

In-situ Gelling System for Mucoadhesive Site-Specific Drug Delivery for Treatment of Recurrent Vaginal Candidiasis

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ABSTRACT

Objectives: The purpose of this work was to design and evaluate bio adhesive films for effective treatment against vaginal candidiasis. Fluconazole, a bis tri-azole agent inhibits ergosterol synthesis leading to the disruption of fungal membrane for use as an antifungal agent. The pharmacokinetic profile of fluconazole reveals minimal metabolization, low protein binding and is largely excreted in the urine. **Methods:** Bioadhesive films were prepared by the solvent evaporation technique. The excipients including chitosan as a mucoadhesive polymer and plasticizer along with polyvinyl alcohol and glycerine were dissolved at a total combined concentration to form optimized formulation. Variables such as swelling index, mucoadhesive strength, percentage drug release, permeation, retention and irritation studies were assessed. **Results:** The prepared films were generally smooth, pliable and transparent/semi-transparent in appearance having an average thickness range from 0.08 ± 0.005 to 0.15 ± 0.006 . *In vitro* release experiments demonstrated that film formulations exhibited higher drug release in comparison to marketed gel, FLUCOS® with maximum percentage cumulative drug release after 8 hr release of $42.563 \pm 0.743\%$. Skin retention studies were carried out to find the amount of drug retained by the skin layers. Percentage drug retention in porcine vaginal tissue for F13 has been found to be 1.15 and 1.09 times higher than F12 and FLUCOS®. The selected formulation showed an appropriate anti-candida activity through appearance of zone of inhibition during anti-fungal activity studies, with promising results to meet the unmet medical need for vaginal delivery that swells and forms a viscous gel upon insertion into the vaginal cavity. Conclusively, the selected formulation of Intravaginal film creates a mucoadhesive gel that spreads rapidly and widely over the vaginal mucosa and is retained for a sufficiently long time to provide therapeutic efficacy via topical action.

Key words: Fluconazole, Vaginal film, Mucoadhesive, Vaginal drug delivery, Vulvovaginal candidiasis.

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INTRODUCTION

The vaginal cavity is a significant area of the reproductive tract and acts as a favourable site for drug administration due to immense permeation area, rich vascularisation and relatively low enzymatic activity. The vaginal physiology is mainly influenced by age, hormonal balance, pregnancy, pH changes and concentration of micro flora. Major changes will take place in vaginal physiology with age, like thickness of epithelium layer, production of vaginal fluid and extent of vaginal discharge. Due to such changes in the

female reproductive organ, females are highly prevalent to fungal infections. Infections sometimes results from disparity or some alterations in the vaginal environment like the pH of vagina. The pH of vagina ranges from 3.5 to 5 in normal adult women and natural production of lactic acid by micro flora of vagina is responsible for the maintenance of this pH. However it shifts to somewhat alkaline in the presence of semen (i.e. pH 7 to pH 8).¹ Throughout out the world and especially in developing countries,



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reproductive tract infection (RTI) is a major public health issue. Annually an estimation of 357 million cases of reproductive tract infection or infections that are transmitted sexually are reported by WHO throughout whole world.² Candidiasis is one of the most persistent genital infections occurring in women of all age groups.³ *Candida albicans* is accountable for 90% of vaginal fungal infection; however, the noxious role in the genesis of vulvovaginal candidiasis has recently been also stressed for other *Candida* species, example *C. glabrata* and *C. parapsilosis*. The manifestations of Vulvovaginal candidiasis are often painful and uncomfortable and can include intense itching, irritation, vaginal discharge and dysuria. Topical, oral and intravaginal routes are used for treatment based on the severity of the infection.⁴ Oral fluconazole is most usually prescribed drug to treat the vaginitis infections. Established vaginal formulations such as suspensions, creams and solutions cannot maintain effective drug concentration for extended period of time inside the vaginal cavity due to their short residence time at the site of administration.

Fluconazole is a bis-tri-azole antifungal agent used in clinical practice, with a broad range of approaches used for treatment of both superficial and systemic mycoses. The pharmacokinetic profile of fluconazole reveals elevated oral bioavailability of the drug with minimal metabolization, low protein binding in plasma and prevailing renal excretion as unchanged drug helps in the management of its dosing. It can cure vaginal candidiasis, tinea infection, cutaneous candidiasis, coccidioid meningitis, fungal keratitis and other systemic fungal infections. Fluconazole is available commercially as tablet, capsule, injection and eye drop formulations. The tablet and capsule dosage forms have well known side effects including nausea, headache, abdominal pain, vomiting and diarrhoea.

Films have been extensively used as drug carriers for the treatment of both local and systemic diseases. They are able to sustain and/or control the release of entrapped drug. Films are also apt for vaginal application. Films are designed to rapidly disperse or dissolve in contact with fluids to form a smooth, viscous and mucoadhesive gel while possessing good appearance (preferably colorless and odorless), softness, flexibility and absence of any sharp edges to avoid mechanical injuries during insertion. These desirable features are expected to facilitate insertion, gather user's acceptability and compliance and result in the immediate formation of a mucoadhesive dispersion that could be retained in the vagina for prolonged periods of time.⁵

Key advantages concerning vaginal administration are the possibility of prolonged *in-situ* residence and intimate

contact with mucus, hence superior and prolonged drug delivery.⁶ Among various polymers, chitosan is favored with respect to safety and mucoadhesiveness. It is a common, natural-origin mucoadhesive polymer, suitable as a stable vehicle for the vaginal administration of drugs.⁷

Current hypothesis is based on the fact that local delivery from an intravaginal mucoadhesive dosage form may provide an efficient approach to oral suppressive therapy. It is being assumed that targeted therapy with the fluconazole may provide pre-exposure prophylaxis against candida infection and may be equally effective in the treatment and suppression of vaginitis.⁸ It may lead to higher drug levels at site of action for prolonged periods of time leading to effective and complete treatment. This approach can decrease the frequency of reoccurrence of vulvovaginal candidiasis which are very common in infected patients and further decreasing the risk of transmission of HIV. Moreover the desired concentrations may be achieved with lesser frequency of doses as compared to the oral dosing thus increasing the efficacy of treatment.⁹

The present study was intended for targeted delivery of fluconazole at intravaginal site in order to improve the drug therapy, patient compliance and minimizing of the systemic side effects. Tablets and gels associated with the major limitation of leakage and messiness causing inconvenience to users, leading to poor patient compliance.¹⁰ Mucoadhesive vaginal drug delivery system tends to avoid these problems of the effective treatment of vaginitis. The formulation should be retained at vaginal mucosa for a better contact of drug with target tissue for sufficient period of time without expulsion from vagina.¹¹

MATERIALS AND METHODS

Materials

Fluconazole was procured as a gift sample from Galpha Labs (Baddi, Himachal Pradesh, India). Polyethylene glycol and Chitosan were purchased from Sigma Alrich Chemicals Pvt. Ltd., (Bangalore, India). Lactic acid and Glycerol were obtained from Loba Chemie (Mumbai, India). *Candida* culture was procured from Microbiology Labs, Panjab University (Chandigarh, India). Polyvinyl alcohol, Urea and Agar were purchased from S.D. Fine Chem. Ltd., (Mumbai, India). FLUCOS[®] gel (Oaknet Healthcare Pvt. Ltd., Bangalore, India). All other chemicals used were of Analytical grade.

Animals

Female Sprague Dawley rats (weighing 200-250gm) were provided by the Central Animal House, Panjab

University, Chandigarh. All protocols were duly approved by institutional animal ethics committee (IAEC) reference no. PU/IAEC/S/15/32 for evaluating the vaginal irritation studies of the prepared formulation. These animals were placed at room temperature and they were provided with food and water *ad libitum*.

Solubility studies

The solubility of fluconazole was determined in various solvents as per following procedure. About 1ml of each solvent was kept in 2 ml vials containing excess amount of fluconazole (50 mg) in thermostatic water shaker bath at $37 \pm 1^\circ\text{C}$ for 48 hr. The process was continued for 48 h till stage of saturation achieved. Similar procedure was followed for the preparation of respective blanks without the addition of drug. The equilibrated samples were then removed; solutions were filtered and were analyzed using UV spectrophotometric method after suitable dilutions.

Drug-Excipient Compatibility Studies

Compatibility studies have been performed to check if there is any interaction between the drug and the polymeric excipients being used in films. These studies have been done using differential scanning calorimetry (DSC, TA Instruments, Waters LLC, USA), using aluminum hermetic pans with pierced lid over range of 30°C to 300°C , at a scan rate of $10^\circ\text{C}/\text{min}$ with nitrogen at the flow of 50 mL/min as a purge gas. The fourier transform infrared spectroscopy (FTIR Spectrum Two®, Perkin Elmer, USA) using KBr pellets were conducted at a scan range of 450-4000 cm^{-1} .

Preparation of vaginal films

Before the preparation of films, various polymers were visually evaluated by for their gelling, swelling, bioadhesive property in the Simulated Vaginal Fluid. The gelling and swelling property were observed visually as well as vaginal toxicity, sensitivity and irritation potential according to the literature data, chitosan was selected as

sustained release polymer along with excellent vaginal adhesion properties. Bio adhesive films were prepared by the solvent evaporation technique. The polymer and the plasticizer were dissolved at a total combined concentration. All the excipients were weighed accurately as per requirement as shown in Table 1. Polyvinyl alcohol solution was made using 1%v/v lactic acid solution as a base solvent as well as acidifier in order to acquire the adequate pH for the formulation.¹² Required quantity of medium molecular weight chitosan was transferred to the beaker. Polyvinyl alcohol solution was added to it. It was heated to 30°C , with continuous stirring. Drug was dispersed into this polymeric solution using glycerine and polyethylene glycol with stirring. These solutions were transferred to the petri dish and it was kept for drying for 24 hr. The films formed were peeled off, studies individually sealed in polyethylene laminated aluminium foils and utilized for further studies.

Physical Properties

Films were evaluated for aesthetic parameters such as color, flexibility and transparency. They were weighed and the thickness of film was measured at different points using a micrometre screw gauge.⁵ The disintegration time of the film was assessed in VFS (vaginal fluid stimulant). The time at which the total film gets converted into gel in the solution is reported as disintegration time.¹³ The prepared films were dissolved in SVF (pH-4.2) for 2-3 min until conversion into gel and was measured for its pH at room temperature by directly immersing the pH electrode into the samples.¹

Drug Content

The prepared films were cut in to $3 \times 3 \text{ cm}^2$ and taken into a 100 mL volumetric flask and dissolved in methanol. Suitable dilution was made and absorbance was checked at 266 nm, using UV spectrophotometer.

Moisture Content

For determination of moisture content, piece of film ($3 \times 3 \text{ cm}$) was weighed and kept in desiccators containing calcium chloride at 40°C for 24 hr. Films were removed from desiccator and reweighed until a constant weight was obtained.¹⁴ The percentage of moisture content was calculated as the difference between initial weight and final weight with respect to initial weight.

Measurement of Swelling Index

Film swelling study was carried out in a simulated vaginal environment using Simulated Vaginal fluid (SVF) as media. Each film sample with a surface area of 9 cm^2 was weighed and placed in a glass measuring cylinder, was submerged into 15 mL medium and initial volume (V_0) was noted.¹⁵

Table 1: Compositions of various intravaginal mucoadhesive fluconazole film formulations.

Name of Ingredients	Quantity of Ingredients			
	F11	F12	F13	F14
Fluconazole	100 mg	100 mg	100 mg	100 mg
Chitosan	0.5%	1%	1.5%	2%
Polyvinyl Alcohol	2%	2%	2%	2%
Glycerine	0.5 mL	0.5 mL	0.5 mL	0.5 mL
Polyethylene glycol	0.5 mL	0.5 mL	0.5 mL	0.5 mL
Lactic acid (1% v/v)	20 mL	20 mL	20 mL	20 mL

The change in the volume of the medium was recorded after 24 hr and it was reported as final volume (Vt). The degree of swelling was calculated as in equation 1:

$$\text{Swelling Index} = \frac{V_t - V_o}{V_o} \dots\dots\dots[1]$$

Rheological Studies

One film from each batch to be tested was placed in glass beaker containing 8 mL of SVF (pH 4.2). Within 10-15 min, film gets converted into gel. The gelled systems were evaluated for the rheological behavior using Anton Paar Rheometer (Rheo QC), which is a type of cup and bob viscometer. The viscosity was determined at various shear rates by subjecting the gel to different torque values. The temperature was kept at 30°C and spindle no.29 was employed for viscosity determination.

Differential Scanning Colorimetry

Differential Scanning Colorimetry (DSC) measures the heat capacity of a system as a function of temperature. Following the change in the heat capacity as a function of temperature, allows for the detection of phase transition of various orders. The thermal behavior of fluconazole was studied by employing the Differential Scanning Colorimetry (DSC, Q20, TA Instruments, Waters LLC, USA) using aluminum hermetic pans with pierced lid over range of 30°C to 300°C, at a scan rate of 10°C/min with nitrogen at the flow of 50 mL/min as a purge gas. Thermogram was plotted between heat flow (mW) and temperature (°C).

Measurement of Mucoadhesive Strength

The mucoadhesive strength of the formulations under investigation was evaluated by measuring the force required to detach the formulation from a mucin disc using Texture Analyzer™ (Stable Micro Systems TA-XT plus Texture Analyzer Godalming, U.K.). Mucin discs were prepared by compression of known weight of mucin (250 mg) using a hydraulic press with a diameter of 20mm. Prior to mucoadhesion testing the mucin disc was hydrated by submersion in 5% solution of mucin for 30s. Excess surface liquid was removed by gentle blotting and they were attached to the lower end of the probe. The films were packed into the lower platform of the texture analyzer. The probe present above, possessing the mucin disc was lowered with a constant speed (0.5 mm/s) and contact force (10g) to the surface of the gel with an ensured intimate contact of 120s. The probe was then progressed upward with a constant speed of 0.1 mm·s⁻¹. The force-distance graph then obtained was for acquisition of the Maximum detachment force (F). From the force-distance plot, area under the curve was

calculated as mucoadhesion (M). The tests were conveyed at 37°C and each experiment was executed three times.¹⁶

In vitro Release Studies

The *in-vitro* release of developed formulations was studied by using simple diffusion cell apparatus. The diffusion cell apparatus consists of a glass tube with an inner diameter of 2.8 cm open at both ends, one end of the tube is tied with cellophane membrane previously dipped in the SVF which serves as a donor compartment.¹⁷ The cellophane membrane was previously soaked in the SVF for 24 hr to activate it and washed thoroughly. Weighed formulation was taken in the tube (to which cellophane was tied) fitted to clamp which was previously attached to an iron stand and tube was placed in 100ml of SVF. The medium was stirred by using the magnetic stirrer and the temperature was maintained at 37±2°C.¹⁸ Periodically 5 ml of samples were withdrawn and after each withdrawal same volume of SVF was replaced. Then the samples were assayed spectrophotometric ally using SVF as blank. The releases of all formulation were compared with marketed preparation.

Ex-vivo permeation studies

Ex vivo permeation study was performed to know the permeation behavior of prepared formulations. Their efficacy was compared with marketed formulation. Porcine vaginal tissue was used to create *ex vivo* condition employing simple diffusion cell apparatus. The diffusion cell apparatus consists of a glass tube with an inner diameter of 2.8 cm, open at both ends, one end of the tube is tied with porcine vaginal tissue, previously dipped in the release medium, which serves as a donor compartment. Weighed formulation was taken in a tube fitted to clamp which was previously attached to an iron stand and tube was placed in 100ml of SVF (pH 4.2). During permeation study temperature was maintained at (37±2°C) and study was continued for 7 hr. Samples were withdrawn at definite time interval with replacement. Then the samples were assayed after suitable dilution by spectrophotometric method using SVF (pH 4.2) as blank. After due time (i.e., 7 hr) drug content in the whole skin was determined.

Permeation Flux

The permeation flux as calculated for all the tested formulations by plotting the amount of drug released per unit surface area vs. time. The slope of the regressed line of the straight portion upto 7 hr of the graph was taken. This slope obtained from regressed line gives the permeation flux.

Skin Retention Studies

The study of retention of drug is a very important parameter as it will determine the concentration of drug present in the layers of the skin and if drug retention is more, it will retain on the skin for longer times, thus, increasing the efficacy of the drug. After permeation experiments, the porcine vaginal skin was removed carefully. The remaining formulation in the donor compartment adhering to the skin was scrapped with the spatula and rinsed 3-4 times with distilled water. Then the skin was dried using lint free cotton swab. Subsequently the skin was cut into small pieces and immersed into 10mL of methanol for 24h. After that, supernatant was filtered through 0.22 μm membrane filter. Subsequently, the filtrate was analyzed after suitable dilutions by UV spectrophotometric method.

Irritation Studies

The rat model has been used to establish the efficacy and/or safety of new microbicide and spermicide formulations. For vaginal administration to the female Sprague Dawley rats, animals were divided into four groups i.e., control (untreated), administered with *in-situ* gels and those administered with FLUCOS®, for vaginal irritation testing with three animals in each group. Animals were dosed intravaginally with *in-situ* gel and the marketed formulation once in group 1, 2, 3 respectively. Each formulation was inserted into the vaginal cavity. On the next day, animals were euthanized; vaginal tissues were excised and fixed with 10% buffered formalin.¹⁹ Fixed tissues were embedded in paraffin and microtoned. The sections were stained with hematoxylin and eosin. Finally, the specimens were evaluated for their integrity. Results were compared with that of control group.

In-vitro Candida albicans Inhibition

The fungus culture of *Candida albicans* was obtained as clinical isolate from Microbiology department (Panjab University, Chandigarh). Sabouraud Dextrose Agar (SDA) was prepared and poured into the plates in the sterile conditions and was allowed to solidify. After solidification agar wells were made in the plate using a sterilized borer and the test samples were poured into the wells and plates were incubated at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 24hr.

Stability Studies

The *in-situ* gel to be kept for stability studies were packed in glass vials. Stability studies have been carried out according to ICH Guidelines. Formulation subjected to accelerated stability studies at $40 \pm 2^{\circ}\text{C}/75\% \pm 5\%$ RH for a period of 3 months to evaluate any physical

or chemical changes on storage. The samples were withdrawn periodically at predetermined intervals (1 month, 2 month, 3 month) and evaluated for physical changes, pH, drug content, bioadhesive strength, film disintegration time and gelation time following the procedures outlined earlier. Zero time samples were used as control.

Statistical analysis

One way ANOVA SPSS (Version 17.0) software was used for analysis of the release study results. A difference below the probability level of 0.05 (95% confidence interval) was considered statistically significant.

RESULTS AND DISCUSSION

Solubility Studies

Based on the solubility data the media for *in vitro* release studies was chosen which maintains the required sink conditions. Solubility of fluconazole in different media was carried out to demonstrate solubility in the order: 0.1 N HCl > SVF (pH 4.2) > Lactic Acid (1%v/v) > Distilled water, possessing values of 6.8, 5, 4.52, 2.51mg/ml respectively.

Drug Excipient Compatibility Studies

Preformulation studies involving the drug excipient compatibility is most helpful in the selection of suitable excipients based upon their compatibility at an initial stage. This prevents major problems later in the formulation development. The techniques of thermal analysis and FTIR were used to determine the drug-excipient compatibility.²⁰

The thermal properties of a physical mixture are the sum of the individual components and this thermo gram can be compared with those of the pure drug and the excipient alone. An interaction on DSC will show any changes in melting point, peak shape and area and/or appearance of any transition. In this case, DSC thermo gram reported no change in the melting point of pure drug in physical mixtures with chitosan (DC), pure drug with HPMC, Polaxamer 407, Polaxamer 188, chitosan (DCH). These polymers can be used further in formulation development suggesting no or minimum interaction between drug and excipients. (Figure 1 A, B). All major peaks in FTIR of pure drug were retained in physical mixtures with HPMC, PVA, chitosan, PLX 407 and PLX 188 suggesting no/minimal chemical interaction between FLZ and HPMC; FLZ and chitosan; FLZ and PVA; FLZ and PLX 407; FLZ and PLX 188. (Figure 1 C, D)

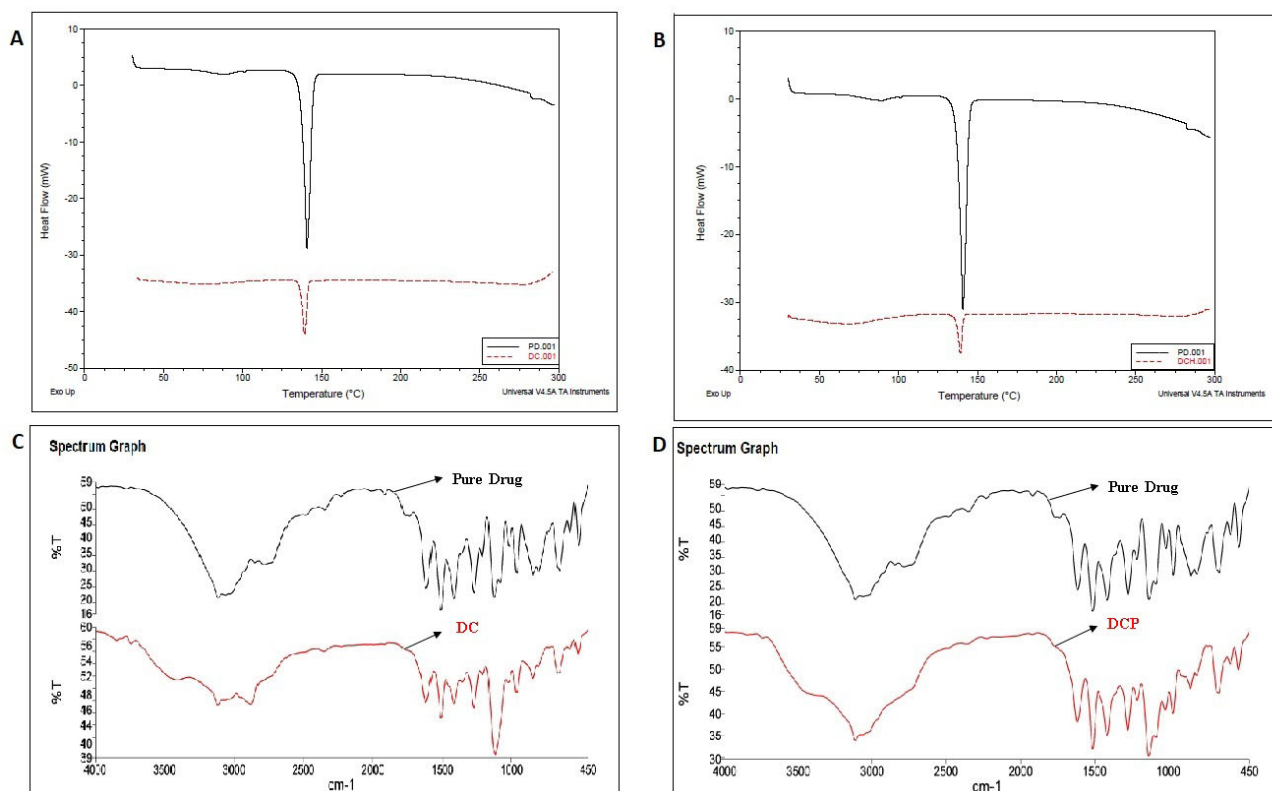


Figure 1: A) DSC thermo gram of PD (pure drug) and DC, B) DSC thermo gram of DCP, C) FTIR spectra of DC, D) FTIR Spectra of DCP.

Physical properties of vaginal films

Mucoadhesive film formulations were evaluated for the physical parameters like aesthetic, physical, mechanical and performance parameters including appearance, colour and surface texture. Various measured parameters are tabulated in Table 2. The films were generally smooth, pliable and transparent/semi-transparent in appearance. The average thickness of the film was found to range from 0.08 ± 0.005 to 0.15 ± 0.006 . The average weight of the film was found to range from 195 ± 3.5 to 287 ± 12.39 mg. The major contributing factor that altered film appearance was the amount of chitosan on the formulation. The films became more textured, rigid and opaque as the ratio of chitosan was increased. The drug was added in the last in order to prevent the drug from precipitating into the aqueous polymer solution. Due to this manufacturing protocol, the films were poured based upon volume and dried under identical conditions; the films all resulted in constant weight, thickness and pliability. Therefore, it is suggested that the physical dimensions of the films are not affected by formulation but by manufacturing process. Cosmetic appearance of the films was only affected by the amount of chitosan in the formulation. As the concentration of chitosan is increased, the films coloration and pliability

gets changed. Thus we can conclude that the excipient concentration affected the physical dimensions.¹³ The average weight of the film was found to range from 195 ± 3.5 to 287 ± 12.39 mg.

Drug Content, Moisture Content and Swelling Index

Drug content, moisture content and swelling index of developed film formulations has been given in Table 3. Drug content of all the formulations has been found in the range of $96.71 \pm 0.89\%$ to $99.22 \pm 1.30\%$. Controlling the water content of films is an important issue contributing towards the stability of formulation over time. The optimum amount of moisture content in formulations helps them to remain stable and prevent them from being a completely dry and brittle film.²¹ The moisture content in the film formulation ranged from 4.65 ± 0.066 to $7.66 \pm 0.15\%$. Vaginal volume has been reported by various researchers to be in the range of 1.89 to 11 mL/day. Owen and Katz had reported that the daily production of vaginal fluid is around 6 g/day, with approximately 0.5–0.75 g present in the vagina at any one time. The ability of films to hydrate upon contact with vaginal fluid is crucial for their mucoadhesive behavior and consequently has a great impact on stability and drug release profiles. The swelling

Table 2: Physical evaluation parameters of mucoadhesive films.

Formulation code	Physical Evaluation Parameters					
	Color	Appearance	Surface Texture	Thickness (mm)	Average Weight (mg)	Pliability
F11	Colorless	Transparent	Smooth and soft	0.08±0.005	192.5±3.5	High
F12	Colorless	Transparent	Smooth and soft	0.09±0.005	226±13.6	Moderate
F13	Colorless	Semi-Transparent	Slightly gritty	0.13±0.01	265±9.08	Moderate
F14	Slightly colored	Opaque	Gritty	0.15±0.006	287±12.39	Low

Table 3: Drug content, Moisture content, Swelling index of various prepared formulations.

Formulation code	Drug content ± S.D ^a	Moisture content ^a (%)	Swelling Index ^a
F11	98.75±0.58	7.66 ± 0.15	0.03
F12	98.98±1.40	5.32 ± 0.045	0.053
F13	99.22±1.30	4.29 ± 0.098	0.06
F14	96.71±0.89	4.65 ± 0.066	0.1

^aData are mean ± S.D, n=4.

index is a commonly used parameter that assesses the weight gain of films with time upon being submerged in VFS. The swelling capability of polymer is crucial for its mucoadhesiveness. Adhesion occurs shortly after beginning of swelling but the bond formed is not very strong.⁵ Films were found to be rapidly swollen within 4 min and thereafter slowly reached a stage of complete gelling/swelling. Maximum swelling was seen with the film containing 2% of chitosan. It was observed that the swelling index increased with increasing concentration of chitosan, with maximum swelling observed at 2% of chitosan. Comparison plots of moisture content and swelling index are depicted in Figure 2.

Rheological studies

Viscosity of formulations in vaginal environment after administration governs the spreading and retention of the

product, which is essential to achieve the desired efficacy.⁵ Higher viscosity is related to prolonged residence time in the vaginal cavity under the normal physiological conditions. However, high viscosity of aqueous solutions does not allow for removal of entrapped air bubbles and uniform spreading on casting surfaces precluding the formation of uniform films.²² Formulation F14 when formed into gel resulted in entrapped bubbles which could not be removed. So, the rheological studies were not performed on the formulation F14 due to practical limitation. Rheological findings for the gel formed from film formulation F11, F12, F13 and marketed product has been presented in the form of rheograms in Figure 3. For obtaining the rheogram for the film formulations, it was placed in 8 mL SVF to make it of semisolid consistency which was suitable for measurement in rheometer. Graphs were plotted between shear rate and shear stress was found to be non-linear which indicates Non-Newtonian flow. The consistency curve begins at the origin thus the flow was pseudo plastic flow and no part of the curve is linear, therefore, the viscosity of the formulation cannot be expressed by single value. Rheograms for all the tested compositions (Figure 3 A-D) including marketed product have proved them to be pseudo plastic in nature, as they started flowing immediately after being submitted to a shear stress (τ). Being pseudo plastic in nature, after vaginal administration, stress imposed by vaginal contractions will lead to decrease in their viscosity

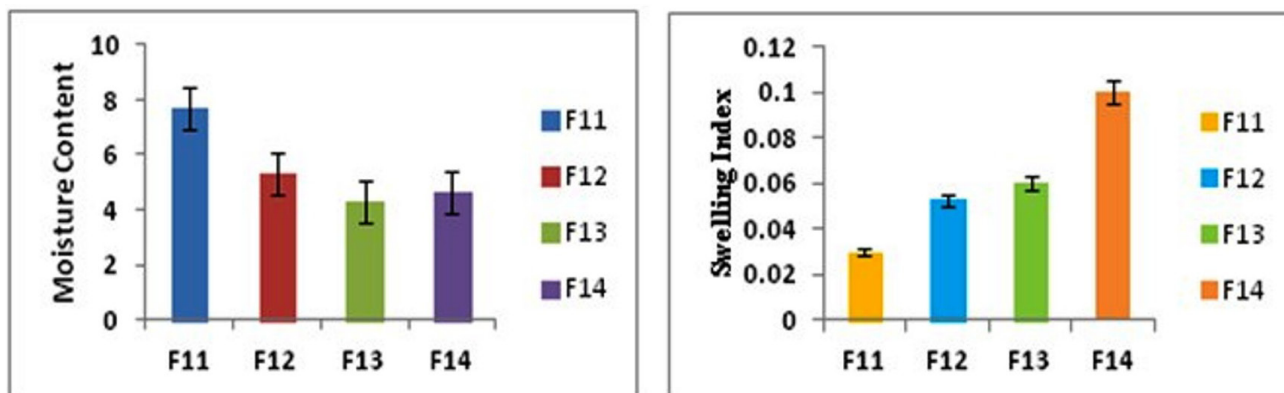


Figure 2: Comparison plots of moisture content and swelling index. Data are mean ± S.D, n=3.

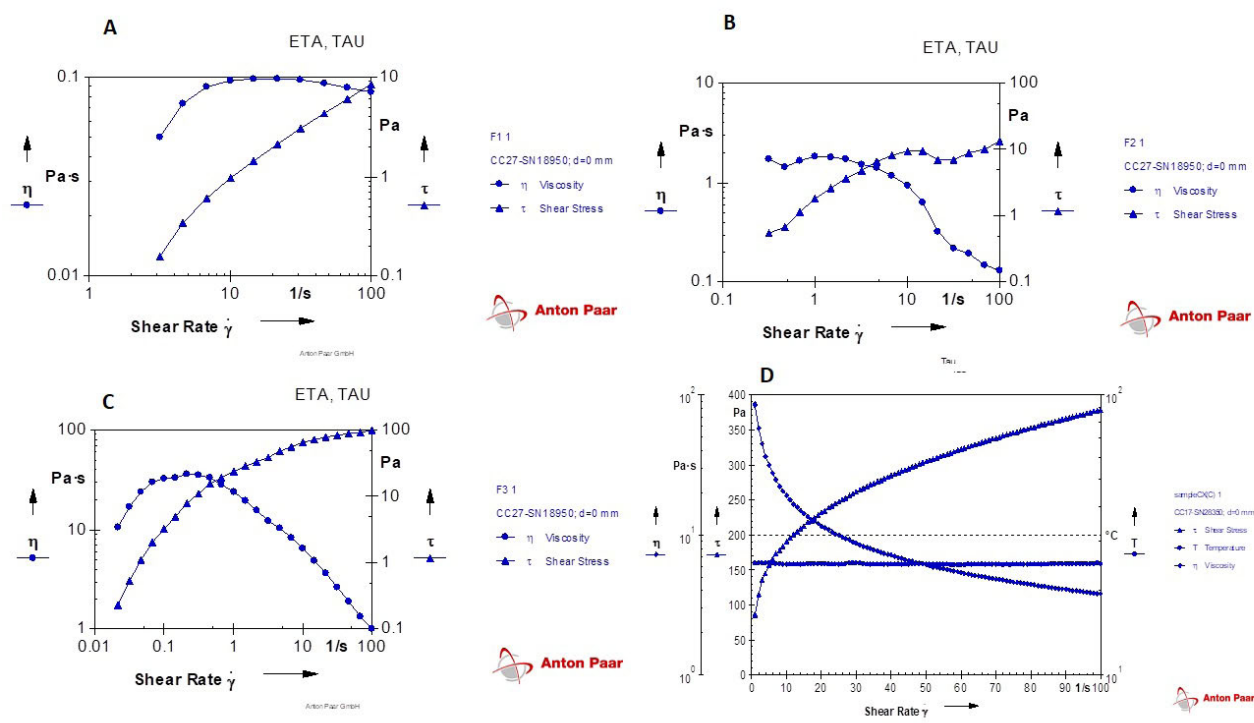


Figure 3: Rheograms for the vaginal film formulation A) F11, B) F12, C) F13, D) marketed intravaginal gel of clotrimazole.

and thus aiding in their dispersion in vaginal cavity. This decrease in consistency helps in dispersion to spread over the cervico-vaginal surface.

Mucoadhesion Studies

The contact time of a formulation on the mucosa is of high importance for vaginal drug deliver. Mucoadhesive formulations have been reported to prolong the residence time of the formulation at the site of application. Quantification of mucoadhesion is important to ensure that the adhesion offered by formulations is sufficient to ensure prolonged retention at the site of application. Mucoadhesion force has been measured using mucin disc in place of mucus membrane. Studies were done using texture analyzer (TA.XT. Microsystems, UK). Data obtained following mucoadhesion studies has been presented in Table 4. Mucoadhesive strength of film formulations has ranged from 0.262 ± 0.006 N to 0.331 ± 0.003 N. It has been observed that maximum mucoadhesive strength was achieved using chitosan i.e., in formulation F13 as compared to F11 and F12. Mucoadhesive strength of all film formulations and marketed preparation were comparable.

Chitosan, a partially deacetylated chitin, which is a biologically safe biopolymer, prolongs the adhesion time of formulations and drug release from them. It is a promising mucoadhesive material at physiological pH. Chitosan provides a prolonged adhesion since it does

Table 4: Mucoadhesive strength (N) of various intra-vaginal film formulations.

Formulation code	Mucoadhesive strength (N) \pm S.D.
F11	0.262 ± 0.006
F12	0.306 ± 0.007
F13	0.331 ± 0.003
Marketed	0.281 ± 0.003

not become inactivated after the first contact and shows no drop in mucoadhesion. Chitosan also possesses antimicrobial property. Chitosan possesses OH and NH_2 groups that give rise to hydrogen bonding. Its linear molecules express sufficient chain flexibility and their conformation is highly dependent on ionic strength. These properties are considered essential for mucoadhesion. Probable explanation for higher mucoadhesive nature of film formulations can be found in the fact that cationic molecules can interact with the mucus surface, since it is negatively charged at physiological pH. So, mucoadhesion of cationic polymers such as chitosan occurs because of the electrostatic interactions of their amino groups with the sialic groups of mucin in the mucus layer.

The developed fluconazole films are mucoadhesive in nature, would be retained in vagina for prolonged intervals, hence are expected to reduce the leakage and messiness, avoid user inconvenience, prolong the contact time in

vaginal cavity and thereby increase the effectiveness of fluconazole.

In vitro release studies

In vitro release studies of intravaginal films have been carried out using diffusion cell assembly with cellophane membrane as the diffusion barrier. SVF (pH 4.2) was used as the release medium. Results obtained from these studies have been presented in Figure 4. For comparison of drug release characteristic, a marketed topical cream of fluconazole (FLUCOS®) was taken.

Data obtained from *in vitro* release experiments have shown that film formulation F12 and F13 exhibited higher drug release in comparison to marketed gel. Vaginal film formulation F12 have shown maximum percentage cumulative drug release after 8 hr release of $42.563 \pm 0.743\%$ followed by F13 > FLUCOS®. Film formulation F12 has shown 1.2 and 2.5 times higher cumulative percentage drug release than F13 and FLUCOS® respectively.

Among film formulations, F12 has shown highest percentage cumulative drug release ($42.563 \pm 0.743\%$), followed by F13 ($35.176 \pm 0.395\%$) with lowest percentage drug release ($29.43 \pm 1.65\%$). This can be explained on the basis of nature of gels formed by these formulations upon contact with SVF. The polymer, viz. Chitosan owes excellent mucoadhesive strength, release rate controlling ability, non-toxicity, non-irritancy and stability at vaginal Ph.²³ Chitosan, a partially Deacetylated chitin, which is a biologically safe biopolymer, prolongs the adhesion time of formulations and drug release from them. Chitosan provides a prolonged adhesion since it does not become inactivated after the first contact and shows no drop in mucoadhesion.

Chitosan has been comprehensively investigated for its suitability for its controlled release characteristics.²⁴ The chitosan formulation presented a high potential for the release of antimicrobial agents in a controlled manner

with mucoadhesion and safety. Drug release rates curves of the formulations showed that with the increase in the polymer (chitosan, HPMC), concentration drug release decreases.¹² In comparison to HPMC films of fluconazole reported by Kumar *et al.* chitosan films in the current work showed a controlled release upto 8hr of *in-vitro* release studies.¹ Chitosan forms strong gel and strong gel network leads to increase in diffusion path length for the drug molecules which results in decreased drug release.

Ex vivo permeation studies

Ex vivo permeation studies of intravaginal films has been carried out using porcine vaginal tissue as the permeability barrier. These studies have been done to illustrate whether or not the developed formulations are able to cross the lipidic physiological barrier to reach the target site.

Results observed were quite in line with *in vitro* release studies without much variation which is presented in Figure 5. With porcine vaginal tissue as the permeability barrier, formulation F13 has shown highest amount of drug permeating per unit area due to the presence of higher concentration of chitosan followed by F12. Marketed topical formulation has shown the lowest amount of drug permeated per unit area. Formulation F13 has shown 1.3 and 2.32 times higher drug amount permeated per unit area in comparison to formulation F12 and FLUCOS® respectively.

Permeation flux and Drug retention

Skin retention studies were carried out to find the amount of drug retained by the skin layers. The skin retention studies give an indication of the amount of the drug that has been retained by the skin and will provide localized drug concentration at the site of administration. Results obtained following drug retention studies have been presented in Table 5. The similarities in structure and function between porcine and human vaginal tissue,

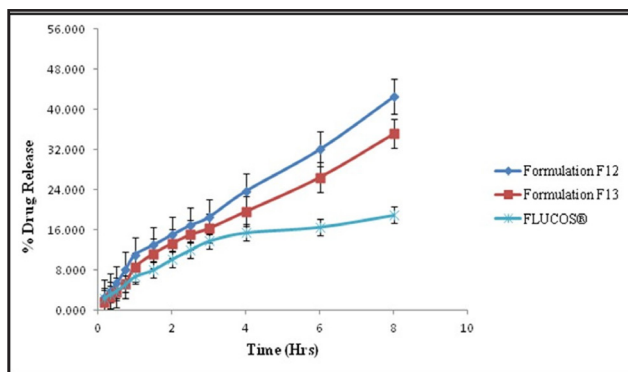


Figure 4: Comparison of cumulative percentage drug release profiles. Data are mean \pm S.D, n=4.

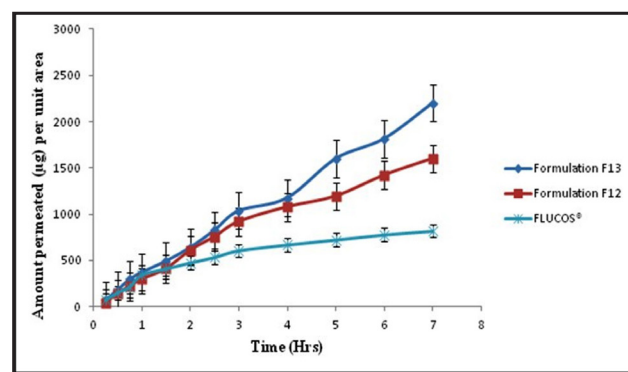


Figure 5: Amount permeated per unit time through porcine vaginal tissue. Data are mean \pm S.D, n=4.

together with similarity in terms of pH, secretions and inflammatory response have led to the use of the pig as an experimental animal model in studies of vaginal irritancy.²⁵ Among film formulations maximum drug retention has been found to be from formulation F13 followed by F12 in porcine vaginal tissue. Percentage drug retention in porcine vaginal tissue for F13 has been found to be 1.15 and 1.09 times higher than F12 and FLUCOS[®]. Drug retention of film formulations was comparable to FLUCOS[®] suggesting that intravaginal local delivery by film formulations will be more effective in comparison to marketed fluconazole gel. Formulation F13 has shown highest drug retention which may be attributed to the presence of chitosan. As stated earlier, chitosan being positively charged may interact with negatively charged biological membranes, leading to opening of junctions, resulting in higher permeation as compared to other formulations. The skin permeation flux values were calculated from the initial linear portion for all the film formulations (F12, F13) and FLUCOS[®]. Data observed after calculation has been presented in the form of Table 5.

Permeation flux values for porcine vaginal tissue related to intravaginal film formulations have been found to be higher than FLUCOS[®]. Permeation flux values for intravaginal mucoadhesive film formulation F13 have been found to be 1.2 and 6.8 times higher than F12 and FLUCOS[®] using porcine vaginal tissue as the membrane barrier. The higher flux values found for film formulations may be attributed to the presence of chitosan. Several studies have reported that chitosan has been found to enhance drug permeation by paracellular transport through electrostatic binding of positively charged chitosan to the negatively charged membranes. Furthermore, chitosan has been shown to improve drug absorption through nasal mucosa through bioadhesion

properties and may be through opening of tight junction effect, so it may be expected that when administered vaginally, chitosan being positively charged may also interact with the negatively charged surface of vaginal mucus by electrostatic interactions and may help in improving drug permeation.

Drug release kinetics

To understand the pattern of drug release from the film formulations (F12 and F13), the data obtained from permeation studies on porcine vaginal tissue has been fitted into various release models such as zero order, first order, Higuchi, Hixson-Crowell, Korsmeyer-Peppas model. Values obtained by model fitting of data have been shown in Table 6.

Pattern of drug release through the gels formed by films and *in-situ* gels was explained by Korsmeyer-Peppas model with regression coefficient of 0.99 and 0.997 for formulation F12 and F13 respectively. The value of release exponent (n) was found to be 0.961, 0.835 for formulations F12, F13 respectively, suggesting the release mechanism to be anomalous (coupled diffusion/polymer relaxation) transport.

Irritation studies

The vaginal mucosa is highly sensitive to the exposed chemicals and therapeutic agents that may result in irritation and/or inflammation and can make women more susceptible to various infections. Hence, the vaginal irritation potential of feminine care formulations and vaginally administered therapeutic agents is a significant public health concern as it is associated with the convenience of the patients. So, not only does a successful antifungal intravaginal formulation have to be effective at blocking the fungal infection, it also needs to be non-irritating to the vaginal mucosa, failure of which may lead to poor patient compliance. Vaginal irritation studies in rodents are a cost-effective way to evaluate the safety of vaginal formulations on multilayered vaginal epithelium *in vivo*, in contrast to studies in rabbits whose single-layer epithelium is very sensitive but also very different from the multilayered human vaginal epithelium. Sprague Dawley rats are a standard toxicology model for vaginal irritation

Table 5: Permeation flux values data through porcine vaginal tissue.

Formulation code	% Drug retained ^a	Permeation flux ^a (µg/cm ² /h)
F12	3.35 ± 0.82	296.43 ± 18.59
F13	3.87 ± 0.56	358.63 ± 17.05
FLUCOS [®]	3.52 ± 0.59	53.36 ± 7.46

Data are mean ± S.D, n=4

Table 6: Model fitting of data obtained from permeation studies through porcine vaginal tissue.

Formulation code	Zero order	First order	Higuchi	Hixson-Crowell	Korsmeyer-Peppas model	
	r ²	r ²	r ²	r ²	r ²	N
F12	0.985	0.98	0.935	0.982	0.99	0.961
F13	0.97	0.974	0.935	0.982	0.997	0.835

studies. For vaginal administration, animal dose (AD) was calculated from equivalent human dose (HED) by referring to US FDA guideline set for dose conversion. In the US FDA guideline, average weight of humans is assumed to be 60 Kg. In the developed intravaginal film and *in-situ* gel dosage form, dose of fluconazole is 100 mg i.e., 6.6 mg/Kg as per US FDA guideline.

$$\text{HED (mg / Kg)} = \frac{\text{Dose(mg)}}{60(\text{Kg})} \dots\dots\dots [2]$$

$$\text{HED (mg / Kg)} = \frac{100}{60} = 1.7\text{mg / Kg} \dots\dots\dots [3]$$

HED dose was converted to AD using equation,

$$\text{HED} = \text{AD} \times 0.16 \dots\dots\dots [4]$$

$$\text{AD} = \frac{1.7}{0.16} = 10.625 \text{ mg / Kg} \dots\dots\dots [5]$$

Average weight of female rats was found to be 250 mg. So, dose was calculated for average weight of animals for preparation of Minitab lets as per following equations.

$$\begin{aligned} \text{Dose to be administered} &= \text{AD} \times \text{average} \\ \text{vaginally} &= \text{wt.of animal} \dots\dots\dots [6] \end{aligned}$$

$$\begin{aligned} \text{Dose to be administered} &= 10.625 \times 0.250 \dots\dots\dots [7] \\ \text{vaginally} &= 2.65 \text{ mg} \end{aligned}$$

Sprague Dawley rats were dosed intravaginally with minifilms (1×1 cm) of composition corresponding to formulation F12 and 0.4 g (0.5% w/w) FLUCOS®. An untreated control group was included in the study and histopathology was analyzed semi-quantitatively. Endpoints which are usually looked for after histopathological examination following rat vaginal irritancy studies are epithelial integrity, epithelial vascular congestion, leukocyte infiltration and oedema.²⁶ Results obtained following histopathological examination has been illustrated in Figure 6. It was observed that fluconazole mini films were very well tolerated by the rats as no significant signs of erythema and/or edema were seen the next day after vaginal administration. Studies indicated that the marketed formulation was also well tolerated by the rats and it did not show any irritation.

From the histopathological examination it has been seen that epithelial membrane is intact, no signs of vascular congestion/leukocyte infiltration/oedema has

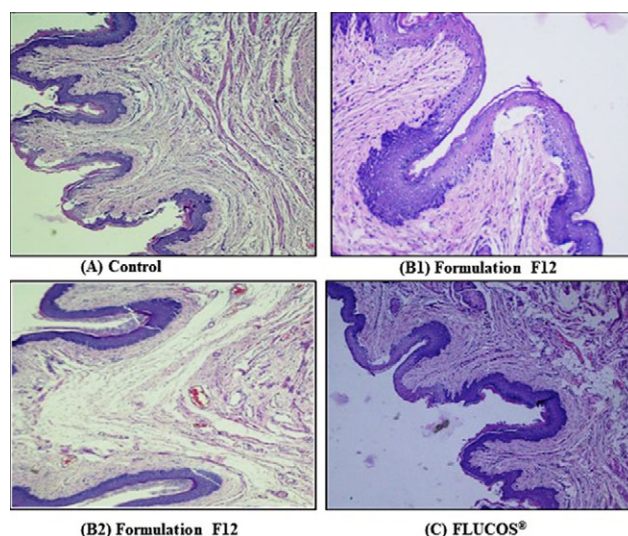


Figure 6: Histopathological evaluation of rat vaginal tissue of various animal groups
A) Untreated/control; B1) and B2) Formulation F12; C) FLUCOS®.



Figure 7: Fungal culture of dilution used in the inoculum preparation.

been seen in any of the groups. It suggests that tested formulation i.e. F12 will be equally safe as marketed formulation upon vaginal administration with regard to vaginal irritancy potential.

In-vitro antifungal studies

Preparation of Fungal inoculum

The fungus culture was subcultured on the Sabouraud dextrose agar (SDA). The incubation temperature was maintained at 35°C throughout. Different dilutions (10^{-1} to 10^{-8}) were made from the culture. The culture was further used from 10^{-4} dilution plate. The plate of the culture was as shown below in Figure 7.

Determination of zone of inhibition

The comparison of the efficacy of different formulations (F12, F13) with FLUCOS® was done by measuring the

zone of inhibition of growth. The formulations were poured in the well made in SDA plates and was allowed to diffuse for an hour and different inhibition zones were observed after 24 hr incubated at 37°C Table 7. Larger the inhibition zone higher is the efficacy against that infection. The efficacy of F12 was higher as compared to FLUCOS® and F13 used against *Candida albicans* as it had higher zone of inhibition (4.8 cm) as compared to FLUCOS® (2.4 cm). The zone of observed for F13 was less as compared to F12 due to higher concentration of chitosan. It may be due to the slow diffusion of the drug into the agar plate from the formulation.

Stability studies

Stability testing of formulations is of paramount importance in formulation development. Stability of the dosage form refers to chemical and physical integrity of the dosage unit, over a period of time. It is defined as the extent to which product retains within specified limits and throughout its period of storage and use (i.e. shelf life), the same properties and characteristics that it possessed at the time of manufacture.

Film formulation F12 was therefore, subjected to accelerated stability testing by keeping them at accelerated conditions of temperature and humidity, i.e., 40°C ± 2°C/75% RH ± 5% RH according to ICH Q1A(R2) guideline for a period of three months. Zero time samples were used as controls. Samples withdrawn at predetermined intervals were analyzed for various performance parameters to access any degradation. Results thus obtained have been summarized in Table 8.

Table 7: Zone of inhibition of various formulations.

Formulation	Plate 1 ^a			Plate 2 ^a		
F12	4.7	4.4	4.8	4.4	4.6	4.5
F13	3.8	4	4.1	3.7	3.8	4.2
FLUCOS®	2.4	2.1	2.2	2.3	2	2.1

^aData are mean ± S.D, n=6

Table 8: Stability studies data for the intravaginal mucoadhesive film formulation F12.

Evaluation parameter	0 Time	1 Month	2 Months	3 Months
Color	Colorless	Colorless	Colorless	Colorless
Appearance	Transparent	Transparent	Transparent	Transparent
^a Film disintegration time (mins)	6.3 ± 1.9	6.5 ± 0.8	7.2 ± 1.4	6.9 ± 1.7
^a Drug Content ± S.D	98.98 ± 1.40	98.39 ± 1.06	97.98 ± 1.56	98.81 ± 0.85
^b pH ± S.D.	4.34 ± 0.015	4.45 ± 0.18	4.37 ± 0.19	4.42 ± 0.22
^b Mucoadhesive strength (N)±S.D.	0.306 ± 0.007	0.279 ± 0.037	0.289 ± 0.019	0.294 ± 0.013

^aData are mean ± S.D, n=4. ^bData are mean ± S.D, n=3.

It was observed that even at elevated conditions of temperature and humidity, there was no physical change, no change in drug content, no change in mucoadhesive strength, no change in film disintegration time or the pH of the gel formed by the film F12. After three months at accelerated conditions, pH of formulation i.e., F12 has remained within the range i.e. 3.5-4.5 suitable for vaginal administration. All the parameters showed no significant difference.

CONCLUSION

In the present study, safe and stable bio adhesive vaginal film dosage forms were successfully developed for Fluconazole. The overall results obtained during this investigation suggest that Fluconazole bio adhesive film possessed desirable aesthetic, pharmaceutical and biological properties, making it a novel delivery system for effective and convenient treatment of vaginal candidiasis. This novel delivery system will offer substantial benefits for improving women's health.

ACKNOWLEDGEMENT

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CONFLICT OF INTREST

The authors declare no conflict of interest.

ABBREVIATIONS

FLZ: Fluconazole; **SVF:** Simulated Vaginal Fluid; **VFS:** Vaginal Fluid Stimulant; **PVA:** Polyvinyl Alcohol; **DSC:** Differential Scanning calorimetry; **h:** Hours, **°C:** Degree Centigrade; **HPMC:** Hydroxy Propyl Methyl Cellulose; **SDA:** Sabouraud Dextrose Agar.

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SUMMARY

The purpose of this work was to design and evaluate bio adhesive films for effective treatment against vaginal candidiasis. Bio adhesive films were prepared by the solvent evaporation technique. The excipients including chitosan as a mucoadhesive polymer along with polyvinyl alcohol and glycerin as plasticizer were dissolved at a total combined concentration to form optimized formulation. Variables such as swelling index, mucoadhesive strength, percentage drug release, permeation, retention and irritation studies were assessed. The prepared films were generally smooth, pliable and transparent/semi-transparent in appearance having an average thickness range. *In vitro* release experiments demonstrated that film formulations exhibited higher drug release in comparison to marketed gel, FLUCOS[®] with maximum percentage cumulative drug release after 8 hr release of 42.563 ± 0.743%. Skin retention studies were carried out to find the amount of drug retained by the skin layers. Percentage drug retention in porcine vaginal tissue for F13 has been found to be 1.15 and 1.09 times higher than F12 and FLUCOS[®]. The selected formulation showed an appropriate anti-candida activity through appearance of zone of inhibition during anti-fungal activity studies, with promising results to meet the unmet medical need for vaginal delivery that swells and forms a viscous gel upon insertion into the vaginal cavity. Conclusively, safe and stable bio adhesive vaginal film dosage forms were successfully developed for Fluconazole. The overall results obtained during this investigation suggest that Fluconazole bio adhesive film possessed desirable aesthetic, pharmaceutical and biological properties, making it a novel delivery system for effective and convenient treatment of vaginal candidiasis. This novel delivery system will offer substantial benefits for improving women's health.

PICTORIAL ABSTRACT

In-situ Gelling System for Mucoadhesive Site- Specific Drug Delivery for Treatment of Recurrent Vaginal Candidiasis

OBJECTIVES	RESULTS																																							
<p>Films are designed because:</p> <ol style="list-style-type: none"> 1. They are able to sustain and/or control the release of entrapped drug. 2. They are also apt for vaginal application. 3. Films are designed to rapidly disperse or dissolve in contact with fluids to form a smooth, viscous and mucoadhesive gel while possessing good appearance (preferably colorless and odorless), softness, flexibility and absence of any sharp edges to avoid mechanical injuries during insertion. 	<p>Table 1: Compositions of various intravaginal mucoadhesive fluconazole film formulations</p> <table border="1"> <thead> <tr> <th rowspan="2">Name of Ingredients</th> <th colspan="4">Quantity of Ingredients</th> </tr> <tr> <th>F11</th> <th>F12</th> <th>F13</th> <th>F14</th> </tr> </thead> <tbody> <tr> <td>Fluconazole</td> <td>100 mg</td> <td>100 mg</td> <td>100 mg</td> <td>100 mg</td> </tr> <tr> <td>Chitosan</td> <td>0.5%</td> <td>1%</td> <td>1.5%</td> <td>2%</td> </tr> <tr> <td>Polyvinyl Alcohol</td> <td>2%</td> <td>2%</td> <td>2%</td> <td>2%</td> </tr> <tr> <td>Glycerine</td> <td>0.5 mL</td> <td>0.5 mL</td> <td>0.5 mL</td> <td>0.5 mL</td> </tr> <tr> <td>Polyethylene glycol</td> <td>0.5 mL</td> <td>0.5 mL</td> <td>0.5 mL</td> <td>0.5 mL</td> </tr> <tr> <td>Lactic acid (1% v/v)</td> <td>20 mL</td> <td>20 mL</td> <td>20 mL</td> <td>20 mL</td> </tr> </tbody> </table>	Name of Ingredients	Quantity of Ingredients				F11	F12	F13	F14	Fluconazole	100 mg	100 mg	100 mg	100 mg	Chitosan	0.5%	1%	1.5%	2%	Polyvinyl Alcohol	2%	2%	2%	2%	Glycerine	0.5 mL	0.5 mL	0.5 mL	0.5 mL	Polyethylene glycol	0.5 mL	0.5 mL	0.5 mL	0.5 mL	Lactic acid (1% v/v)	20 mL	20 mL	20 mL	20 mL
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