Research Article

iMedPub Journals
http://www.imedpub.com

Journal of Biomedical Sciences
ISSN 2254-609X

2017
Vol. 6 No. 1:12

DOI:10.21767/2254-609X.100056

A Potential *In Situ* Gel Formulation Loaded with Novel Fabricated PLGA Nanoparticles for Enhancing and Sustaining the Ophthalmic Delivery of Ketoconazole

Tarek A Ahmed[1,2] and Bader M Aljaeid[1]

1 Department of Pharmaceutics, Faculty of Pharmacy, King Abdulaziz University, Jeddah, KSA
2 Department of Pharmaceutics & Industrial Pharmacy, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt

Corresponding author:
Dr. Tarek Abdelnapy Ahmed

dr_tarek_nour@yahoo.com

Associate Professor of Pharmaceutics and Industrial Pharmacy, Department of Pharmaceutics, Faculty of Pharmacy, King Abdulaziz University, Jeddah, KSA.

Tel: 96626400000/22250

Citation: Ahmed TA, Aljaeid BM. A Potential *In Situ* Gel Formulation Loaded with Novel Fabricated PLGA Nanoparticles for Enhancing and Sustaining the Ophthalmic Delivery of Ketoconazole. J Biomedical Sci. 2017, 6:1.

Abstract

Oral ketoconazole therapy is commonly associated with serious hepatotoxicity. Improving ocular drug delivery could be sufficient to treat eye fungal infections. The purpose was to develop an optimized ketoconazole poly lactide-co-glycolide (PLGA) nanoparticles (NPs) with subsequent loading into *in situ* gel (ISG) formulation for ophthalmic drug delivery. Three formulation factors were optimized for their effect on particle size (Y1) and entrapment efficiency (Y2) utilizing central composite experimental design. Interaction among components was studied using differential scanning calorimetry (DSC) and fourier transform infrared (FTIR). Ketoconazole crystalline state was studied using powder X-ray diffractometer (XRD). Six different polymeric ISG formulations were prepared and loaded either with optimized NPs or pure drug. The prepared ISG formulations were characterized for *in vitro* gelation, drug release and antifungal activity. The permeation through human epithelial cell line was also investigated. Results revealed that all the studied formulations parameters were significantly affecting Y1 and Y2 of the developed NPs. DSC and FTIR studies illustrated compatibility among NPs components while there was change from the crystalline to amorphous state in the NPs. The *in vitro* release from the ISG formulations loaded with drug NPs showed sustained and enhanced drug release when compared to pure drug formulations. Also, ISG loaded with NPs showed enhanced anti-fungal activity when compared to pure drug formulations. Alginate-chitosan ISG formulation loaded with optimized ketoconazole NPs illustrated higher drug permeation through epithelial cell lines and is considered as an effective ophthalmic drug delivery in treatment of fungal eye infections.

Keywords: Ketoconazole; PLGA; Nanoparticles; ISG; Ophthalmic delivery

Received: December 07, 2016; **Accepted:** December 27, 2016; **Published:** January 03, 2017

Introduction

Ketoconazole is a synthetic imidazole drug used primarily to treat different types of body fungal infections. The drug works by inhibiting the enzyme cytochrome P450 14 α-demethylase which contributes in the synthesis of the fungal ergosterol [1]. Recently, the U.S. Food and Drug Administration (FDA) announced a warning that oral treatment with ketoconazole can cause severe liver damages and also adrenal gland problems [2]. Unlike the oral administration, topical drug formulations have not been associated with these adverse effects. When ketoconazole is administered for treatment of fungal eye infection, it is characterized by a very short duration of action since its elimination half-life in the aqueous humor and cornea is about 19 and 43 minutes, respectively [3,4]. Although ketoconazole characterized by its high lipid solubility (log P=4.74), which may support permeation through biological membranes especially through the corneal epithelium, yet the drug high molecular weight (531.44 Da) hinder its transport. Moreover, it is difficult for the drug to present in a solubilized form in the aqueous corneal surface due to the limited aqueous solubility [5].

Ocular drug administration offers lower incidence of systemic drug side effects and drug-drug interactions that are common during systemic treatment. However, due to the unique anatomy and physiology of the eye, ocular drug delivery represents a major challenge during development of effective ophthalmic preparations. The eye is supported with static and dynamic barriers, and efflux pumps that impede drug delivery particularly to the posterior eye segment [6]. Nanoparticulate drug delivery systems have been reported to deliver the administered drug successfully across the different eye segments including the posterior part [5,7]. Polymeric nanoparticles (NPs) especially those that have been developed using biocompatible-biodegradable polymers, such as polycaprolactone, poly (alkyl cyanocaprylate) and poly lactide-co-glycolide (PLGA), are promising ocular delivery system due to enhanced bioavailability and reduced frequency of administration [8,9]. PLGA based NPs are the most suitable polymer nanoparticulate ocular drug delivery owing to ease of fabrication and approval by the FDA for drug delivery [10,11].

The aim of this work was to develop ketoconazole PLGA NPs utilizing novel *in vitro* solvent exchange technique with subsequent loading into ISG formulation to enhance and sustain the ocular drug delivery. The prepared ISG preparations were characterized for *in vitro* gelation, drug release, antifungal activity and permeation through human epithelial cell lines.

Materials and Methods

Materials

Poly (DL-lactide-co-glycolide), ester terminated, inherent viscosity range: 0.55-0.75 dL/g from Lactel absorbable polymers, Duracet corporation (Birmingham, AL USA). Polyvinyl alcohol, cold water soluble, Mol. Wt: 140,000 was purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Ketoconazole was a kind gift from Deef Pharmaceutical Industries Co. (Alqassim, KSA). Poloxamer 407 was supplied from Xi'an Lyphar Biotech Co., LTD (Shaanix Province, China). Chitosan of low molecular weight, sodium alginate and dimethyl sulphoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). Carbopol 940 and hydroxypropyl methyl cellulose (HPMC) MW 86,000, viscosity 4000 cp (2% solution) were procured from Acros Organics (New Jersey, USA).

Methods

Central composite experimental design: Three formulation factors were optimized for their effects on the particle size (Y1) and drug entrapment efficiency (Y2) of the developed NPs utilizing StatGraphics Centurion XV version 15.2.05 software (StatPoint Technologies Inc, Warrenton, VA, USA). **Table 1** illustrates the independent variables, their levels and studied responses. A preliminary study was conducted to identify the levels of the independent variables.

Preparation of the formulation: Based on the composition of the sixteen formulations, obtained from the experimental design, which is depicted in **Table 2**, Drug NPs were prepared by dissolving the calculated amount of PLGA in the specified volume of DMSO to prepare 3-6% organic polymer solution. A constant concentration of ketoconazole (0.1% w/v) based on the total formulation, was dissolved in this organic solution. Aqueous PVA solutions (1-3%) were prepared by dissolving the specified amount of PVA in distilled water under magnetic stirring. The organic drug polymer solution was gradually added through a micropipette into the aqueous PVA solution on a magnetic stirrer. The milky colloidal dispersion obtained was kept stirring for 30 minutes at 1200 rpm and then was probe sonicated (Sonics Vibra cell, VCX 750, USA) for 10 min at 60 magnitudes for better colloidal dispersion. Drug loaded NPs were separated by centrifugation at 15000 rpm for 1 h at 4°C using (Sigma Laboratory centrifuge, 3K30, Ostrode, Germany). The precipitated NPs were subjected to two cycles of washing with double distilled water and centrifugation to remove the remaining of PVA and DMSO. Finally, the separated NPs were freeze dried using (alpha 1-2 LD plus, Christ lyophilizer, Germany).

Characterization of the Prepared NPs

Measurement of NPs particle size

Known weight of the freeze-dried NPs was suspended in double distilled water by vortex and the particle size of the suspended particles was determined by dynamic light scattering using a Zetatrac (Microtrac Inc., York, PA).

Entrapment efficiency

Following centrifugation, the supernatant was filtered through 0.2 µm cellulose acetate membrane filter and the concentration of the drug in the filtrate was determined spectrophotometrically at 231 nm using Jenway 6715 UV-Visible spectrophotometer (Jenway, Stone, UK). The entrapment efficiency (EE) was calculated using the following equation:

$$EE\ (\%)=[(D_{total}-D_{free\ drug})/D_{total}] \times 100$$

Where D_{total} is the total concentration of the drug loaded, and $D_{free\ drug}$ represents the concentration of the free drug in the supernatant.

Central Composite Experimental Design Statistical Analysis and Optimum Desirability

The observed values for both Y1 and Y2, tabulated in **Table 2**, were introduced into the response columns of the StatGraphics optimization software and analyzed to identify the statistical significance of the relationships between the studied variables and obtained responses. A p-value<0.05 was considered significant following statistical analysis of the data. The optimum desirability was calculated, and an optimized formulation was proposed. This formulation, containing the proposed optimum levels for X1, X2 and X3, was prepared and characterized for Y1 and Y2 as previously described, and the differences between predicted and observed values were calculated.

Tables 1 Ketoconazole PLGA NPs independent and dependent variables used in the central composite experimental design.

Independent Variables	Level		
	Low	Medium	High
% PLGA in organic phase (X1)	3.0	4.5	6.0
% Organic to aqueous phase (X2)	4.0	5	6.0
% PVA in aqueous phase (X3)	1.0	2	3.0
Dependent Variables	**Goal**		
Particle Size in nm (Y1)	Minimize		
% Entrapment efficiency (Y2)	Maximize		

Table 2 Experimental runs along with the observed and fitted values for the studied responses.

Run	X1 (%)	X2 (%)	X3 (%)	Y1 (nm)		Y2 (%)	
				Observed	Fitted	Observed	Fitted
1	3.0	6.0	3.0	457.0 ± 0.014	441.092	61.627 ± 8.66	61.0401
2	4.5	5.0	3.682	729.0 ± 0.040	711.058	70.023 ± 6.96	67.7879
3	3.0	4.0	3.0	417.0 ± 0.011	463.84	64.277 ± 0.47	66.7802
4	6.0	6.0	3.0	995.0 ± 0.024	1090.13	67.354 ± 1.11	68.227
5	4.5	3.318	2.0	483.0 ± 0.019	526.614	83.453 ± 3.08	78.0304
6	1.977	5.0	2.0	328.0 ± 0.018	302.979	55.624 ± 0.44	54.9099
7	6.0	4.0	1.0	477.0 ± 0.012	520.366	69.122 ± 1.19	72.6754
8	3.0	4.0	1.0	446.0 ± 0.017	378.33	64.362 ± 0.54	66.4575
9	7.023	5.0	2.0	982.0 ± 0.083	968.19	69.666 ± 0.78	66.182
10	4.5	6.682	2.0	945.0 ± 0.043	862.554	67.524 ± 3.06	68.7485
11	4.5	5.0	0.318	536.0 ± 0.055	515.111	68.765 ± 4.28	66.802
12	6.0	6.0	1.0	962.0 ± 0.066	942.618	66.912 ± 0.51	67.3773
13	3.0	6.0	1.0	356.0± 0.017	457.081	60.911 ± 0.99	60.505
14	6.0	4.0	3.0	843.0 ± 0.047	769.377	69.938 ± 1.19	73.3126
15	4.5	5.0	2.0	885.0 ± 0.183	893.331	67.847 ± 3.10	67.5697
16	4.5	5.0	2.0	895.0 ± 0.174	893.331	66.572 ± 0.96	67.5697

X1: % PLGA in organic phase; X2: % Organic to aqueous phase; X3: % PVA in aqueous phase; Y1: Particle size in nm; Y2: % Entrapment efficiency.

Physicochemical Characterization

Differential scanning calorimetry (DSC)

The thermal behavior of pure ketoconazole, PLGA, PVA and the optimized formulation was studied using Shimadzu DSC TA-50 ESI DSC apparatus (Shimadzu, Tokyo, Japan). Aluminium crucibles containing approximately 2 mg of each sample under a dynamic nitrogen atmosphere (flow rate: 50 mL/min) and at a heating rate of 10°C/min in a temperature range from 25-950°C were used.

Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared (FTIR) spectra of the samples used in the DSC technique were recorded using Nicolet iS10, Thermo Scientific Inc. (Waltham, MA, USA) in the range of 4000-400 cm^{-1}.

X-ray powder diffraction (XRPD)

The crystalline state of the prepared drug NPs compared to that of the pure ketoconazole powder was studied using powder X-ray diffractometer (D/max 2500, Rigaku, Tokyo, Japan). The diffraction patterns of the studied samples were recorded at a scan speed of 0.5000 degree/min.

Preparation of *In Situ* Gel (ISG) Formulations

Different ISG base solutions were prepared by simple dispersion of the polymeric material, specifically sodium alginate (1%), chitosan (0.5%), poloxamer 407 (16%), or carbopol 940 (0.5%) in deionized water with continuous mixing on a magnetic stirrer until complete dissolving of the polymer. Polymeric solution of chitosan was prepared by dispersion the polymer in slightly acidified solution. HPMC in a concentration of 0.5% (w/v) was added to each solution as a viscosity enhancer. A quantity equivalent of NPs, containing 0.3% ketoconazole, was added to the prepared homogenous polymeric dispersion solutions. For comparative study, the same gel base solutions were prepared and loaded with 0.3% pure ketoconazole. ISG formulations containing mixture of the studied polymers were also prepared. A total of twelve ISG preparations were prepared and the composition is illustrated in **Table 3**.

Characterization of the Prepared ISG Formulations

In vitro gelation

The prepared ISG formulations were characterized for *in vitro* gelation by mixing 25 µL of each formulation with 7 µL simulated tear fluid (0.67 g sodium chloride, 0.2 g sodium bicarbonate, 0.008 g calcium chloride dehydrate in deionized water quantity sufficient to 100 ml and the pH was adjusted to 7.4 using hydrochloric acid) at 37°C. A ratio of 25:7 was used to mimic the *in vivo* ocular conditions since the volume of tear in human is estimated to be 7 µL, and the cul-de-sac can transiently contain around 30 µL of fluid [12]. The *in vitro* gelation was evaluated as previously reported [13] by recording the time required for gelation.

In vitro release study

The diffusion of ketoconazole in the form of NPs or pure drug from the prepared ISG formulations was carried out using automated franz diffusion cell apparatus (MicroettePluss™, Hanson Research, Chatsworth, CA, USA). The apparatus is adapted with 1.76 cm^2 of diffusion area. A quantity of ISG equivalent to 400 mg was placed in position, donor chamber, and covered with a synthetic nylon membrane of 0.45 µm pore size. Simulated tear fluid of pH 7.4 was used as a receiver medium in the receptor chamber, the temperature was kept at 32 ± 0.5°C and the stirring rate was adjusted at 400 rpm. Samples from the receptor chamber were automatically withdrawn at 0.25, 0.5, 1, 2, 4, 6 and 8 h with replacement and analyzed for drug content spectrophotometrically. The release patterns of ketoconazole from the studied ISG formulations were determined by plotting the cumulative amount of the drug permeated (Q) per unit area as a function of time and the drug steady-state flux (JSS) for each formulation was determined from the slope. The permeability coefficient (Pc) was estimated by dividing the drug flux by the initial drug load (C_0). Another plot of the Q versus square root of time was constructed and the diffusion coefficient (D) was obtained from the following equation: $D=[slope/2Co]^2 \times \pi$

Anti-fungal activity

The antifungal activity of the prepared ISG formulations was tested against standard strain of Candida albicans ATCC 76615 that was obtained from the microbiology laboratory, King Abdulaziz University Hospital, Jeddah, KSA. Preliminary screening of the antifungal activities was conducted using agar diffusion technique. Briefly, Petri dishes (150 mm) were filled with 50 mL Muller-Hinton agar containing 1 mL fungal culture (1 × 106 CFU/mL) and the strain was inoculated separately. Holes of 12 mm in diameter were made in the seeded agar plates and filled with 200 µL of each formula. Dishes were then incubated for 4 h at 37°C. Inhibitory activity was defined as the absence of fungal growth in the area surrounding the holes, inhibition zone that was measured using a caliper.

Trans-corneal Permeation

The human corneal epithelium represents the main barrier for transcorneal drug permeation. To determine the impact of ocular barriers on the ophthalmic drug delivery, cell culture models can be used [14]. In this study, evaluation of ketoconazole transcorneal permeability from ISG formulation loaded with either optimized drug NPs or pure drug (F9 and F10) was performed on human epithelial cell line. Cells (10^5) were seeded on T-25 flasks for 24 h and then were divided into two groups. Group I was exposed to F9 (0.3 mg/ml ketoconazole in the form of NPs) while, group II was exposed to F10 (0.3 mg/ml of pure ketoconazole). Cells were collected after 2, 4, 6, 12 and 24 h (n=3) and washed twice with ice cold phosphate buffer saline. The collected cell pellets were ruptured by subjecting to two repeated cycles of freeze and thawing followed by ultrasonication for 10 minutes. Cell lysates were centrifuged using Sigma Laboratory centrifuge ($3K_30$, Ostrode, Germany) at 15000 rpm for 1 h at 4°C and the concentration of the drug in the supernatant was determined using high-performance liquid chromatography (HPLC) method previously reported [15] except for slight modifications. An isocratic HPLC chromatographic method using C18, 4 × 250 mm, 5 µm, Thermo Fischer Scientific analytical column. The mobile phase composed of methanol and water 80:20 (v/v). Its flow rate was adjusted at 1.2 ml/min. The detection wave length was 240 nm. Drug standards in the intracellular concentrate containing known amounts of the drug were prepared and analyzed before determination of the unknown drug concentrations in the samples.

Results and Discussion

Preparation of drug nanoparticles based on the in-vitro solvent exchange congealing method is a novel approach to develop polymeric drug delivery system by easily achievable and facile technique. The term nanoparticle laden *in situ* gel has been previously reported to describe the incorporation of PLGA nanoparticles, prepared by nanoprecipitation process, into *in situ* gel formulation suitable for ocular retention [16]. In the nanoprecipitation process, the drug and polymer in a specified ratio are dissolved in organic solvent at room temperature and the resulting polymeric drug solution is slowly injected in aqueous PVA solution while stirring and, the organic solvent and some water are evaporated.

In this study, the in-vitro solvent exchange congealing technique which depends on solvent exchange mechanism was employed to develop PLGA NPs. PLGA is soluble in both water miscible solvents such as N-methyl pyrrolidone and DMSO, and in partially miscible ones such as triacetin, ethyl acetate [17]. When PLGA solution is injected into an aqueous medium, the solvent dissipate into the aqueous environment and the polymer solidify [18-20]. PVA was added to the aqueous phase as a stabilizer to produce small size and stable NPs [21,22]. The possible formulation parameters affecting the preparation of the NPs were optimized to achieve the optimum concentration of PLGA in the organic

solvent, organic to aqueous phase ratio and PVA concentration in the aqueous phase.

Effect on Particle Size

Results of the statistical analysis, using multiple regression analysis and two-way ANOVA, for the effect of the studied independent variables on particle size indicated that the main effects of X1, X2 and X3 along with their quadratic effects were significantly Y1 as illustrated in **Table 4**. When the sign of the estimated effect denotes a positive value, this is an indication of a synergistic effect of this variable on the selected response, while antagonistic effect is expected when a negative sign is obtained. F-ratio is used to compare between the actual and expected variation of variable averages; an F-ratio greater than 1 is an indication of a location effect and hence the P-value is used to report the significance level. A factor is considered to significantly influence a selected response if the P-value differs from 0 and is less than 0.05. As the concentration of PVA was increased, particle size of the prepared particles was increased. This behavior could be attributed to two reasons. First, the increase in particle size is attributed to exceeding the PVA concentration required to provide complete coverage of the nanoparticles surface and start formation of PVA micelle molecules. Above this critical micelle concentration, there is a less surfactant surface adsorption since the micelles molecules begin to compete for the adsorption at the solid particles, this effect results in destabilization of the prepared nanoparticles and therefore contributed to increase in the particle size [21-24]. Second, increase in the concentration of PVA leads to increase in the viscosity of the aqueous phase which in turn decreases the rate of PLGA solidification and so, formation of large size particles. Results also revealed that, as the concentration of PLGA was increased, the particle size of the prepared NPs was increased. This finding is in good agreement with previous study indicated an increase in the average particle size of PLA nanoparticles loaded with 5-Fluorouracil when the concentration of the polymer was increased. The authors attributed this finding to high polymer load in the aqueous medium during the solidification process [25]. High percent of the organic phase also increase the polymer load and so attributed to larger particle size. The standardized pareto chart that illustrates the effect of the X1, X2, X3, their interaction and quadratic effect of Y1 confirmed the above finding as depicted in **Figure 1**. A vertical reference line at a significant p-value of 0.05 is represented in the chart in which an effect that exceeds this line is statistically significant. The polynomial equation that relates the independent variables and the particle size is as follow:

Particle Size=-2009.41+155.604 X1+595.679 X2+458.835 X3-40.501 X12+57.25 X1X2+27.25 X1X3-70.2679 X22-25.375 X2X3-99.0823 X32.

Table 3 Composition of the prepared ISG formulations, *in vitro* release parameters, antifungal activities and gelation time.

ISG Formula	Composition			Release parameters			Zone diameter (mm)	*In vitro* gelation (min)
	Polymer	Conc. (%)	Drug form	J_{ss} (µg/cm² min)	P (cm/h)	D		
1	Na-Alginate	1	NPs	9.88×10^{-2}	8.98×10^{-5}	7.13×10^{-6}	24	56
2	Na-Alginate	1	Pure drug	3.73×10^{-2}	3.39×10^{-5}	9.74×10^{-7}	22	55
3	Carbopol	0.5	NPs	15.04×10^{-2}	13.67×10^{-5}	1.50×10^{-5}	21	86
4	Carbopol	0.5	Pure drug	12.51×10^{-2}	11.37×10^{-5}	1.10×10^{-5}	20	88
5	Poloxamer	16	NPs	39.45×10^{-2}	35.86×10^{-5}	10.9×10^{-5}	20	65
6	Poloxamer	16	Pure drug	45.67×10^{-2}	4.15×10^{-5}	1.47×10^{-6}	18	65
7	Chitosan	0.5	NPs	75.18×10^{-2}	68.35×10^{-5}	4×10^{-4}	25	91
8	Chitosan	0.5	Pure drug	75.16×10^{-2}	68.33×10^{-5}	39.28×10^{-5}	22	93
9	Chitosan-Alginate mixture	0.5-0.5	NPs	1.41	12.76×10^{-4}	13.65×10^{-4}	30	47
10	Chitosan-Alginate mixture	0.5-0.5	Pure drug	55.66×10^{-2}	50.59×10^{-5}	22.11×10^{-5}	26	48
11	Chitosan-Poloxamer mixture	0.5-16	NPs	29.89×10^{-2}	27.18×10^{-5}	6.47×10^{-5}	22	46
12	Chitosan- Poloxamer mixture	0.5-16	Pure drug	14.81×10^{-2}	13.47×10^{-5}	1.54×10^{-5}	20	47

PF127: Poloxamer F127; NPs: nanoparticles; J_{ss}: steady state flux; P: permeability coefficient; D: diffusion coefficient.

Table 4 Estimated effects of factors, F-ratio, and associated P-values for the ketoconazole PLGA NPs particle size (Y1) and entrapment efficiency (Y2).

Factor	Y1			Y2		
	Estimated effect	F-ratio	P-value	Estimated effect	F-ratio	P-value
X1	395.537	72.32	0.0001	6.70238	10.27	0.0185
X2	199.752	18.44	0.0051	-5.51903	6.96	0.0386
X3	116.511	6.27	0.0462	0.58618	0.08	0.7887
X1X1	-182.254	10.42	0.0180	-4.96651	3.82	0.0983
X1X2	171.75	7.99	0.0301	0.32725	0.01	0.9086
X1X3	81.75	1.81	0.2272	0.15725	0.00	0.9560
X2X2	-140.536	6.19	0.0473	4.11522	2.63	0.1563
X2X3	-50.75	0.70	0.4356	0.10625	0.00	0.9702
X3X3	-198.165	12.31	0.0127	-0.194251	0.01	0.9415

X1: % PLGA in organic phase; X2: % Organic to aqueous phase; X3: % PVA in aqueous phase; Y1: Particle size in nm; Y2: % Entrapment efficiency.

3D response surface plots were constructed to illustrate the effect of changing two independent variables, when the third one was kept at the intermediate level, on Y1 were constructed and are represented in **Figure 2**.

Effect on Entrapment Efficiency

Statistical analysis of the obtained data for entrapment efficiency indicated that the main effects of X1 and X2 were significantly affecting Y2 as tabulated in **Table 4** and graphically represented in the pareto chart **(Figure 1)**. As the concentration of the organic phase (DMSO) was increased, the entrapment efficiency was decreased owing to the higher solubility of ketoconazole in DMSO that leads to escape and diffusion of the drug within the organic phase during the solvent exchange process. This behavior was more pronounced when the concentration of the organic phase was increased. The drug entrapment efficiency was increased when the concentration of PLGA was increased which is in agreement with previous work for PLA nanoparticles but with water soluble drug [25]. This behavior could be attributed to rapid solidification and formation of the nanoparticles that enclose higher percent of the drug when the concentration of PLGA was increased. The polynomial equation that relates the independent variables and the entrapment efficiency is as follow:

Entrapment Efficiency=102.887+11.5169 X1-23.9328 X2+0.180091 X3-1.10367 $X1^2$+0.109083 X1X2+0.0524167 X1X3+2.05761 $X2^2$+0.053125 X2X3-0.0971253 $X3^2$.

To illustrate the effect of changing two independent variables on Y2, when the third one was kept at the intermediate level, 3D response surface plots were constructed and are graphically represented in **Figure 2**.

To prepare an optimized formulation characterized by smaller particle size and high entrapment efficiency, the optimum desirability that achieve an optimum combination of factor levels was identified and the optimum levels for the independent variables along with predicted, observed and residual values are tabulated in **Table 5**.

Physicochemical Characterization

The DSC thermogram of ketoconazole showed a characteristic sharp endothermic peak at 151°C as illustrated in **Figure 3**. No characteristic drug peak was observed in the drug loaded PLGA NPs formulation which is an evidence that there was no crystalline drug material in the optimized drug formulation. This is an indication of the change in the drug crystallinity and homogenous dispersion of the drug in the PLGA matrix. The same finding has been previously reported for PLGA nanoparticles loaded capecitabine [26] and monensin loaded PLGA nanoparticles [27], and the authors attributed this behavior to the existence of the drug in an amorphous or disordered-crystalline phase which is distributed as a molecular dispersion or a solid solution state in polymeric matrix.

The FTIR spectroscopy of pure ketoconazole displayed a characteristic drug peaks of carbonyl group C=O stretching vibration at 1647.26 cm^{-1}, C-O stretching of the drug aliphatic ether group at 1031.95 cm^{-1} and C-O stretching of cyclic ether at 1244.13 cm^{-1} [28]. The spectra of the optimized NPs loaded with drug exhibited the same characteristic peaks, **Figure 4**, except for slight shift or decrease in the intensity of the peaks due to slight overlapping between the drug and the polymers characteristic peaks or due to the drug-polymer ratio.

XRPD patterns of pure ketoconazole and optimized NPs formulation are illustrated in **Figure 5**. The diffraction spectrum of the pure drug was crystalline in nature as indicated by numerous distinct peaks. The spectrum of drug NPs was different from that of pure ketoconazole which demonstrated peaks of lower intensity, disappearance, and formation of some new peaks. This finding indicates crystalline state transformation of ketoconazole in the NPs into the amorphous form and absence of interaction between the drug and the polymers used.

Characterization of the Prepared ISG Formulations

Previous studies have indicated that 1% sodium alginate, 16% poloxamer and 0.5% carbopol were suitable as *in situ* vehicles for ophthalmic delivery [25,29,30]. Ionic gelation, thermosensitive and pH induced transformation are the mechanisms involved during phase transition for alginate, poloxamer and carbopol, respectively. HPMC was added to all the prepared formulation as viscosity enhancing agent. Chitosan is a muco-adhesive polymer that possesses viscosity enhancing properties and is soluble in aqueous acidic solution. The pH of the simulated tear fluid (STF), the presence of alkaline substances in the STF, and addition of HPMC to the chitosan based ISG formulation could attribute to increase in the formulation viscosity upon contact with the STF. It was noticed that ISG formulations 7 and 8 showed the longest *in vitro* gelation time among all the studied ISG formulations. ISG formulations containing combination of chitosan and other polymers were also prepared except for carbopol which was incompatible with chitosan as indicated by formation of white precipitate during preparation. Mixing chitosan with alginate or poloxamer leads to decrease in the time of *in-vitro* gelation. When all the prepared formulation solution was added to STF, the viscosity was increased owing to gel formation by ionic, thermosensitive or pH interaction. If higher sheer rate is applied, the high shear rate of eye (4250-28,500 1/s) during blinking, the viscosity usually decreased due to the pseudo-plastic behavior of the formulation as previously described by Nagarwal et al which suggests suitability of these formulation for ocular delivery [25]. Faster gelation of formulation ISG 9 and 10 could be attributed

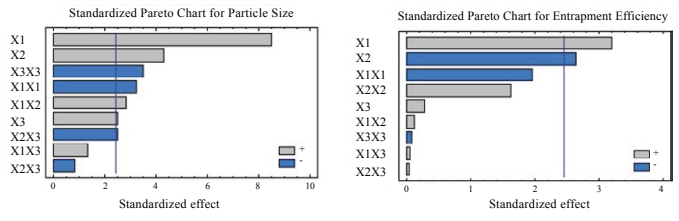

Figure 1 Standardized Pareto charts for the effect of the independent variables on Y1 and Y2.

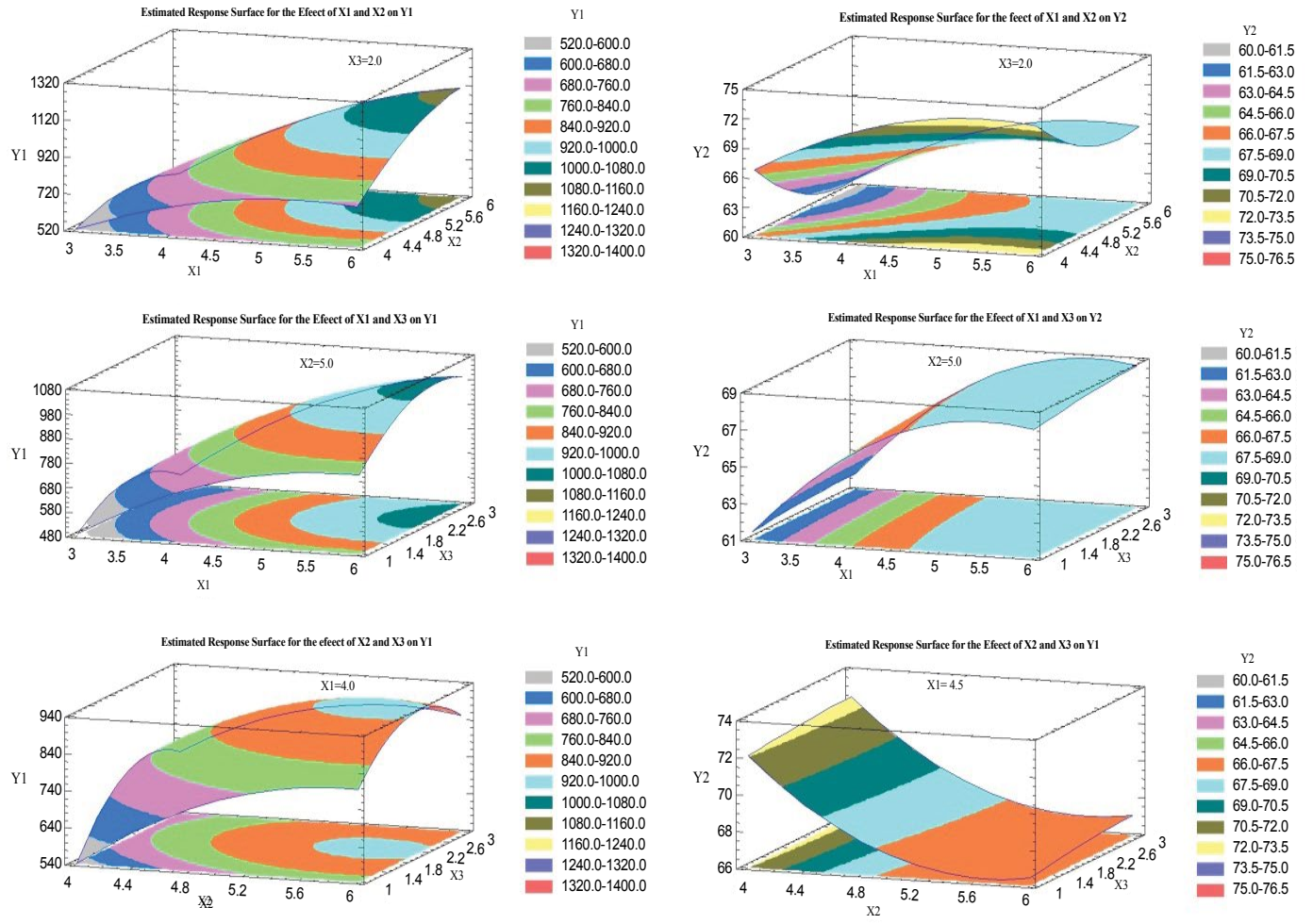

Figure 2 3D Response surface plots for the effect of the independent variables on Y1 (left side) and Y2 (right side).

Table 5 Multiple response optimization of ketoconazole PLGA NPs.

Factor	Low level	High level	Optimum
X1	1.977	7.023	5.494
X2	3.318	6.682	3.318
X3	0.3182	3.682	1.105
Response	**Predicted value**	**Observed value**	**Residual**
Y1	328.0	331.0	+ 3
Y2	78.67	77.32	-1.35

X1: % PLGA in organic phase; X2: % Organic to aqueous phase; X3: % PVA in aqueous phase; Y1: Particle size in nm; Y2: % Entrapment efficiency.

to the presence of double viscosity enhancing agent, HPMC and chitosan, and to the rapid ionic gelation of alginate with Ca^{++} in the STF.

The *in vitro* release study revealed superior drug release from NPs formulations when compared to the same formulations containing pure drug as indicated by the calculated release parameters illustrated in **Table 3**. Development of drug PLGA NPs results in improvement in the drug permeation owing to a decrease in the drug particle size. The release from ISG formulation 9 and 10 was the highest, the effect that could be attributed to rapid gelation and easy diffusion of the drug from the chitosan-alginate system.

Results for the antifungal activity, represented as the inhibition zone diameter, were in direct relation to the release data. Large inhibition zone diameter was noticed with ISG formulation 9, contains drug nanoparticles in chitosan-alginate mixture, which could be related to high drug permeation from this formulation when compared with others with subsequent inhibition effect on the studied fungal strain.

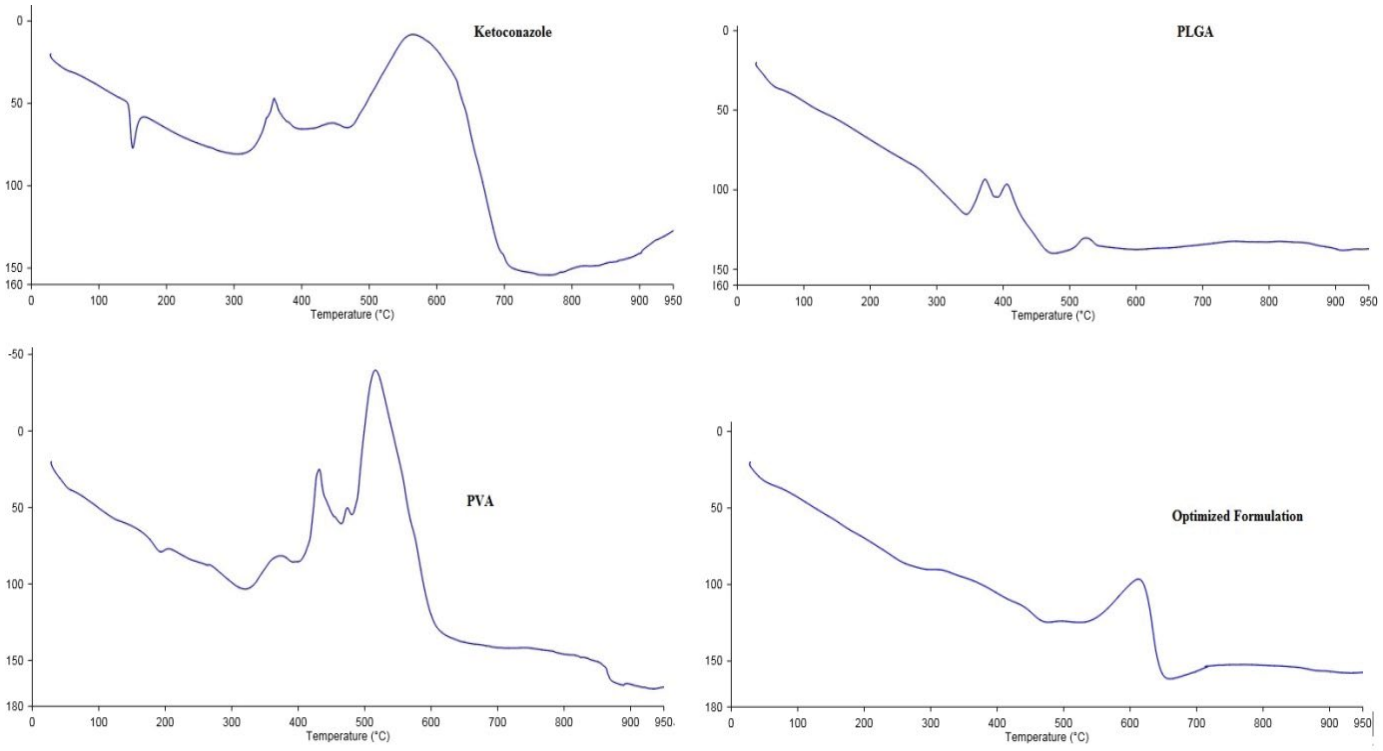

Figure 3 DSC thermograms of pure ketoconazole, PLGA, PVA and optimized NPs loaded with drug.

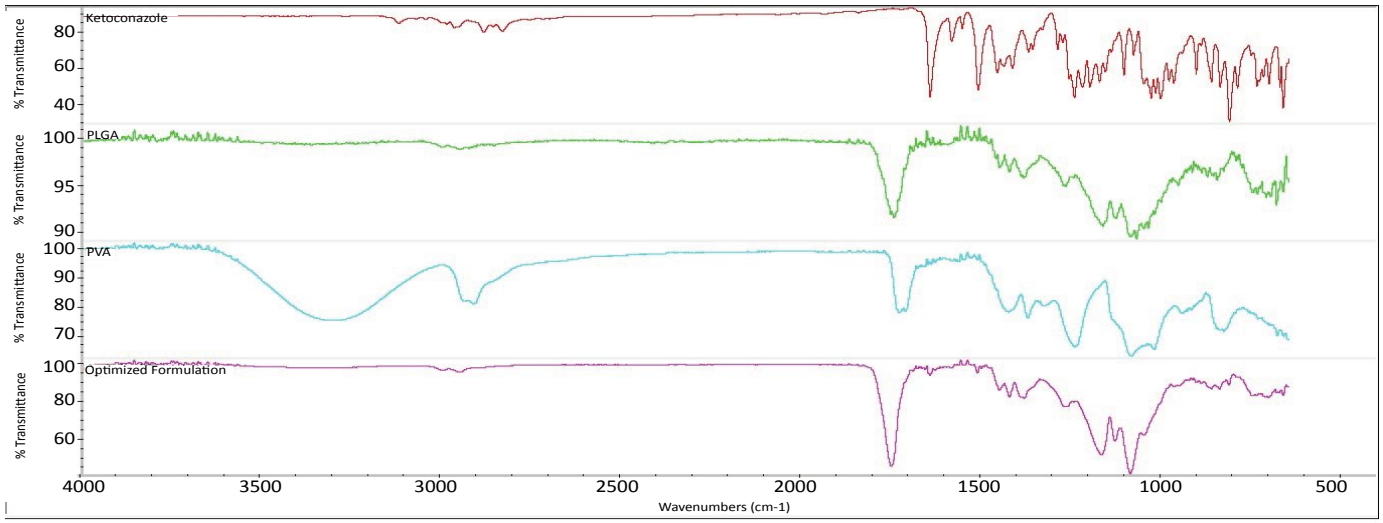

Figure 4 FTIR-spectroscopy of pure ketoconazole, PLGA, PVA and optimized NPs loaded with drug.

Trans-Corneal Permeation

Fungal eye infections can be very serious if not treated. Infection of the clear, front layer of the eye (the cornea) is known as keratitis while, infection in the interior part of the eye is called endophthalmitis. Eye infections may be attributed to different types of fungi that result in both keratitis and endophthalmitis. Candida albicans, a type of yeast that normally lives on human skin and on the protective mucous membranes lining inside the body, has been reported to be the most common cause of endogenous endophthalmitis. Mycotic keratitis is usually attributed to filamentous fungi such as *Lasiodiplodia theobromae* although other species such as Fusarium, Alternaria, and Aspergillus have been also mentioned. Keratitis usually occurs in conjunction with trauma to the cornea with vegetable matter. Keratitis that is attributed to yeasts such as Candida is most likely to occur, and always happen in abnormal eyes, e.g., patients suffering dry eye, chronic corneal ulceration, or corneal scarring

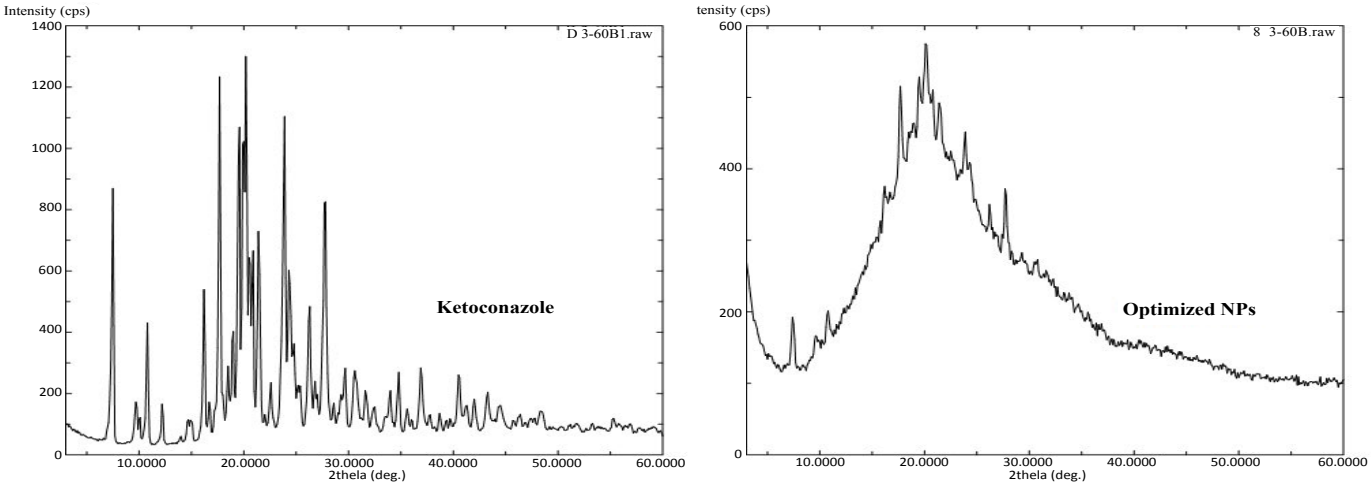

Figure 5 X-ray diffraction patterns of pure ketoconazole and optimized NPs loaded with drug.

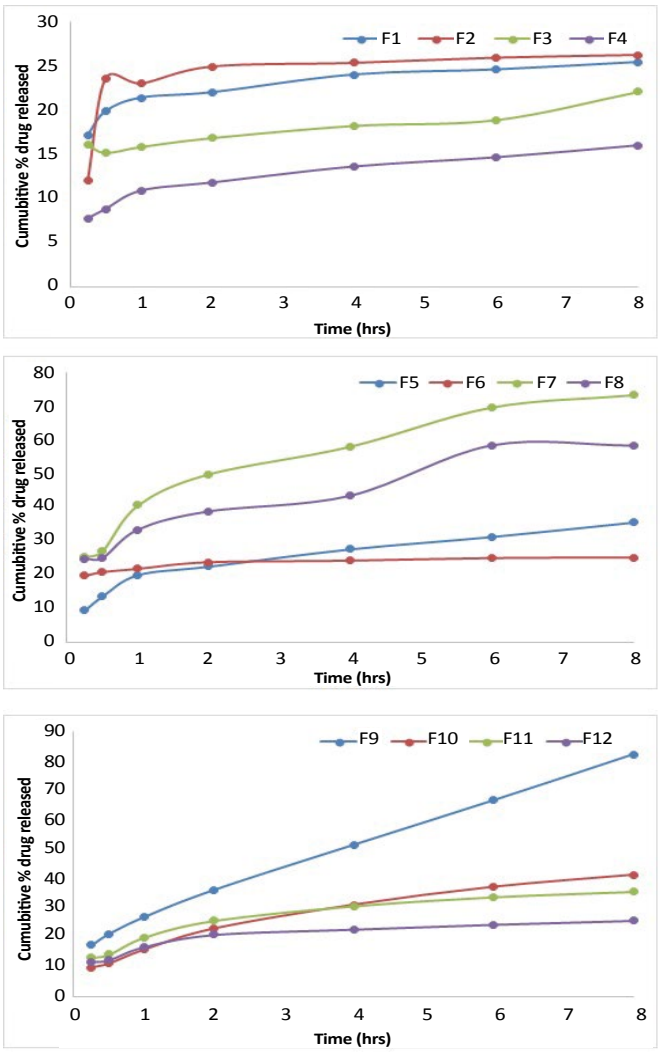

Figure 6 Diffusion profile of ketoconazole from the prepared ISG formulations.

[14,31]. In this study, the permeation of ketoconazole in the form of drug NPs from ISG system was studied to evaluate the usefulness of the formulation in treatment of both keratitis and endophthalmitis. Effective permeation of the drug from the human epithelial cells coupled with the antifungal activity will be considered as a successful delivery system in management of the above mentioned eye inflammation conditions.

Results of the trans-corneal permeation indicated higher and sustained drug permeation from formulation F9 when compared to F10 as represented in **Figure 6**. The prepared optimized drug PLGA NPs augmented the drug permeation, owing to the smaller particle size, and sustained the drug release due to entrapping the drug inside the polymeric matrix. It was previously stated that drug release from PLGA polymeric matrix is attributed to diffusion followed by polymer erosion [32]. Loading the drug or the prepared optimized NPs into ISG vehicle represents another factor that could assist in sustaining the drug release but the effect of the drug release mechanism from PLGA, diffusion and erosion, remains superior **(Figure 7)**.

Conclusion

In this study, development of ketoconazole PLGA NPs based on solvent exchange technique was successfully achieved after optimization the formulation factors affecting the development process. Combination of alginate and chitosan was found to be an effective ISG system as indicated by *in vitro* drug permeation and gelation, antifungal activity and trans-corneal permeation. Hence, ketoconazole PLGA NPs loaded into alginate-chitosan ISG formulation could be used as an effective antifungal medication in treatment of both keratitis and endophthalmitis. Combination of alginate and chitosan was found to be an effective ophthalmic drug delivery system as indicated by *in vitro* drug permeation and gelation, antifungal activity and trans-corneal permeation. Hence, ketoconazole PLGA NPs loaded into alginate-chitosan ISG formulation could be considered as a successful ophthalmic

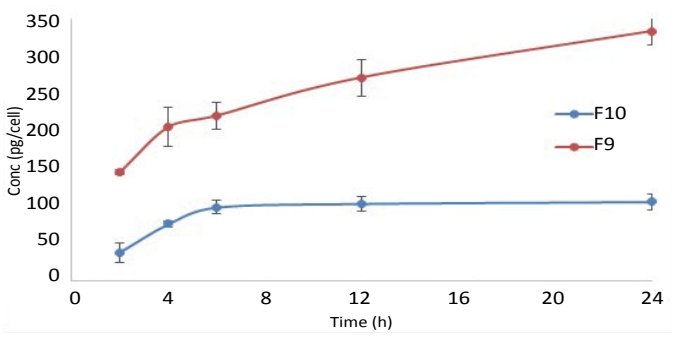

Figure 7 Permeation of ketoconazole through human epithelial cell line.

antifungal medication in treatment of both keratitis and endophthalmitis.

Conflict of Interest

All authors of this work declare that there are no conflicts of interest.

Acknowledgment

This paper was funded by the Deanship of Scientific Research (DSR), King Abdulaziz University, Jeddah, under grant no. 166-822-D1435. The authors, therefore, acknowledge with thanks DSR for technical and financial support.

Reference

1 Loose DS, Kan PB, Hirst MA, Marcus RA, Feldman D (1983) Ketoconazole blocks adrenal steroidogenesis by inhibiting cytochrome P450-dependent enzymes. J Clin Invest 71: 1495-1499.

2 FDA Drug Safety Communication: FDA limits usage of Nizoral (ketoconazole) oral tablets due to potentially fatal liver injury and risk of drug interactions and adrenal gland problems (2013) U.S. Food and Drug Administration.

3 De Logu A, Fadda AM, Anchisi C, Maccioni AM, Sinico C, et al. (1997) Effects of in-vitro activity of miconazole and ketoconazole in phospholipid formulations. J Antimicrob Chemother 40: 889-893.

4 Zhang J, Wang L, Gao C, Zhang L, Xia H (2008) Ocular Pharmacokinetics of Topically-Applied Ketoconazole Solution Containing Hydroxypropyl Beta-Cyclodextrin to Rabbits. J Ocul Pharmacol Ther 24: 501-506.

5 Kakkar S, Karuppayil SM, Raut JS, Giansanti F, Papucci L, et al. (2015) Lipid-polyethylene glycol based nano-ocular formulation of ketoconazole. Int J Pharm 495: 276-289.

6 Aungst B, Svensson CK, Gaudana R, Ananthula HK, Parenky A, et al. (2010) Ocular Drug Delivery. AAPS J 12: 348-360.

7 Kaur IP, Kakkar S (2014) Nanotherapy for posterior eye diseases. J Control Release 193: 100-112.

8 Vega E, Gamisans F, García ML, Chauvet A, Lacoulonche F, et al. (2008) PLGA nanospheres for the ocular delivery of flurbiprofen: Drug release and interactions. J Pharm Sci 97: 5306-5307.

9 Mandal B, Halder KK, Dey SK, Bhoumik M, Debnath MC, et al. (2009) Development and physical characterization of chloramphenicol loaded biodegradable nanoparticles for prolonged release. Pharmazie 64: 445-449.

10 Jain RA (2000) The manufacturing techniques of various drug loaded biodegradable poly (lactide-co-glycolide) (PLGA) devices. Biomaterials 21: 2475-2490.

11 Ahmed TA, Ibrahim HM, Samy AM, Kaseem A, Nutan MTH, et al. (2014) Biodegradable Injectable In Situ Implants and Microparticles for Sustained Release of Montelukast: In Vitro Release, Pharmacokinetics, and Stability. AAPS Pharm SciTech 15: 772-780.

12 Aldrich DS, Bach CM, Brown W, Chambers W, Fleitman J, et al. (2013) Ophthalmic Preparations, USPNF.

13 Jaiswal M, Kumar M, Pathak K (2015) Zero order delivery of itraconazole via polymeric micelles incorporated in situ ocular gel for the management of fungal keratitis. Colloids Surf B Biointerfaces 130: 23-30.

14 Kaur and Kakkar Barar J, Asadi M, Abdolreza Mortazavi-Tabatabaei S, Omidi Y (2009) Ocular Drug Delivery; Impact of in vitro Cell Culture Models. J Ophthalmic Vis Res 4: 238-252.

15 Vertzoni MV, Reppas C, Archontaki HA (2006) Optimization and validation of a high-performance liquid chromatographic method with UV detection for the determination of ketoconazole in canine plasma. J Chromatogr B Analyt Technol Biomed Life Sci 839: 62-67.

16 Gupta H, Aqil M, Khar RK, Ali A, Bhatnagar A, et al. (2013) Nanoparticles laden in situ gel for sustained ocular drug delivery. J Pharm Bioallied Sci 5: 162-165.

17 Ahmed TA, Ibrahim HM, Ibrahim F, Samy AM, Kaseem A, et al. (2012) Development of Biodegradable In Situ Implant and Microparticle Injectable Formulations for Sustained Delivery of Haloperidol. J Pharm Sci 101: 3753-3762.

18 Al-Tahami K, Singh J (2007) Smart polymer based delivery systems for peptides and proteins. Recent Pat Drug Deliv Formul 1: 65-71.

19 Ibrahim HM, Ahmed TA, Hussain MD, Rahman Z, Samy AM, et al. (2013) Development of meloxicam in situ implant formulation by quality by design principle. Drug Dev Ind Pharm 9045: 1-8.

20 Kurakula M, Ahmed TA (2016) Co-delivery of atorvastatin nanocrystals in PLGA based in situ gel for anti-hyperlipidemic efficacy. Curr Drug Deliv 13: 1-11.

21 Ahmed T (2016) Preparation of finasteride capsules-loaded drug nanoparticles: formulation, optimization, in vitro, and pharmacokinetic evaluation. Int J Nanomedicine 11: 515-527.

22 Ahmed TA, El-Say KM (2016) Transdermal film-loaded finasteride microplates to enhance drug skin permeation: Two-step optimization study. Eur J Pharm Sci 88: 246-256.

23 Wang H, Pan Q, Rempel GL (2011) Micellar nucleation differential microemulsion polymerization. Eur Polym J 47: 973-980.

24 Wang Y, Zheng Y, Zhang L, Wang Q, Zhang D (2013) Stability of nanosuspensions in drug delivery. J Control Release 172: 1126-1141.

25 Nagarwal RC, Kumar R, Dhanawat M, Pandit JK (2011) Modified PLA nano in situ gel: a potential ophthalmic drug delivery system. Colloids Surf B Biointerfaces 86: 28-34.

26 Sun SB, Liu P, Shao FM, Miao QL (2015) Formulation and evaluation of PLGA nanoparticles loaded capecitabine for prostate cancer. Int J Clin Exp Med 8: 19670-19681.

27 Surolia R, Pachauri M, Ghosh PC (2011) Preparation and Characterization of Monensin Loaded PLGA Nanoparticles: In Vitro Anti-Malarial Activity Against Plasmodium Falciparum. J Biomed Nanotechnol. 8: 1-10.

28 Kumar P, Mohan C, Srinivasan MK, Shankar U, Gulati M (2011) Physiochemical Characterization and Release Rate Studies of Solid Dispersions of Ketoconazole with Pluronic F127 and PVP K-30. Iran J Pharm Res 10: 685-694.

29 Gupta S, Vyas SP (2010) Carbopol/Chitosan Based pH Triggered In Situ Gelling System for Ocular Delivery of Timolol Maleate. Sci Pharm 78: 959-976.

30 Başaran B, Bozkir A (2012) Thermosensitive and pH induced in situ ophthalmic gelling system for ciprofloxacin hydrochloride: Hydroxypropyl-β-cyclodextrin complex. Acta Pol Pharm-Drug Res 69: 1137-1147.

31 Klotz SA, Penn CC, Negvesky GJ, Butrus SI (2000) Fungal and parasitic infections of the eye. Clin Microbiol Rev 13: 662-685.

32 Husmann M, Schenderlein S, Lück M, Lindner H, Kleinebudde P (2002) Polymer erosion in PLGA microparticles produced by phase separation method. Int J Pharm 242: 277-280.