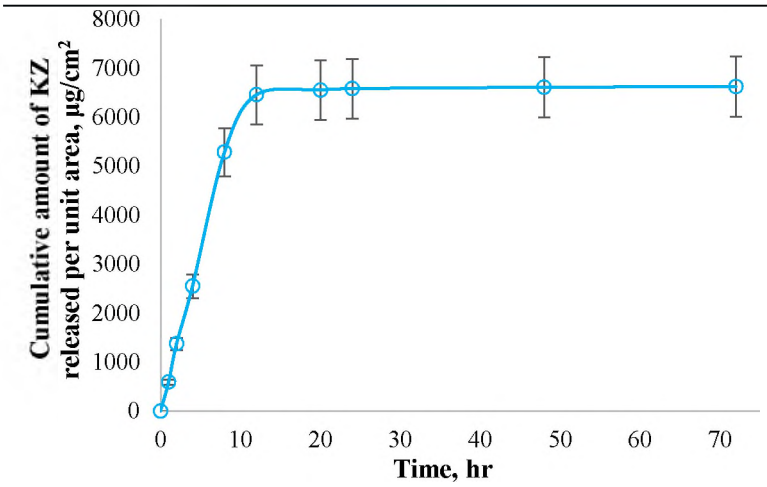
Product: Ketoconazole thermosetting vaginal gel
Batch Size: 50 g
Batch Number: S4

Start of Manufacture: 26/06/2015 09:00
End of Manufacture: 29/06/2015 11:00

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	17.00	8500		
Poloxamer 188	RM000307	7.36	3680		
Poloxamer 237	RM000313	6.38	3190		
Sodium metabisulphite	RM1004933	1.50	250		
Ketoconazole	RM000242	2.00	1000		
Solvent system (pH 3.50):		65.76	33380		
Polysorbate 80	1043695	0.99	495		
Ethanol	-	6.25	3125		
Citric acid	241460 184	1.98	990		
Disodium hydrogen phosphate	35716	0.79	395		
HPLC grade water	-	55.75	27875		

IN VITRO RELEASE PROFILE



GEL RESPONSES

KZ content: 103.05%

Viscosity 22°C: 3966 cP

Viscosity 37°C: 289450 cP

pH: 4.07

Biopharmaceutics Research Laboratory
Department of Pharmaceutics, Faculty of Pharmacy
Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

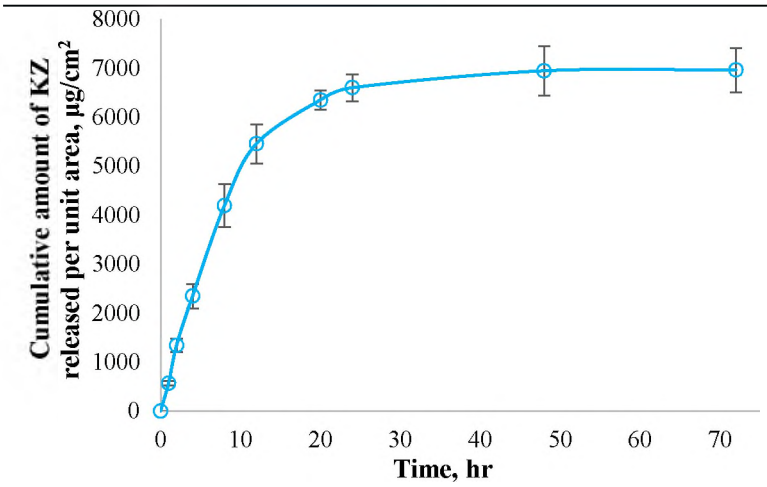
Product: Ketoconazole thermosetting vaginal gel
Batch Size: 50 g
Batch Number: S5

Start of Manufacture: 26/06/2015 09:10
End of Manufacture: 29/06/2015 11:10

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	17.00	8500		
Poloxamer 188	RM000307	7.36	3680		
Poloxamer 237	RM000313	6.38	3190		
Sodium metabisulphite	RM1004933	1.50	250		
Ketoconazole	RM000242	2.00	1000		
Solvent system (pH 3.00):		65.76	33380		
Polysorbate 80	1043695	0.99	495		
Ethanol	-	6.25	3125		
Citric acid	241460 184	2.80	1400		
Disodium hydrogen phosphate	35716	0.79	395		
HPLC grade water	-	54.93	27465		

IN VITRO RELEASE PROFILE



GEL RESPONSES

KZ content: 99.87%

Viscosity 22°C: 4138 cP

Viscosity 37°C: 333867 cP

pH: 3.73

Biopharmaceutics Research Laboratory
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Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

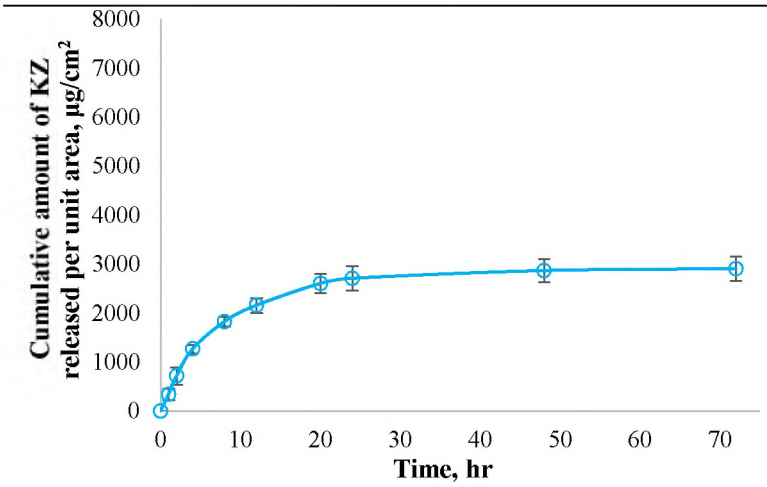
Product: Ketoconazole thermosetting vaginal gel
Batch Size: 50 g
Batch Number: S6

Start of Manufacture: 06/08/2015 09:00
End of Manufacture: 09/08/2015 11:00

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	17.00	8500		
Poloxamer 188	RM000307	7.36	3680		
Poloxamer 237	RM000313	6.38	3190		
Sodium metabisulphite	RM1004933	0.50	250		
Ketoconazole	RM000242	2.00	1000		
Solvent system (pH 2.50):		66.76	33380		
Polysorbate 80	1043695	1.00	500		
Ethanol	-	6.34	3170		
Citric acid	241460 184	3.67	1835		
Disodium hydrogen phosphate	35716	0.81	405		
HPLC grade water	-	54.94	27470		

IN VITRO RELEASE PROFILE



GEL RESPONSES

KZ content: 100.21%

Viscosity 22°C: 8905 cP

Viscosity 37°C: 341550 cP

pH: 3.34

Biopharmaceutics Research Laboratory
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Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

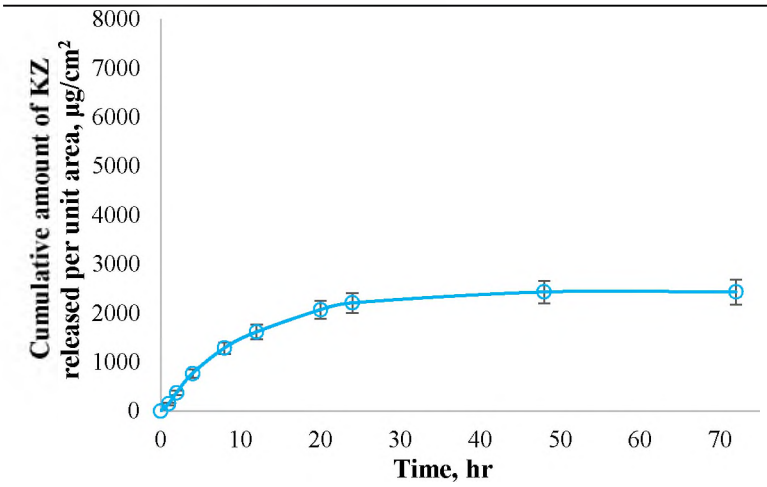
Product: Ketoconazole thermosetting vaginal gel
Batch Size: 50 g
Batch Number: S7

Start of Manufacture: 06/08/2015 09:10
End of Manufacture: 09/08/2015 11:10

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	17.00	8500		
Poloxamer 188	RM000307	7.36	3680		
Poloxamer 237	RM000313	6.38	3190		
Sodium metabisulphite	RM1004933	1.50	250		
Ketoconazole	RM000242	2.00	1000		
Solvent system (pH 2.50):		65.76	33380		
Polysorbate 80	1043695	0.99	495		
Ethanol	-	6.25	3125		
Citric acid	241460 184	3.62	1810		
Disodium hydrogen phosphate	35716	0.79	395		
HPLC grade water	-	54.11	27055		

IN VITRO RELEASE PROFILE



GEL RESPONSES

KZ content: 104.22%

Viscosity 22°C: 8102 cP

Viscosity 37°C: 306400 cP

pH: 3.74

Biopharmaceutics Research Laboratory
Department of Pharmaceutics, Faculty of Pharmacy
Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

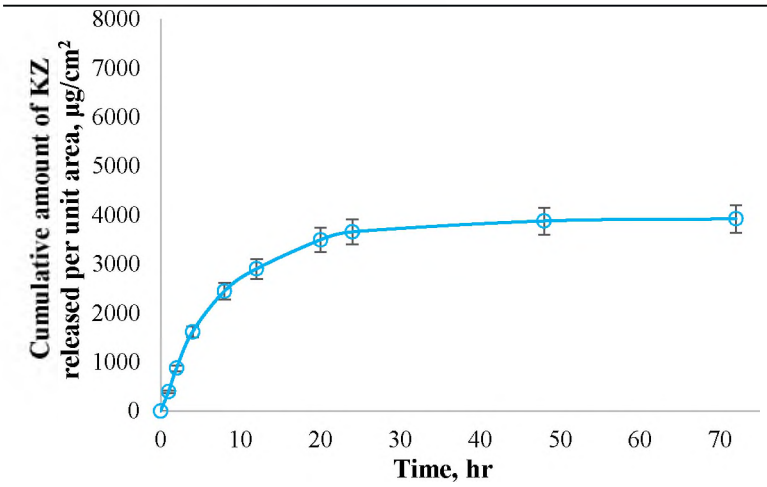
Product: Ketoconazole thermosetting vaginal gel
Batch Size: 50 g
Batch Number: S8

Start of Manufacture: 11/08/2015 10:00
End of Manufacture: 14/08/2015 12:00

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	17.00	8500		
Poloxamer 188	RM000307	7.36	3680		
Poloxamer 237	RM000313	6.38	3190		
Sodium metabisulphite	RM1004933	1.00	500		
Ketoconazole	RM000242	2.00	1000		
Solvent system (pH 2.50):		66.26	33130		
Polysorbate 80	1043695	0.99	495		
Ethanol	-	6.29	3145		
Citric acid	241460 184	3.65	1825		
Disodium hydrogen phosphate	35716	0.80	400		
HPLC grade water	-	54.53	27265		

IN VITRO RELEASE PROFILE



GEL RESPONSES

KZ content: 97.86%

Viscosity 22°C: 7725 cP

Viscosity 37°C: 328968 cP

pH: 3.51

Biopharmaceutics Research Laboratory
Department of Pharmaceutics, Faculty of Pharmacy
Rhodes University
Grahamstown 6140, South Africa

BATCH SUMMARY RECORD 1/1

Formulation Scientist: Ashmita Ramanah

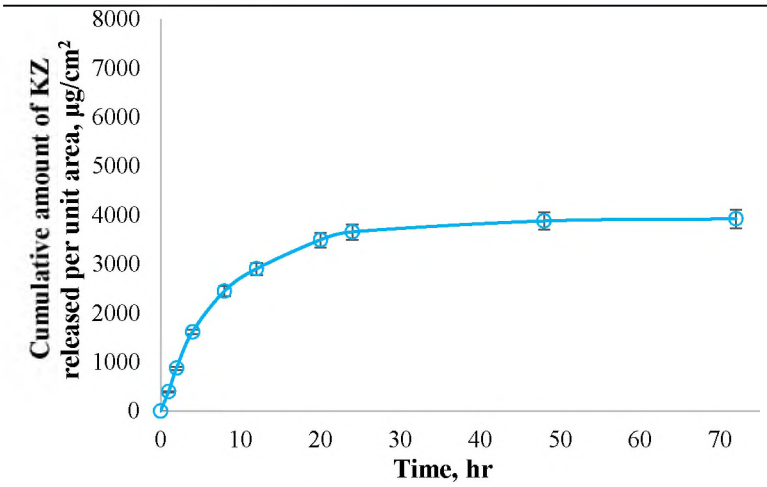
Product: Ketoconazole thermosetting vaginal gel
Batch Size: 50 g
Batch Number: S9

Start of Manufacture: 11/08/2015 10:00
End of Manufacture: 14/08/2015 12:00

FORMULATION

Materials	Batch No.	Original Formula (% m/m)	Working Formula (mg)	Dispensed by	Checked by
Poloxamer 407	RM000175	17.00	8500		
Poloxamer 188	RM000307	7.36	3680		
Poloxamer 237	RM000313	6.38	3190		
Sodium metabisulphite	RM1004933	1.00	500		
Ketoconazole	RM000242	2.00	1000		
Solvent system (pH 3.50):		66.26	33130		
Polysorbate 80	1043695	0.99	495		
Ethanol	-	6.29	3145		
Citric acid	241460 184	1.99	995		
Disodium hydrogen phosphate	35716	0.80	400		
HPLC grade water	-	56.19	28095		

IN VITRO RELEASE PROFILE



GEL RESPONSES

KZ content: 100.67%

Viscosity 22°C: 5297 cP

Viscosity 37°C: 264500 cP

pH: 4.08

APPENDIX J

3D SURFACE PLOTS FROM STABILITY STUDIES

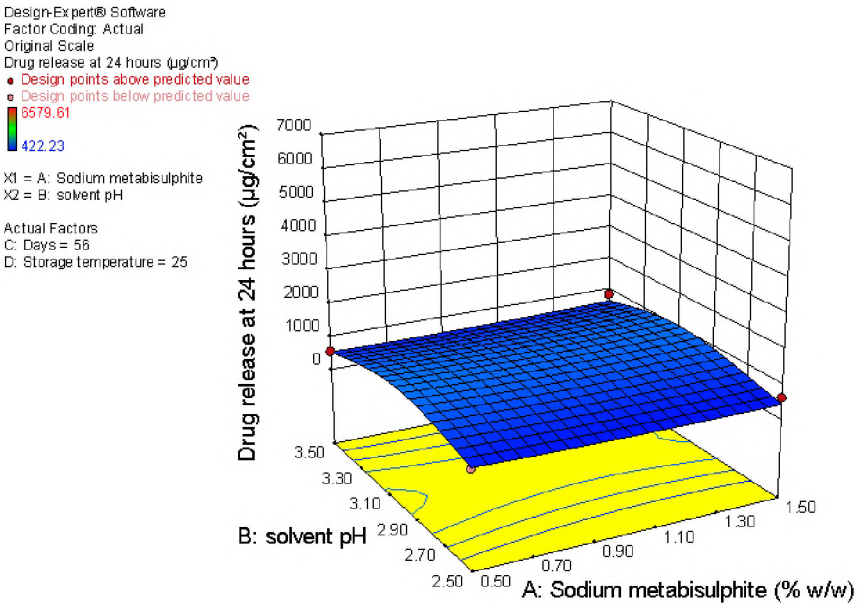


Figure J1 3D response surface plot depicting the impact of SM content and solvent pH at 25 ºC on KZ release at 24 hours.

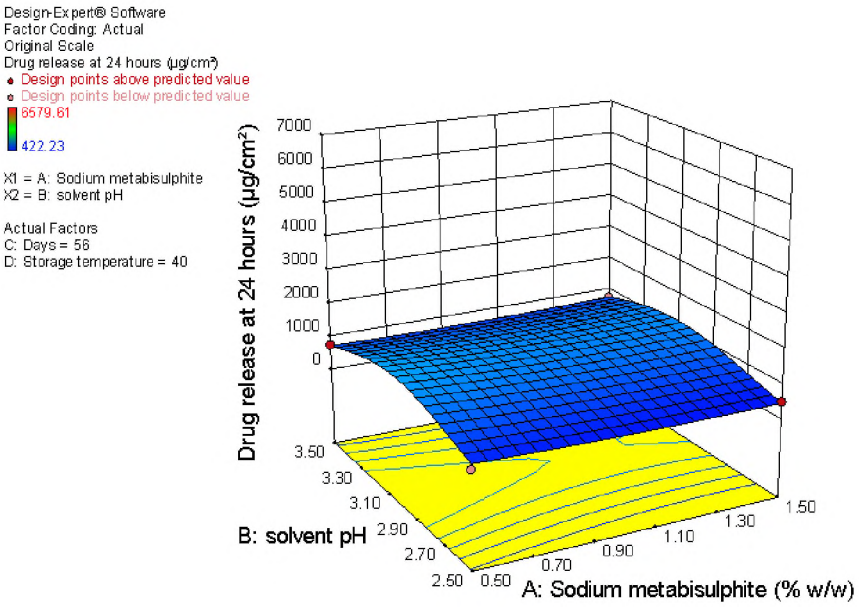


Figure J2 3D response surface plot depicting the impact of SM content and solvent pH at 40 ºC on KZ release at 24 hours.

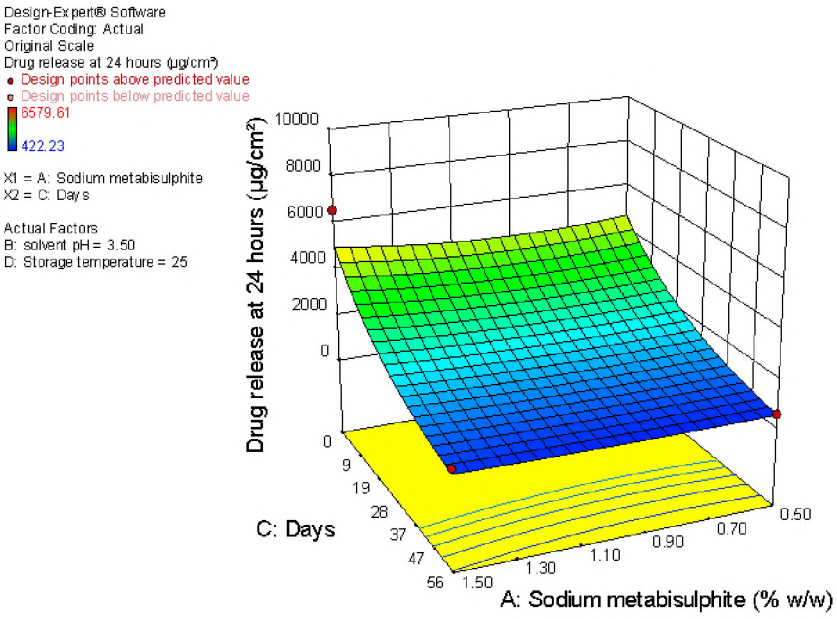


Figure J3 3D response surface plot depicting the impact of SM content and length of storage at 25 ºC on KZ release at 24 hours.

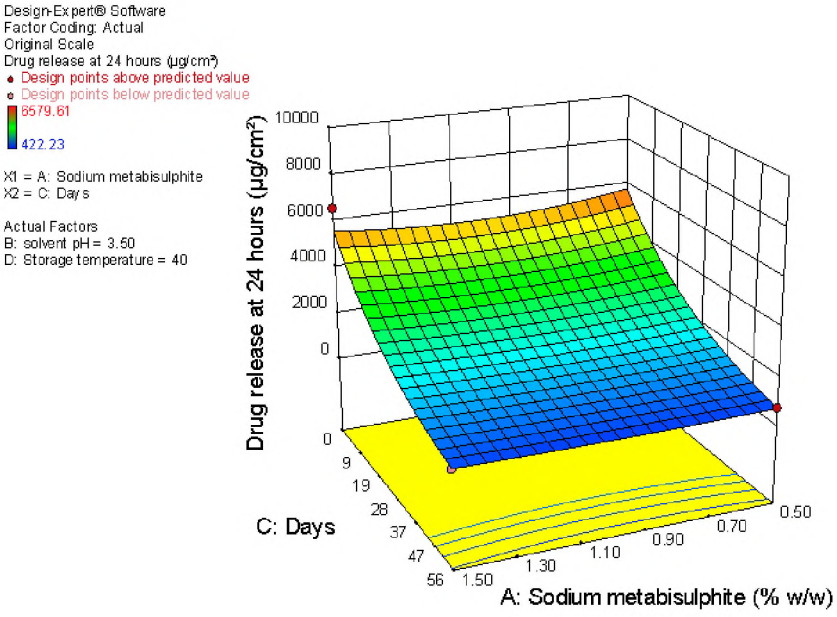


Figure J4 3D response surface plot depicting the impact of SM content and length of storage at 40 ºC on KZ release at 24 hours.

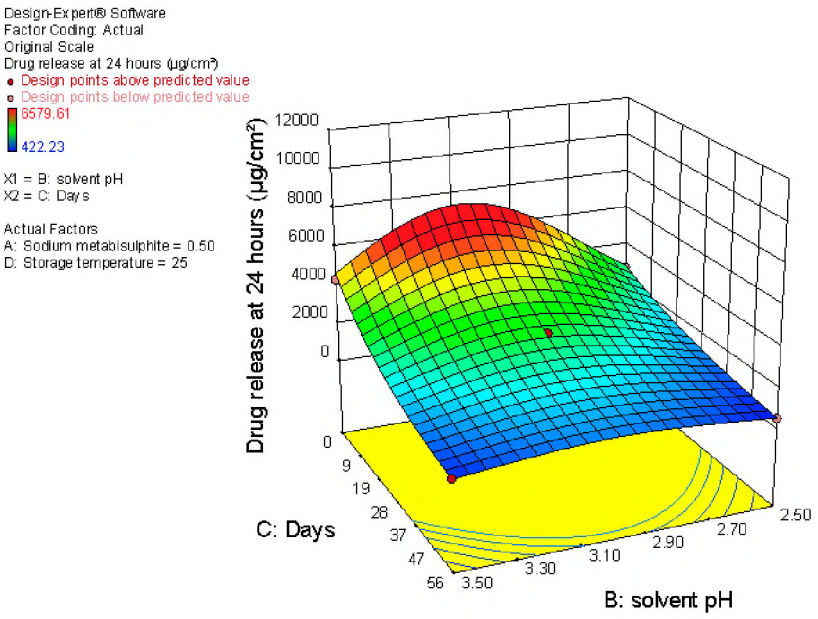


Figure J5 3D response surface plot depicting the impact of solvent pH and length of storage at 25 ºC on KZ release at 24 hours.

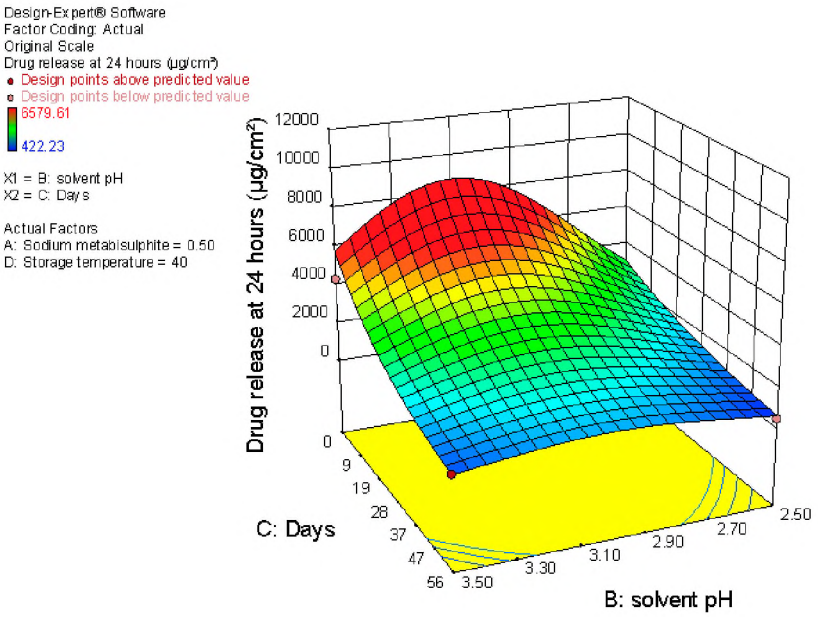


Figure J6 3D response surface plot depicting the impact of solvent pH and length of storage at 40 ºC on KZ release at 24 hours.

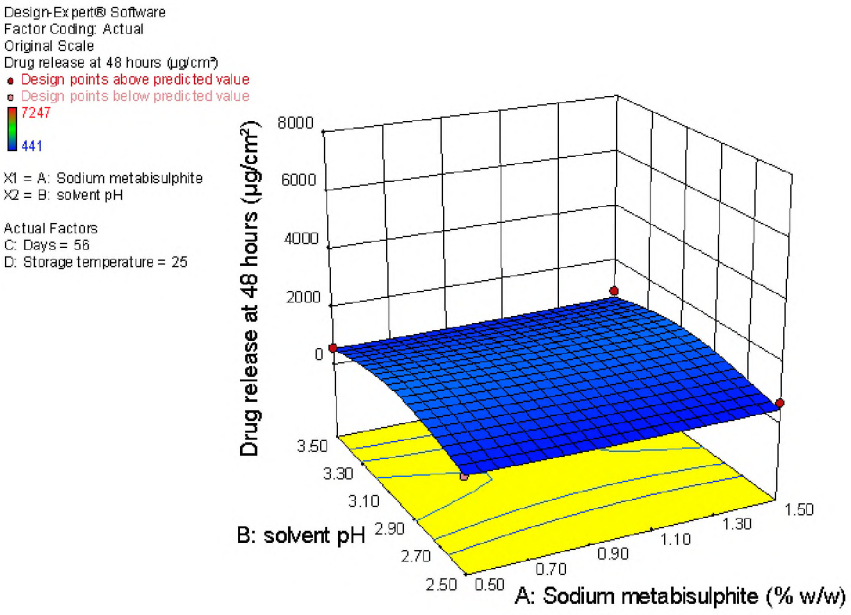


Figure J7 3D response surface plot depicting the impact of SM content and solvent pH at 25 ºC on KZ release at 48 hours.

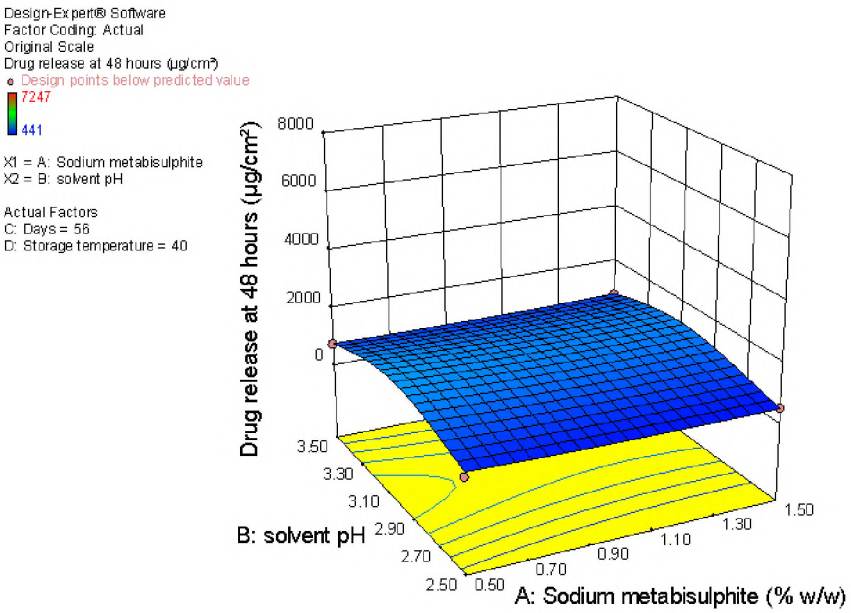


Figure J8 3D response surface plot depicting the impact of SM content and solvent pH at 40 ºC on KZ release at 48 hours.

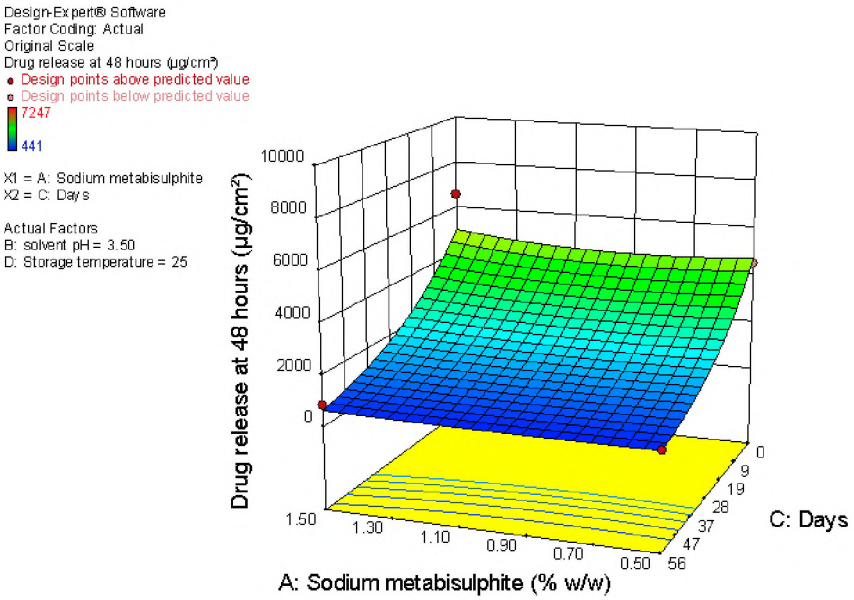


Figure J9 3D response surface plot depicting the impact of SM content and length of storage at 25 ºC on KZ release at 48 hours.

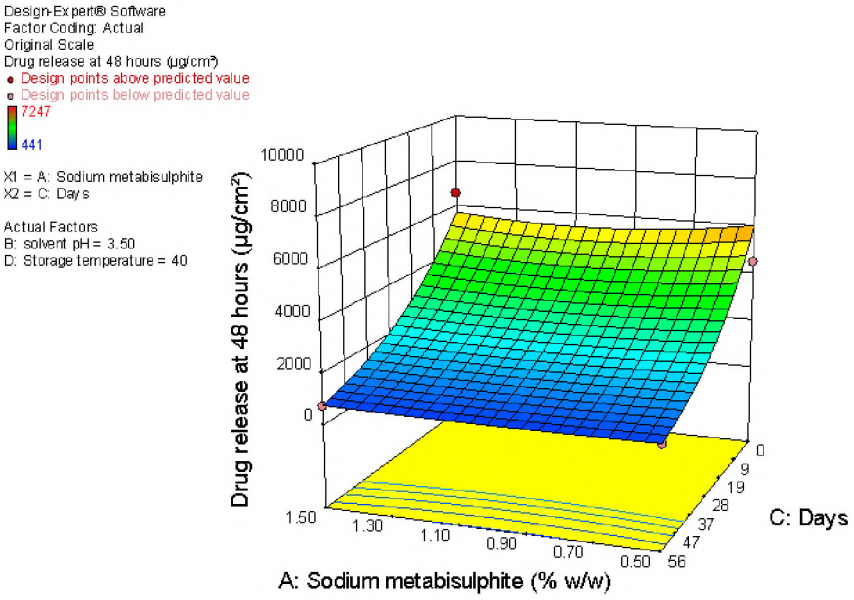


Figure J10 3D response surface plot depicting the impact of SM content and length of storage at 40 ºC on KZ release at 48 hours.

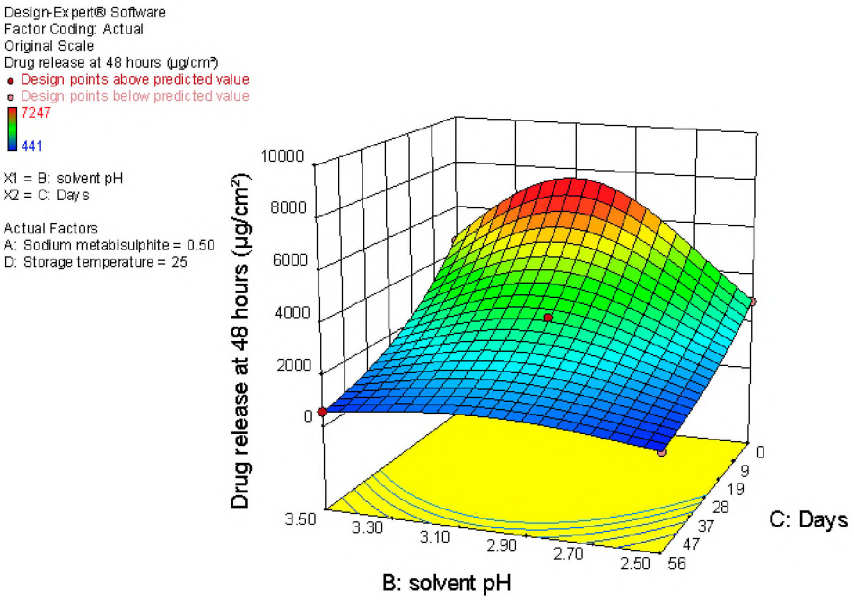


Figure J11 3D response surface plot depicting the impact of solvent pH and length of storage at 25 ºC on KZ release at 48 hours.

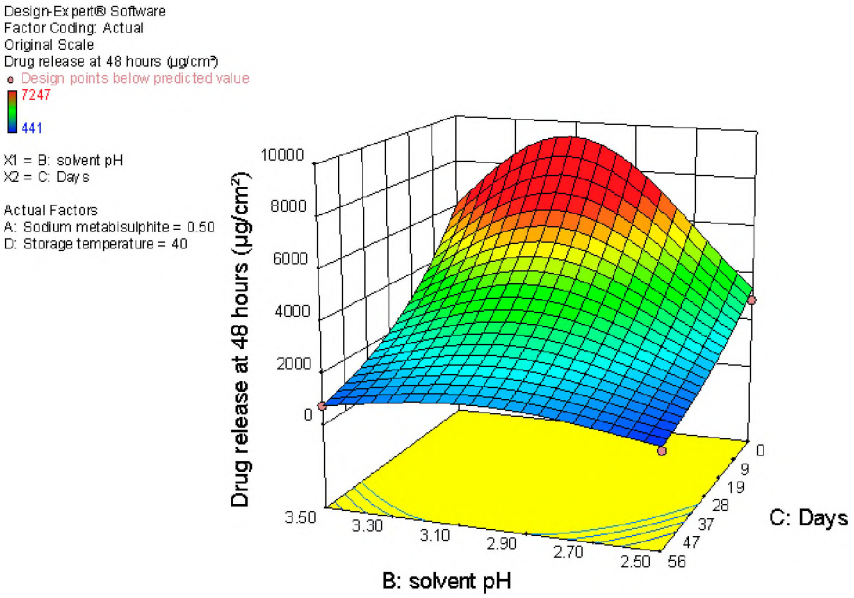


Figure J12 3D response surface plot depicting the impact of solvent pH and length of storage at 40 ºC on KZ release at 48 hours.

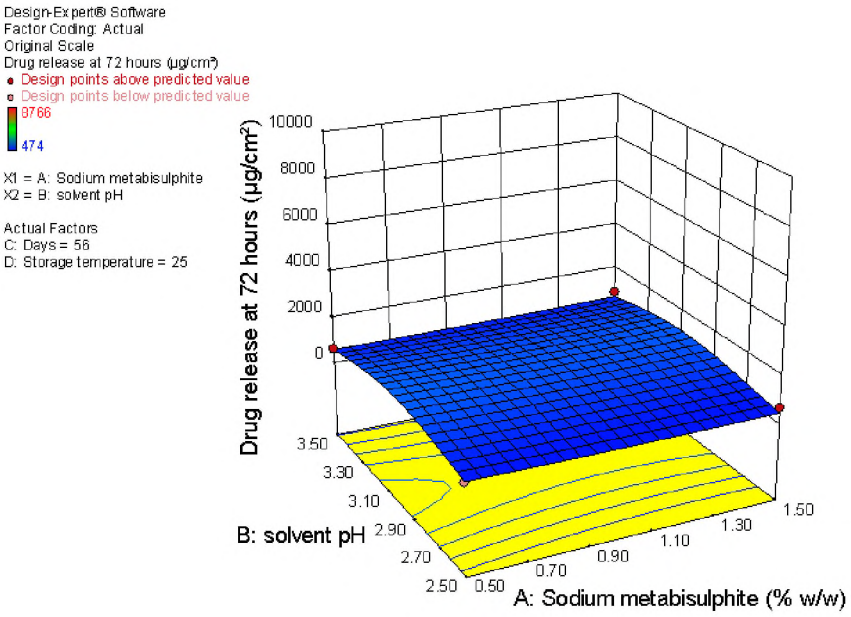


Figure J13 3D response surface plot depicting the impact of SM content and solvent pH at 25 ºC on KZ release at 72 hours.

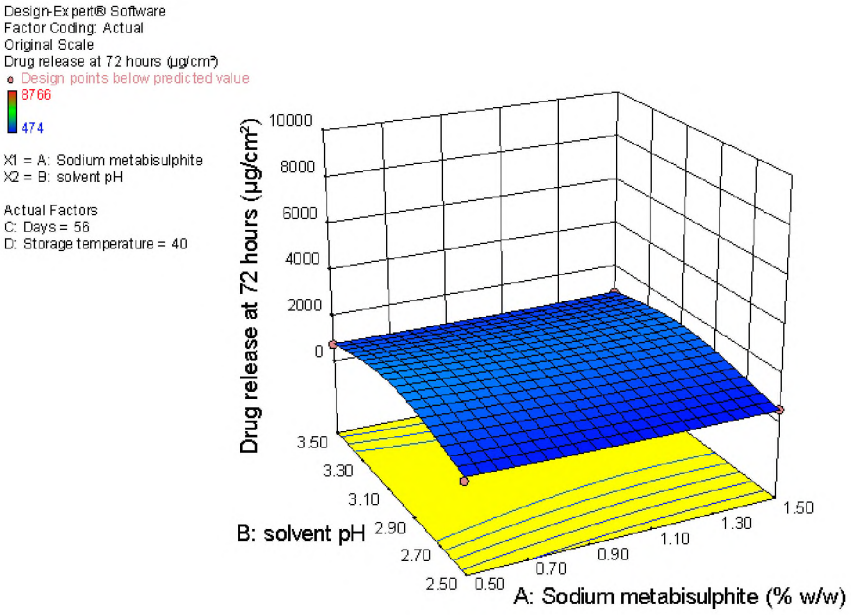


Figure J14 3D response surface plot depicting the impact of SM content and solvent pH at 40 ºC on KZ release at 72 hours.

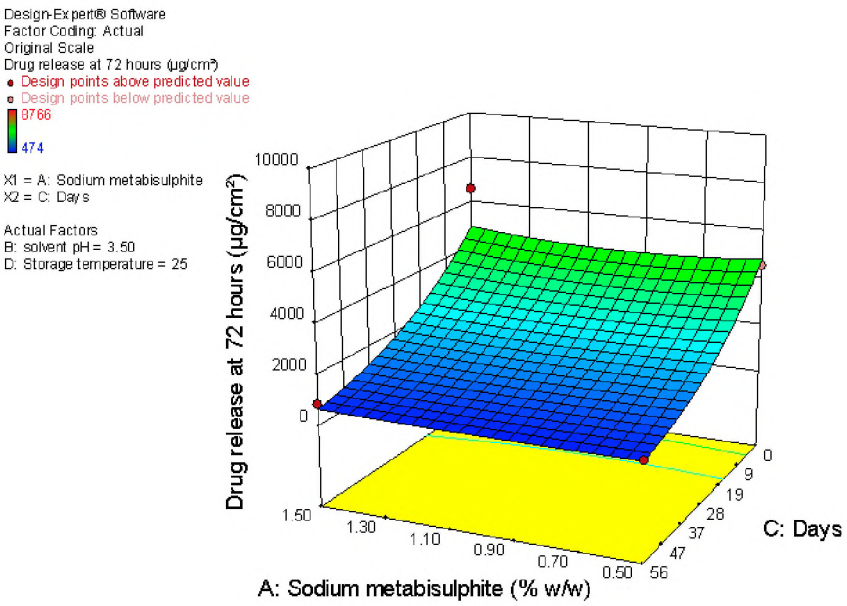


Figure J15 3D response surface plot depicting the impact of SM content and length of storage at 25 ºC on KZ release at 72 hours.

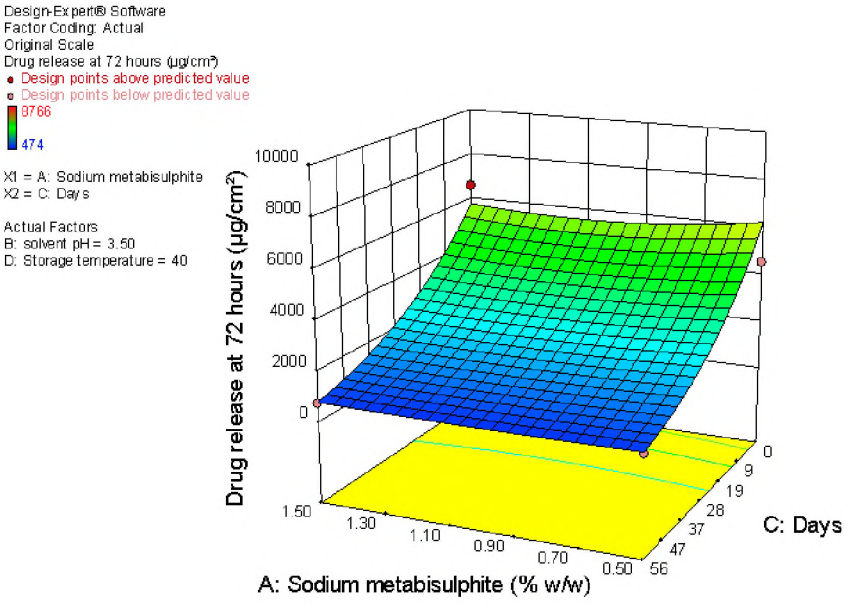


Figure J16 3D response surface plot depicting the impact of SM content and length of storage at 40 ºC on KZ release at 72 hours.

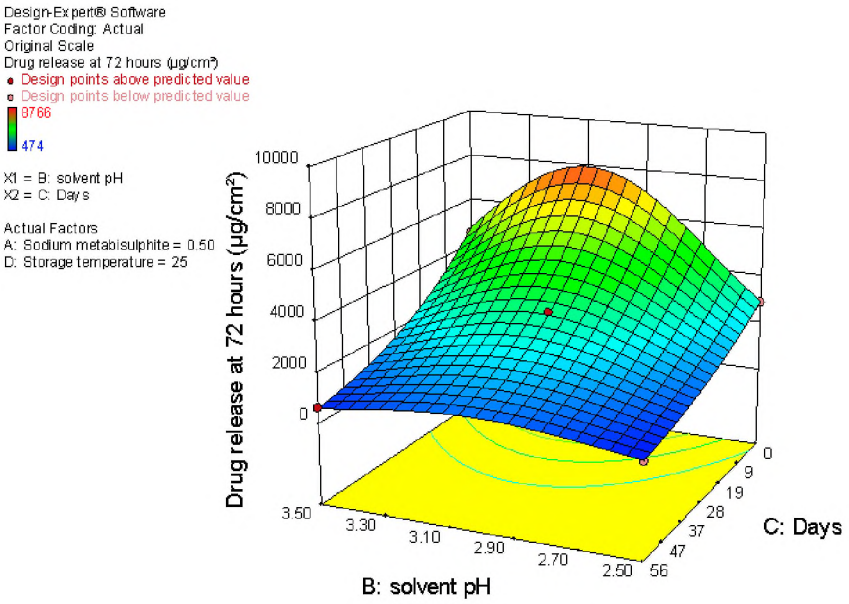


Figure J17 3D response surface plot depicting the impact of solvent pH and length of storage at 25 ºC on KZ release at 72 hours.

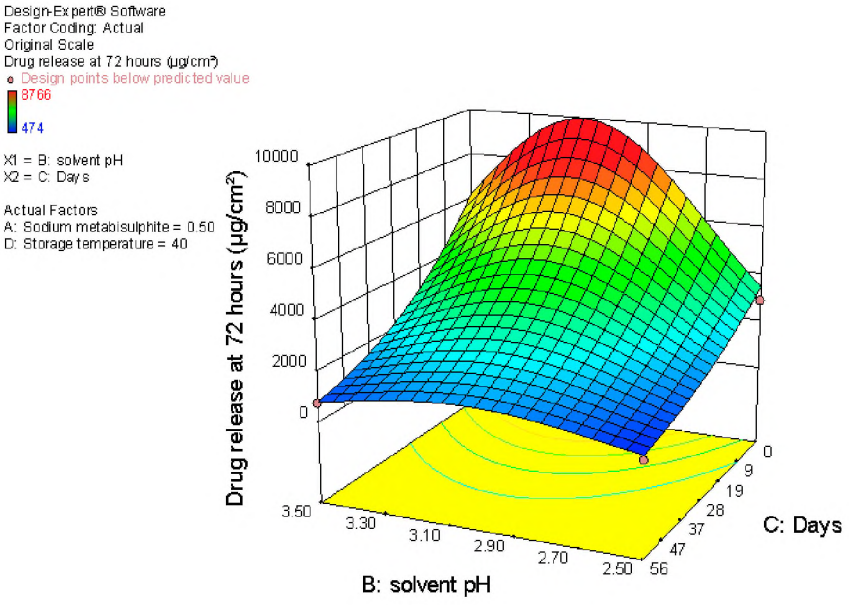


Figure J18 3D response surface plot depicting the impact of solvent pH and length of storage at 40 ºC on KZ release at 72 hours.

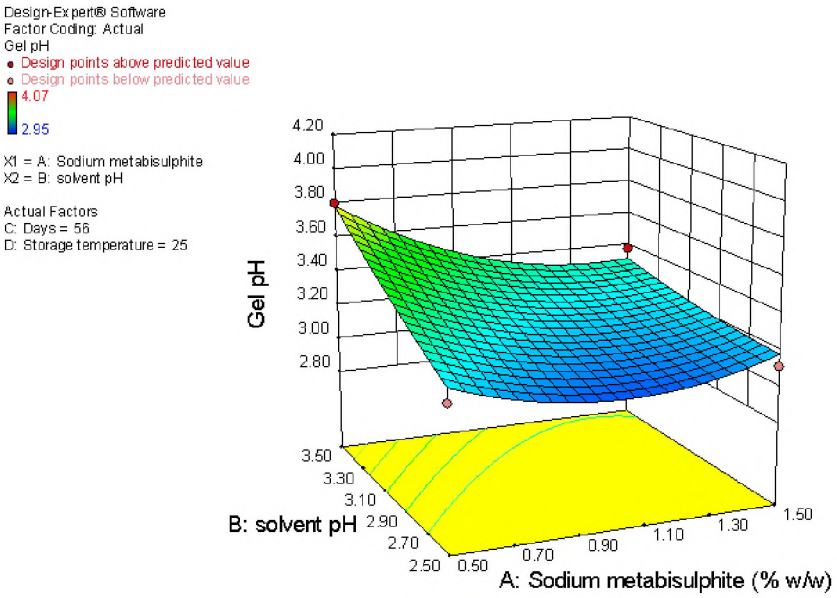


Figure J19 3D response surface plot depicting the impact of SM content and solvent pH at 25 ºC on gel pH.

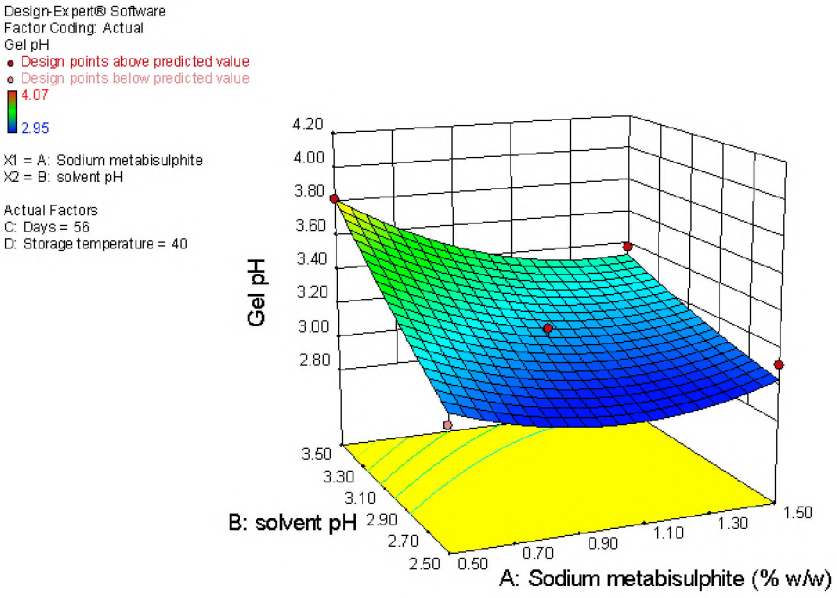


Figure J20 3D response surface plot depicting the impact of SM content and solvent pH at 40 ºC on gel pH.

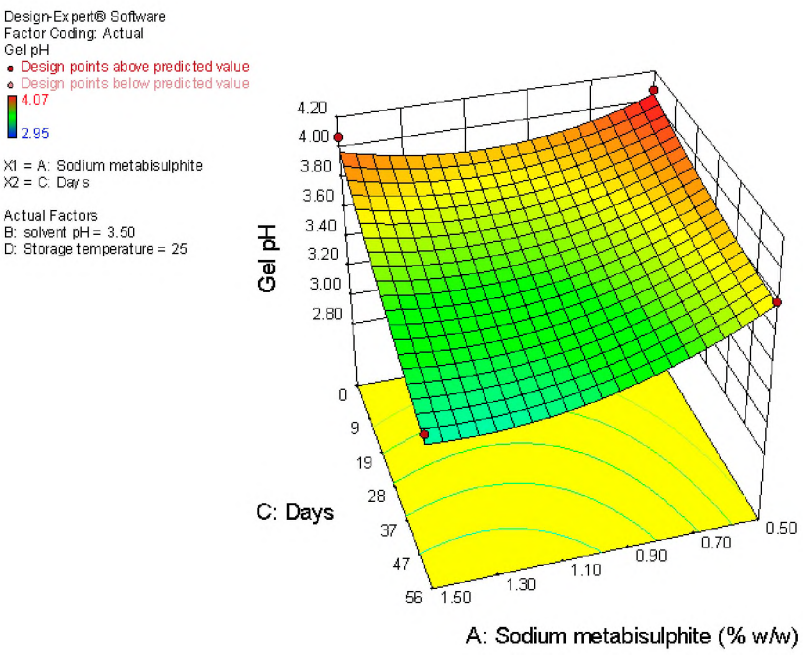


Figure J21 3D response surface plot depicting the impact of SM content and length of storage at 25 ºC on gel pH.

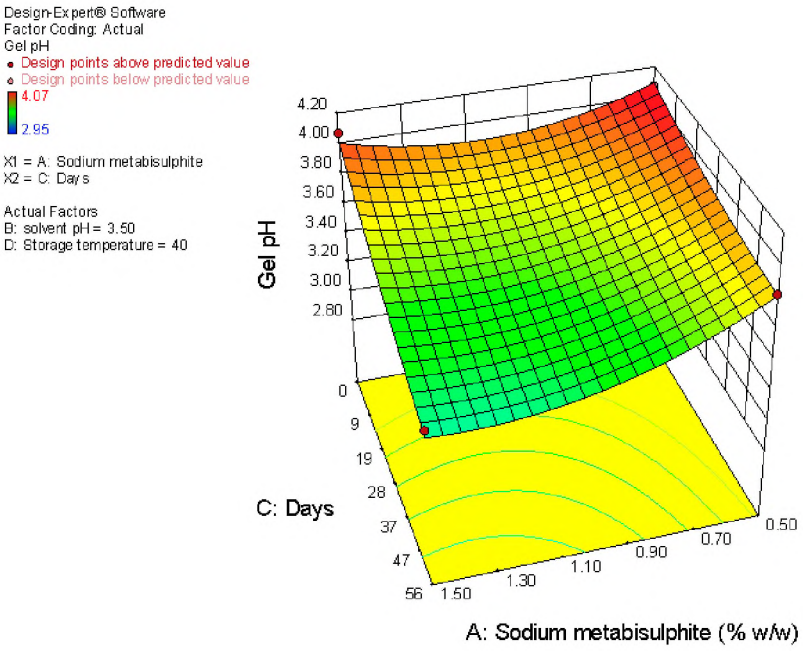


Figure J22 3D response surface plot depicting the impact of SM content and length of storage at 40 ºC on gel pH.

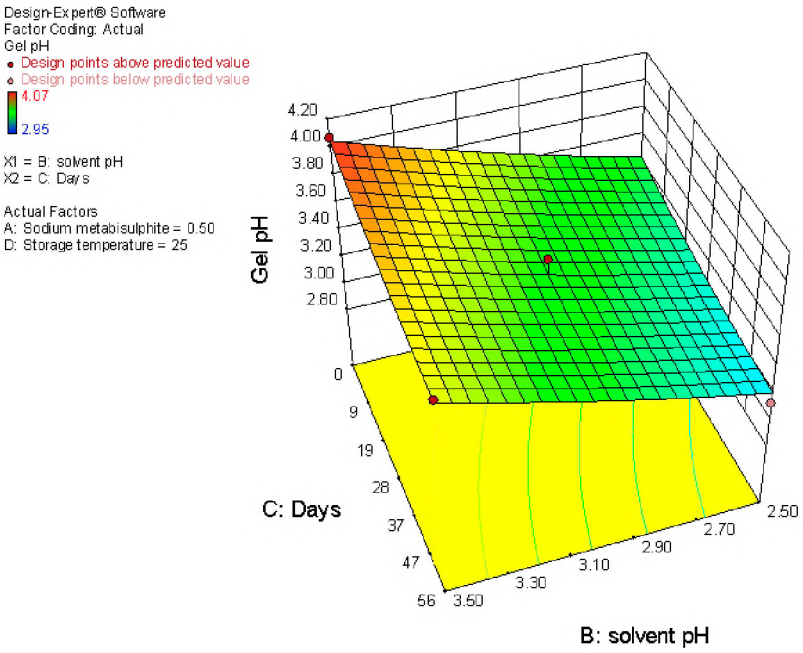


Figure J23 3D response surface plot depicting the impact of solvent pH and length of storage at 25 ºC on gel pH.

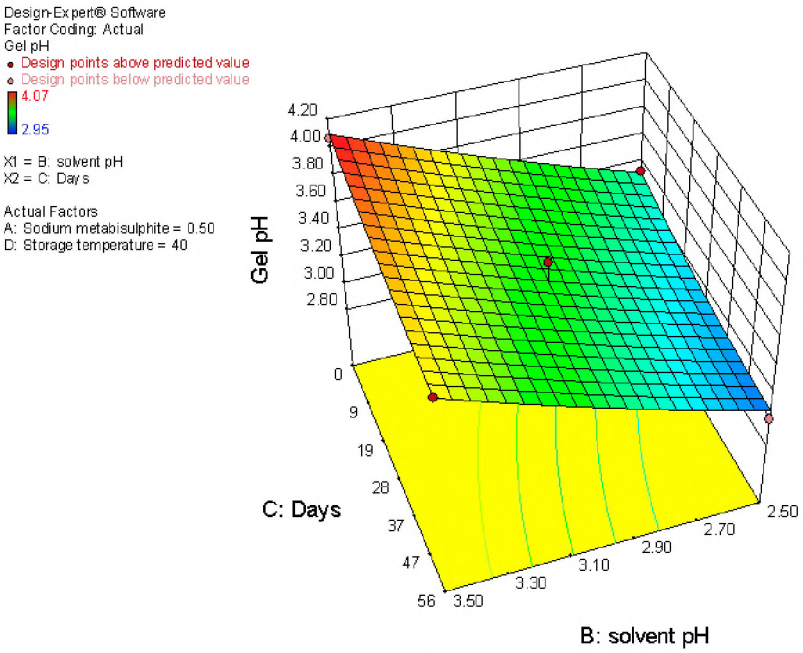


Figure J24 3D response surface plot depicting the impact of solvent pH and length of storage at 40 ºC on gel pH.

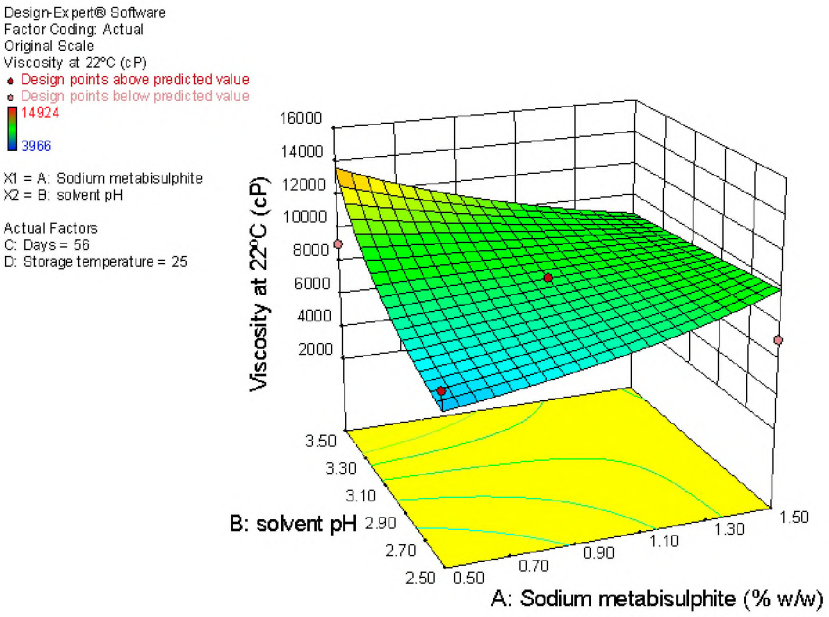


Figure J25 3D response surface plot depicting the impact of SM content and solvent pH at 25 ºC on viscosity at 22 ºC.

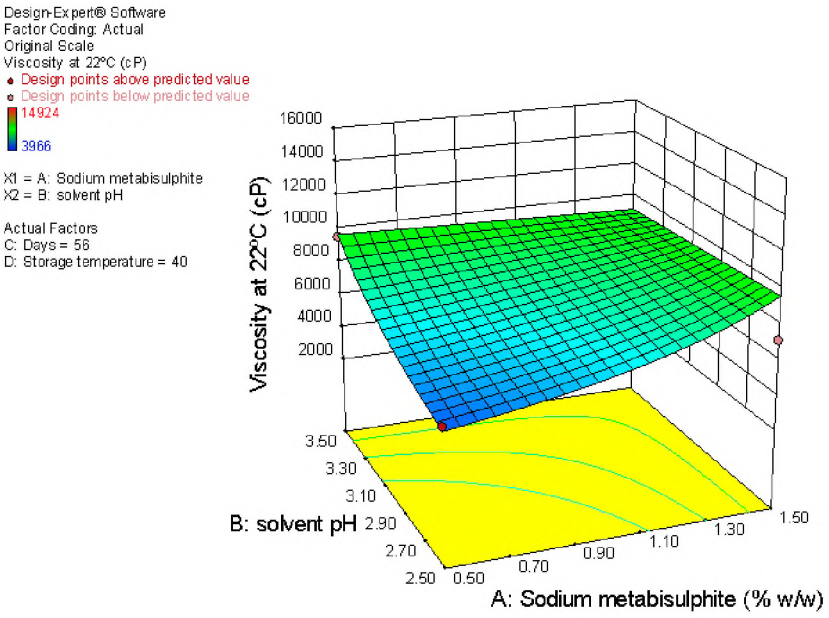


Figure J26 3D response surface plot depicting the impact of SM content and solvent pH at 40 ºC on viscosity at 22 ºC.

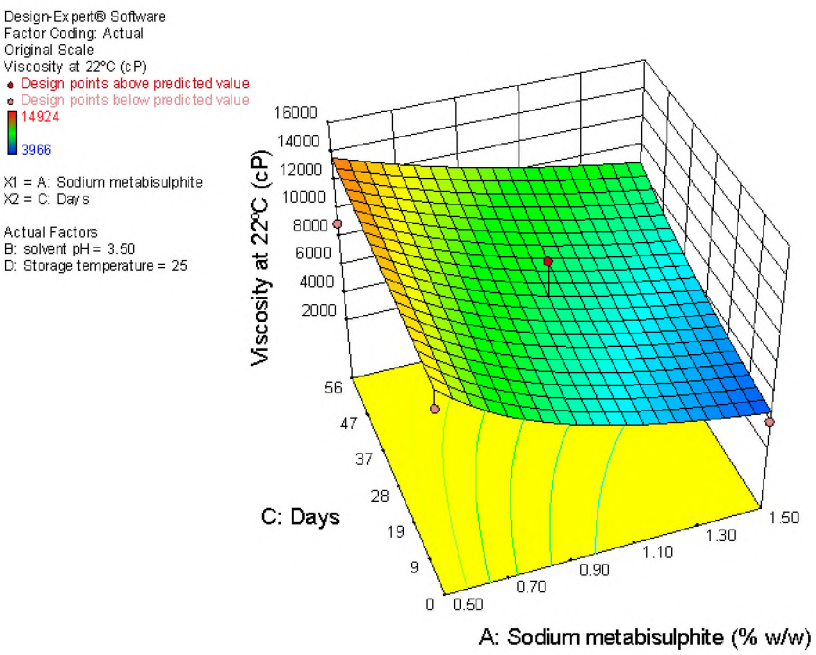


Figure J27 3D response surface plot depicting the impact of SM content and length of storage at 25 ºC on viscosity at 22 ºC.

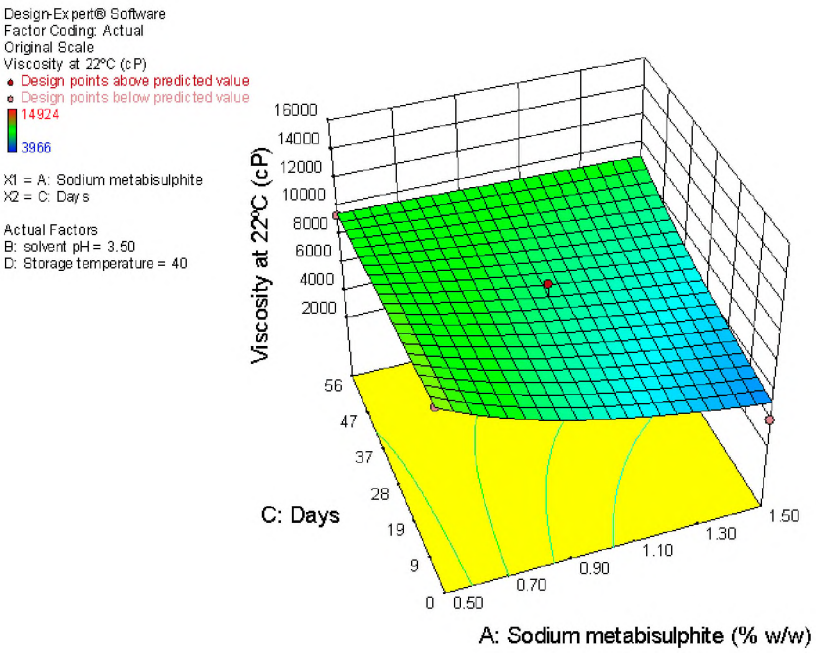


Figure J28 3D response surface plot depicting the impact of SM content and length of storage at 40 ºC on viscosity at 22 ºC.

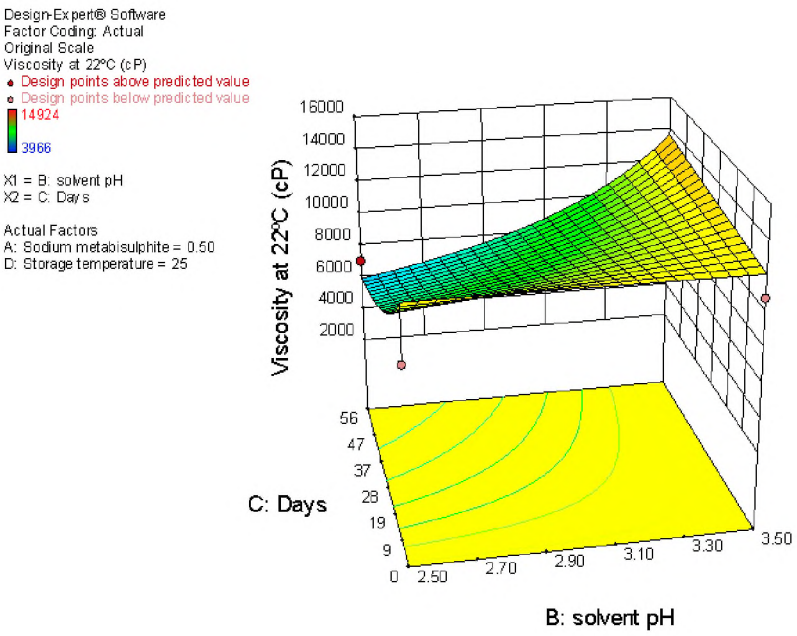


Figure J29 3D response surface plot depicting the impact of solvent pH and length of storage at 25 ºC on viscosity at 22 ºC.

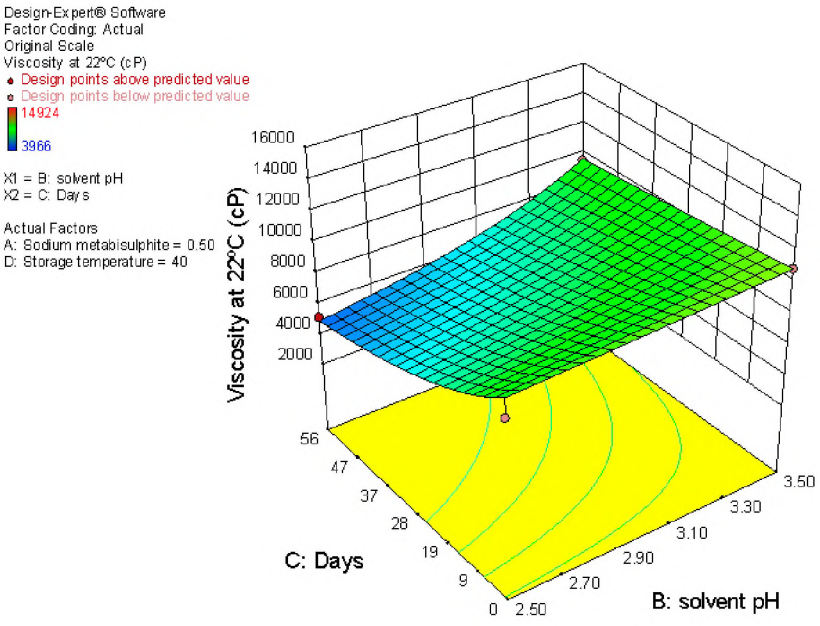


Figure J30 3D response surface plot depicting the impact of solvent pH and length of storage at 40 ºC on viscosity at 22 ºC.

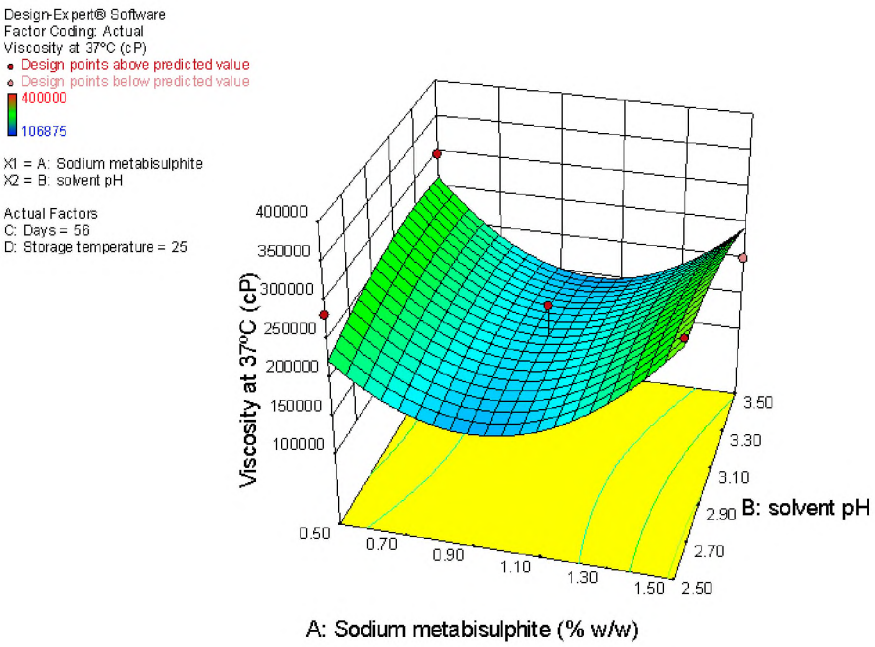


Figure J31 3D response surface plot depicting the impact of solvent pH and SM content at 25 ºC on viscosity at 37 ºC.

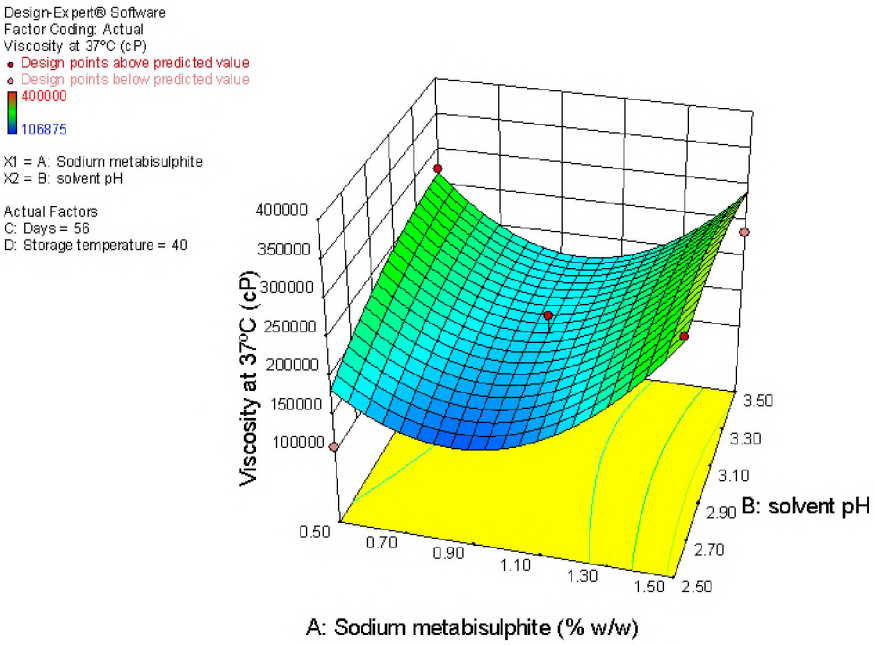


Figure J32 3D response surface plot depicting the impact of solvent pH and SM content at 40 ºC on viscosity at 37 ºC.

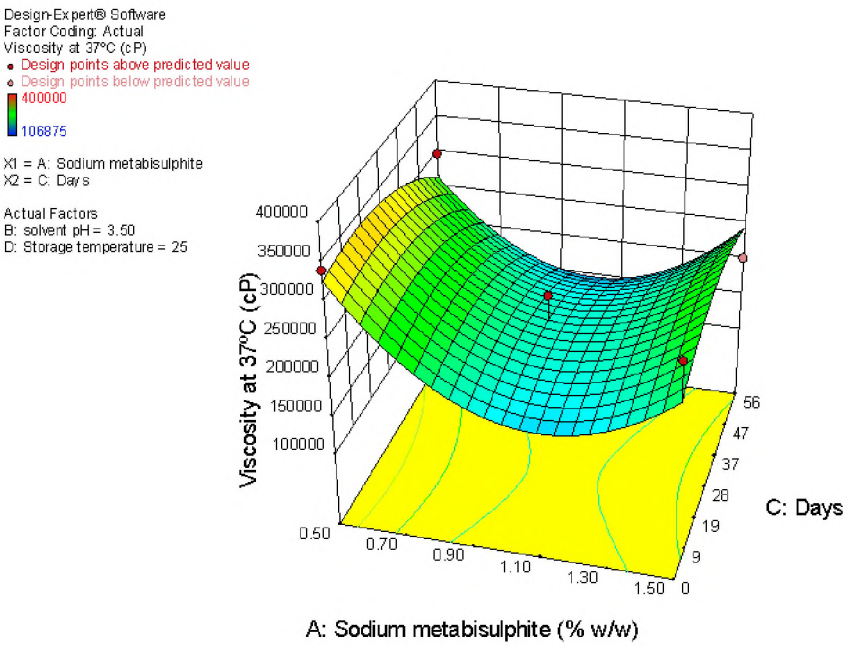


Figure J33 3D response surface plot depicting the impact of SM content and length of storage at 25 ºC on viscosity at 37 ºC.

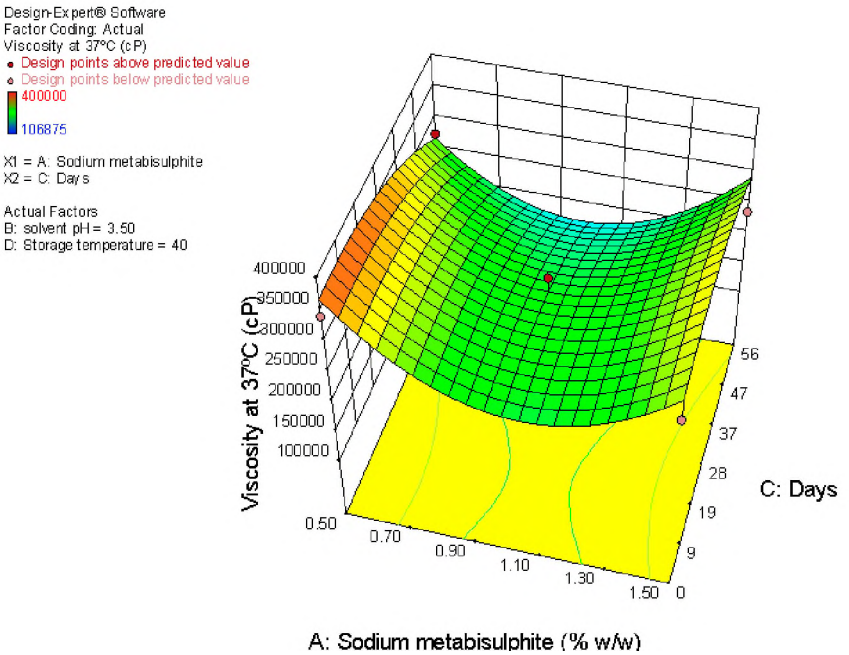


Figure J34 3D response surface plot depicting the impact of SM content and length of storage at 40 ºC on viscosity at 37 ºC.

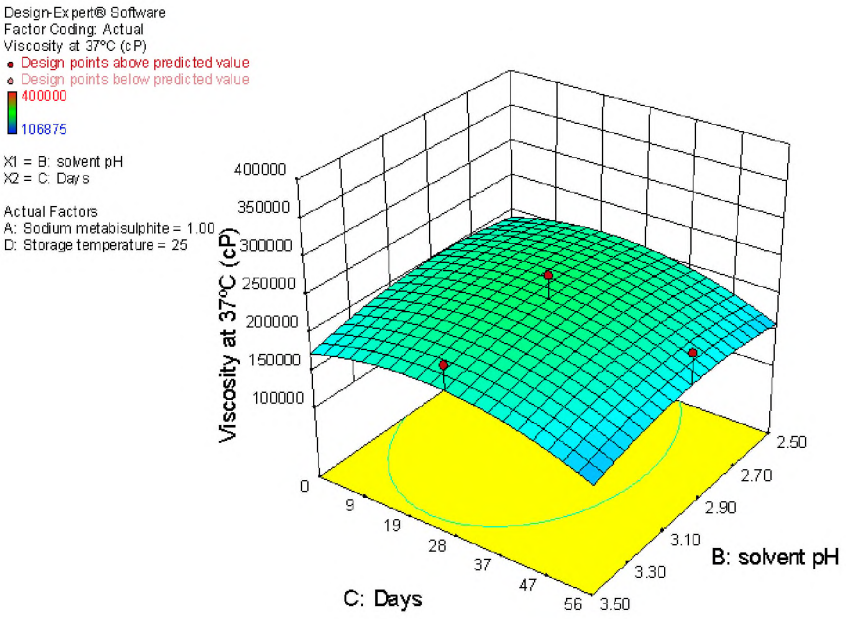


Figure J35 3D response surface plot depicting the impact of solvent pH and length of storage at 25 ºC on viscosity at 37 ºC.

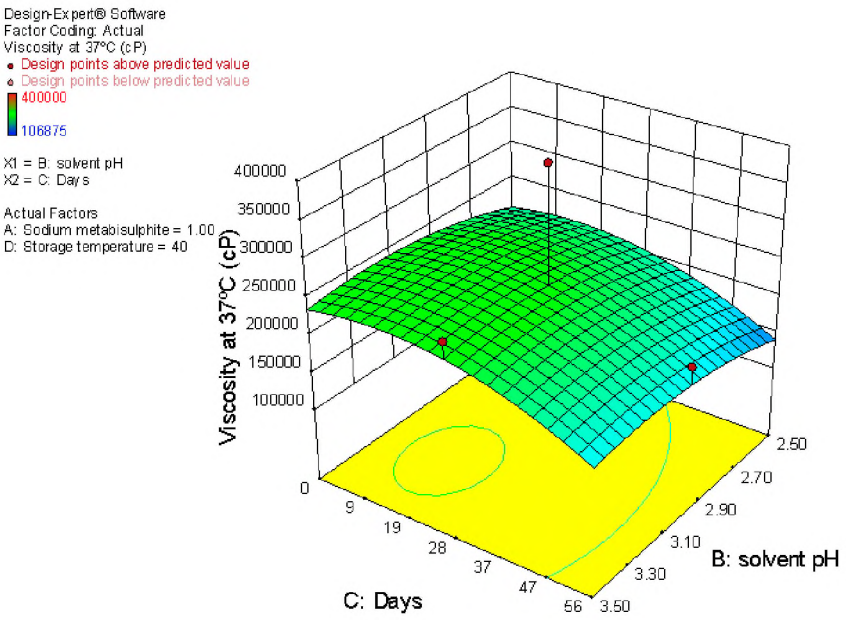


Figure J36 3D response surface plot depicting the impact of solvent pH and length of storage at 40 ºC on viscosity at 37 ºC.