CHAPTER - V

ITRACONAZOLE CONTAINIING BIOADHESIVE FILM FOR VAGINAL DELIVERY

5.1 BACKGROUND

An important part of the product development strategy in the area of female healthcare is designed of broad line products that provide the unmet medical needs of women. The vast majority of gels, foams, creams, suppositories and tablets are presently available conventional formulations which possess poor bioadhesive properties.

Bioadhesive drug delivery

Bio-adhesion refers to any bond formed between two biological surfaces or between a biological and synthetic surface as shown in **Fig.** 5.1. As one would expect, this concept has received considerable attention in the pharmaceutical field due to the potential for applications in drug delivery. In bioadhesive delivery system, bioadhesive molecules are incorporated into some type of pharmaceutical formulations with active compound that are capable of delivering the active compound for an extended period at a predictable rate. The formulations will be held on/at close to the vaginal mucosa with consequent to achieve high drug level (Methiowitz E. *et al.*, 1999). The vagina is a highly suitable site for delivery of bioadhesive formulations. Among the different types of polymers, biodegradable polymers are more suitable to design bioadhesive delivery system over the non-biodegradable ones as they can be degraded to nontoxic monomers inside the vaginal cavity. Thus, obviate the need of removal of the device after its depletion.

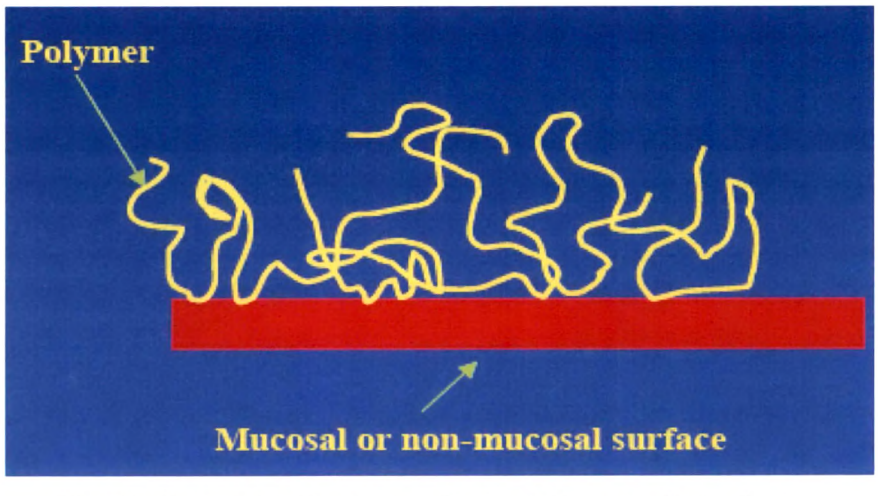

Fig. 5.1 Interaction between polymer and mucosal membrane.

The interaction of the molecular chain with vaginal mucosa and variation in chain length may affect the bioadhesive properties of polymers such as dextran, hyluronic acid, chitosan, polycarbophil, sodium alginate etc. In addition to bioadhesive vaginal formulation, polycarbophil 934P is a commonly used bioadhesive polymer that exhibited pH-dependent bioadhesive properties (Blanco-Fuente H. *et al.*, 1996). The necessary assemblies have been designed to measure the bioadhesion characteristics of polymers and formulations in a

simulated vaginal environment (Lee C.H. *et al.*, 1996). List of different bioadhesive polymers can be used in bioadhesive formulation are given below.

Bioadhesive polymer	
Polycarbophil	Sodium alginate
Polyethylene oxide	Chitosan
Sodium Alginate	Tragacanth
Carbopol 974P-NF	Sodium Carboxy Methyl Cellulose
HPMC	Hydroxy Propyl Cellulose
	or other cellulose derivatives

Solid bioadhesive formulation: As a progressive hydration approach to solid bioadhesive delivery system, the product absorbs moisture, outside becomes a gel and releasing medication in time-controlled process as shown in **Fig.** 5.2.

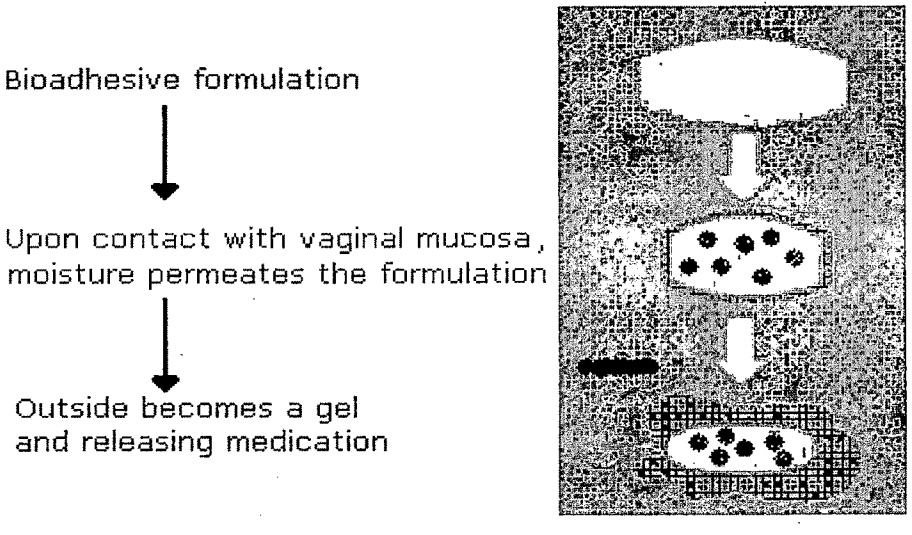

Fig. 5.2 Progressive hydration approach to solid bioadhesive delivery system

Solid bioadhesive formulations achieve bioadhesion via dehydration of the local mucosa. A polymeric film that is placed directly between the vaginal mucosal surfaces has been demonstrated to be excellent bioadhesive formulation. For example, bioadhesive films of polystyrene sulfonate under the toxicological and clinical evaluation will offer substantial benefits for improving women's health by preventing sexsual transmitted diseases and AIDS. Upon contact with mucosal surface, the persistent gel layer formed surrounding the film formulations may prevent irritation to the vaginal mucosa (Lee J.W. *et al.*, 2000). In another study, water soluble or dispersible films are being used to deliver drugs directly to a mucosal surface as they have to form close contact with mucosal membrane (A Robert Neurath *et al.*,

2003). Chitosan and sodium alginate based bioadhesive tablets were found to released 100% of metronidazole over a period of 8 hours in buffer pH 4.8. Aesthetic properties of vaginal formulation are also crucial to ensure proper compliance and regular use.

Semi-solid bioadhesive formulation: Gel- forming bioadhesive polymers include cross-linked polyacrylic acid, tragacanth, carbomer 934P, polycarbophil, acacia, carrageenan, sodium carboxymethyl cellulose and Polyvinyl alcohol that has been used to adhere to mucosal surface for extend period of time and provide controlled release of drugs. Mucoadhesive gels based on polycarbophil, Repelen gel was to be retained in the genital cavity for 3-4 days (Robinson J.R. *et al.,* 1994). **Fig.** 5.2 shows the progressive adhesion of semisolid bioadhesive formulation to vaginal mucosa.

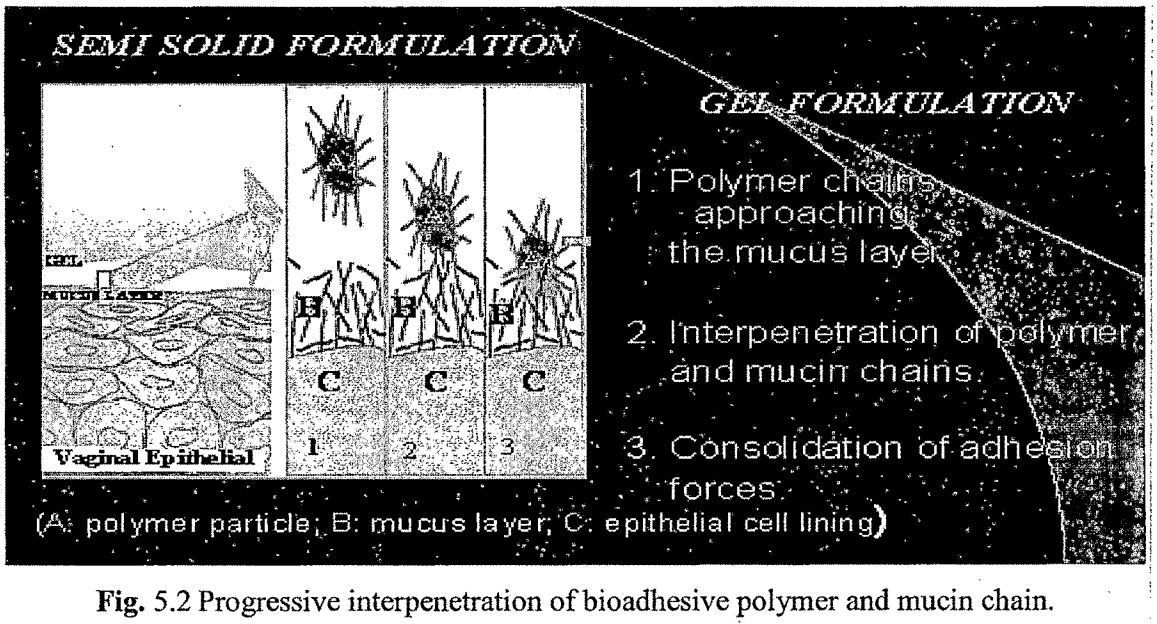

Fig. 5.2 Progressive interpenetration of bioadhesive polymer and mucin chain.

Bioadhesive formulation based on carbomers and polycarbophil, gives satisfactory drug delivery within vaginal cavity for the application of a single dose. For example, Prochieve ^{TM} is a bioadhesive gel that is satisfactorily used in the therapy of hormone replacement in female. Francois *et al.,* (2003) reported a mucoadhesive, cyclodextrin-based vaginal cream formulation of ITR, was found to form a thin bioadhesive layer over the genital tract surface. A clinical study reveals that this innovative formulation of ITR was well tolerated and effective in combating vaginal candidiasis. The controlled release drug delivery system can be formulated by incorporation of time-release additives. For example, polycarbophil based bioadhesive vaginal gel, Crinone (Columbia Laboratories, Rockville Center, NY) to ensure a controlled release of progesterone for at least 48 hours after a single vaginal application.

5.2 INTRODUCTION

Vulvovaginal candidiasis (Nyririesy P. *et al.*, 2001) is one of the most frequent genital infections occurs in women once in their lifetime, most commonly during pregnancy or after treatment with antibiotics. The increasing incidence of vulvovaginal candidiasis has highlightened urgent need of appropriate therapeutic strategies aimed to successful eradication of infectious agent, short-term treatment, achievement of high drug levels at the target site, avoidance of first-pass metabolism and safety (Ghelardi E. *et al.*, 1998). From such a perspective, topical antifungal chemotherapy could represent a rational choice for treatment of vaginal candidiasis, because of the toxicity of antifungal drug after systemic administration. The introductions of potent imidazole and triazole antifungal agents have significantly altered the duration of treatment of acute vaginal candidiasis (Bloch G. *et al.*, 1988). ITR is a new triazole antifungal drug with a broad spectrum of activity (Stein G.E. *et al.*, 1993). This agent appears to be an attractive alternative for treatment of vaginal candidiasis because of its enhanced activity against candida species, leading to short courses of therapy (Sanz F. *et al.*, 1987).

The vagina has been explored as a favourable site for the local and systemic delivery of drugs used for the treatment of female-specific conditions. Vaginal administration of drugs is mainly used for the treatment of local infections such as bacterial vaginosis, candidiasis and other infections. All of the existing conventional vaginal formulations have poor bioadhesive properties. They exhibit limited effectiveness due to rapid, uncontrolled release of the active agents (Kirschner M.I. *et al.*, 2005). Conventional dosage forms frequently discharge a leakage and drippage (Garg K. *et al.*, 2000). Therefore, it is an urgent need of development of innovative vaginal formulation technology that can fulfills certain criteria such as desirable product dispersion throughout vagina and retention for prolong time intervals which may help to get desire therapeutic action. These features can be achieved by the application of bioadhesive vaginal drug delivery system (BVDDS) (Robinson J. *et al.*, 1994).

Vaginal films are more preferred over gels (Coggins C. *et al.*, 1998) by women in different area of the world due to its aesthetic appeal. In addition, the film formulation possess several advantages of easy storage and handling, ease of application (applicator not required for administration) and improved stability of drug at tropical climate. Hence, efforts were made to develop a BVDDS in the form of film for ITR.

ITR possess very poor water solubility (~4 µg/ml at acidic pH) that makes it difficult to formulate in bioadhesive delivery system. One approach, which has been applied for producing BVDDS of ITR, is use of solid dispersion of ITR (SDITR) and HPMC E15 in

which drug particles are homogeneously distributed throughout the hydrophilic polymer. SDITR can improve wettability of ITR which may help in development of bioadhesive delivery system. Also, SDITR forms colloidal dispersion in simulated vaginal fluid (SVF) is essential for local action on vaginal mucosa. Therefore, SDITR was used in present investigation for preparation of film formulation. SDITR was prepared by spray drying method as reported in chapter IV and characterized by differential scanning calorimetry and X- ray diffraction technique.

In the present study, we are reporting the design and *in vitro, ex vivo* and *in vivo* characterization of ITR containing bioadhesive vaginal film. The vaginal films were targeted to retain on vaginal mucosa up to 8h and should possess aesthetic appeal such as flexibility, softness and free of any sharp edges to avoid mechanical injuries during insertion. These desired features of film formulation can not only improve the patient's compliance, but also provides good bioadhesion and retained in the vaginal cavity for prolong time periods.

5.3 MATERIALS

ITR was procured as gift sample from Intas Pharmaceutical Limited (Ahmedabad, India). HPMC (hydoxypropylmethyl cellulose) E15 was gifted by Colorcon Asia Pvt. Ltd. (Goa, India). Hydroxypropyl cellulose (HPC) (MW: 1 40 000) was purchased from Innovative Chemicals (Mumbai, India). Carrageen, hydroxyethyl cellulose (HEC), sabouraud dextrose broth, MRS broth and sabouraud dextrose agar media were purchased from Himedia (Mumbai, India). Methanol, polyethylene glycol 400 (PEG 400) and propylene glycol (PG) were purchased from S. D. Fine Chemicals (Mumbai, India). HPLC grade acetonitrile (ACN) and methanol (MeOH) were purchased from Spectrochem Lab. Tetrabutyl ammonium butyl hydrogen sulphate (TBAHS) was purchased from spectrochem pvt. Ltd. Mumbai. Double distilled water was used for preparing mobile phase solutions. All other chemicals used were of analytical grade.

All in vitro tests was carried out in simulated vaginal fluid (SVF). The detail composition of SVF was as follows (Owen D.H. *et al.*, 1999): 5.0 gL^{-1} glucose, 3.51 gL^{-1} NaCl, 1.40 gL^{-1} KOH, 0.22 gL^{-1} $Ca(OH)_2$, 0.018 gL^{-1} bovine serum albumin, 2.0 gL^{-1} lactic acid, 1gL^{-1} acetic acid, 0.16 1 gL^{-1} glycerol, and 0.40 gL^{-1} urea. Final pH was adjusted to 4.20 ± 0.02 using 0.1 M HCl

5.4. EXPERIMENTAL

5.4.1 Analytical Method

5.4.1.1 High-Performance Liquid Chromatography (HPLC) Method

A high-performance liquid chromatography (Shimadzu, Kyoto, Japan) was composed of a LC-20AT Prominence solvent delivery module, a manual rheodyne injector with a 20µl fixed loop and a SPD-20A Prominence UV–visible detector.

Separation was performed on a Hypersil BDS C18 column (particle size 5µm; 250mm×4.6mm i.d.) at an ambient temperature. Chromatographic data were recorded and processed using a Spinchrom Chromatographic Station® CFR Version 2.4.0.195 (Spinchrom Pvt. Ltd., Chennai, India).

Standard stock solution was prepared by dissolving 100mg of ITR into 100ml of methanol and further diluting 1 ml of this solution upto 10ml with mobile phase. The mobile phase was prepared by mixing MeOH, ACN and 0.01N TBAHS in 70:20:10 proportion and filtered through 0.2 µm membrane filter to remove any particulate matter and degassed by sonication before use. For calibration curve, solution of 5, 10, 15, 20, 25, 30 and 35µg/ml were prepared by diluting 0.5 to 3.5ml of standard stock solution to 10ml with mobile phase. Then, sample was analyzed by HPLC method. Mobile phase flow rate was set at 0.7ml/min and detection was carried out at 261nm. Sample solutions (20µl injection volume) were individually injected and peak area for respective solution was determined from the chromatogram. The calibration curve for HPLC analysis was constructed by plotting the peak area of the drug against the drug concentration. The calibration plot was generated by replicate analysis (n = 5) at all concentration levels and the linear relationship was evaluated using the least square method within Microsoft Excel® program. The proposed method was validated as per ICH Guidelines for linearity, specificity, precision, and accuracy (Walfish S., 2006). Specificity of the method was checked by using placebo samples (excipents blend).

5.4.2 Formulation Development and Optimization

5.4.2.1 Selection of Film Forming Polymer and Plasticizer

Development of an ideal vaginal formulation with desired characteristics in terms of safety, patient compliance, aesthetics, acceptability to regulatory authorities and efficacy requires a careful and meaningful selection of the active ingredients and excipients. Preliminary trial was carried out for selection of film forming polymer and plasticizer. Different water soluble plasticizers and polymers were explored for preparation of ITR film.

To ascertain effect of each polymer and plasticizer on the physical characteristics of film, trials $ITRF_1$ to $ITRF_6$ (**Table** 5.1) were conducted. Polymeric film prepared in the preliminary trials was evaluated for their mechanical and bioadhesive properties. These are the key physicodynamic properties of films which are imparting aesthetic appeal and prolong retention of formulation in vaginal cavity. From these trials, the HPC and PEG 400 was

found most suitable polymer and plasticizer for preparation of ITR film respectively, and selected for further optimizations of the film formulation.

Table 5.1 Composition of preliminary formulation trials.

INGREDIENTS (mg/ film of 2.5 cm × 2.5 cm)	$ITRF_1$	$ITRF_2$	$ITRF_3$	$ITRF_4$	$ITRF_5$	$ITRF_6$
SDITR (eq. to 100mg ITR)	250	250	250	250	250	250
d-sorbitol	50	0	0	0	0	0
Glycerol	0	50	0	0	0	0
PEG 400	0	0	50	50	50	50
HEC	0	0	0	100	0	0
Carragenan	0	0	0	0	100	0
HPC	0	0	0	0	0	100

5.4.2.2 Drug Excipients Compatibility Study

Compatibility of ITR with excipients to be used for development of film formulation was studied by thermal and isothermal stress analysis. For thermal analysis, samples of pure ITR, excipients, $ITRF_{65}$ and their physical mixture were characterized by differential scanning calorimeter (DSC 60, Shimadzu, Japan). Instrument was calibrated using indium as the reference standard. Approximately, 2mg samples were crimped in aluminum pans and analyzed at a nitrogen flow of 30 ml/min and heating rate of 15°C/min from 35°C to 300°C. For isothermal stress testing, drug excipients blends were stored at stress conditions (50°C) for 3 weeks. The blends were examined periodically for any unusual changes in color and physical form. After 3 weeks of storage, the samples were analyzed quantitatively for content of ITR by HPLC method. Sample stored at refrigerator were considered as control.

5.4.2.3 Factorial Design and the Desirability Function

Factorial designs of experiment and desirability function have been extensively useful approach in pharmaceutical field for systemic study of the effect of formulation variables and their interaction on response variable (Li *et al.* 2003; Narendra *at el.,* 2006). Conventional approach in formulation development was not able to give the desired formulation. Also it is

unpredictable and sometimes even may be unsuccessful. Hence, to study all possible combinations of all factors at all levels, full factorial design was used as an optimization tool (Mashru R.C. *et al.*, 2005).

In the present work, 3^2 full factorial designs were constructed (two factors, three levels) and were conducted in a fully randomized order. The dependent variables to be measured are tensile strength (TS), percentage elongation at break (%EB) and % drug retained on the vaginal mucosa up to 8 h (Y_{8h}). Formulation designing by 3^2 factorial designs is shown in **Table** 5.2. Two independent factors, concentration of HPC (X_1) and PEG 400 (X_2) were set at three different levels. High and low levels of each factor were coded as 1 and -1, respectively, and the mean value as zero. The range of a factor was choosing in order to adequately measure its effects on the response variables. This design was selected as it provides sufficient degrees of freedom to resolve main effects as well as the factor interactions. Composition of full factorial designs batches are shown in **Table** 5.3.

Table 5.2 Formulation designing by 3^2 full factorial designs

Batches	**Formulation variables**	
	X_1	**X_2**
$ITRF_{61}$	-1	-1
$ITRF_{62}$	-1	0
$ITRF_{63}$	-1	1
$ITRF_{64}$	0	-1
$ITRF_{65}$	0	0
$ITRF_{66}$	0	1
$ITRF_{67}$	1	-1
$ITRF_{68}$	1	0
$ITRF_{69}$	1	1

Formulation variables	**Levels**		
	Low (-1)	**Medium (0)**	**High (+1)**
X_1 = Amount of hydroxypropyl cellulose (mg)	80	100	120
X_2 = Amount of polyethylene glycol 400 (mg)	40	50	60

The desirability function was used for optimization of formulation composition (Moore J.W. *et al.,* 1996). In addition, the responses have to be combined in order to produce a product of desired characteristics. The applications of desirability function combines all the responses in one measurement and give possibility to predict an optimum levels for the independent variables(Derringer G. *et al.,* 1980). The combination of responses in one desirability function requires the calculation of individual functions. An ideal film should have a moderate tensile strength, high %EB and high % drug retained on vaginal mucosa. The individual desirability for each response was calculated using the following methods.

In this study, there were no specific requirements for TS of optimum formulation. Therefore, the range of values of produced formulations was selected. As moderate TS desired, the formulations that have its value within the range of 7.0-10.0, their desirability was considered 1. The formulations have values of TS out side this range, their desirability was considered zero. These can be described by the following equations:

$$d_1 = 0 \quad for\ Y_i < Y_{\min}$$
$$d_1 = 1 \quad for\ Y_{\min} < Y_i < Y_{\max}$$
$$d_1 = 0 \quad for\ Y_i > Y_{\max}$$

Where, d_1 is individual desirability of TS.

In addition, optimum vaginal film formulation should have high %EB and high Y_{8h} values. Desirability functions of these responses were calculated using the following equation:

$$d_2 \text{ or } d_3 = \frac{Y_i - Y_{\min}}{Y_{target} - Y_{\min}} \quad for\ Y_i < Y_{target}$$
$$d_2 \text{ or } d_3 = 1 \quad for\ \ Y_i > Y_{target}$$

Where d_2 is individual desirability of %EB and d_3 is the individual desirability of Y_{8h}. The values of Y_{target} and Y_{min} for %EB are 70.08 and 51.42 and the values of Y_{target} and Y_{min} for are 18.63 and 9.77 and Y_i is the experimental result. The overall desirability values were calculated from individual values by using the following equation:

$$D = (d_1 d_2 d_3)^{1/3}$$

Table 5.3 Composition of full factorial design batches

INGREDIENTS (mg/ film of 2.5 cm × 2.5 cm)	$ITRF_{61}$	$ITRF_{62}$	$ITRF_{63}$	$ITRF_{64}$	$ITRF_{65}$	$ITRF_{66}$	$ITRF_{67}$	$ITRF_{68}$	$ITRF_{69}$
SDITR (eq. to 100mg ITR)	250	250	250	250	250	250	250	250	250
HPC	80	80	80	100	100	100	120	120	120
PEG 400	40	50	60	40	50	60	40	50	60

5.4.3 Preparation of Films

Bioadhesive films were prepared by solvent evaporation technique (Garg S. *et al.*, 2005), using specific amount of SDITR (250mg) and various ratios of film forming polymers to plasticizer. SDITR was added in distilled water and sonication was done for 30 min to obtain an aqueous dispersion. Subsequently film forming polymer and plasticizer was added to this dispersion. This polymeric dispersion was stirred on magnetic stirrer (Remi equipments ltd., Mumbai, India) for 30 min followed by sonication for 15 min and kept for 2h to remove all the entrapped air bubbles. Polymeric dispersion of ITR was uniformly spread onto a plastic plate of defined area (50 cm^2) and dried in vaccum oven at 50 °C for 16h. Dried films were carefully peeled off from the plate surface, cut into pieces of defined size (2.5 cm ×2.5 cm). Film was sealed in sachets prepared from polyethylene laminated alluminium foil and stored at temperature of 30 ± 2°C and relative humidity 60 ± 5% until further analysis.

5.4.4 Pharmaceutical Characterization

Bioadhesive film formulation (BFF) was characterized for various aesthetic (appearance, odor, color, flexibility and peelability) and physicodynamic properties such as moisture content, pH and viscosity of polymeric dispersion, swelling index, TS, %EB, film thickness, content uniformity, % drug retention on vaginal mucosa and bioadhesion (Jin-Wook Y. *et al.*, 2006). Thickness of each sample was measured using a thickness tester (Model 110, 0.01 mm capacity, Mitutoyo Manufacturing Corporation Ltd., Japan) at five locations (center and four corners) and mean thickness was calculated.

Viscosity of film dispersion was determined by Brookfield cone and plate rheometer LVDVIII (Brookfield engineering, Middleboro, MA, USA). For studying viscosity and pH of film, one unit of formulation (2.5 cm × 2.5 cm size film) was dispersed in 10ml each of

distilled water and SVF. Viscosity of BFF was measured at 33.8°C by keeping speed 5 RPM and shear rates 10 sec^{-1}. The pH of film dispersion was measured with pH meter (LABINDIA Pvt. Ltd., New Mumbai). All the parameters were measured in triplicates.

5.4.5 Morphology Study

Morphology of prepared film was observed under scanning electron microscope (SEM) (Model JSM 5610LV, Jeol, Japan). The samples were attached to slab surfaces with double sided adhesive tapes and scanning electron photomicrograph was taken at 1000X magnification.

5.4.6 Moisture Content

For determination of moisture content, unit of film (size 2.5 cm ×2.5 cm) was weighed and kept in desiccator's containing calcium chloride for 24h. Films were removed from desiccator and reweighed until a constant weight obtained. The percentage of moisture content was calculated as difference between initial weight and final weight with respect to initial weight (Jin-Wook Y. *et al.,* 2006). Moisture content was measured in triplicates.

5.4.7 Measurement of Swellings Index

Film swelling study was carried out in simulated vaginal environment using SVF as media. Each film sample with surface area 2.5 cm × 2.5 cm was weighed (Wo) and placed in a preweighed stainless steel basket with 200 mesh aperture. Then, basket containing film sample was submerged into 15 ml SVF medium in glass beaker. Basket was removed from glass beaker at preset time intervals and reweighed until no further change in weight of film (Wt). Swellings index was measured in triplicates. The degree of swelling was calculated as follows. (Gannu R. *et al.,* 2007).

$$\text{Swelling index} = (Wt - Wo) / Wo$$

5.4.8 Measurement of Mechanical Properties

Mechanical properties of film were evaluated using Instron Universal Testing Instrument (Model 1121, Instron Limited, UK) equipped with 100 kg load cell. Samples with air bubbles, nicks or tears and having mean thickness variations greater than 5% were excluded from analysis. Film was cut into narrow strips in dimension of 40 mm × 10 mm. Film strip was placed between two clamps positioned at a distance of 10 mm in same plane. During measurement, lower clamp was fixed and strip was pulled by top clamp at a rate of

100 mm/min. The force and elongation at a moment of break were recorded and TS as well as %EB was calculated by using the following equations (Peh K.K. *et al.,* 1999).

$$Tensile\ strength = \frac{Force\ at\ break\ (N)}{Initial\ cross\ sectional\ area\ of\ the\ sample\ (mm^2)}$$

$$\%\ Elongation\ at\ break\ (\%mm^{-2}) = \frac{Increase\ in\ length}{Original\ length} \times \frac{100}{cross-sectional\ area\ (mm^2)}$$

Results of film samples, which broke at and not between the clamps were not included in calculations. Measurements were run in triplicates for each film.

5.4.9 Determination of Average Drug Content in Films

To ensure uniformity of distribution of ITR in BFF, average drug content in film was measured. In addition, samples (2.5 cm ×2.5 cm size of film) were collected from five different locations (centre and four corners) within film, weighed and dispersed in 20ml of water and were sonicated for 10min to destroy any agglomerates. Subsequently, diluted with mobile phase and filtered through 0.45µm membrane filter. After filtration, sample was analyzed by HPLC method. The content of ITR was calculated using a preconstructed calibration curve for ITR (5-35 µg/ml) in mobile phase. No polymeric interference was observed under conditions of assay procedure.

5.4.10 *Ex-vivo* Bioadhesion Measurement

The bioadhesive property of vaginal film was assessed in simulated vaginal environment (Alam M.A. *et al.,* 2007; Lei W. *et al.,* 2008) using a texture analyzer equipped with 2.0 kg load cell ((Model 1121, Instron Limited, UK). Isolated sheep vaginal mucosa free from supporting tissues was stored in a deep freezer at –20 °C. For experiments, vaginal tube (thawed in normal saline with 0.1% w/v sodium azide preservative) was incised longitudinally and held on lower platform of the texture analyzer. Film was applied to upper probe with the help of double-sided adhesive tap. The vaginal mucosa was moistened with SVF. Mucosal membrane was kept in contact with film for 5 min to allow formation of adhesive bond. Upper probe of texture analyzer was moved at speed of 0.1mm/sec. The force required to detach the film from mucosal surface was determined as bioadhesive strength. Each measurement was repeated in triplicate.

5.4.11 *Ex-Vivo* Retention Measurement

Retention of ITR films on vaginal mucosa was measured by a new model using simulated dynamic vaginal system. Simulated dynamic vaginal system consisted of closed glass cell with 30° angle slope and flow rate pump as shown in **Fig.**5.3. The glass cell had two small side arms in which lower act as inlet and upper act as outlet for circulating warm water. Warm water circulated through side arms into the glass cell by using a peristaltic pump (Miellins, India). This assembly was a modified version of the C.H. Lee apparatus (Jin-Wook Yoo, et al., 2006). The study is based on principle of measuring weight of dispersion falling down (or retained) as function of time. Sheep vaginal tube was obtained from local slaughterhouse immediately after sacrifice of animal. Before commencement of the experiments, sheep vaginal tube was thawed in normal saline. The sheep vaginal mucosa was cut into 5cm × 5cm pieces and mounted on simulated dynamic vaginal system with mucosal side up. The polymeric film (2.5cm ×2.5cm) was placed on mucosal membrane and SVF was applied on the film with a flow rate of 5 ml/hr. At predetermined time intervals, dispersion was collected into a receiver beaker and was analyzed by spectrophotometrcally.

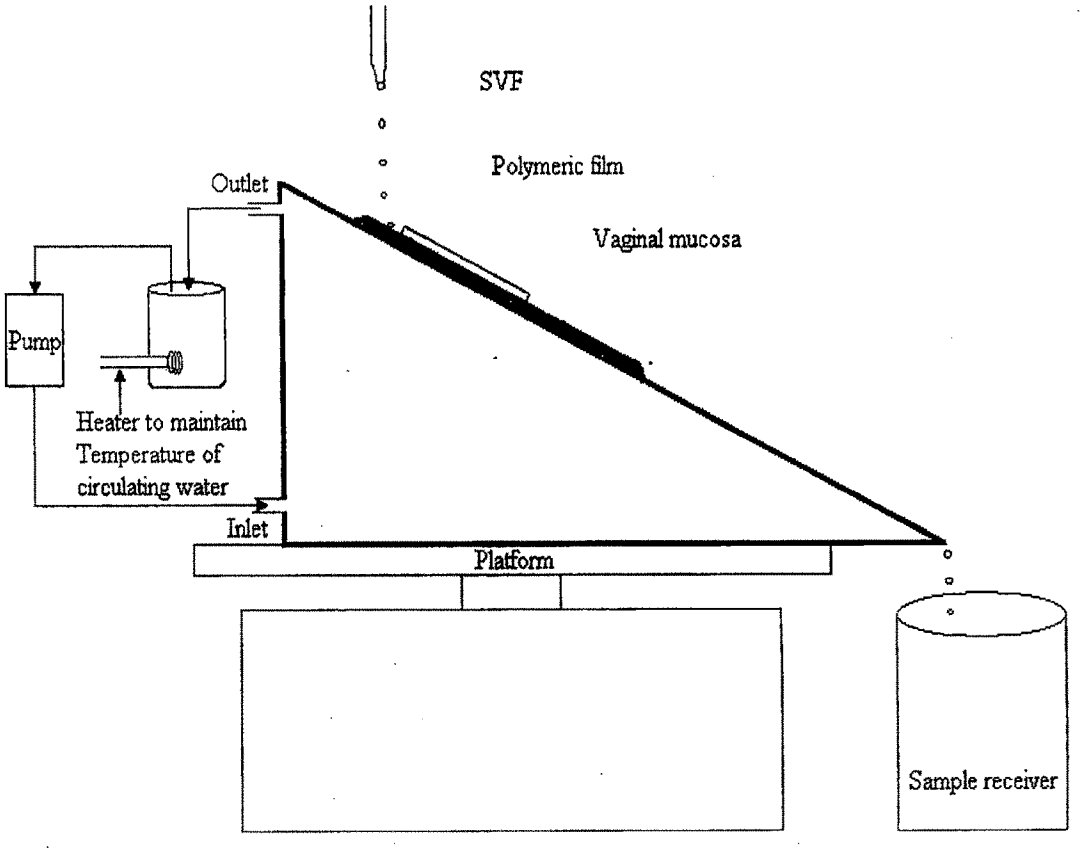

Fig.5.3 Out line diagram of simulated dynamic vaginal system for ex- vivo retention of drug measurement.

5.4.12 *In Vitro* Lactobacillus Inhibition

In vitro activities exerted by ITR, placebo film and BFF against lactobacillus acidophilus could be estimated by using cup plate method (Huang L. *et al.*, 2006). The bacterial strains of lactobacillus acidophilus were obtained from MTCC (Microbial Type Culture Collection and Gene Bank, Chandigarh, India) and subcultured in MRS broth two to three times before commencement of experiment. After autoclaving, first base agar (3% w/v) was poured into sterile petridish (15 cm in diameter) and allowed to solidify. Five milliliter of standardized suspension of lactobacillus acidophilus (10^5cells/ml) was uniformly mixed with 50 ml of 1 % w/v MRS agar (top agar) and then plated on previously solidify base agar plate. Wells were made in the plate using 8 mm borer.

Test samples were prepared by separately dispersing placebo film, pure ITR and $ITRF_{65}$ film in SVF. Concentration of ITR was same in all the test samples except placebo. Test samples (1.0 ml) were poured into the wells and plates were incubated at 37±2 °C for 24h. The mean diameter of zone of inhibition for each sample was calculated.

5.4.13 *In Vitro* Antimicrobial Activity

Preliminary experiments were designed to evaluate whether differences in the *in vitro* activities exerted by ITR alone, SDITR and BFF against *C. albicans.* It could be performed by using cup plate method (Hire N.N. *et al.*, 2007). The fungal strains of *C. albicans* (ATCC 1023) were freshly subcultured into sabouraud dextrose broth and incubated at 30±2°C for 24h followed by measurement of optical density at 530 nm. After autoclaving, base agar (3% w/v) was poured into sterile petridish (15 cm in diameter) and allowed to solidify. Five ml of standardized suspension of *C. albicans* (10^7cells/ml) was mixed with 50 ml of 1% w/v sabouraud dextrose agar (top agar) and then poured onto previously solidify base agar. Wells were made in the plate using 8 mm borer.

Test samples prepared from placebo film, pure ITR, SDITR and $ITRF_{65}$ in SVF were poured into wells and incubated at 30±2°C for 24 hrs. The mean diameter of zone of inhibition for each sample was calculated.

5.4.14 *In Vivo* Studies

5.4.14.1 Animals. Female wistar rats weighing 180 to 200 gm were used for these studies. Total 15 female rats were used in this study and divided in three groups, one each for excipients (control), pure ITR and BFF. Vaginitis in rodents is inducible only under the conditions of pseudoestrus. Therefore, female wistar rats were maintained in pseudoestrous

condition by weekly injection (0.5mg subcutaneously) of estradiol valerate (Sobel J.D. *et al.*, 1984). (Permission was taken from institutional animal ethics committee for carrying out the study).

5.4.14.2 Inoculation of *C. albicans*: Suspension of *C. albicans* was prepared by inoculating 10 ml of sterile sabouraud dextrose broth with 100 µl of a stock blastospore suspension of *C. albicans* and incubating the preparations at 30±2°C for 24 hrs. The resulting culture was resuspended in 1 to 2 ml of sterile phosphate-buffered saline (PBS). The number of blastospores in this suspension was counted by haemocytometer. At 72 hrs after the initial estrogen injection, infection was induced in rats by vaginal inoculation of 20µl of PBS suspension containing 1.04×10^4 viable blastoconidia of *C. albicans* (Ryley J.F. *et al.*, 1986).

5.4.14.3 Vaginal Lavage Fluid Collection: Vaginal lavage fluid was collected from each animal on alternate days by pipetting 20µl portions of PBS in and out of the vagina several times to get total of 100 µl. The time course of infection was monitored in individual rat by serial dilution of the retrieved lavage fluid and plating on sabouraud dextrose agar supplemented with 400 mg of chloramphenicol/liter and 40 mg of gentamicin/liter (Ghelardi E. *et al.*, 1998). All plates were incubated at 30±2°C for 48 hrs for each series of dilutions. The plate containing 30 to 300 colonies was used to calculate the colony forming unit (c.f.u.) per 100 µl.

Infected rats (5 days post inoculation with *C. albicans*) were topically treated with ITR (PG used as vehicle, 1.8 mg/20 µl) or BFF eq. to 1.8 mg of ITR or with placebo film for consecutive five days. Vaginal tissues were isolated from each group of an animal at end of the study and fixed in 10% formaldehyde and cut into slices. The slices were stained with hematoxylin-eosin and observed under a light microscope (Primo star- 415500-1500-000, Zeiss, Germany.) attached with microscope image projection system (Magnus MIPS – 700223, New Delhi, India).

5.4.15 Stability Studies

The films were subjected to stability testing according to the International Conference on harmonization (ICH) guidelines for zones IV countries like India. The developed films were individually sealed in sachets prepared from polyethylene laminated alluminium foil and stored at intermediate stability (30±2°C and 65±5% RH) and accelerated stability conditions (40±2°C and 75±5% RH). A visual inspection (for change in color and odor), pH

of film dispersion, mechanical properties of film and ITR content estimation was carried out periodically at the end of 1, 2, 3 and 6 months of the stability study..

5.5 RESULTS AND DISCUSSION

5.5.1 Analytical Method

5.5.1.1 HPLC Method

The chromatographic separation was achieved with reverse-phase C18 column (250mm×4.6mm i.d., 5µm particle size), using a mixture of MeOH, ACN and 0.01N TBAHS in the ratio of 70:20:10 (%v/v) at a flow rate of 0.7 ml/min with UV detection at 261 nm and quantified based on drug peak area. The mobile phase was prepared freshly everyday. The mobile phase was premixed, filtered through a 0.2 µm membrane filter to remove any particulate matter and degassed by sonication before use. This mobile phase composition was found to be optimal for good peak with desirable asymmetry. Prior to injecting solutions, the column was saturated for at least 45 min with the mobile phase flow rate 0.3 ml/min through the system. Experimental set (5, 10, 15, 20, 25, 30 and 35µg/ml) was analyzed in triplicate by using HPLC method. **Table.** 5.4 represent peak area response of three experimental set. The calibration curves for ITR (**Fig.** 5.4a) were constructed by plotting area of the peaks vs. concentration; this was found to be linear over an analytical range of 5-35 µg/ml. The calculated value for the determination of coefficient (r^2 = 0.9996) indicated linear relationship between the variables. **Fig.** 5.4b represent overlay HPLC chromatogram of various concentrations of ITR. Spectral and validation data of proposed HPLC method are illustrated in **Table** 5.5.

Table 5.4 Measurement of peak area of ITR in different experimental set by HPLC

Concentration (µg/ml)	Experimental Set			Mean	SD	%RSD
	I	II	III			
5	205.85	198.8	201.12	201.9233	3.59	1.78
10	397.66	395.95	384.82	392.81	6.97	1.77
15	572.32	563.24	561.81	565.79	5.70	1.01
20	778.13	791.16	766.71	778.6667	12.23	1.57
25	941.85	970.21	958.06	956.7067	14.23	1.49
30	1156.91	1181.51	1150.98	1163.133	16.19	1.39
35	1355.61	1346.86	1344.91	1349.127	5.70	0.42

Table 5.5 Spectral and validation data of the proposed HPLC method

Parameter	Value
Absorption maxima, λ_{max} (nm)	261
Linearity range (µg/ml)	5-35
Coefficient of determination	0.9996
Slope	38.380
Intercept	4.996
LOD (µg/ml)	0.88
LOQ (µg/ml)	2.93
Interday precision (%RSD)	1.26
Intraday precision (%RSD)	1.39
Accuracy (mean ± %RSD)	99.14 ± 0.78

(a)

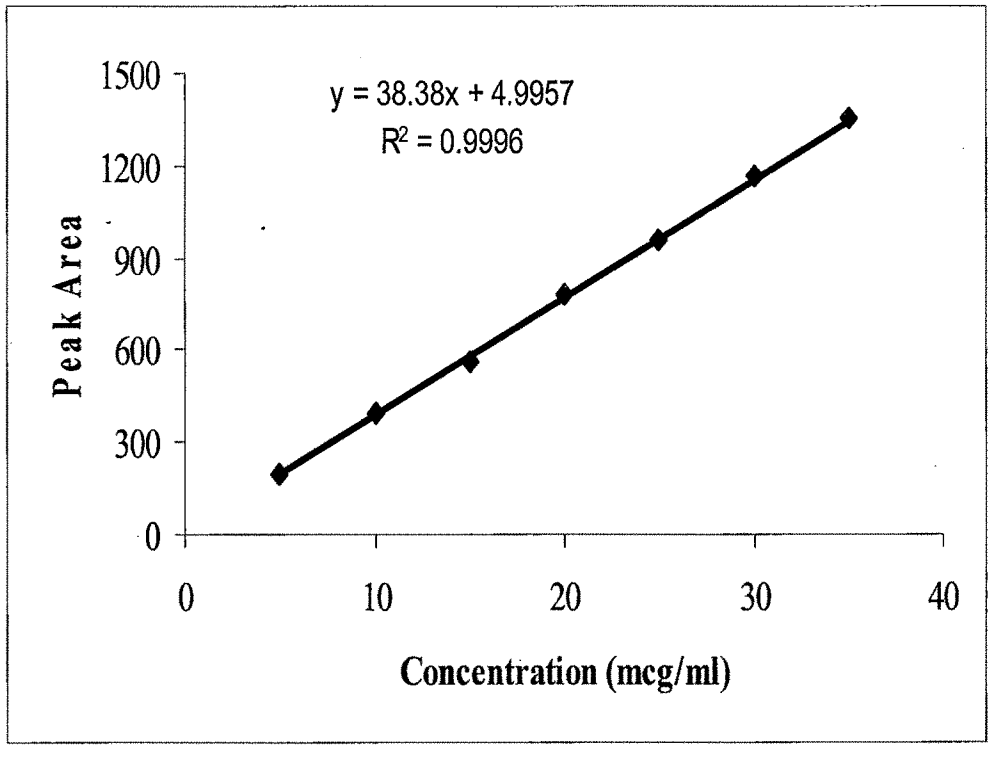

Fig.5.4 (a) Calibration curve of ITR

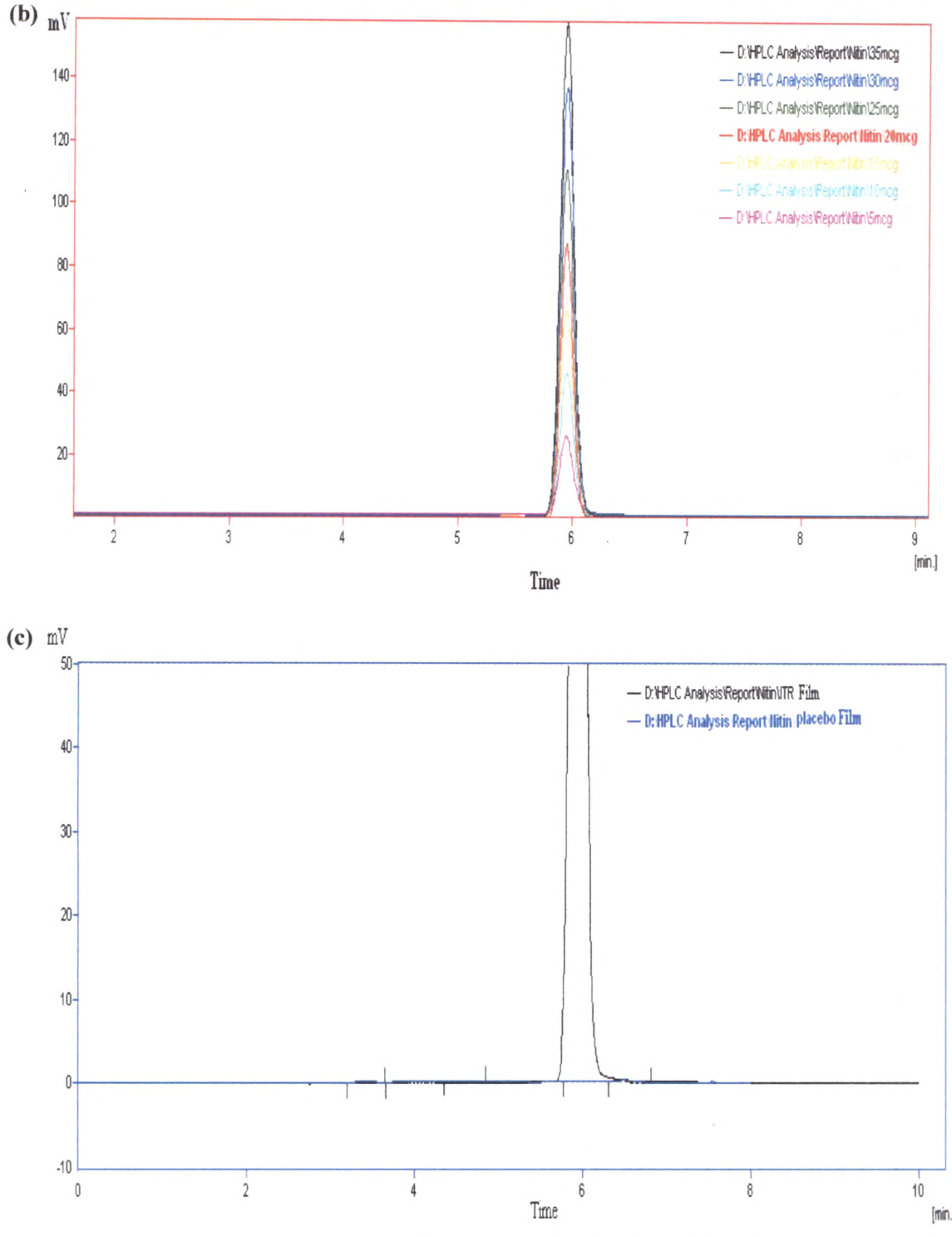

Fig.5.4 (b) Overlay chromatogram of various concentrations of ITR
(c) Overlay chromatogram of ITR film and placebo film

The specificity of method was confirmed from **Fig.** 5.4(c) as excipients did not shows any interference peak at retention time corresponds to ITR peak.

5.5.2 Development and Optimization Film Formulation

5.5.2.1 Selection of Film Forming Polymer and Plasticizer

In preliminary experiments, various polymers and plasticizers were explored for preparation of ITR film formulation. The physical characteristics of ITR film prepared with various polymers and plasticizers are given in **Table** 5.6. Different water soluble plasticizer such as glycerol, PEG 400 and d- sorbitol were explored for development of BFF. Film containing glycerol and d- sorbitol as plasticizers could not removed from casting surface. On other hand, films containing PEG 400 as plasticizer were formed, but not easily peelable. It was concluded that HPMC based SDITR with plasticizer could not formed film, necessitating the addition of another film forming polymer to increase peelability and mechanical strength.

Table 5.6 Composition of ITR film prepared with various polymers and plasticizers and their physical characteristics.

Formulation	Polymer	Plasticizer	Composition (SDITR:Polymer: Plasticizer)	Physical characteristic of film
$ITRF_1$	0	d-sorbitol	2.5:0:0.5	Could not be removed from casting surface
$ITRF_2$	0	Glycerol	2.5:0:0.5	Could not be removed from casting surface
$ITRF_3$	0	PEG 400	2.5:0:0.5	Could not be easy to removed from casting surface
$ITRF_4$	HEC	PEG 400	2.5:1.0:0.5	Non homogeneous surface, more soft, easy to peel
$ITRF_5$	Carragenan	PEG 400	2.5:1.0:0.5	Brittle, Hard to peel
$ITRF_6$	**HPC**	**PEG 400**	**2.5:1.0:0.5**	**Homogeneous surface and Easy to peel**

Three different water-soluble film forming polymers such as HEC, HPC and carrageen were investigated for preparation of ITR film. Film containing carrageen as film-forming polymer was found hard to peelable from casting surface and brittle in nature. In addition to HEC, high viscosity of polymeric dispersion causes difficult to remove entrapped

air bubbles. Non homogeneous surface of films with HEC was posing the problems, such as unequal distribution of drug in film. This problem was overcome by using HPC as film forming polymer. Polymeric dispersion of SDITR with HPC was found to be less viscous and uniform spreading on casting surface. Physical characteristics and other mechanical properties of ITR film containing HPC and PEG 400 was found acceptable. Both, HPC and HPMC E15 were also responsible for imparting bioadhesion property of the formulation (Vermani K. *et al.,* 2002). Hence, these were selected as formulation variables and effect of different levels was studied by 3^2 full factorial designs.

Preparation of homogenous aqueous dispersions of drug is preliminary processing step for development of film formulation. But, ITR possess very less aqueous solubility (~4 µg/ml at acidic pH). Therefore, it was very difficult to prepare homogenous aqueous dispersions of ITR. Solid dispersions can improve wettability of ITR and produce homogenous aqueous dispersions. Also, SDITR forms colloidal dispersion in simulated vaginal fluid (SVF) is essential for local action in vaginal cavity. Therefore, SDITR has been used in present investigations for development of film formulation.

5.5.2.2 Drug Excipients Compatibility

Drug-excipients compatibility studies were conducted with the objective of selecting a reasonable composition for vaginal bioadhesive film. Any kind of incompatibility between ITR and film forming polymer affects its performance to a significant extent. Results of ITR-excipients compatibility study performed by DSC are shown in **Fig.** 5.5. DSC thermogram for pure ITR shows sharp endotherms at 166 °C that corresponds to melting point of ITR. In DSC thermogram of solid dispersion, ITR endotherm was disappeared indicating transform the drug from the crystalline to partially amorphous state. DSC thermogram of film formulation also shows complete absence of endothermic peak which indicates no risk of transforming physical state of ITR from an amorphous to crystalline state during preparation of film. Amorphous state of drug lead to high energy state resulting in enhanced solubility.

In addition to isothermal stress testing, the samples kept under stress condition (50°C) were analyzed by HPLC for estimation of ITR content after three weeks. The content of ITR was found same as in control which was stored at refrigerator condition. No any additional peak was found in chrmatogram of ITR film which stored under stress condition (50°C). **Fig.**5.6 confirmed the compatibility between ITR and excipients to be used for preparation of film.

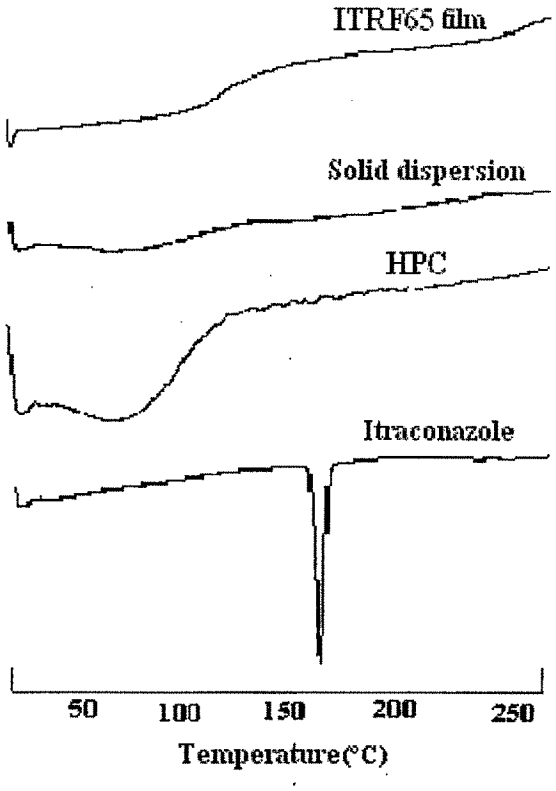

Fig.5.5 Representative DSC thermogram for ITR-excipients compatibility study

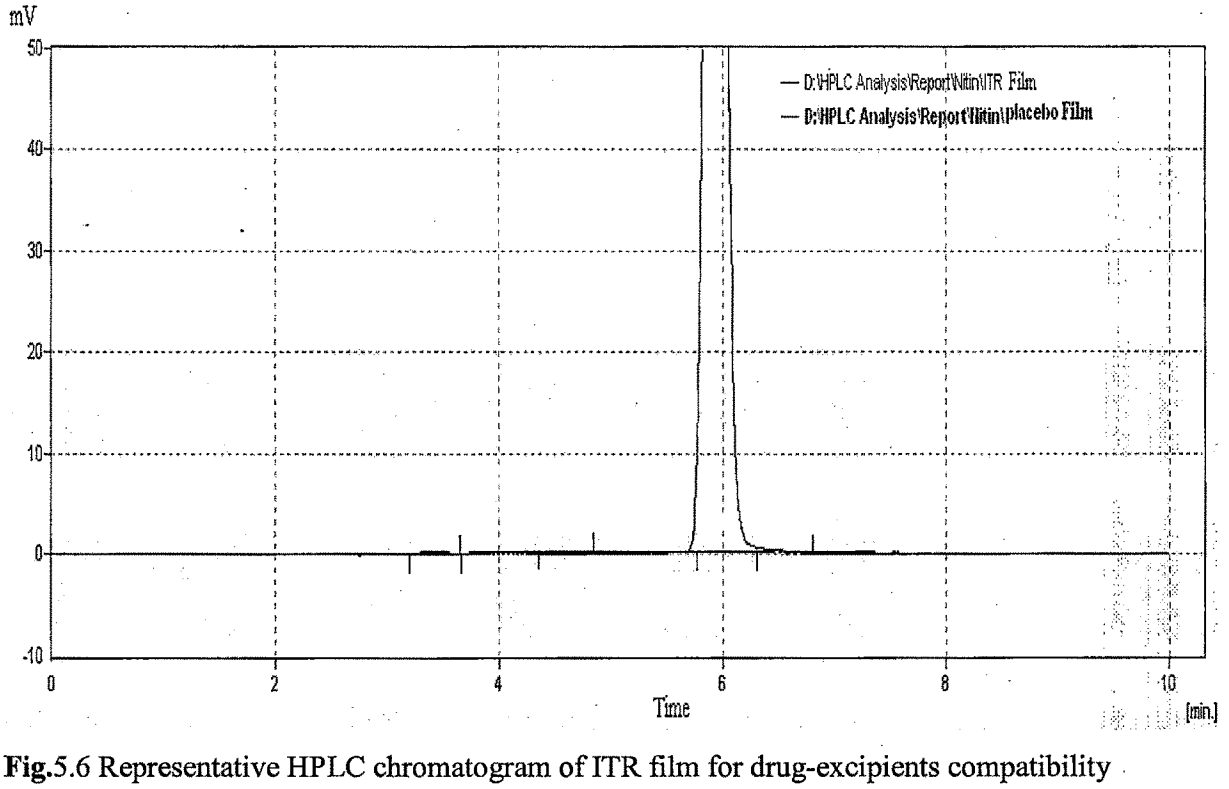

Fig.5.6 Representative HPLC chromatogram of ITR film for drug-excipients compatibility study.

5.5.3 Mechanical Properties

The mechanical properties such as TS and %EB indicate the strength and elasticity of the film. Repka A.M. *et al.,* (2000) reported that mechanical properties of polymeric films were dependent on the plasticizer/polymer composition of the extruded films. Gutierrez-Rocca and McGinity reported that plasticizers would increase the workability, flexibility, and distensibility of a polymer (ASTM Standards, 1995). For all the designed film formulation, TS was found between 6.99 and 10.46 N/mm^2. The results of mechanical properties are shown in **Table** 5.7. Counter plots shown in **Fig.** 5.7 represent that an increase in HPC content resulted in a higher TS and %EB, while increase in amount of PEG 400 reflects decrease in TS and increase in % EB. An interesting finding was decrease in TS and increase in %EB of film as a function of plasticizer by weakening the intermolecular interactions between polymer chains. This is in agreement with work of Gutierrez-Rocca and McGinity who investigated plasticizers modify the physical-mechanical properties, by lowering the melt viscosity and glass transition temperature of a polymeric film. Therefore, the optimum level of HPC and PEG 400 was desired because high content of PEG 400 produced film more flexible and softer. ITR films with HPC are tougher and softer than those without HPC.

Desirability function was utilized to find out optimum level of HPC and PEG 400 out of nine batches. Desirability function was calculated for TS, %EB and Y_{8h} Batch $ITRF_{65}$ showed highest overall desirability of 0.92. Therefore, this batch was considered to be optimized batch and values of formulation variables of this batch were considered to be optimum values for BFF. Excipients used in films are GRAS listed and approved for vaginal use (Garg S. *et al.,* 2001). The optimized composition of film containing ITR is given in

Table 5.8 Optimized Composition of film formulation containing ITR.

Formulation variable	Optimum value (mg per film of 2.5 cm ×2.5 cm area)
SDITR e.q. to 100mg of ITR.	250
HPC	100
PEG 400	50

Table 5.7 Experimental result of response variables and overall desirability of formulations

Batches	Independent Variables		Response values			Overall Desirability
	X_1	X_2	TS (N/mm^2)	% EB	Y_{8hr}	
$ITRF_{61}$	-1	-1	7.83	51.42	15.14	0.00
$ITRF_{62}$	-1	0	7.23	54.37	12.25	0.00
$ITRF_{63}$	-1	1	6.99	55.91	9.77	0.00
$ITRF_{64}$	0	-1	10.46	61.28	18.63	0.81
$ITRF_{65}$	**0**	**0**	**9.64**	**67.56**	**17.75**	**0.92**
$ITRF_{66}$	0	1	9.15	70.08	14.06	0.78
$ITRF_{67}$	1	-1	10.31	59.84	15.03	0.65
$ITRF_{68}$	1	0	9.85	60.61	15.41	0.68
$ITRF_{69}$	1	1	9.27	63.64	13	0.62

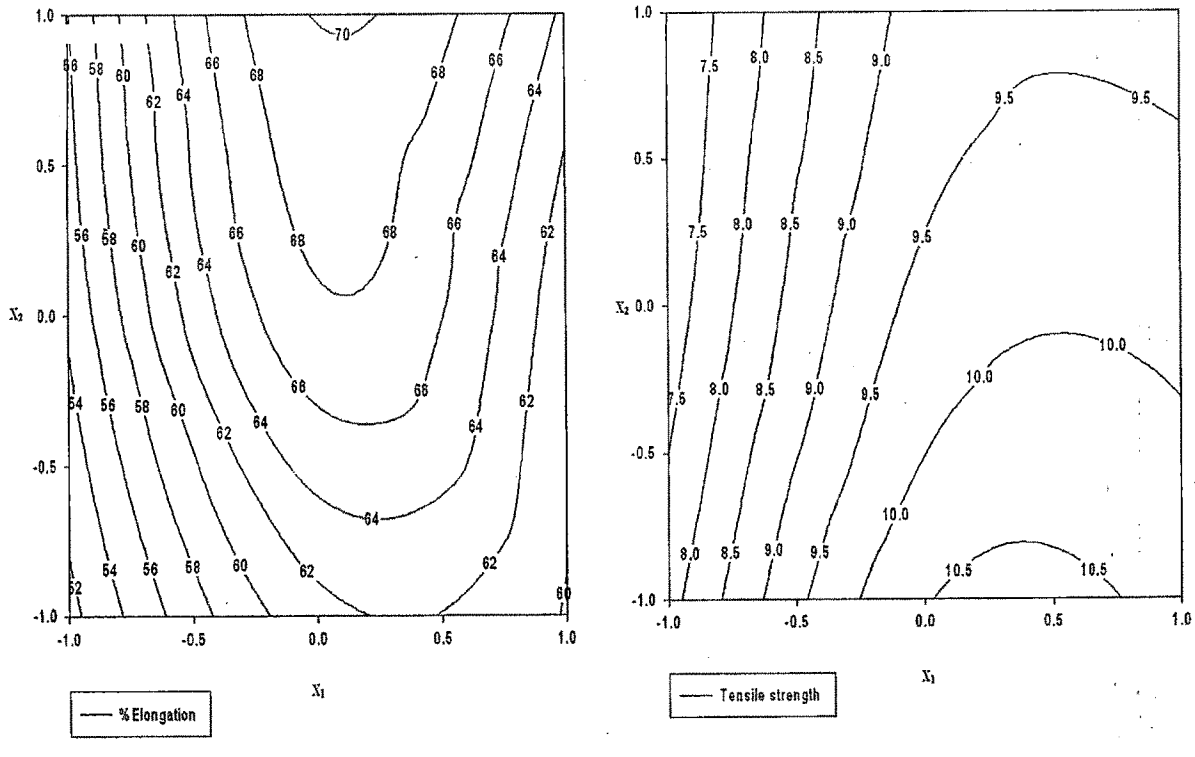

Fig.5.7 Counter plots showing effect of X_1 and X_2 on mechanical properties of film

The prediction profiles were obtained for the measured responses using JMP 5.1, statistical discovery software. The relationship between formulation variables and dependent response value of ITR film can be further explained by using prediction profile as shown in **Fig.**5.8. Among tested variables, the HPC concentration seems to be the most prominent factor in determining response value of film. An interesting observation of these profiles was improved TS, %EB and Y_{8h} as the content of HPC in film increased. The high concentration of PEG 400 can decrease Y_{8h} and TS value of bioadhesive film and increased %EB value of film. Fluid uptake capacity of film depends on amount of HPC present in the formulation. The prediction profile indicates that swelling index of film increases as the content of HPC in film increased.

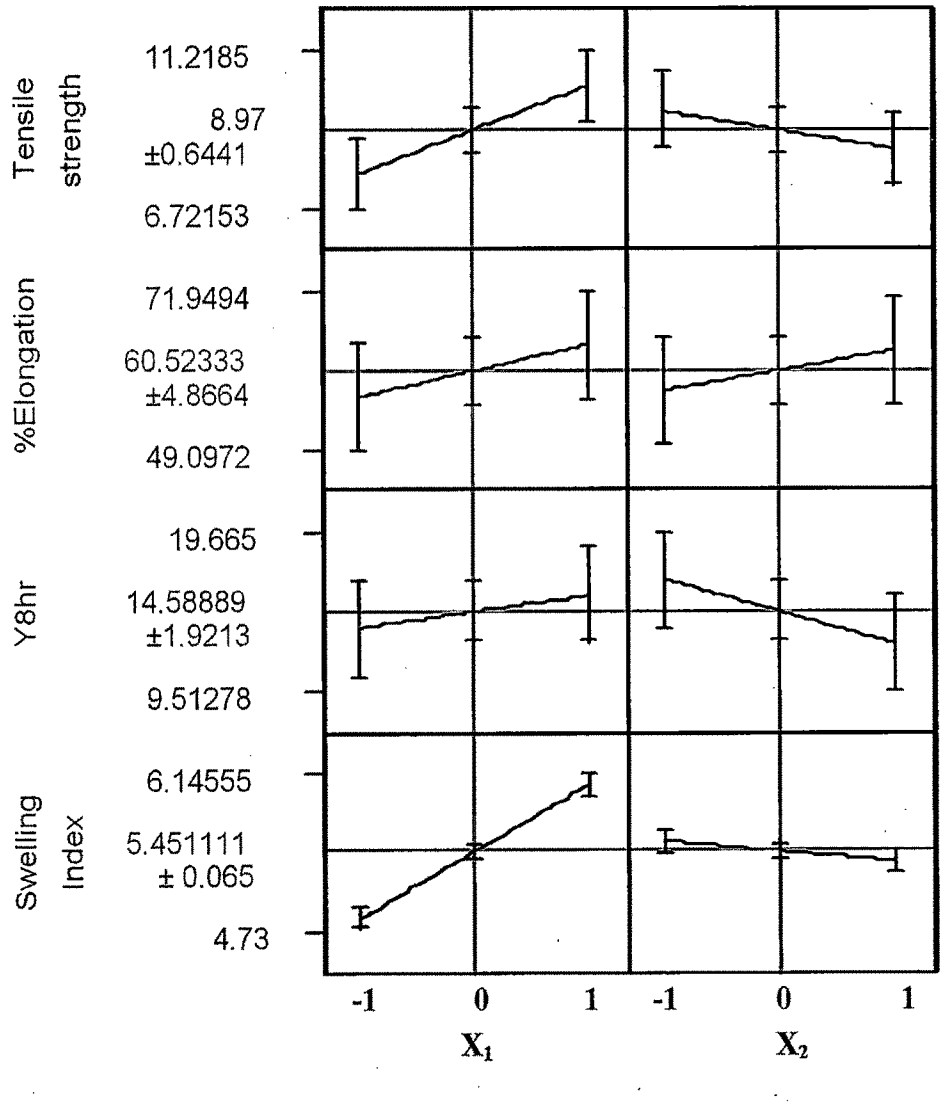

Fig.5.8 Predictions profile correlating all the dependent variables against independent variables

5.5.4 Pharmaceutical Characterization of Bioadhesive Vaginal Film

A newly developed ITR films are colorless, odorless, flexible, uniform and possesses smooth surface. Films of three different batches of $ITRF_{65}$ (optimized batch) were found to have similar aesthetic, mechanical and other physicodynamic properties. This suggests that films with desired properties can be prepared consistently and reproducibly. All the performance parameters of film ($ITRF_{65}$) have been given in **Table** 5.9.

Average drug content was found between 94.5 to 98.9% of added amount of ITR per film (2.5 cm ×2.5 cm).

Table 5.9 Performance parameter of bioadhesive ($ITRF_{65}$) films.

Parameter	Optimum values
Size (sq. cm)	2.5 × 2.5
Weight[a] (mg)	431.5 ± 8.78
Thickness[a] (mm)	0.46 ± 0.026
Moisture content[a] (%w/w)	7.66 ± 0.51
Tensile strength[b] (N/mm^2)	9.64 ± 0.21
% Elongation at break[b]	67.56 ± 2.13
pH of dispersion[c]	7.04 ± 0.07
In water	4.90 ± 0.04
In SVF	
Viscosity (cp) of dispersion[d]	7.57 ± 0.31
In water	7.84 ± 0.25
In SVF	0.368 ± 0.02
Bioadhesive strength[e] (N)	
Retention on isolated sheep vaginal mucosa	17.75% retained amount of drug on vaginal mucosa after 8hr

[a] Mean ± S.D., n = 3
[b] Determined by texture analyzer, Mean ± S.D., n = 5
[c] pH of dispersion (2.5 × 2.5 cm^2 film dissolve in 10ml each of water and SVF at 30°C), Mean ± S.D., n = 3
[d] Viscosity of dispersion measured by Brookfield Viscometer (2.5 ×2.5 cm^2 film dissolved in 10ml each of water and SVF at 33.8°C), Mean ± S.D., n = 3
[e] Bioadhesive strength determined by texture analyzer, Mean ± S.D., n = 5

The viscosity of polymeric dispersion of film was found to be more in SVF (7.84 ± 0.25) as compared to water (7.57 ± 0.31). Viscosity and dispersibility of formulation in vaginal

environment after administration governs the spreading and retention characteristics of the formulations which is essential to achieve desired efficacy. The developed film was dispersed rapidly in SVF and would formed bioadhesive layer over vaginal mucosa in order to bring drug in contact with the target tissue for sufficient periods of time.

The pH of the healthy female genital tract is acidic (pH 3.5–4.5) and is maintained by bacterial conversion of glycogen from exfoliated epithelial cells to lactic acid. The acidic pH of genital tract can prevent growth of unwanted micro organism, but favour the growth of normal microflora (lactobacillus acidophilus). Hence, pH of the formulation was studied as it impact on the acidic pH of the female genital tract and microflora. The pH of ITR film was found to be slightly acidic (4.90 ± 0.04) in SVF and alkaline (7.04 ± 0.07) in water reveals that vaginal pH as well as micro flora may remains unaffected after administration of film. The moisture content in the film was found to be 7.66 ± 0.51 % (w/w). The little amount of moisture content in formulations helps them to remain stable and prevent from being a completely dry and brittle film.

5.5.5 Morphology of Film

The purpose of scanning electron microscopy was to obtain morphological characterization of film. **Fig.** 5.9 illustrated the scanning electron photomicrographs of the film at 1000X magnification confirm that film surface was free from any scratches or transverse striations. The smooth surface of film may reduce chance of mechanical injury during insertion in vaginal cavity and improving patient compliance.

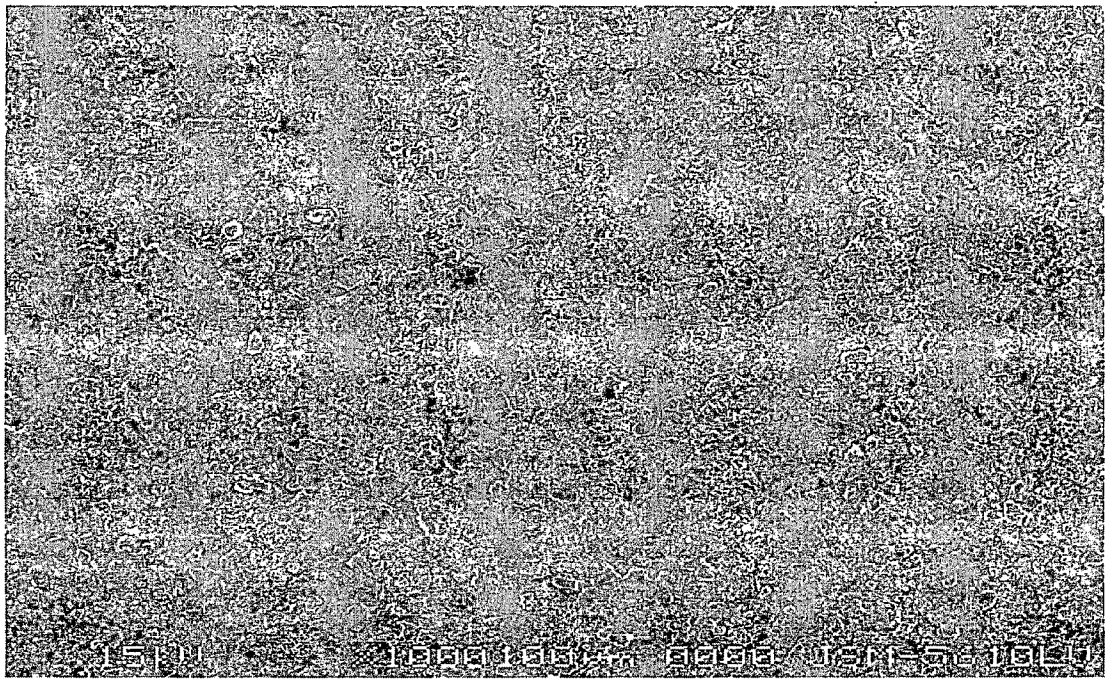

Fig. 5.9 Scanning Electron Photomicrographs of the ITR film ($ITRF_{65}$).

5.5.6 Swelling Capability of Films

After coming in contact with SVF, ITR film absorbed water and the film gets hydrated. Films were found to be rapidly swollen within 4 min and thereafter slowly reached to plateau. Results of the swelling index of films with various compositions are shown in **Fig.**5.10. Both of HPMC E15 and HPC have property to absorb water and get hydrated. Therefore, swelling index (fluid uptake capacity) of film depends on the amount of these components present in the formulation. $ITRF_{67}$ containing highest amount of these component having swelling index 6.2, while $ITRF_{62}$ containing lowest amount has value of 5.35. Maximum swelling was seen with film containing high content of HPC. The swelling index of film increased as the concentration of HPC increased. The swelling capability of polymer is crucial for its bioadhesive character. Adhesion occurs shortly after the beginning of swelling but the bond formed is not very strong.

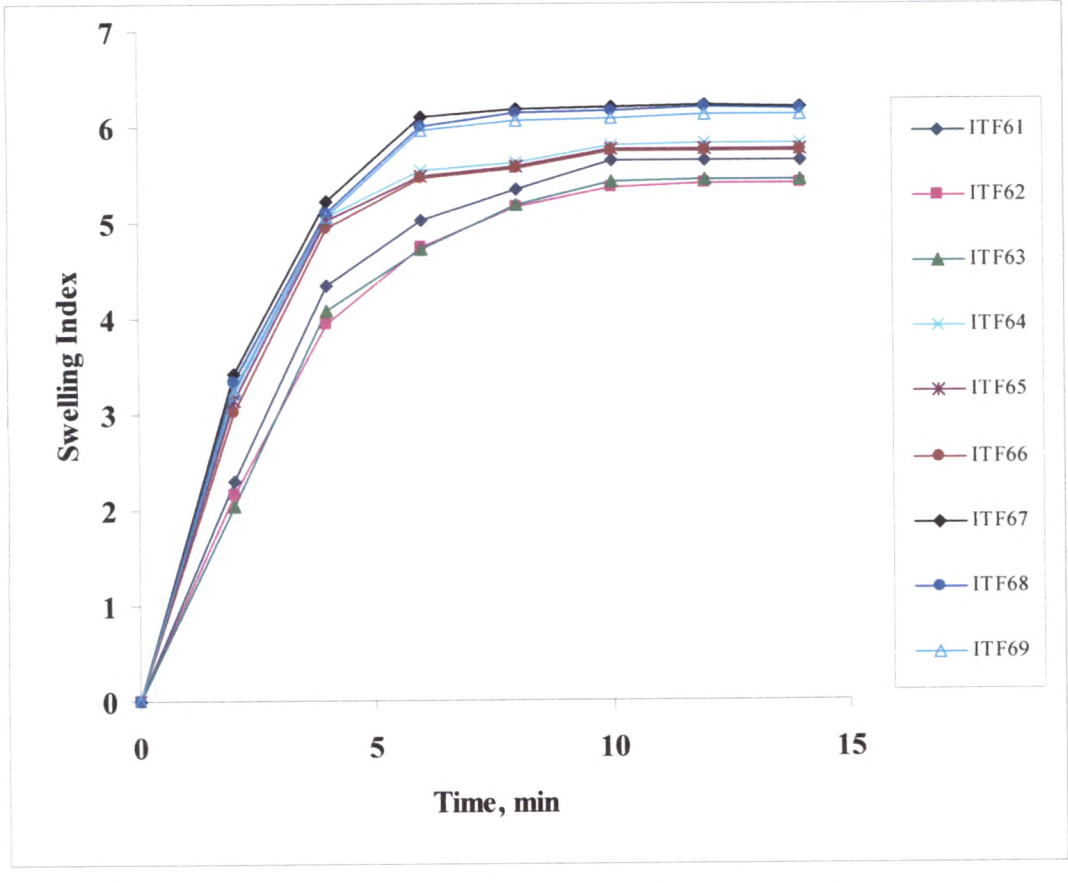

Fig.5.10 Comparisons of swelling index of films ($ITRF_{61}$ to $ITRF_{69}$).

5.5.7 Bioadhesive Strength

Bioadhesion is generally defined as the ability of a biological or synthetic material to 'stick' to a mucosal membrane. This results in the adhesion of the material to the tissue for a prolonged period of time (Ohio Bulletin, 1994). Thus, bioadhesion is very important aspect

for maintaining high drug levels at the site of administration and prevent expulsion of formulation. The swelling capability is prerequisite for bioadhesion, since it concerns wetting, uncoiling and spreading of the polymer over vaginal mucosa. **Fig.**5.11 shows the effect of various polymers/plasticizer ratios on bioadhesive strength of film. HPC has very good bioadhesive property. This may be explained by the fact that the HPC contain a large number of hydroxyl groups that provide the ability to form hydrogen bonds with the mucosal membrane (Mortazavi S.A. *et al.,* 1995). Bioadhesive strength of ITR film may be due to the formation of either physical or hydrogen bonding with vaginal mucosa. This is in agreement with work of De Ascentiis and co-workers who investigated the influence of hydrogen bonding functional groups of hydrophilic polymers on the mucoadhesion of these materials to rat intestinal mucosa. The bioadhesive strength of film was improved with increased in content of HPC up to certain extent, and then decreased. This result might be obtained due to wetting theory. This finding suggest that adhesion will improve with extent of hydration until an optimum point where overhydration leads to a decrease in adhesive force due to disentanglement at the polymer/tissue interface. Amongst nine films, $ITRF_{65}$ showed good bioadhesion (0.368 ± 0.02 N) under simulated vaginal environment.

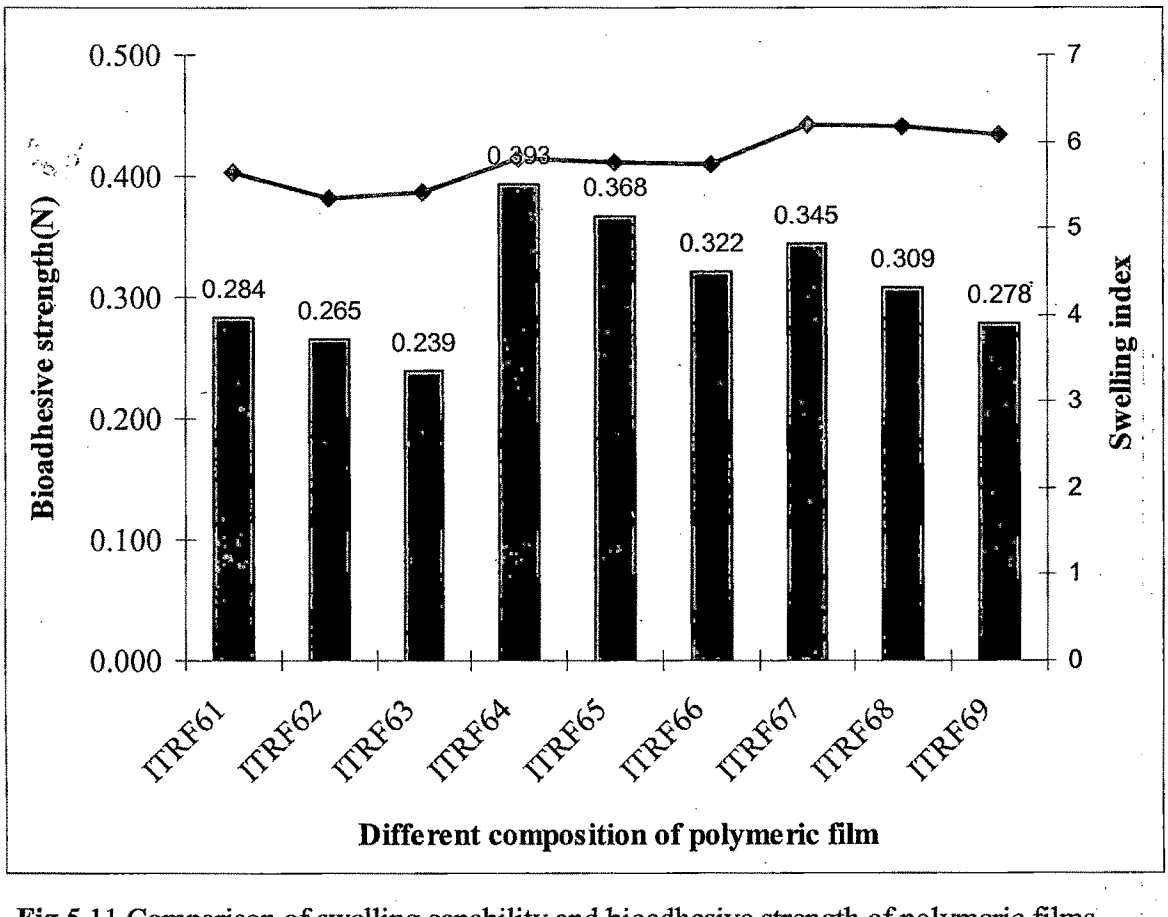

Fig.5.11 Comparison of swelling capability and bioadhesive strength of polymeric films.

5.5.8 *Ex-Vivo* Retention of Bioadhesive Vaginal Film

C. albicans resides in vaginal mucosa. Complete eradication of these infectious agents can achieve by longer exposure of therapeutic moiety over infectious site. Keeping in mind this necessity, development aspect in this research work was focused on imparting prolong retention feature to the formulation. Retention performance of film was studied using simulated dynamic vaginal system which mimics the physicodynamic conditions of vagina. Initially, the film softened on the vaginal mucosa after absorbing SVF and became a swollen structure, helping it to adhere to the vaginal mucosa. Film would form bioadhesive layer over vaginal mucosa which may help to achieve high drug levels at infectious site for longer time. Films were eroded as function of time. The remaining percentage of ITR on vaginal mucosa expressed as a function of time is demonstrated in **Table** 5.10. Films were eroded slowly within first 2h and then gradually increase upto 8h. Time required for entire removal of polymeric film from vaginal mucosa varied with the compositions of film. As the ratio of HPC to PEG 400 in film increased, the residence time of film increased until an optimum point and then it decreased. This indicates retention properties of polymeric film can be controlled by varying HPC/PEG 400 ratio. Drug retained over vaginal mucosa at 8h varied from 9.76% to 18.63 for the designed experiments. Results of this study clearly indicate that amount of HPC and PEG 400 is an integral factor in retention of BF over vaginal mucosa.

Table 5.10 Percentage retention of ITR on vaginal mucosa as a function of time.

Time (hr)	Cumulative Percentage Retained of Itraconazole, Mean ± SD (n = 3)								
	$ITRF_{61}$	$ITRF_{62}$	$ITRF_{63}$	$ITRF_{64}$	$ITRF_{65}$	$ITRF_{66}$	$ITRF_{67}$	$ITRF_{68}$	$ITRF_{69}$
1	93.38±0.13	93.03±0.11	92.72±0.09	93.79±0.16	93.59±0.10	93.31±0.10	94.40±0.16	94.03±0.13	93.79±0.11
2	86.07±0.11	85.02±0.14	83.21±0.12	88.80±0.13	87.93±0.08	86.32±0.08	89.67±0.11	88.45±0.07	86.37±0.10
3	68.37±0.16	67.83±0.12	66.64±0.12	72.24±0.11	71.15±0.13	66.67±0.23	73.34±0.17	71.90±0.14	70.05±0.19
4	52.72±0.16	49.71±0.20	50.68±0.12	55.97±0.25	55.15±0.2	52.61±0.12	57.52±0.26	55.72±0.18	53.48±0.21
5	39.00±0.30	35.82±0.26	39.18±0.26	39.43±0.44	42.20±0.27	39.83±0.20	42.87±0.30	42.45±0.26	40.49±0.28
6	28.45±0.27	27.47±0.27	25.72±0.30	33.20±0.30	31.71±0.32	28.08±0.29	34.07±0.31	30.26±0.23	27.58±0.34
7	21.34±0.32	20.84±0.28	17.46±0.46	26.05±0.32	24.07±0.43	21.42±0.31	25.87±0.38	22.55±0.36	17.47±0.39
8	15.14±0.44	12.25±0.60	9.76±0.55	18.63±0.41	17.75±0.32	14.06±0.36	15.03±0.39	15.41±0.40	13.00±0.48

5.5.9 *In Vitro* Antimicrobial Activity

Anti-fungal activity of ITR against *C. albicans* manifested that mean diameter of inhibition zone produced by SDITR and ITR film was found larger than that of pure ITR (**Table** 5.11). ITR film prepared with SDITR shows larger size of inhibition zone as compared to pure ITR. (as shown in **Fig.** 5.12). These **higher antifungal activities of SDITR may be due to an increased solubility of ITR in SVF**. The placebo formulation did not exert any inhibitory activity against *C. albicans* reference strain indicates no antimicrobial action of bioadhesive polymers. SDITR and ITR film showed almost similar mean diameter of zone of inhibition. It reveals that bioadhesive polymers did not interfere with antifungal activity of ITR.

Table 5.11 Antifungal activity for ITR

Samples	Zone of inhibition, mm			mean ± SD (n=3)
	I	II	III	
ITR bulk powder	15.67	16.33	16.67	16.22 ± 0.51
SDITR	19.67	21.67	20	20.45 ± 1.07
$ITRF_{65}$ film	20.33	21	21.33	20.89 ± 0.51
Placebo film	0	0	0	0

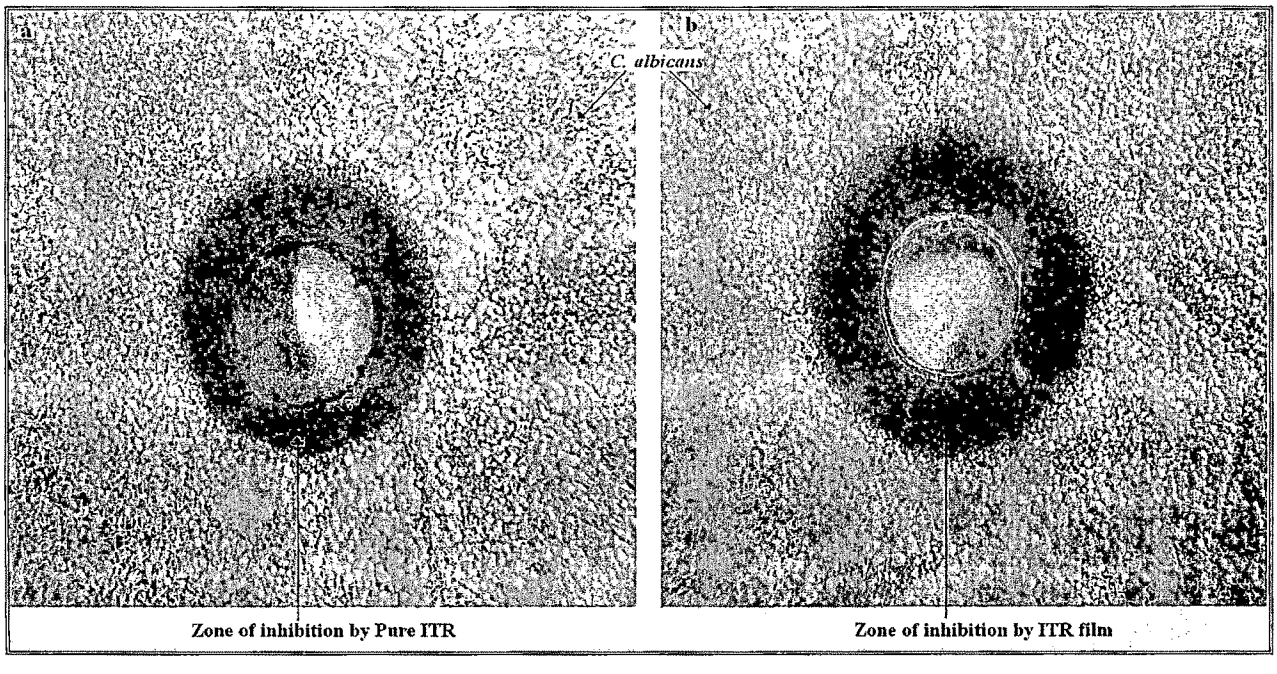

Fig. 5.12 Zone of inhibition showing antifungal activity of pure ITR and ITR film against *C. albicans.*

5.5.10 Lactobacillus Inhibition

However, ITR have inhibitory effect against *Candida albicans*, but it should not affect the growth of lactobacillus which is a normal component of vaginal flora. The results of lactobacillus inhibition against ITR bulk powder, $ITRF_{65}$ film and placebo film are shown in **Table** 5.12. ITR bulk powder and $ITRF_{65}$ film at a concentration of 10mg per ml did not show any considerable inhibition effect on growth of lactobacillus acidophilus after 24h.

Table 5.12 *In Vitro* Lactobacillus Inhibition Activity

Samples	Zone of inhibition, mm			mean ± SD (n=3)
	I	II	II	
ITR bulk powder	2	2	1.5	1.83 ± 0.29
$ITRF_{65}$ film	2.00	1.5	1.5	1.67 ± 0.29
Placebo film	Nil	Nil	Nil	Nil

5.5.11 *In Vivo* Antimicrobial Activity

Experimentally induced vaginal candidasis model in rats (Jung Yun C. *et al.*, 2002) were used in the present study. This study was to investigate whether BFF would improve therapeutic benefit of ITR in the topical treatment of vaginal candidasis which was induced in rats. Results obtained during study were expressed in terms of mean of three observations. Statistical analysis was performed by two-tailed student's *t*-test with the significance level set at a p value of 0.05.

After incubation, optical density of subcultured suspension of *C. albicans* was found 0.750. The value of optical density indicates growth of *C. albicans* was in lag phase. The resulting culture was resuspended in 1 to 2 ml of sterile phosphate-buffered saline (PBS) and the number of blastospores counted by haemocytometer. It was found 5.2 x 10^5 viable blastoconidia of *C.* albicans per ml.

At two days of post inoculation, colony formation of *C. albicans* in sabouraud dextrose agar plate for serial dilution of the retrieved lavage fluid is systematically represented in **Fig.** 5.13. Infected rats developed high titer vaginal infections resulted with 4.72 ± 0.062 (log_{10} c.f.u./100 µl) at two days post inoculation. Complete clearance of *C. albicans* was rarely observed. This was most likely due to the dominant pharmacological effect of estrogen administration which produces an environment facilitating persistence and proliferation of *Candida* organisms.

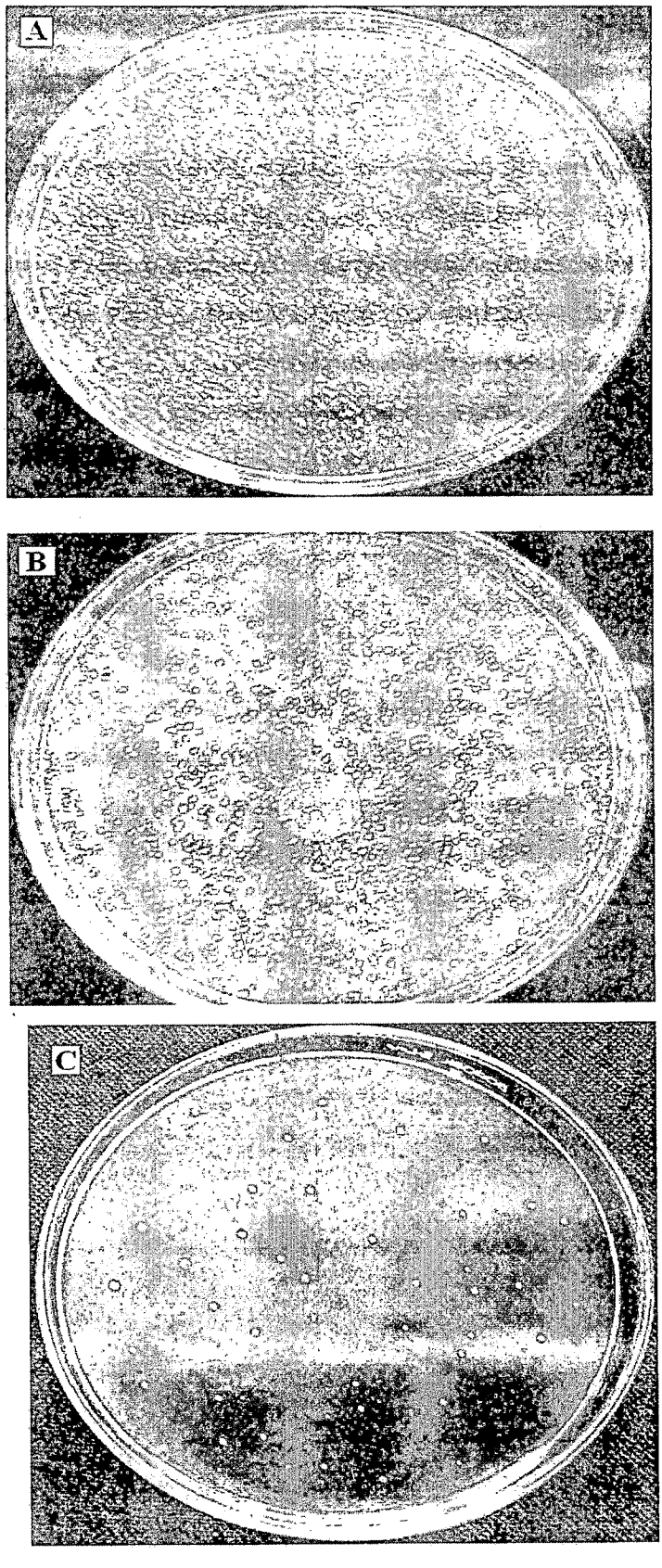

Fig. 5.13 Colony formation of *C. albicans* in sabouraud dextrose agar plate for serial dilution of the retrieved lavage fluid (collected after two days post inoculation).
A) first dilution, B) second dilution, C) third dilution

No appreciable reduction in the vaginal *C. albicans* burden was observed in control group rat receiving only excipients. It shows that excipients did not have therapeutic benefits. Infected rat receiving ITR showed moderately decline in *C. albicans* levels immediately after day three of postadministration, but did not completely eradicate *C. albicans* from the vagina. The mean colony counts of BFF treated group continuously decreased from day three post dose and showed significant differences ($P < 0.05$) from ITR treated group on day six. Moreover, the burdens of *C. albicans* in vaginal cavity were 10^3 folds lower in formulation treated group than control on day 6 of the treatment. **Fig.** 5.14 represents the $\log_{10}$ c.f.u./100 µl of *C. albicans* as function of time, suggesting that BFF showed sharp declines in vaginal *C. albicans* burden as compared to ITR alone (given in PG vehicle).

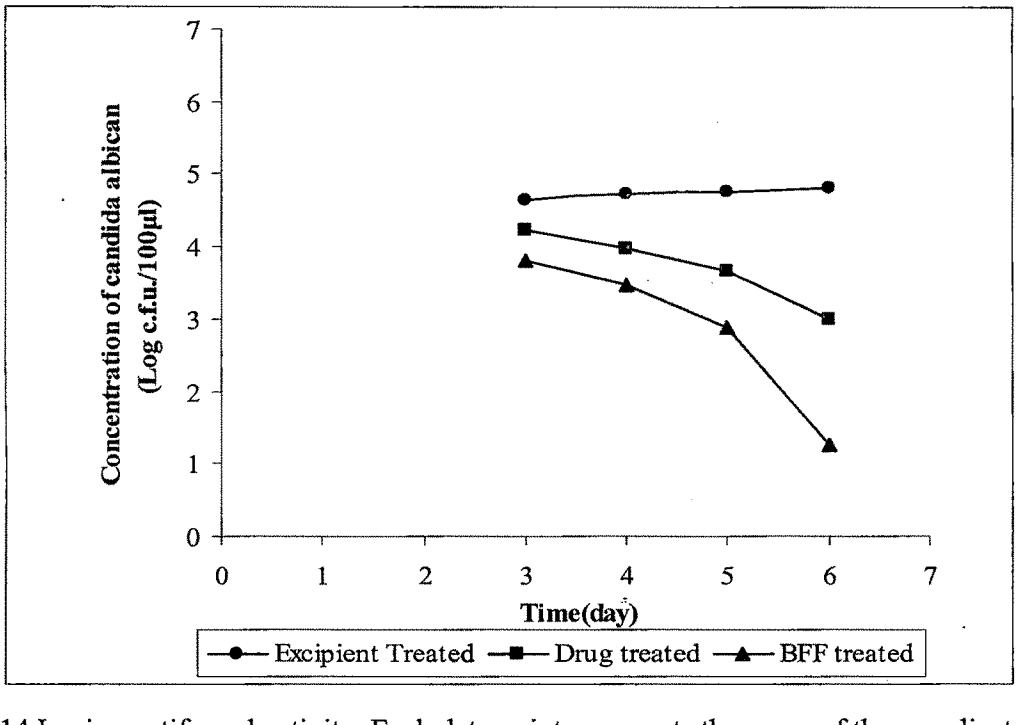

Fig. 5.14 In vivo antifungal activity. Each data point represents the mean of three replicates.

In contrast, improvement in the therapeutic efficacy of ITR, when delivered as BFF could be convincingly explained by assuming prolonged residence time of BFF on vaginal mucosal surface. Such a hypothesis was supported by the findings that successful clearance of *C. albicans* was achieved only in rats which receive film formulation. It was making the therapeutic benefit of ITR containing bioadhesive film significantly higher ($P < 0.041$) than that exerted by ITR alone as shown in **Table** 5.13. Indeed, infected rat receiving ITR showed improvement in clearance but no complete eradication of *C. albicans* from the vagina. In contrast, infected rat treated with BFF exhibited either marked improvement or complete clearance of *C. albicans* infection.

Fig. 5.15 Shows histopathology of vaginal mucosa after intravaginal administration of itraconazole containing BFF. Bioadhesive film did not alter the morphology of vaginal tissues. As compared to the control (**Fig.** 5.15a), the formulation treated group showed no visible sign of inflammation or necrosis that indicates the compatibility of bioadhesive polymer with vaginal mucosa.

Table 5.13 *In vivo* clearance of *Candida albicans* after the administration of excipients, ITR alone and BFF

Group	***C. albicans* burden (cfu/100µl) in each group on different days after treatment, mean ± S.D (n=3)**		
	Day 4	**Day 5**	**Day 6**
Excipients treated	$5.3\times10^{4}\pm3.51\times10^{3}$	$5.7\times10^{4}\pm6.0\times10^{3}$	$6.5\times10^{4}\pm5.9\times10^{3}$
Pure ITR treated	$9.4\times10^{3}\pm4.6\times10^{2}$	$4.8\times10^{3}\pm4.0\times10^{2}$	$1.0\times10^{3}\pm5.8\times10^{1}$
BFF treated	$3.3\times10^{3}\pm2.6\times10^{2}$	$7.7\times10^{2}\pm4.6\times10^{1}$	$1.8\times10^{1}\pm1.7\times10^{1}$

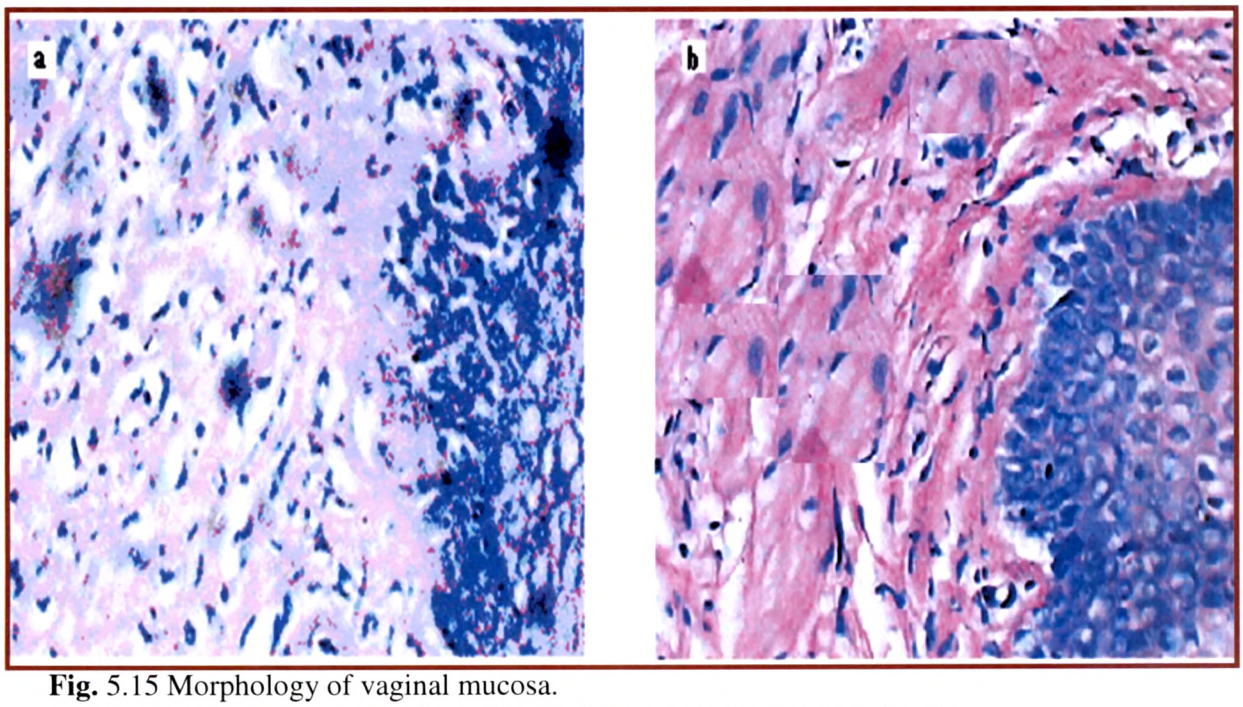

Fig. 5.15 Morphology of vaginal mucosa.
a) Vaginal mucosa of candidiasis induced rat (untreated animal).
b) Vaginal mucosa of film formulation treated rat

5.5.12 Stability Studies

Stability samples were analyzed at 0, 1, 2, 3 and 6 months time intervals for pH, mechanical properties and assay content of ITR as per the procedure described in method section. The

result of the stability study of ITR film is shown in **Table** 5.14. The results of accelerated storage condition demonstrate the influence of relative humidity (75% RH) on the TS and % EB of the ITR films. The tensile strength determined at 75% RH decreased in all films tested when compared to that at 60% RH. All films exhibited an increase in % EB when stored at 75% RH as compared to store at 60% RH. This observation was anticipated due to the higher moisture content of the films stored at the higher relative humidity. But, assay of content remains unaffected at accelerated storage condition. During stability studies, no significant change in odor, color, pH of polymeric dispersion, mechanical properties and assay of films were observed at the end of 6 months of storage at intermediate stability condition as well as accelerated conditions. During stability studies, films were found to be physically and chemically stable under accelerated conditions suggesting their suitability for tropical climates. The pH of polymeric dispersion of ITR film in SVF was found acidic (pH 4.90) suggesting that vaginal pH and microflora remains unaffected.

Table 5.14 Results of the stability studies of ITR film.

Sample storage condition	**Testing interval (Month)**	**pH of polymeric dispersion** mean ± S.D (n=3)	**Mechanical properties of film**		**ITR Content of Film (%)** mean ± S.D (n=3)
			TS (N/mm^2) mean ± S.D (n=3)	**%EB** mean ± S.D (n=3)	
-	0	4.87 ± 0.06	9.7 ± 0.28	66.69 ± 1.46	96.58 ± 1.88
Intermediate stability condition (30±2°C and 65±5% RH)	1	4.88 ± 0.05	9.78 ± 0.41	69.72 ± 1.52	98.23 ± 1.61
	2	4.99 ± 0.02	9.84 ± 0.22	72.41 ± 1.29	96.76 ± 1.77
	3	4.89 ± 0.03	9.79 ± 0.36	70.08 ± 1.57	94.76 ± 1.59
	6	4.98 ± 0.06	9.88 ± 0.27	70.08 ± 1.36	99.87 ± 1.53
Accelerated stability conditions (40±2°C and 75±5% RH)	1	4.81 ± 0.02	9.61 ± 0.23	76.82 ± 1.39	97.44 ± 1.43
	2	4.92 ± 0.04	9.55 ± 0.42	75.12 ± 1.48	99.18 ± 1.88
	3	5.03 ± 0.06	9.51 ± 0.31	77.63 ± 1.43	96.18 ± 1.62
	6	4.99 ± 0.05	9.43 ± 0.36	77.63 ± 1.28	97.98 ± 1.66

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