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IN-SITU GEL BASED MUCOSAL SITE-SPECIFIC DRUG DELIVERY SYSTEMS: FORMULATION AND EVALUATION

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ABSTRACT: The aim of this study was to prepare a suitable mucoadhesive *in-situ* gel formulation of clotrimazole for the treatment of vaginal candidiasis. *The in-situ* gel-forming systems are viscous liquids that turn to a gel phase upon exposure to physiological conditions. The lower retention of conventional formulations in the vagina, mucoadhesive formulations would prolong the retention time. For this study mixture of poloxamer (Plx) 407 and 188 were used. 3^2 full factorial design was applied for the optimization. Selected independent variables were concentration of carbopol 974 (X1), and Hydroxypropyl methylcellulose (HPMC) E50 (X2) was added to in situ gel for improve the mucoadhesive properties of the formulation, and prolong the residence time in vaginal cavity. Selected dependent variables were viscosity (Y1) release at 1 h (Y2), release at 12 h (Y3). After the preparation of mucoadhesive *in-situ* gels, gelation temperature/time, viscosity, mucoadhesive strength, spreadability, drug content, *in-vitro* drug release, and *in-vitro* antimicrobial efficacy were determined. Based on the obtained results and according to desirability, it was found that gel prepared with 20% Plx 407, 10% Plx 188, 1.5% HPMC (E50), and 1.5% carbolop 974 suitable for vaginal administration of clotrimazole.

INTRODUCTION: Vaginitis is a common gynecological problem in women of all age groups. It may result from microbial infections, contact dermatitis, atrophic vaginitis, or allergic reactions [1]. The infectious vaginitis is of three types: candidiasis, trichomoniasis, and bacterial vaginosis. Vaginal infections are usually characterized by vaginal discharge, vaginal irritation or vulvar itching, and vaginal odor [2, 3].

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The vaginal route has been traditionally used for the conventional delivery of several locally acting drugs like antimicrobial agents [4]. Vaginal candidiasis is a common condition, and up to 75% of all women suffer at least one episode of this infection during their lifetime.

Clotrimazole is an azole antifungal agent with a broad spectrum of activity against many fungal species, including *Candida albicance*, mainly responsible for vaginal *Candidiasis* [5]. Clotrimazole is interfere with ergosterol synthesis, which is a main component maintaining the fluidity and integrity of the fungal cell membrane. It shows lower mucosal toxicity and excellent safety profile. Hence, it remained one of the most prescribed drugs for the treatment of vaginal Candidiasis [6].

Topical application of clotrimazole has been reported to reduce the side effects as compared to its oral administration [7].

The topical vaginal drug delivery systems include a large variety of pharmaceutical dosage forms such as semi-solids, tablets, capsules, pessaries, liquid preparations, vaginal films, vaginal rings, foams, and tampons. Most preferred conventional dosage forms for the treatment of infection include semi-solid preparations like creams, ointments, and gels. These semisolid preparations may suffer from the limitation of messiness, difficulty in spreading, and leakage from the vaginal cavity due to self-cleansing action of vagina [8].

These limitations can be overcome by formulating a mucoadhesive gel with good gel strength and resistance to dilutions. Mucoadhesive polymers are able to swell rapidly when placed in an aqueous environment. The polymer chains interpenetrate across the mucus layer of vaginal mucosa, which results in adhesion; thus, the formulation is retained at the biological surface for a longer time, and the drug is released in a controlled manner close to the absorptive membrane, with a consequent enhancement of bioavailability and decreased side effect of the drug and ultimately improved patient compliance [9, 10].

Considering the unique anatomy and physiology of vagina, for effective treatment of infection, an ideal mucoadhesive gel should easily spread in the vaginal cavity and cover the entire infected mucosa. At the same time, it should retain its viscosity in the presence of vaginal fluids and resist the dilution due to stress applied by squeezing action of elastic vaginal walls, which otherwise can cause leakage through vaginal cavity [8].

Similarly, the formulation should be stable and robust in the vaginal acidic pH (4.2-5.5) range, preventing faster erosion of the gel matrix. Thus, it is required to evaluate the reported mucoadhesive polymers for their potential as gel matrices for vaginal drug delivery.

The objective of the present study was the development of mucoadhesive vaginal gel for clotrimazole for the treatment of Vaginal *Candidiasis*. For this work, we selected poloxamer (plx) 407 and 188, Carbopol 974 (C974), hydroxyl propyl methyl cellulose E 50 (HPMC); various mucoadhesive gel formulations of clotrimazole were prepared using these polymers, and comparative evaluation of gel formulations was done for various *in-vitro* characteristics, *viz*. appearance, gelation temp., mucoadhesive strength, spreadability, viscosity, and *in-vitro* drug release. The best suitable formulations were optimized on the basis of these *in-vitro* characteristics and the optimized formulations were evaluated for *in-vitro* antifungal activity.

MATERIALS AND METHODS:

Materials: Clotrimazole was obtained as a gift sample from Vadis Pharma Pvt. Ltd. Mandadi. poloxamer 407 and 188 were obtained from Camper health care, Kherva. Hydroxyl methyl cellulose E 50 and carbopol 974 were obtained from ambix health care, Gojariya. All other ingredients used were of analytical grade.

Experimental Method:

Infrared (IR) Spectroscopic Analysis: Fourier-transform infrared (FT-IR) spectra of moisture free powdered samples were obtained using a spectrophotometer (FTIR-8300, Shimadzu Co., Japan) by potassium bromide (KBr) pellet method (app. 5 mg sample in 200 mg KBr). The scanning range was 400-4000 cm^{-1} and the resolution was 1 cm^{-1}.

Differential Scanning Calorimetry (DSC) Analysis: DSC scans of the powdered samples were recorded using DSC- Shimadzu 60 with TDA trend line software. All samples were weighed (8-10 mg) and heated at a scanning rate of 10°C/min under dry nitrogen flow (100 ml/min) between 50 and 300 °C. Aluminium pans and lids were used for all samples. Pure water and indium were used to calibrate the DSC temperature scale and enthalpic response.

Measurements of Sol/Gel Transition Temperature: Method given below was used for the determination of sol-gel transition temperature Experiments were carried out at least three times.

A 20 ml transparent vial containing a magnetic bar and 10 g of Plx mixture was placed in a low-temperature thermostat water bath. A digital thermo sensor connected to a thermistor was immersed in the Plx gel.

Plx gel was heated at the rate of 1°C/min with continuous stirring at 30 rpm. When the magnetic bar stopped moving due to the gelation, the temperature displayed on the thermo-sensor was determined as a gelation temperature [11].

Preparation of Calibration Curve: Solutions ranging from 50 to 300 µg/ml were prepared using citrate buffer Solution. Absorbance was measured separately for each solution at λ_{max} of 263.00 nm using Shimadzu UV/visible 1700 spectrophotometer. The calibration curve was constructed by plotting concentration versus absorbance at 263.00.

Preliminary Trial for Preparation of Thermosensitive Poloxamer Formulations: Poloxamer 407 and 188 combinations with different ratios were prepared by using the cold method. The certain amount of Poloxamer 407 and 188 were added to distilled water at 4 °C with gentle mixing and then allowed to dissolve overnight at the same temperature [11]. The composition of the formulations is shown in **Table 1**.

TABLE 1: PRELIMINARY TRIAL FOR PREPARATION OF THERMOSENSITIVE POLOXAMER FORMULATIONS

Formulation no.	Total polymer (%)	Poloxamer 407 (%)	Poloxamer 188 (%)	Gelation temp. °C	pH
F1	30	5	25	≥ 40	5.6
F2	30	8	22	≥ 40	5.5
F3	30	10	20	≥ 40	5.6
F4	30	15	15	39 ± 0.3	5.4
F5	30	18	12	38 ± 0.3	5.7
F6	30	20	10	36 ± 0.3	5.6
F7	30	22	8	21 ± 0.3	5.7
F8	30	25	5	23 ± 0.3	5.7
F9	30	30	-	21 ± 0.3	5.5
F10	30	-	30	≥ 40	5.6

Preparation of Formulations by Cold Method: Vaginal mucoadhesive *in-situ* gel formulations of clotrimazole were prepared with different ratio of Plx 407: Plx 188 mixture (20:10 %), adding the different concentration of HPMC E50 and carbopol 974 (0.5, 1, and 1.5%) as mucoadhesive agent. Plx mixture ratio was decided according to our above study. Gels were prepared by a modification of the cold method [12]. Distilled water was cooled to 4 °C. Plx 188 and 407 were then slowly added to the distilled water containing benzalkonium chloride with continuous agitation. The gels were left at 4 °C until a clear solution was obtained. Then, HPMC E50 and carbopol 974 was gradually added and these gels were left at room temperature for 24 h. Finally, 1% clotrimazole was added with vigorous stirring. The compositions of gels are given in **Table 2**.

TABLE 2: COMPOSITION OF DRUG AND EXCIPIENTS IN FACTORIAL BATCHES OF IN SITU GEL

Ingredients (%)	Formulation								
	$F6_1$	$F6_2$	$F6_3$	$F6_4$	$F6_5$	$F6_6$	$F6_7$	$F6_8$	$F6_9$
Poloxamer 407	20	20	20	20	20	20	20	20	20
Poloxamer 188	10	10	10	10	10	10	10	10	10
Carbopol 974	0.5	1	1.5	0.5	1	1.5	0.5	1	1.5
HPMC E50	0.5	0.5	0.5	1	1	1	1.5	1.5	1.5
Clotrimazole	1	1	1	1	1	1	1	1	1
Benzylkonium chloride	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
D.W	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s

Experimental Design: A 3^2 randomized full factorial design was employed in the present study. In this design, 2 factors were evaluated, each at 3 levels, and experimental trials were performed for all 9 possible combinations. The concentration of carbopol 974 (X1) and concentration of HPMC (X2) were chosen as independent variables and viscosity, % cumulative drug release at 1 h. (Q1) and % cumulative drug release at 12 h. (Q12) was taken as dependent variables Shown in **Tables 3** and **4**.

TABLE 3: SELECTION OF LEVELS FOR INDEPENDENT VARIABLES

Level	Variable	X 1 (Concentration of carbopol 974) %w/v	X 2 (Concentration of HPMC E 50) %w/v
Low	-1	0.5	0.5
Medium	0	1	1
High	+1	1.5	1.5

TABLE 4: FORMULATION LAYOUT FOR 3^2 FACTORIAL BATCHES

Batches	Coded value		Actual value	
	X1	X2	X 1 (Concentration of carbopol 974)	X 2 (Concentration of HPMC)
$F6_1$	-1	-1	0.5	0.5
$F6_2$	0	-1	1	0.5
$F6_3$	+1	-1	1.5	0.5
$F6_4$	-1	0	0.5	1
$F6_5$	0	0	1	1
$F6_6$	+1	0	1.5	1
$F6_7$	-1	+1	0.5	1.5
$F6_8$	0	+1	1	1.5
$F6_9$	+1	+1	1.5	1.5

Evaluation Parameter:

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

Gelling Ability: For this take, about 2-3 ml of each of the sol formulation was in glass test tubes. The test tube was exposed to gradually increasing temperature in the range of 32 to 38 °C. Increase the temperature of °1 every 5 min, and the temperature at which sol was transformed to semisolid gel was note down. The formulations were graded as, No Phase transition (-), formation of gel after 60 S and collapsed rapidly within 1 h (+), formation of gel after 60 S and gel collapsed within 3 h (++), formation of gel within 60 S and gel remained stable for more than 6-7 h (+++). The average of three readings was recorded.

Gelling Temperature: 2 ml of the sample solution and a magnetic bead were put into a 30 ml transparent vial that was placed in a low-temperature digital water bath. A thermometer was placed in the sample solution. The solution was heated at the rate of 1°C/min with continuous stirring at lower rpm.

The temperature was determined as gelation temperature. The experiment was repeated in triplicate, and average of three readings was taken.

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

Viscosity: [13] The viscosity of the liquid formulations (at 10 °C) as well as of the preformed gels (at 37 °C) was determined using a programmable Viscometer (Brookfield, RVDV pro II, USA). For the determination of solution viscosity, 5 ml of the formulation was transferred into the sample cell, which was placed carefully within a small volume sample adaptor. The guard leg was placed around the adaptor, and the sample was continuously stirred. The viscosity of the sample was measured at different RPM ranging from 0.5 to 100 RPM at temperature 10 °C. For the determination of viscosity of gel at 37 °C, the formulations were equilibrated at a temperature of 37 °C for 24 h.

The viscosity of the gel was determined using a Brookfield Viscometer with T-bar spindle. The helipad movement was controlled to avoid touching of the spindle to any part of the sample holder especially the bottom. A typical run involved changing the angular velocity from 0.5 to 100 RPM after every 10 s at a controlled speed. The viscosity

values at each RPM were noted. For the same gel sample, the experiment was repeated thrice, and the average reading was noted.

Effect of Dilution on Viscosity: [14] Vaginal formulations after administration might get diluted with the vaginal fluid. The dilution of *in-situ* gel might lead to an early erosion of gel. In order to assess the effect of dilution on the viscosity of the gel, 0.25 ml citrate buffer (pH 5.2) was added per 2 g of gel. The viscosity of the diluted gel was determined using the Brookfield Viscometer.

Drug Content: 1 ml of the formulation was diluted to 100 ml with buffer solution. The solution was filtered, and clotrimazole content was analyzed by a UV spectrophotometer (Shimadzu, UV-1700, Japan) at λ_{max} of 263 nm. For each formulation, the experiment was repeated thrice.

Mucoadhesive Strength: [15] For determination of mucoadhesive strength of gel an apparatus was fabricated in the laboratory. The apparatus was modified dispensing balance consisting of a central lever, on one arm with pan and another arm with a glass vial. Another glass vial in inverted position was fixed to the wooden base of the balance using double-sided adhesive tape. The membrane was fitted in the gap between lower surface suspended vial and upper surface of the fixed vial.

Weighed quantities (0.5 g) of individual samples of gel formulations were applied to the base of inverted glass vial using double-sided adhesive tape to secure the gel in position. The distance between two vials was adjusted in such a way that the gel sample remained adhered to mucosal membrane. Sufficient pressure was applied on both of the vials for 10 s to allow proper adhesion of gel to mucosa. A constant weight was added to the pan to pull the vial away from the vial. The weight required for detaching the two vials was noted down. The mucoadhesive force, expresses as the detachment stress in dyne/cm^2.

$$\text{Detachment stress} = mg/A$$

Where, m is the weight required for detaching the two vials, g is the acceleration due to gravity, taken as 980 cm/s^2, and A is the area of the membrane exposed and is equal to $A = \pi r^2$. (r- is the radius of the exposed membrane).

In-vitro **Dissolution:** The *in-vitro* release of clotrimazole from the formulations was studied through a cellophane membrane using a modified USP II dissolution testing apparatus. The dissolution medium was citrate buffer (pH 5.2). The cellophane membrane, previously soaked overnight in the dissolution medium, was tied to one end of a specifically designed glass cylinder (open at both ends and of 5 cm diameter). A selected volume of the formulation was accurately pipetted into this assembly.

The cylinder was attached to the metallic driveshaft and suspended in 50 ml of dissolution medium maintained at 37 ± 0.5 °C so that the membrane just touched the receptor medium surface. The dissolution medium was stirred at the required 50 rpm using a magnetic stirrer. Aliquots, a sample was withdrawn at regular intervals and replaced by an equal volume of the receptor medium. The aliquots were diluted with the receptor medium and were analyzed by UV-VIS spectrophotometer.

$$M_t / M = kt^n$$

M_t/M represents a fraction of drug released at time t, k is release constant, and n represents a mechanism of drug release. The value of n as 0.5 indicates drug release by fickian diffusion. The value of n between 0.5 to 0.9 indicates non-fickian or anomalous drug release, whereas if n = 0.9, it indicates zero-order drug release[16].

In-vitro **Antimicrobial Efficacy:** The microbiological studies were carried out to ascertain the biological activity of the optimized formulation and marketed product (Candid) against microorganisms. Fungus Candida albicance was used as the test microorganisms. A layer of nutrient agar (20 ml) seeded with the test microorganism (0.2 ml) was allowed to solidify in the petri plate. Cups were made on the solidified agar layer with the help of a sterile borer of 4 mm diameter. Then, the volume of the formulations (optimized formulation and marketed product (Candid)) containing equivalent amounts of the drug was poured into the cups. After keeping petri plates at room temperature for 4h, the plates were incubated at 37 °C for 24 h. The diameter of the zone of inhibition was measured by using an antibiotic zone reader [17].

RESULTS AND DISCUSSION:

Infrared (IR) Spectroscopic Analysis:

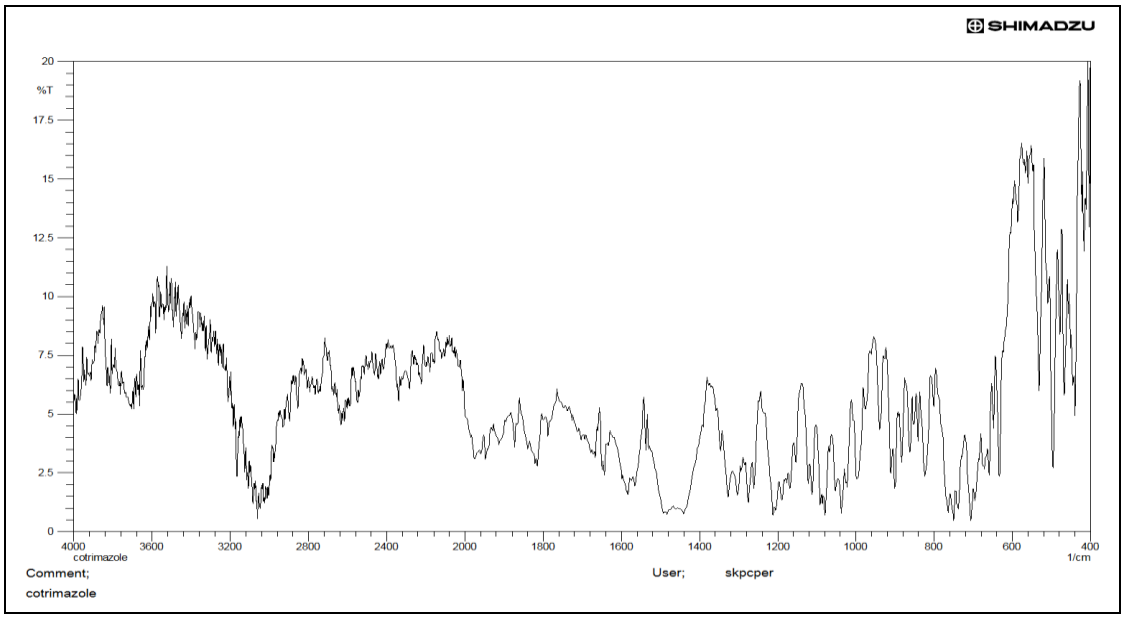

FIG. 1: CLOTRIMAZOLE IR SPECTRA

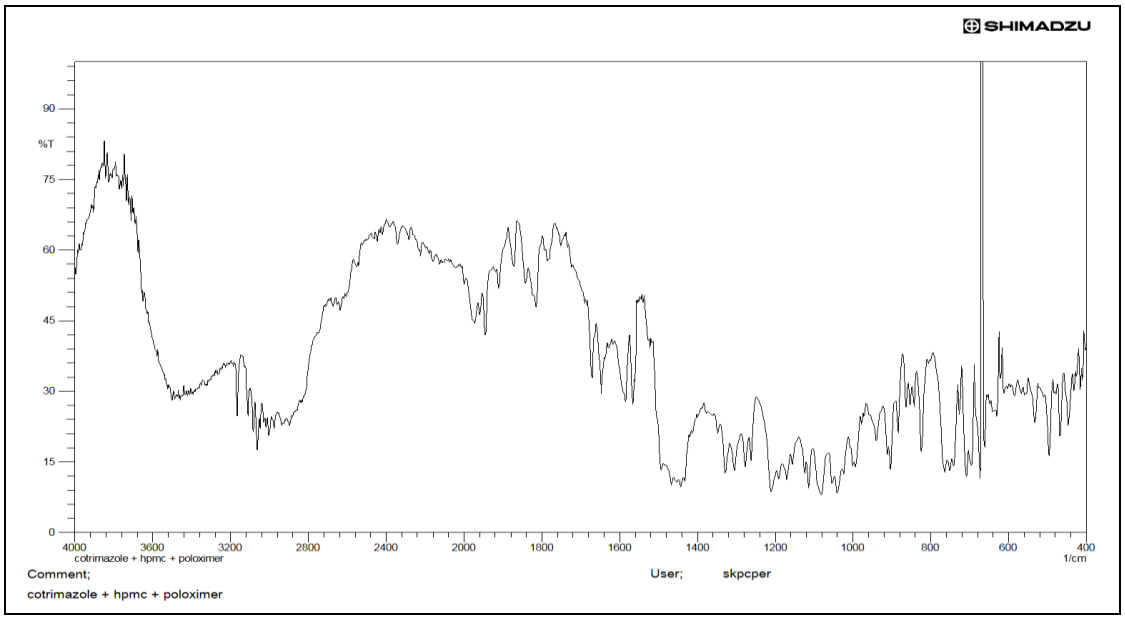

FIG. 2: CLOTRIMAZOLE + EXCIPIENTS IR SPECTRA

Differential Scanning Calorimetry (DSC) Analysis: Differential Scanning Calorimetry enables the quantitative detection of all processes in which energy is required or produced (*i.e.* endothermic or exothermic phase transformations). The thermograms of Clotrimazole Drug and Clotrimazole & Excipients are shown in Figure below. The melting peak of Clotrimazole is at 148.77 ºC shown in the figure below, and in the physical mixture was present at position *i.e.* near 145.03 ºC. This confirmed the physicochemical stability of drugs with the formulation excipients used in the study. It was observed from the above thermogram that there was no appearance of new peaks, no change in peak shapes. The results indicated that there was no interaction with the drug. Hence, the drug was compatible with excipients. Based on DSC data, the selected materials were also used for the preparation of *in-situ* gel because no possible interaction of the drug with polymers and copolymer was observed.

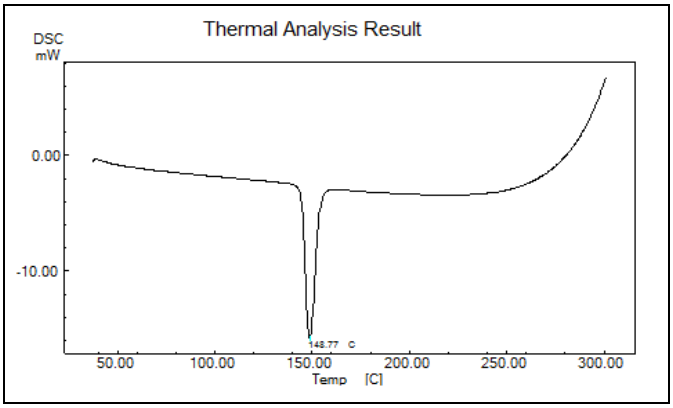

FIG. 3: DSC STUDY OF CLOTRIMAZOLE

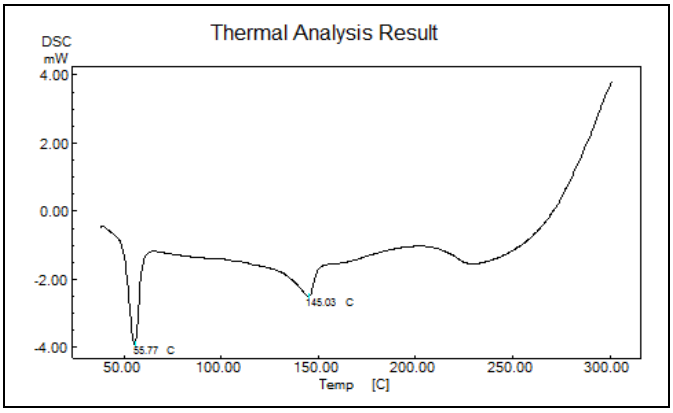

FIG. 4: DSC STUDY OF CLOTRIMAZOLE DRUG + EXCIPIENTS

Measurements of Sol-Gel Transition: Plx molecules exhibit a zigzag configuration transforming into a close-packed meander configuration, and they transform into a viscous gel due to the increasing temperature [18]. The sol-gel transition temperature is the temperature at which the liquid phase makes the transition into a gel. The gelation temperature range suitable for mucosal formulations is 30-36 ºC [19, 20]. If the gelation temperature is high, the formulation stays in liquid form at physiological temperature, and leakage increases. Lower gelation temperature provides the solution to become a gel at room temp. which is in conflict with the aimed formulation type.

Plx 407 or Plx 188 alone showed higher or lower sol-gel transition temperature than body temperature. Sol-gel transition temperature could be modified using additives that interfere with the Plx micellization and alter the dehydration of hydrophobic PO blocks. To avoid the unexpected effect of the additives, Plx mixtures in different ratios were prepared and examined to obtain the proper gelation temperature for the mucosal administration.

Sol-gel transition temperature must be determined sensitively because of the great importance of the gelation temperature to design the proper purposed formulation. There are simple experimental procedures to evaluate the gelation temperature.

One procedure, a well-known process, is mostly used for the determination of sol-gel transition temperature. It was observed that the viscosity was low at the beginning but increased drastically with increasing temperature as a result of the gel-forming process. The formulations which contained higher amounts of Plx 407 (F7, F8, F9) showed lower sol-gel transition temperatures than the gel bases containing only Plx 188 with a high ratio (F1, F2, F3, F10). The sol-gel transition temperature increased when the Plx 407 concentration was decreased. This observation was in accordance with the data available in the literature [34]. When the mixtures were compared according to the ratio of Plx 407/Plx 188, it was observed that the w/w percent ratio of Plx 407/Plx 188was important to reach the desirable gelation temperature, and the result was compatible with the literature [21]. Briefly, the sol-gel transition temperature of F6 was found suitable for administration **Table 1**.

The determination of the gelation time of a gelling sol requires knowledge of the viscosity of the sol as a function of time. The short gelation time was advantageous to prevent the drainage from the site of application leading to prolonged retention of the active substance on the mucosal tissue [22].

All the pH values of the Plx mixtures were found in physiological limitations. The gelation temperature and pH values of the prepared formulations of Plx 407 and Plx 188 are shown in **Table 1**. In light of the observations above, F6 was proven suitable for further studies.

Preparation of Standard Calibration Curve:

TABLE 5: STANDARD CALIBRATION CURVE OF CLOTRIMAZOLE

S. no.	Concentration (µg/ml)	Absorbance
1	50	0.210
2	100	0.320
3	150	0.410
4	200	0.540
5	250	0.620
6	300	0.730

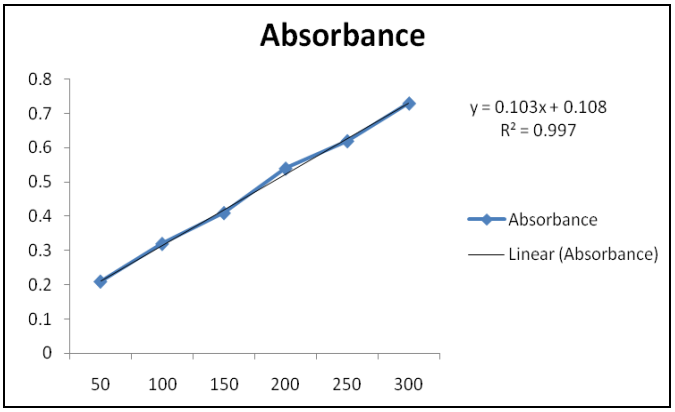

FIG. 5: CALIBRATION CURVE OF CLOTRIMAZOLE

Appearance, Clarity, pH and Drug Content: All the formulations of P407 & P188 alone and with non-ionic polymers, HPMC E 50 were found to be very clear without any precipitation. The formulations containing C 974 was found to be slightly turbid initially, but the turbidity disappeared after storage of the formulations for 48 h. This might be because C974 formed aqueous colloidal dispersions initially, which imparted a slight turbid appearance to the formulation. After equilibration of the formulations, due to complete swelling of the polymer, the turbidity disappeared. pH of the formulations was found to be in the range of 5.2 to 5.8. This pH range is acceptable to the vaginal cavity as the vaginal pH is in the range 4.0 to 5.5 [23]. The drug content of formulations was found to be in the range of 96.90 ± 0.34 to 99.50 ± 0.77.

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

When mucoadhesive polymers (HPMC, and C 974) were incorporated in w/w of Poloxamer formulations, this suggests an increase in the gel strength with the addition of these mucoadhesive polymers. It can be assumed that the addition of mucoadhesive polymers might form hydrogen bonds with PEO blocks in Poloxmer solutions, which might cause dehydration of Poloxamer molecules, leading to aggregation of molecules at a lower temperature. Thus, sol to gel phase transitions was obtained at lower temperature [24].

The addition of C974 reduced the gelation temperature drastically, which might be attributed to the high hydrophilic interaction of Carbopol with water. Also, the carbopol backbone is hydrophobic, which lead to the formation of crosslink between the hydrophobic chain, which subsequently increases the viscosity of formulation resulting in a quick sol to gel transition [25].

Appearance and Clarity: (-) Turbid, (+) slightly turbid, (++) clear and transparent.

Gelling Ability: (-) No phase transition,

(+) Formation of the gel after 60 S and gel collapsed within 1h, (++) formation of the gel after 60 S and gel collapsed within 3 h, (+++) Formation of gel within 60 S and gel remained stable for more than 6-7 h. Mean + SD, n=3.

Characterization of 3^2 Full Factorial Batches: In the present study, 3^2 factorial designs were applied in which carbopol 974 and HPMC E 50 polymer in combination were fixed from the preliminary trials. In this design, two factors were evaluated, each at three levels and experimental trials were carried out at all nine possible combinations. The factors were selected based on a preliminary study. The concentration of carbopol 974 (X1) and concentration of HPMC E50 (X2) were selected as dependent variables. Viscosity (cps), Drug release at 1 h. (Q1) (%) and Drug release at 12 h. (Q12) (%) were selected as independent variables. The evaluation of factorial batches ($F6_1$ to $F6_9$) has been shown in **Table 6**.

Spreadability: The spreadability of formulations was measured in terms of distance traveled by the formulations before the transition to gel. The increase in Polymer concentration decreased the spreadability of the formulations due to a decrease in gelation temperature. The addition of muco-adhesive polymers also lowered the spreadability.

TABLE 6: EVALUATION OF FACTORIAL BATCHES

Formulation	Appearance & clarity	Gelling ability	Gelation temperature	Spreadability (cm)	Drug content	Mucoadhesive strength (dyne/cm^2.10^3)	Viscocity (p)
$F6_1$	+	++	25.3±0.2	2.1±0.2	98.89±0.75	22.34±0.20	350
$F6_2$	+	++	22.3±0.1	2.3±0.1	98.20±0.34	24.12±0.12	380
$F6_3$	+	++	21.2±0.2	2.5±0.2	97.30±0.75	27.20±0.13	410
$F6_4$	++	+++	28.3±0.2	2.2±0.3	96.90±0.34	26.00±0.18	360
$F6_5$	++	+++	27.1±0.3	2.4±0.1	98.22±0.31	29.22±0.17	400
$F6_6$	++	+++	25.4±0.2	2.6±0.2	98.35±0.76	30.32±0.22	450
$F6_7$	++	+++	36.2±0.2	2.1±0.1	98.86±0.45	32.22±0.24	430
$F6_8$	++	+++	37.1±0.1	2.1±0.2	98.60±0.56	34.77±0.21	450
$F6_9$	++	+++	37.5±0.3	2.0±0.2	99.50±0.77	35.23±0.32	480

Mucoadhesive Strength: Mucoadhesion relies on the interaction of a polymer and the mucin coat covering the vagina. Structurally, the mucin consists of a protein or polypeptide core with carbohydrate side chains branching off the core. The polymer with many hydrophilic functional groups (*e.g.*, carboxyl group and a hydroxyl group) can establish electrostatic interactions and hydrophobic interactions, and hydrogen bonds with the underlying surface. These non-covalent forces, hydrogen bonding, appears to be the most important.

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

Viscosity: The viscosity of prepared formulation at Storage condition as well as applied condition. As per the below representation, Viscosity drastically increased at body temperature. The viscosities of clotrimazole formulations both in solution as well as gel forms, were determined using a Brookfield Viscometer.

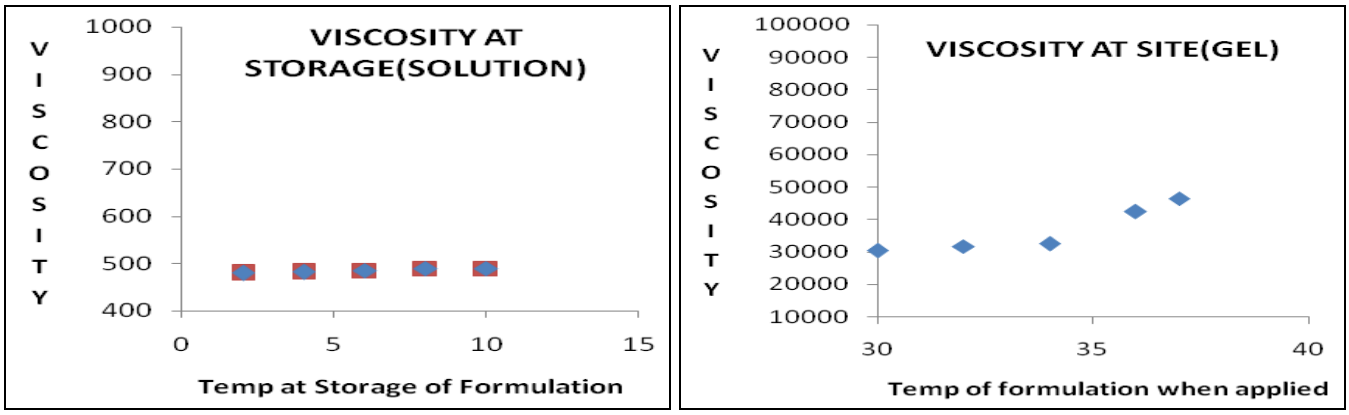

FIG. 6: VISCOSITY OF SOLUTION AT LIQUID AND GEL STATE

Effect of Dilution on Viscosity: *In-situ* gelling vaginal formulation is intended to retain its gel structure and release the drug in a sustained manner. When the formulation is applied to the vaginal cavity, there are chances of the formulation getting diluted with vaginal fluid. Thus, it is required to develop a formulation that is insensitive to dilution and be capable of releasing the drug in a sustained manner for a prolonged period of time.

Generally, 2 to 5 g of the formulation is applied to the vaginal cavity, which might dilute with 0.25 to 0.5 ml of vaginal secretions. Hence, 0.25 ml of citrate buffer (pH 5.2) per 2 g of the formulation was added, and the viscosity of the diluted formulation was determined. It was observed that there was a significant dilution of PLX gels, and there was an almost 20 % drop in the viscosity **Fig. 7**.

This might be due to dilution with buffer, the concentration of PLX in a formulation (20% w/w) decreased to 18% w/w, which decreased its viscosity drastically, and viscosity resembled the viscosity of $F6_9$ (16% w/w) formulation. The drop in the viscosity was lower with the addition of mucoadhesive polymer.

The formulation containing HPMC (1.5%) and Carbopol 947 (1.5%) was found to be a more robust formulation amongst all the formulations.

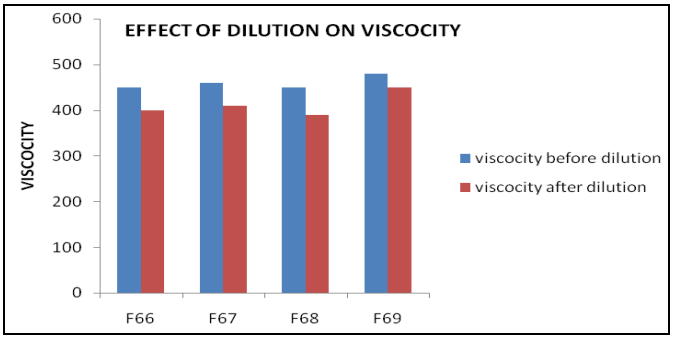

FIG. 7: EFFECT OF DILUTION ON VISCOSITY OF *IN-SITU* GELLING FORMULATIONS

TABLE 7: % *IN-VITRO* DRUG RELEASE

Time (hrs)	Formulation								
	$F6_1$	$F6_2$	$F6_3$	$F6_4$	$F6_5$	$F6_6$	$F6_7$	$F6_8$	$F6_9$
0	0	0	0	0	0	0	0	0	0
1	14.28	12.15	11.89	12.50	11.01	10.50	12.01	11.00	10.9
2	28.57	25.22	23.09	24.34	22.21	21.34	23.14	19.11	18.2
3	42.08	37.21	34.20	36.23	34.23	32.83	33.15	29.64	24.6
4	56.36	49.60	45.31	48.10	46.09	43.15	44.21	39.12	33.5
5	70.64	61.89	53.42	61.36	54.76	52.32	52.23	52.00	41.6
6	84.92	73.86	62.23	73.12	67.1	61.11	64.16	61.16	49.8
7	97.56	88.32	74.20	84.87	78.00	72.20	75.56	72.56	57.3
8		97.94	83.78	96.80	86.43	80.92	84.06	81.00	65.9
9			96.56		97.50	88.77	98.00	88.5	73.4
10						98.34		98.10	83.9
12									99.02

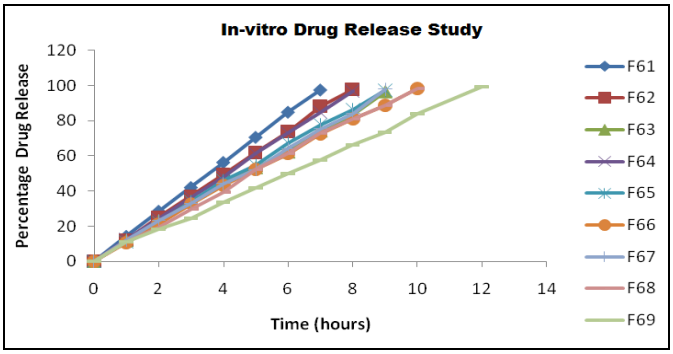

FIG. 8: *IN-VITRO* DRUG RELEASE

The *in vitro* drug release study was carried out for all formulated *in situ* gels containing clotrimazole. All batches showed a sustained drug release profile for 12 h.

Formulations $F6_9$ showed 99.02 % release of Clotrimazole **Fig. 8**. The R^2 value for the formulation was good for zero order. Therefore, the release rate of drugs was in accordance with the zero-order kinetic release. It was noted that the release of drug is not only affected by poloxamer concentration but also the concentration of viscosity modifiers. The drug release retarding effect of polymers could be attributed of their ability to increase overall product viscosity as well as their ability to distort the extra micellar aqueous channels of polymer micelles through which drug diffuses thereby delaying the drug release.

Kinetic Modeling of Drug Release Data: Based on the R^2-value, the best-fit model was selected. The n value was 0.5-1, so the drug transport mechanism was non Fickian diffusion. The highest R^2value was obtained in the zero-order so the release kinetics followed by Zero-order as best fit model shown in **Table 8**.

TABLE 8: KINETIC MODELING OF DRUG RELEASE DATA

Batch no.	Zero-order	First-order	Higuchi	Korsmeyer
$F6_9$	R^2	R^2	R^2	R^2
	0.998	0.977	0.995	0.729

***In-vitro* Antimicrobial Efficacy:** The optimized gel showed antimicrobial activity when tested microbiologically by cup plate technique. Clear zones of inhibition were obtained in the case of formulation $F6_9$ and in the marketed product (candid). The diameter of the zone of inhibition produced by formulation against test organisms was similar to those produced by the marketed

product. The antimicrobial effect of the *in-situ* gelling formulation is probably due to a fairly constant release of drug from the cross-linked hydrogel drug reservoir, which permits the drug to be released to the target site relatively slowly.

TABLE 9: ZONE OF INHIBITION PRODUCED BY THE OPTIMIZED FORMULATION $F6_9$

Microorganism	Area of zone of inhibition (mm2) after 48 hrs of incubation		
	Formulation $F6_9$	Marketed gel (CANDID ®)	Control
Candida Albicance	585±2.0	580±2.0	575±3.0

Stability Studies: Stability study was performed only on the optimized batch at 40 ± 2 ºC temp. and 75 ± 5% RH conditions. The results obtained after the time period are shown in the table.

TABLE 10: PRODUCT INTENDED FOR STORAGE IN GENERAL CONDITION

Study	Storage condition	Time period
Accelerated	40°C ± 2°C / 75% RH ± 5% RH	6 months

TABLE 11: ACCELERATED STABILITY STUDIES OF OPTIMIZED BATCH $F6_9$

Time (month)	Clarity	pH	Gelation temp.	CPR
Initial	Clear	5.6±0.04	36.22±0.24	99.50
After 1 month	Clear	5.57±0.06	35.17±0.13	99.25
After 2 month	Clear	5.61±0.04	34.36±0.33	98.10
After 4 month	Clear	5.60±0.08	35.16±0.23	97.21
After 6 month	Clear	5.8±0.23	35.12±0.23	97.12

TABLE 12: DESIRABILITY FUNCTION

S. no.	D1	D2	D3	D4	D5	D
	Gelation temp.	Mucoadhesive strength	Viscocity	Drug release	Drug strength	
$F6_1$	0.26875	0.026153846	0.166667	0.5625	0.6	0.160219
$F6_2$	0.08125	0.163076923	0.333333	0.68720	0.4	0.26783
$F6_3$	0.0125	0.4	0.5	0.8765	0.2	0.00173
$F6_4$	0.45625	0.307692308	0.222222	0.6782	0.5	0.003932
$F6_5$	0.38125	0.555384615	0.444444	0.7675	0.3	0.571996
$F6_6$	0.275	0.64	0.722222	0.8032	0.1	0.619168
$F6_7$	0.95	0.786153846	0.611111	0.8534	0.7	0.715254
$F6_8$	0.81875	0.882307692	0.722222	0.8876	0.2	0.873006
$F6_9$	0.7625	0.92394781	0.888889	0.9345	0.1	0.889176

The desirability function was utilized in order to find out the best batch out of all 9 batches of the central composite design. Formulation $F6_9$ showed the highest overall desirability of 0.889176. Therefore, this formulation was considered to be the best formulation, and the values of independent variables of this formulation were considered to be optimum values for the preparation of *in-situ* gel.

The results of the stability study show no remarkable change in the release profile, assay, and other evaluation parameters of the vaginal in-*situ* gel after exposure to accelerated stability conditions.

CONCLUSION: Mucoretention of the gel was aimed to develop a long term release formulation in routine and widely occur infection in a woman nowadays. It was made successful by using biocompatible, inert and body temperature-sensitive polymers to get the desired effect over the site of application. In this study, temperature-sensitive in situ gel by the cold method of clotrimazole for vaginal delivery was formulated using a combination of Plx 407: Plx 188, HPMC, and Carbopol. The developed formulation was clear, spreadable, with shear-thinning properties, forming a quick and stable gel having resistance to dilution and excellent mucoadhesion to the vaginal mucosa. The formulation exhibited sustained release behavior and indicated strong antifungal activity against *Candida albicans.* All the formulations showed sustained drug release for a period of 12 h. The desirability function was utilized in order to find the best batch out of all 9 batches. Formulation $F6_9$ showed the highest overall desirability of 0.889176.

Therefore, this formulation was considered to be the best formulation, and the values of independent variables of this formulation were considered to be optimum values for the preparation of *in-situ* gel. The results of the stability study show no remarkable change in the release profile, assay, and other evaluation parameters of the *in-situ* gel after exposure to accelerated stability conditions.

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CONFLICTS OF INTEREST: Nil

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