International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 8, Issue 1, 2016

Original Article

DEVELOPMENT OF THERMOSENSITIVE GEL OF FLUCONAZOLE FOR VAGINAL *CANDIDIASIS*

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Received: 09 Oct 2015 Revised and Accepted: 02 Dec 2015

ABSTRACT

Objective: The aim of the present study was to develop *in-situ* gelling formulations of Fluconazole (FCZ) using thermosensitive polymer for treatment of vaginal *Candidiasis*.

Methods: *In-situ* gelling formulations of FCZ (1 % w/w) were prepared with different concentrations of Poloxamer 407 (P 407, 15-20% w/w) using the cold dispersion method. Similarly, formulations were also prepared by adding mucoadhesive polymers like hydroxyethyl cellulose, Polycarbophil, Carbopol 974 and Hydroxypropyl methylcellulose E 50 LV (0.4 % w/w) to the P 407 formulations. These formulations were evaluated for appearance, clarity, pH, gelling ability, gelling time, gelling temperature, viscosity (in sol and gel forms), spreading time, *ex-vivo* mucoadhesion, *in-vitro* dissolution, morphological characteristics by SEM and *in-vitro* antifungal efficacy against *Candida albicance. In-vivo* vaginal irritation of developed formulation was assessed in New Zealand female rabbits.

Results: *In-situ* gelling formulation of FCZ, prepared using 18 % w/w P407 and 0.4 % hydroxyethyl cellulose, was optimized since this formulation was found to be clear, transparent, forming a quick and stable gel with shear thinning behaviour and excellent mucoadhesion. The developed formulation released 74.21% of FCZ after 8 h of dissolution in 5.2 pH citrate buffer. *In-vitro* antifungal activity against *Candida albicance* showed the stronger antifungal activity of formulation as compared to a marketed formulation. *In-vivo* vaginal irritation study in rabbits demonstrated no significant irritation after 10 d of exposure to the formulation.

Conclusion: The study demonstrated that *in-situ* gelling formulation of FCZ prepared using thermosensitive polymer had improved activity against *Candida albicance* and would be efficacious for the treatment of vaginal *Candidiasis.*

Keywords: Fluconazole, Vaginal drug delivery, *In-situ* gel, Poloxamer, *Candidiasis*

INTRODUCTION

Vaginal route of drug delivery is gaining the attention of scientists in recent years due to its unique features including, large surface area, high perfusion rate, the feasibility of uterine drug targeting and good permeability of many drugs [1]. The potential of the vaginal route has been investigated for local as well as systemic delivery of drugs, especially microbicides [2], contraceptives [3, 4], anticancer drugs [5] anti-infectives [6], drugs used for hormone replacement therapy [7] and mucosal vaccination [8]. Despite all the advantages of the vaginal route, the factors like vaginal secretions, pH, microflora and cyclic changes need to be considered during development of vaginal formulations [9, 10].

For the effective local therapy, the vaginal drug delivery system should be easy to administer, distribute in the vaginal cavity, release drug in a sustained manner for an extended period of time and remain in contact with the vaginal mucosa for prolonged time so as to minimize the earlier clearance of the drug [8, 11]. Mucoadhesive gels have several advantages over other dosage forms such as increase contact with the mucosal surface, cause lubrication of the dry vagina, which helps in drug uptake and permeation and does not produce irritation to the mucosa. But it may also suffer from the limitations like messiness, difficulty in administration and difficulty in spreading of gel in the vagina [12]. In the case of vaginal infections, the direct application of gels onto the infected sites of the vagina might be difficult as well as inconvenient. To overcome this problem, recently, *in-situ* gelling liquid dosage forms have been investigated for vaginal delivery [13-16]. These liquids when applied to vaginal cavity turn into gels as a result of chemical/physical change induced by physiological environments. This transition is induced by a shift in pH or temperature as in Poloxamers [17]. Poloxamer, a block copolymer made of polyoxyethylene (PEO) and polyoxy-propylene (PPO), is known for its excellent compatibility with other chemicals, and high solubility capacity for various drug [18-20]. At low temperature, both PEO and PPO blocks of the Poloxamer are soluble in water. As a result, the PEO-PPO-PEO chains exist as unimers and form cage-like structures in water and exist as a liquid. At higher temperatures, the cage-like structures are broken, and the hydrophobic PPO blocks are exposed. Therefore, they can associate to form micelles and thus forms gels at higher temperature [21].

One of the major drawbacks of Poloxamer is its weak mechanical strength which leads to rapid erosion. Considering the anatomy and physiology of the vagina, there are chances of Poloxamer gel dilution due to the presence of vaginal fluid and continuous mechanical stress applied due to squeezing action of elastic vaginal walls leading to leakage from vaginal cavity [22]. The blending of Poloxamer with other polymers is reported to increase not only the mechanical strength of the formulation but also its surface interaction with the mucosal tissue and consequently, contact time. To date, the most commonly studied mucoadhesive polymers are synthetic poly acrylates like carbopols (C 974 and C980), polycarbophil (PC) [1], cellulose polymers like Hydroxyethyl cellulose (HEC) [23, 24], hydroxyl propyl cellulose (HPC) and Hydroxypropyl methyl cellulose (HPMC) [7]. Mucoadhesive polymers are able to swell rapidly when placed in the aqueous environment. The polymer chains interpenetrate across the mucus layer of the vaginal mucosa, which results in adhesion, thus the formulation is retained at the biological surface for a longer time and the drug is released in a controlled manner close to the absorptive membrane, with a consequent enhancement of bioavailability [10, 12].

In the present study, an attempt has been made to develop and evaluate mucoadhesive thermo sensitive *in-situ* gelling formulations of Fluconazole (FCZ) for the treatment of vaginal *Candidiasis.* FCZ is a triazole with a wide spectrum of antifungal activity, high efficacy and is comparatively safer as compared to other imidazole derivatives. FCZ acts on lanosterol 14-alpha-demethylase and inhibits the synthesis of ergosterol, which leads to loss of fluidity and integrity of the fungal cell

membrane. Azoles also reduce the adhesion of fungal cells to host tissue and reduce multiplication and growth of fungi [25]. In the present study, *in-situ* gel formulations were formulated using thermo sensitive polymer, poloxamer 407 and evaluated for clarity, gelling time, gelation temperature, and viscosity, spread ability, *ex vivo* mucoadhesion, *in-vitro* dissolution, *in-vitro* antifungal efficacy and *in-vivo* vaginal irritation.

MATERIALS AND METHODS

Materials

Fluconazole (FCZ) was a generous gift from IPCA laboratories, Mumbai, India. Kolliphor P 407 (P 407) was received from BASF, Mumbai, India. Carbopol 974 (C974) and Polycarbophil (PC) were received as gift samples from Lubrizol, Mumbai, India. Natrosol 250 (Hydroxyethyl cellulose, HEC) and Methocel E50 LV (Hydroxy propyl methyl cellulose, low viscosity, HPMC E) were received from Ashland, Mumbai, India and Colorcon Asia Pvt Ltd, Mumbai, India respectively. Deionized water and the solvents of analytical grade were used for the study.

Methods

Preparation of *in-situ* gelling formulations of FCZ using P407

In-situ gelling solutions were prepared on weight basis using the cold method [26]. The required amount of P s407 (15 to 20 % w/w) was dispersed in water containing benzalkonium chloride (0.01 % w/w) and 5 % w/w glycerol at 5+3 °C. The partially dissolved P407 solutions were stored in the refrigerator for 24 h until the complete dissolution of the polymer. FCZ (1 % w/w) was dissolved in 10 % w/w propylene glycol (PG). This solution was added dropwise to the P 407 solution and mixed uniformly to obtain the final formulation (table 1).

For the preparation of *in-situ* gelling formulations of P 407 with mucoadhesive polymers, viz., HEC, HPMC E 50, C 974 and PC, the procedure was slightly modified. For the formulations with C 974 and PC, the required amount of C 974 and PC were dispersed in water with continuous mechanical stirring for 1 hour. For HPMC E 50 and HEC formulations, the polymers were dispersed in the water at 80 °C with continuous stirring till the dissolution. To these polymer solutions, glycerol, benzalkonium chloride and FCZ solution in propylene glycol were added followed by addition of P 407. The dispersion was kept for complete solubilization of P 407 at refrigerated temperature for 24 to 48 h to obtain *in-situ* gelling formulations (table 1).

Evaluation of *in-situ* gelling formulations

Appearance and clarity

The formulations were observed carefully for colour, odour and presence of suspended particulate matter if any. The clarity of the solutions was further assessed by observing them against a dark and white background. The formulations were graded as, turbid (-), slightly turbid (+), clear and transparent (++).

Table 1: Composition for *in-situ* gelling formulations of FCZ

Code	P 407 (%w/w)	Mucoadhesive polymer (%w/w)	PG (%w/w)	Glycerol (%w/w)	FCZ (%w/w)
P1	15	-	10	5	1
P2	16	-	10	5	1
P3	17	-	10	5	1
P4	18	-	10	5	1
P5	19	-	10	5	1
P6	20	-	10	5	1
PE	18	0.4 % HEC	10	5	1
PH	18	0.4 % HPMC E 50	10	5	1
PC	18	0.4 % C974	10	5	1
PP	18	0.4 % PC	10	5	1

*All above formulations contained 0.01% w/w of benzalkonium chloride and distilled water sufficient to make 20 g of solution.

Gelling ability [27]

The formulations (0.5 ml) were slowly added into 2 ml of citrate buffer pH 5.2 (37±1 °C) contained in glass vials without shaking. The transition of the formulation to viscous gel was observed visually, and numerical scores were assigned depending upon the quickness of gel formation and time taken for the collapse of gel structure on shaking the vials. The formulations were graded as, No Phase transition (-), Formation of gel after 60 S and collapsed rapidly within 1 h (+), Formation of gel after 60 S and gel collapsed within 3 h (++), Formation of gel within 60 S and gel remained stable for more than 6-7 h (+++). The average of three readings was recorded.

Gelling temperature [28]

2 ml of the formulation was placed in a test tube which was immersed in a cryostatic water bath (Modern scientific, MIC 1500, India) at 4 °C. The temperature of the bath was gradually increased with the increment of 1 °C. The sample is allowed to equilibrate at each temperature. The sample was observed by tilting the test tube by an angle of 90 °. The temperature at which sol to gel transition took place was noted as gelation temperature and was indicated when meniscus would no longer move after tilting the test tube. The experiment was repeated in triplicate and average of three readings was taken.

Spread ability [29]

For determining the spread ability of *in-situ* gelling formulations, a specially fabricated apparatus was used. The apparatus consisted of a rectangular glass slide mounted on a triangular glass box making an angle of 45 ° to the horizontal surface. The temperature of the glass slide was maintained at 37 °C. One drop of the formulation was placed on the slanting glass slide and the distance travelled by the drop before getting gelled was noted as spreading time.

Viscosity [30]

The viscosity of the liquid formulations (at 10 °C) as well as of the preformed gels (at 37 °C) was determined using programmable Viscometer (Brookfield, RVDV pro II, USA). For determination of solution viscosity, 5 ml of formulation was transferred into the sample cell which was placed carefully within a small volume sample adaptor. The guard leg was placed around the adaptor, and the sample was continuously stirred. The viscosity of the sample was measured at different RPM ranging from 0.5 to 100 RPM at temperature 10 °C. For the determination of viscosity of gel at 37 °C, the formulations were equilibrated at a temperature of 37 °C for 24 h. The viscosity of the gel was determined using a Brookfield Viscometer with T-bar spindle. The helipad movement was controlled to avoid touching of the spindle to any part of the sample holder especially the bottom. A typical run involved changing the angular velocity from 0.5 to 100 RPM after every 10 s at a controlled speed. The viscosity values at each RPM were noted. For the same gel sample, the experiment was repeated thrice, and the average reading was noted.

Effect of dilution on viscosity [31]

Vaginal formulations after administration might get diluted with the vaginal fluid. The dilution of *in-situ* gel might lead to an early erosion

of gel. In order to assess the effect of dilution on the viscosity of the gel, 0.25 ml citrate buffer (pH 5.2) was added per 2 g of gel. The viscosity of the diluted gel was determined using the Brookfield Viscometer.

Drug content

1 ml of formulation was diluted to 100 ml with Citrate buffer pH 4.0. The solution was filtered, and FCZ content was analysed by a UV spectrophotometer (Shimadzu, UV-1700, Japan) at λ_{max} of 260 nm. For each formulation, the experiment was repeated thrice.

***In-vitro* dissolution [32]**

The *in-vitro* release of FCZ from various formulations was carried out using a dialysis tube (Mol size cut off 10000 to 12000 Da). Formulation (2 g) was placed into the dialysis tube which was previously soaked in citrate buffer pH 5.2. Both the ends of the tube were tied, and the tube was placed in a beaker containing 100 ml of citrate buffer pH 5.2. The dissolution assembly was further placed in a water bath maintained at 37 °C and the content of beaker was stirred at 100 RPM. Aliquots (5 ml) were withdrawn from the release medium at one h interval and replaced by an equal volume of the release medium. The aliquot withdrawn was filtered using 0.45 µm syringe filters and FCZ content was analysed at 260 nm by the UV spectrophotometer. The release experiments were repeated in triplicate and average of three readings were recorded. The data obtained from release experiments were fitted to the Korsemayer Peppas model to find out the mechanism of drug release (table 2).

$$M_t/M = k\,t^n$$

M_t/M represents a fraction of drug released at time t, k is release constant, and n represents a mechanism of drug release. The value of n as 0.5 indicates drug release by Fickian diffusion. The value of n between 0.5 to 0.9 indicates non Fickian or anomalous drug release, whereas if n = 0.9, it indicates zero order drug release [33].

Scanning electron microscopy [34]

To study the changes in the morphology of P 407 gels, after addition of mucoadhesive polymers, selected gels were lyophilized (Labconco, Free Zone 2.5, USA) and morphological characteristics were studied by SEM (JEOL, JSM-6360A, Japan).

Mucoadhesive strength [35]

Mucoadhesive properties of *in-situ* gel formulations were assessed using a Texture Analyzer (Brookfield, CT3 Texture Analyzer, USA). A sample of goat vaginal tissue was collected immediately after slaughter of the animal and was separated from the underlying tissues. After proper washing with distilled water, the tissue was rapidly frozen (−20 °C) and stored in the de Jalon solution. Before testing, vaginal tissue was defrosted at room temperature. The tissue was placed on the base of the texture analyser. The gel formulations (previously equilibrated at 37 °C) were applied to the aluminium probe of Texture Analyzer using double sided adhesive tape. The vaginal tissue was moistened with citrate buffer pH 5.2. The probe of Texture Analyzer was lowered until contact with tissue was made. After establishing the contact for 20 S with contact load of 10 g, the probe was withdrawn at a rate of 1 mms^{-1}. The force required to detach the gels from goat vaginal tissue was measured as mucoadhesive strength (g). The readings were taken in triplicate on the same vaginal mucosa.

***In-vitro* antifungal activity [34]**

The antifungal activity of the optimized formulation was evaluated against *Candida albicance*. The viability of *Candida* in Sabouraud's Dextrose (SD) broth (pH 5.2) was compared with its viability in the presence of the developed *in-situ* gel formulation. Briefly, *Candida* suspension, prepared from a broth culture of *Candida albicans,* was added to a flask containing 100 ml of SD broth (pH 5.2). The final strength of *Candida* was adjusted to 6 log of CFU/ml. Before the addition of the formulations, T_0 reading was taken. To each flask, 5 g of different formulations were added and incubated aerobically at 37 °c for 24 h. The viable count of *Candida* was determined at 0 (T_0), 6 (T_6) and 24 (T_{24}) hours in triplicate for each flask. Appropriate dilutions were plated on SD agar plates and resulting colony forming units per ml of the culture was determined and compared against control and standard. *Candida* in SD broth (pH 5.2) was used as a control, Marketed gel formulation of FCZ as standard and gel base without drug as a solvent control. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests (Graph pad Instat, version 3.1, USA) wherein each test sample was compared with the control.

Vaginal irritation study

Vaginal irritation study of the optimized formulation (PE) was performed, using the rabbit as the animal model, in National Toxicology Centre, Pune. The study was conducted with prior approval from the animal ethical committee as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India, guidelines. The rabbits were provided with food pellets and water *ad libitum* and temperature and humidity were controlled at 20-22 °C and 40-60 % RH respectively. A 12 h light/dark cycle was maintained. The study was performed on 6 female New Zealand white rabbits of weight 2.5 to 3.0 Kg, used in 2 replicates of 3 rabbits per group. The *in-situ* gel formulation, PE (0.5 g), containing 1 % w/w of FCZ, was applied to the vagina of the first group of rabbits with a syringe applicator. The placebo formulations were applied to the second group of rabbits. The dose of 0.5 g/rabbit twice daily was administered for 10 d. The animals were examined visually at preselected time intervals for any signs of vaginal or vulval irritation. The findings were recorded in terms of the numerical scores for each animal on following grades, no irritation (0), minimal (1), mild (2), moderate (3), severe (4). The average score of six rabbits was considered for determination of any irritation by gel formulation.

RESULT AND DISCUSSION

Appearance, clarity, pH and drug content

All the formulations of P407 alone and with non-ionic polymers, HPMC E 50 LV and HEC were found to be very clear without any precipitation. The formulations containing C 974 and PC were found to be slightly turbid initially, but the turbidity disappeared after storage of the formulations for 48 h. This might be because C974 and PC formed aqueous colloidal dispersions initially, which imparted slight turbid appearance to the formulation. After equilibration of the formulations, due to complete swelling of the polymer, the turbidity disappeared. pH of the formulations was found to be in the range of 5.2 to 5.8. This pH range is acceptable to the vaginal cavity as the vaginal pH is in the range 4.0 to 5.5 [12]. The drug content of formulations was found to be in the range of 94.0+0.34 to 98.6+0.97.

***In-situ* gelling ability and gelation temperature of formulations**

An ideal *in-situ* gelling system should be a free flowing liquid with low viscosity at the storage conditions (at 25 °C) to allow reproducible administration into the vaginal cavity. It should also undergo *in-situ* phase transition to form a strong gel capable of withstanding shear forces in the vaginal cavity and sustain drug release at physiological conditions in vaginal fluid (pH 4.0 to 5.5 and 37 °C). Hence, formulations were evaluated for their *in-situ* gelling ability in citrate buffer pH 5.2 at 37 °C and temperature of sol-gel transition was also evaluated.

The gelation temperature obtained (table 2) for different P 407 concentrations (15% w/v to 20% w/v) were found to be in accordance with the results obtained in the literature [36] and confirmed that it was dependent on polymer concentration. It is evident from the results that the increase in P 407 concentration resulted in a decrease in gelation temperature. The gelation of P 407 is the result of the formation of cubic micellar structures[37]. Poloxamer is made up of PEO and PPO units. PEO units are hydrophilic, and PPO units are less hydrophilic. When the temperature and concentration of P407 increase above a critical point, the less hydrophilic PPO units undergo dehydration. This dehydration causes aggregation of PPO units leading to micelle formation. The increase in the P407 concentration leads to enlargement of micelles and tight packing of adjacent micelles leading to gel formation at lower temperatures. The gel is more

entangled at higher concentration of P 407 [38, 39]. The formulation containing P 407 at concentration 15% w/w (P1) did not form a gel at physiological conditions in citrate buffer (pH 5.2; temperature 37 ºC). This might be attributed to its higher gelation temperature (41 °C). Formulations with 16 % (P2) and 17% w/w (P3) of P 407 formed gels after 60 S and the resultant gel collapsed within 1 h and 3 h respectively. The formulations containing P407 at concentration 18% w/w (P4) or more were found to be forming gels quickly within 60 S (gelation temperature equal to or less than 28.2 °C) and had a stable gel structure after formation. Hence, for further study, 18% w/w concentration of P 407 was considered as optimum.

When mucoadhesive polymers (HEC, HPMC, C 974 and PC) were incorporated in 18% w/w of P 407 formulations, the gel formation was quick and gelation temperature was lowered to a significant extent. This suggests an increase in the gel strength with the addition of these mucoadhesive polymers. It can be assumed that, the addition of mucoadhesive polymers might form hydrogen bonds with PEO blocks in P 407 solutions, which might cause dehydration of P 407 molecules, leading to aggregation of molecules at a lower temperature. Thus, sol to gel phase transitions was obtained at lower temperature [40]. The addition of C974 and PC reduced the gelation temperature drastically, which might be attributed to the high hydrophilic interaction of Carbopol with water. Also, the carbopol backbone is hydrophobic, which lead to the formation of crosslink between hydrophobic chain which subsequently increases the viscosity of formulation resulting into a quick sol to gel transition [36].

Table 2: Physical characteristics of *in-situ* gelling formulations of FCZ

Code	Appearance and clarity*	Gelling ability**	Gelation temperature (°C)***	Spreadability (cm)***	Release kinetics		
					Release exponent (n)	Kinetic constant (k)	Regression coefficient (R^2)
P1	++	-	40.2+0.2	--	--	--	--
P2	++	-	36.1+0.2	4.5+0.2	0.4928	37.77	0.9903
P3	++	+	32.0+0.1	3.5+0.1	0.5028	31.24	0.9759
P4	++	++	28.2+0.3	3.3+0.2	0.5290	27.75	0.9923
P5	++	+++	26.9+0.2	2.5+0.2	0.5357	25.18	0.9938
P6	++	+++	24.7+0.1	2.0+0.3	0.5820	20.02	0.9880
PE	++	+++	27.10.3	2.9+0.1	0.6177	20.44	0.9986
PH	++	+++	27.7+0.2	3.1+0.1	0.5421	25.45	0.9970
PC	+	++	25.9+0.2	2.2+0.2	0.5668	23.14	0.9982
PP	+	++	25.6+0.1	2.2+0.1	0.5722	23.24	0.9994

*Appearance and clarity: (-) Turbid, (+) slightly turbid, (++) clear and transparent. **Gelling ability: (-) No phase transition, (+) Formation of gel after 60 S and gel collapsed within 1h, (++) Formation of gel after 60 S and gel collapsed within 3 h, (+++) Formation of gel within 60 S and gel remained stable for more than 6-7 h. ***Mean+SD, n=3.

Spreadability

The spreadability of formulations was measured in terms of distance travelled by the formulations before the transition to gel. The increase in Poloxamer concentration decreased the spreadability of the formulations due to a decrease in gelation temperature. The addition of mucoadhesive polymers also lowered the spreadability.

Viscosity

In-situ gelling systems are expected to undergo shear thinning (decrease in viscosity with increasing shear rate) in liquid as well as gel state due to the pseudoplastic behaviour of the gels formed. The viscosities of FCZ formulations both in solution as well as gel forms were determined using a Brookfield Viscometer. The results are reported in fig. 1 and fig. 2.

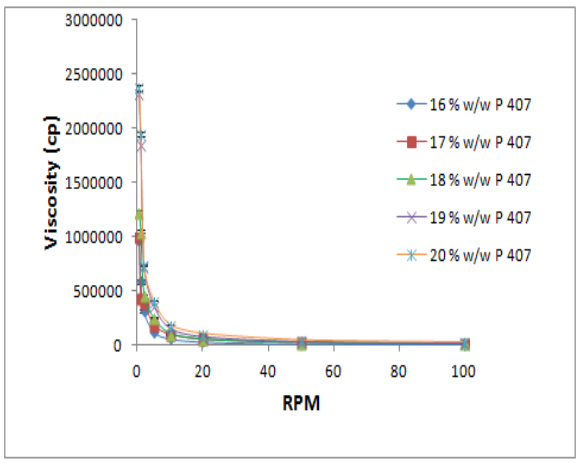

Fig. 1: Effect of P 407 concentration on viscosity of gel at 37 °C
***Average of three readings+SD**

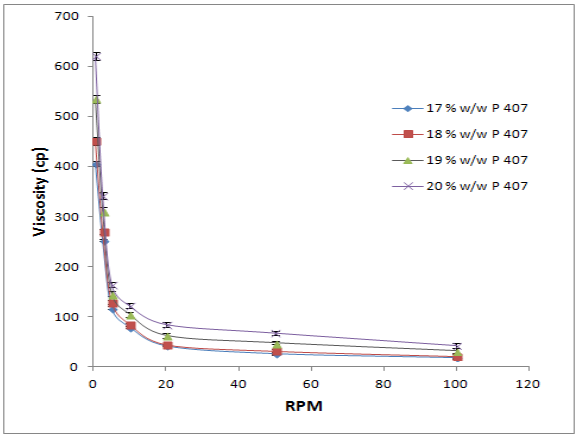

Fig. 2: Viscosity of *in-situ* gelling formulations at 10 °C
***Average of three readings+SD**

The gel form of all the formulations of P 407 was found to be exhibiting shear thinning behaviour (decreasing viscosity at increasing RPM). It was observed that the viscosity of P 407 formulations after gelation (at 37 °C) (fig. 1) was much higher than that of solution form (10 °C) (fig. 2), suggesting the occurrence of phase transition between two conditions. This increase in viscosity for P 407 formulations, from non-physiological to physiological conditions, was mediated by temperature and was the result of micelle formation leading to sol-gel transition.

It was also observed that as the concentration of P 407 was increased, the viscosity of resulting gel was increased. This is because, the increase in P 407 concentration resulted in increase micelle size and micelle number, thus leading to a decrease in the distance between the adjacent micelles. This further leads to increased interaction of the micelles resulting information of viscous gels.

All the gels were found to be exhibiting shear thinning behaviour (decreasing viscosity at increasing RPM). This decrease in viscosity is due to the breakdown of three-dimensional network structures due to high stress developed because of the high rate of shear. When mucoadhesive polymers were added to the P 407 gel, the viscosity of the resultant gel was found to be increased (fig. 3). This might be the result of the formation of hydrogen bonding between the oxygen atom of PEO and hydrogen group of cellulose or poly (acrylic acid) derivatives. This increase in hydrogen bonding increased the gel strength and viscosity of the resultant formulation to a greater extent [28].

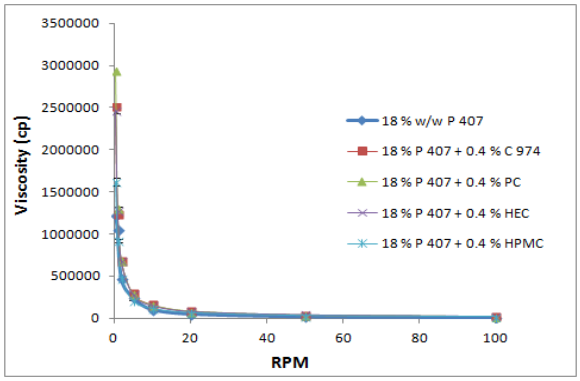

Fig. 3: Viscosity of P407 formulations with mucoadhesive polymers at 37 °C, *Average of three readings+SD

Effect of dilution on viscosity

In-situ gelling vaginal formulation is intended to retain its gel structure and release the drug in a sustained manner. When the formulation is applied to vaginal cavity, there are chances of formulation getting diluted with vaginal fluid. Thus, it is required to develop a formulation which is insensitive to dilution and be capable of releasing the drug in a sustained manner for a prolonged period of time. Generally, 2 to 5 g of the formulation is applied to the vaginal cavity which might get diluted with 0.25 to 0.5 ml of vaginal secretions. Hence, 0.25 ml of citrate buffer (pH 5.2) per 2 g of the formulation was added, and viscosity of diluted formulation was determined. It was observed that there was a significant dilution of 18 % P 407 gels, and there was almost 50 % drop in the viscosity (fig. 4). This might be due to dilution with buffer, the concentration of P 407 in formulation P4 (18 % w/w) decreased to 16 % w/w which decreased its viscosity drastically and viscosity resembled the viscosity of P2 (16 % w/w) formulation. The drop in the viscosity was lower with the addition of mucoadhesive polymer. The formulation containing HEC was found to be a more robust formulation amongst all the formulations whereas the effect of dilution was more pronounced with C 974 and PC. This might be because of the presence of unfavourable pH conditions for ionization of Carbopol [41].

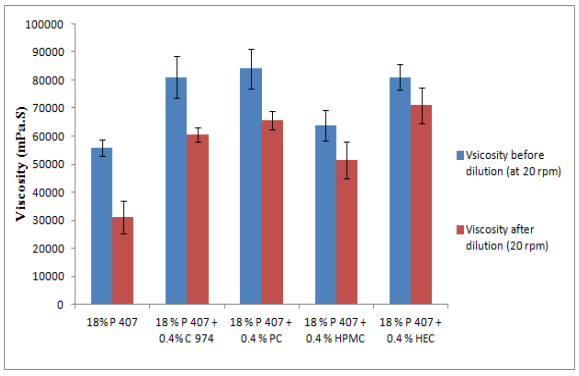

Fig. 4: Effect of dilution on viscosity of *in-situ* gelling formulations, *n=3, Mean+SD

In-vitro drug release

All the formulations showed burst release at the start of the release experiment. This might be because of the lag time required for sol to gel transition of the formulations. It was observed that there was a corresponding decrease in the release of FCZ from the gels with an increase in the concentration of P 407 from 16% w/v to 20% w/v (P2-P6) (fig. 5). After 0.5 h of dissolution, formulation P4 (18% w/v of P 407) released 20.90+1.9 % of FCZ, whereas formulation P6 (20% w/v of P 407) released 14.93+1.1 % of the drug. This indicated a decrease in burst release of drug with an increase in the concentration of polymer. At higher concentration of P 407, sol to gel transition was quicker, which resulted in a decrease in burst release.

After 8 h of dissolution, formulation P4 (18% w/v of P 407) released 88.31+0.8 % of FCZ and P6 (20% w/v of P 407) released 75.45+2.0 % of the drug. The probable mechanism for such retardation of release, with the increase in P 407 concentration, may be a reduction in number and dimensions of the channels in the gel structure. Decrease in the inter-micellar distance within the gel leads to bridging between the neighboring micelles and increase in viscosity of gels, thus decreasing the drug release [26].

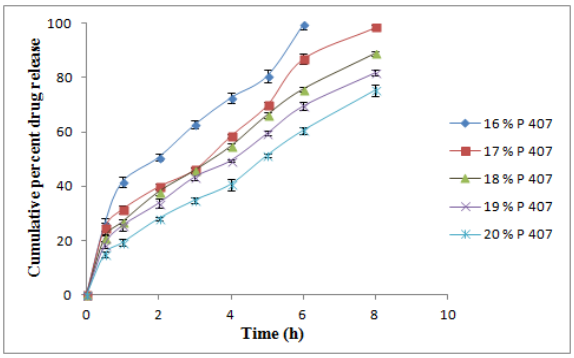

Fig. 5: Effect of P 407 concentration on FCZ release *n=3, Mean+SD

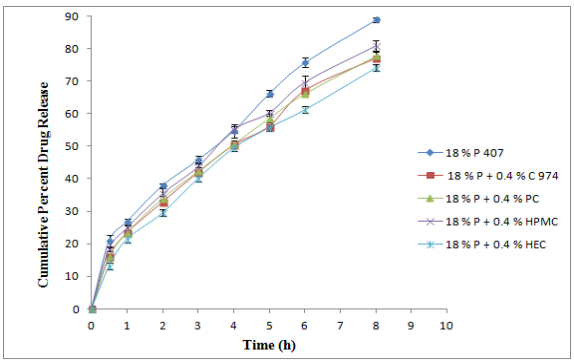

Fig. 6: Release profile of P 407 gel with mucoadhesive polymers *n=3, Mean+SD

The addition of mucoadhesive polymer to the formulations led to decrease in the release of FCZ (fig. 6). The drug release from P 407 gel is mainly through extra cellar channels filled with water. The presence of mucoadhesive polymer would decrease the amount of free water in these channels as well as lead to hydrogen bonding with water. This would result in an increase in the viscosity and subsequent decrease in the drug release [28]. The release of FCZ from formulations containing mucoadhesive polymers was found to be in the following order:

HPMC E 50 LV>PC>C 974>HEC.

The faster release from HPMC E 50 formulation was because of lower viscosity of the formulation. Although Carbopol and PC formulations indicated higher viscosity, the drug release was faster from these formulations. This might be attributed to a decrease in the viscosity of Carbopol or PC polymer in the presence of dissolution medium with pH 5.2. Carbopol requires a favourable pH (above 6.0) for ionization of carboxylic group which results in de-coiling and relaxation of the polymer network, enhancing the viscosity of its gel. As pH of the vagina is acidic, at lower concentration Carbopol would undergo rapid erosion resulting in a higher FCZ release. The release of FCZ was found to be more sustained, with Poloxamer-HEC formulation (PE). After 8 h of dissolution, formulation PE was found to release 74.21+1.0 % of FCZ.

Kinetics of drug release

The mechanism of drug release from gel matrix is complex and is based on diffusion of drug through a hydrated portion of the gel matrix and erosion of the outer fully hydrated polymer on the surface of the matrix. Due to permeation of excess water into the core of gel matrix, there is an increase in hydration of gel matrix which provides a diffusion barrier to drug release. As gel matrix becomes fully hydrated, the polymer chains become completely relaxed and can no longer maintain the integrity of the gel leading to disentanglement and erosion of polymer from the surface of the gel matrix.

For P 407 formulations P1 and P2, n value closely approximated to 0.5 indicating Fickian diffusion (table 2). Thus, the drug release from the gel formulation was mostly governed by diffusion of FCZ through a gel matrix. With the increase in the P 407 concentration, there was a decrease in the rate of drug release and initial burst release. This was evident from n value 0.52 to 0.58. The addition of mucoadhesive polymers to P 407 also changed the n value to 0.54 to 0.61 which indicated non-Fickian/anomalous behaviour. Thus, the drug release in these formulations was mostly governed by both drug diffusion through gel matrix and disentanglement or erosion of polymer chains.

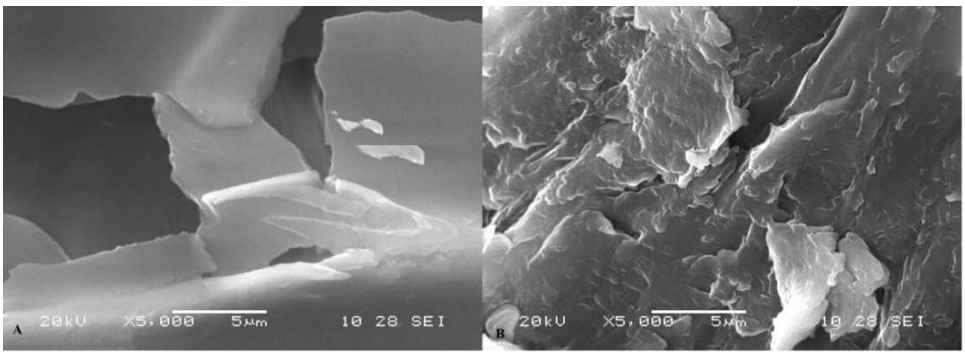

Fig. 7: Scanning electron micrographs of (A) lyophilised P 407 gel (B) lyophilised P 407 with HEC gel

Scanning electron microscopy

Fig.7 indicates morphological changes that occurred after the addition of mucoadhesive polymer to the P 407. The lyophilised P 407 gel exhibited plate-like structure with a smooth surface, whereas the surface of P 407-HEC gel represented more compact structure with an irregular surface. The change in the morphology of the gel might affect the release of drug from the gel matrix.

Mucoadhesive strength

Mucoadhesion relies on the interaction of a polymer and the mucin coat covering the vagina. Structurally, the mucin consists of a protein or polypeptide core with carbohydrate side chains branching off the core. The polymer with many hydrophilic functional groups (e. g. carboxyl group, a hydroxyl group and sulphate) can establish electrostatic interactions and hydrophobic interactions and hydrogen bond with the underlying surface. Of these non-covalent forces, hydrogen bonding appears to be the most important [42]. The mucoadhesive potential of all gel formulations was evaluated by using goat vaginal mucosa (fig. 8).

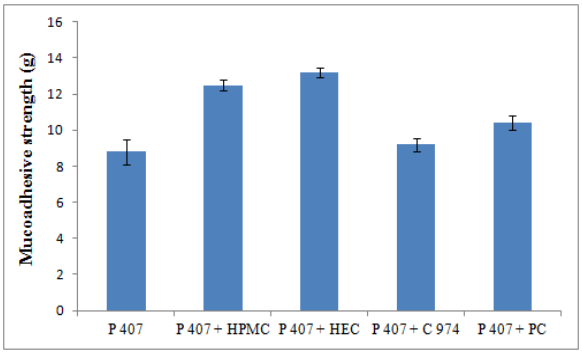

Fig. 8: Mucoadhesive strength of *in-situ* gels, *Average of three readings+SD

It could be noted that when mucoadhesive polymers were added to P 407 gel, the adhesion was increased. The increase was significant with the addition of HPMC and HEC. This can be attributed to the interaction of hydroxyl group of cellulose derivatives with a hydroxyl/carboxyl group of mucin leading to the formation of hydrogen bonds between gel formulation and mucous membrane. This might have resulted in an increase in mucoadhesion. Thus, indicating the chances of prolonged retention of formulation on vaginal mucosa.

Considering the results of mucoadhesion and in-vitro drug release from various formulations, PE formulation with HEC as mucoadhesive polymer was selected as optimized formulation for further study.

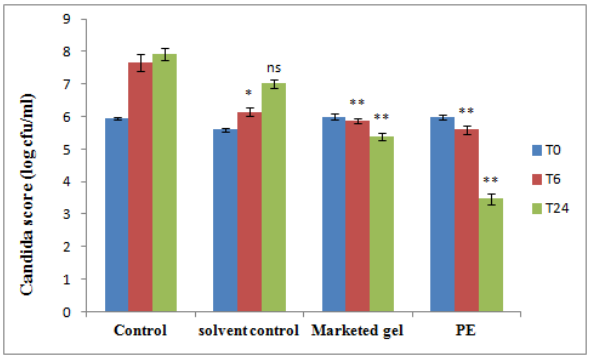

Fig. 9: Comparison of *antifungal in-vitro* activity of developed formulations with the marketed formulation. *n=3, Mean+SD

***In-vitro* antifungal activity**

The viability of *Candida albicance* in the presence and absence of the developed formulation was evaluated after 6 h and 24 h of

incubation and shown in fig. 9. The data in fig. 9 is represented as the average of three observations with a standard error mean (* indicates $p<0.05$ and ** $p<0.01$, NS–non significant). The cell count of *Candida* was found to be decreased with marketed formulation, ($p<0.01$) after 6 h (5.88+0.11 log cfu/ml) and 24 h (5.39+0.10 log cfu/ml) of incubation (survival 89.83 %) as compared to T_0 reading (6+0.1 log cfu/ml). The developed formulation (PE) exhibited strong antifungal activity (**$p<0.01$) with a reduction in *the Candida count* at 6 h (5.60+0.16) and 24 h (3.47+0.22) with the survival of 57.8 % of *Candida.* Thus, the study suggested that the developed *in-situ* gelling formulation showed inhibitory activity against *Candida.* This showed the possibility of the developed formulation to be used for effective treatment of *Candidiasis.*

In-vivo vaginal irritation study

The rabbit model was selected for vaginal irritation study since the vaginal epithelium of rabbit is columnar which is very sensitive, hence considered to be the standard model for irritation study. After application of developed drug loaded formulation and placebo formulation for 10 d, there was no sign of irritation on the vulval or vaginal region of the rabbit as evident from the average score assigned as 0. Thus, the developed formulation was found to be safe for vaginal application.

CONCLUSION

In this study, an *in-situ* forming gel of FCZ for vaginal delivery was formulated using a combination of P 407 and HEC. The developed formulation was clear, spreadable, with shear thinning properties, forming a quick and stable gel having resistance to dilution and excellent mucoadhesion to the goat vaginal mucosa. The formulation exhibited sustained release behaviour and indicated strong antifungal activity against *Candida albicance*. The formulation did not cause irritation to vaginal mucosa when tested on the rabbit. Thus, the study demonstrated the potential of thermosensitive polymer in vaginal delivery of FCZ for effective treatment of vaginal *Candidiasis*.

ACKNOWLEDGEMENT

Authors are thankful to BASF, India, Colorcon India Pvt Ltd and Lubrizol, India for providing the gift samples of polymers, Poloxamer, HPMC and Carbopol respectively. The authors would like to thank Zim laboratories, Nagpur, for providing the gift samples of Fluconazole. We are thankful to APT Research centre, Pune for providing the facilities and guidance for conducting the animal studies.

CONFLICT OF INTERESTS

The authors declare no conflict of interest

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