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Ion activated bioadhesive in situ gel of clindamycin for vaginal application

Himanshu Gupta[1*], Aarti Sharma[2]

***Corresponding author:**

Himanshu Gupta

[1]Department of Pharmaceutics,

Faculty of Pharmacy,

Jamia Hamdard

(Hamdard University),

New Delhi,

India

Email:
himanshu18in@yahoo.com

[2]School of Pharmacy,

Jaipur National University,

Jaipur, India

Abstract

Vaginal preparations, although generally perceived as safer most , still they are associated with a number of problems, including multiple days of dosing, dripping, leakage and messiness, causing discomfort to users and expulsion due to the self-cleansing action of the vaginal tract. These limitations lead to poor patient compliance and failure of the desired therapeutic effects. For effective vaginal delivery of antimicrobial agents, the drug delivery system should reside at the site of infection for a prolonged period of time. In our present work, we have developed and optimized a chitosan (bioadhesive and permeation enhancer) and gellan gum (ion activated gelling polymer) based in situ gel system of clindamycin for vaginal application. The developed formulation was characterized for various in-vitro parameters e.g. clarity, refractive index, pH, isotonicity, sterility, viscosity, drug release profile, statistical release kinetics, bioadhesive force, retention time, microbial efficacy, irritation test and stability studies. To simulate vaginal conditions, a synthetic membrane (cellophane hydrated with modified simulated vaginal fluid) and sheep vaginal mucosa were used as model membranes. The developed formulation was found to be non irritant, bioadhesive with good retention properties. Developed formulation shows matrix model release kinetic by PCP disso software. The developed formulation is thus a viable alternative to conventional vaginal dosage forms.

***Keywords*:** sol-to-gel system; chitosan; gellan gum; vaginal; clindamycin

Introduction

The vagina, unlike other systems is highly dynamic with respect to absorption of drugs, their metabolism and their elimination. The vaginal defense i.e. epithelium, flora, immune cells and pH make it favorable site for local and systemic delivery of drugs that are used specifically for the treatment of female-related conditions. The vaginal cavity has traditionally been used for local delivery of drugs such as prostaglandins, steroids, antibiotics, antifungal, antiprotozoal, antichlamydial, antiviral, and spermicidal agents [1]. Recently, there has been increased interest in vaginally administered agents and formulations known as ‘‘microbicides,’’ which provide effective contraception and protection against transmission of various sexually transmitted infections (STDs), including acquired immunodeficiency syndrome (AIDS) [2]. In addition, vaginal preparations include products meant for the maintenance of reproductive hygiene, enhancement of sexual pleasure, and moisturizing the vaginal mucosa in case of dry vagina in postmenopausal women.

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Several kinds of vaginal dosage forms have been developed, such as sponges, rings, suppositories, tablets, tampons, foams, films, gels, and creams. Vaginal preparations, although generally perceived as safer most but still these are associated with a number of problems, including multiple days of dosing, dripping, leakage and messiness, causing discomfort to users and expulsion due to the self-cleansing action of the vaginal tract. These limitations lead to poor patient compliance and failure of the desired therapeutic effects. For effective vaginal delivery of antimicrobial agents, the drug delivery system should reside at the site of infection for a prolonged period of time [3].

The conventional dosage forms i.e preformed gel and solution have a number of lacunas, which has limited their use in vaginal drug delivery. Direct application of gels onto the infected sites of the vagina might be difficult, inconvenient as well as have frequent dosing because the conventional gels do not remain for long time at the site of application. A new and recent approach is to try to combine advantages of both gels and solution so that an accurate dose can be administered with ease of administration i.e in-situ gel system. These formulations remain to a solution state before administration but however transforms to gel after administration in to vaginal cavity. Some researcher explored efficacy of in situ vaginal gel. The liquid applied to topical areas turns into gels as a result of physical and/or chemical change induced by physiological environments such as pH for cellulose acetate phthalate, the concentration of calcium ions for gellan gum, temperature for poloxamers, etc. [4].

Bioadhesion and retention at the site of application for a sufficient period of time can be achieved by incorporating bioadhesive polymers in the formulations. Till date, only a limited number of studies have been reported on bioadhesive drug delivery systems for vaginal administration. Chitosan is a natural polymer obtained by deacetylation of chitin. Chitosan act both as bioadhesive and permeation enhancer. It also possesses antimicrobial and wound-healing properties [5]. Gellan gum is an ion activated polymer, which gel when comes in contact with ions i.e vaginal fluid.

Clindamycin phosphate is a water soluble semi-synthetic antibiotic. Clindamycin inhibits bacterial protein synthesis at the level of the bacterial ribosome. The antibiotic binds preferentially to the 50S ribosomal subunit and affects the process of peptide chain initiation. Clindamycin is indicated for the treatment of bacterial vaginosis (formerly referred to as *Haemophilus* vaginitis, *Gardnerella* vaginitis, non-specific vaginitis, *Corynebacterium* vaginitis, or anaerobic vaginosis) in non-pregnant women. We are using clindamycin as a model drug for our present study to check the efficacy of the developed formulation.

Hence, in our present work, we are developing and optimizing a bioadhesive chitosan and gellan gum based in situ gel system of clindamycin for vaginal application. The optimized gel was evaluated for various physicochemical properties, in vitro drug release, bioadhesive force, retention time, microbial efficacy, irritation test, and stability studies.

Materials and Method

Clindamycin phosphate was obtained as gift samples from Glenmark Pharmaceuticals, Mumbai, India. Chitosan (practical grade, 75-85% deacetylated, molecular weight 150 kDa) was obtained as kind gift from M/s, India Sea Foods, Cochin, India. Gellan gum (Gelrite ® CP Kelco, US) was obtained as a gift from Applied Biosciences, Mumbai, India. All other chemicals and solvents used were purchased from local suppliers and of analytical grade unless mentioned.

Preparation of simulated vaginal fluid (SVF)

The simulated vaginal fluid was prepared from 3.51 gL^{-1} NaCl, 1.40 gL^{-1} KOH, 0.222 gL^{-1} $Ca(OH)_2$, 0.018 gL^{-1} bovine serum albumin, 2 gL^{-1} lactic acid, 1 gL^{-1} acetic acid, 0.16 gL^{-1} glycerol, 0.4 gL^{-1} urea and, 5 gL^{-1} glucose. pH of the mixture was adjusted to 4.5 ±0.02 using 0.1 M HCl [6].

Interaction studies

Liquid solutions of chitosan, gellan gum and clindamycin phosphate was prepared individually and in combinations and were autoclaved at 121°C for 20 min at 15 psi. The ultraviolet spectra were taken before and after autoclaving using double beam ultraviolet-visible spectrophotometer. Both spectra were compared for any possible change due to interactions between different ingredients.

Preparation of placebo Gel System

Different concentrations of chitosan and gellan gum were prepared and evaluated for their gelling capacity in order to identify the compositions suitable for use as in *in situ* gelling systems (Table 1). Chitosan, a pH sensitive polysaccharide was dissolved in 1% v/v acetic acid diluted further with phosphate buffer system, pH adjusted to 5.0. Gellan gum, an ion activated polymer was dissolved in phosphate buffered saline, pH 5.0. The gelling capacity was determined by placing a drop of the system in a vial containing 2 ml of freshly prepared simulated vaginal fluid and visually assessing the gel formation, noting the time taken for gelation and time taken by gel formed to dissolve.

Medicated *in- situ* gel formulation

For antibacterial activity clindamycin phosphate was available as 2% of vaginal cream. Hence a dose of 2% was used in present formulation. The weighed quantity of drug was placed in volumetric flask and dissolved in normal saline under aseptic conditions. 0.1% of methyl paraben was added as a preservative. The resultant polymeric solutions were mixed well and kept undisturbed at room temperature for 24 hours to ensure proper mixing. Osmolarity of the formulation was determined by osmometer (Fiske Associate, USA) and required amount of sodium chloride was added and mixed thoroughly to make solution isotonic. Complete formula of the developed formulation was given in Table 2. The solution was transferred into amber-colored bottles and sealed till further use. The resulting solutions were sterilized by autoclaving at 121°C for 20 min at 15 psi

Physicochemical Characterization

Formulations were tested for different physicochemical properties as described and results are shown in Table 3

Clarity and Refractive Index

The clarity of the formulations after and before gelling was determined by visual examination of the formulations under light alternatively against white and black backgrounds. Refractive index of the formulations were determined by Abbe`s refractometer.

pH Evaluation

The pH of the formulation was recorded with a glass microelectrode (Mettler Instruments, Germany), and allowing it to equilibrate for 1 min. Experiments were performed in triplicate.

Rheological measurements

Rheological measurements were performed by a Brookfield Model DVIII+ Digital Rheometer (Brookfield Engineering Laboratories Inc., MA, USA). Brookfield Rheometer was used in which the test material is placed between two surfaces, one surface is rotated, and the torque resisting flow is measured. Simulated vaginal fluid (SVF) of pH 4.5 was added in increments of 25 ml to 200 ml of the formulations, and the viscosity at which gelation occurred were recorded. This allows the determination of relationship between applied shear rate and shear stress experienced by the test material [7, 8]. All the measurements were conducted using SC4-14 spindle using about 4 ml sample volume at 20rpm. The tests were performed in triplicate, with a coefficient of variation of less than 5% being found. Since, in vivo, vaginal formulations will experience the dilution with vaginal fluids and it has been reported that the rheological behavior of gels could be affected by various factors such as copolymer compositions and solutes [9].

In-vitro release kinetics

The in vitro drug release was, performed in sink conditions, by means of a Franz diffusion cell, (PermeGear, Inc. Bethlehem, PA.) diameter 20 mm, with a water jacketed receptor chamber (15 ml) and

a donor chamber thermostated at 37°C. The receptor solution was constantly stirred at 600 rpm. The two chambers were separated by a cellulose membrane (Filter paper Whatman 41, 20-25µm, Whatman GmbH, Dassel, Germany) and each formulation (100 mg) was spread on a circular portion of the membrane. Drug release was determined by previously reported method of HPLC. [10] Briefly, The method uses a Hypersil ODS, 5 µm, 250×4.6 mm i.d. column maintained at 45°C. The mobile phase comprises acetonitrile–phosphate buffer (1.35% v/v phosphoric acid, adjusted to pH 6.0 with ammonium hydroxide)–water (35:40:25, v/v) at a flow rate of 1.0 ml/min. Drug was quantified according to previously determined calibration curve (r= 0.9998), and reported as an average of 3 determinations. % cumulative drug released was plotted against each time point.

Statistical Analysis

The results of in vitro data were analyzed by statistical software PCP Disso, version 3.0, to obtain the best fit kinetic model for in vitro drug release from optimized formulation.

Bioadhesion Measurement

The assemblies developed for in vitro measurement of bioadhesive strength in a simulated vaginal environment are a modification of the previously reported bioadhesion test assembly [11]. The method is based on the measurement of tensile strength or shear stress required to break the adhesive bond between a model membrane and the test formulation. The test formulation is sandwiched between two model membranes fixed on flexible supports in the assemblies for a sufficient period of time. After the adhesive bond has formed, the force (weight) required to separate the bond was measured and calculated as bioadhesive strength.

Ex- Vivo Retention Measurement

Another desirable property of vaginal formulations is retention in the vaginal cavity for a desired period of time. An intact tubular piece of sheep vagina (cut to 3 in long; thawed in normal saline containing 0.1% w/v sodium azide) was procured from a slaughter house. Vagina was suspended vertically with the help of a loop of wire and a stand [12]. The tissue was surrounded with a cotton pad moistened with normal saline, further surrounded by aluminum foil in order to keep the tissue moist for the duration of the experiment. An electronic balance was placed below the suspended tissue to measure the weight of gel falling down. The room temperature was maintained at 37±2ºC. A test sample (4mL) was introduced into the isolated vaginal tube with the help of a 10mL syringe (without needle), taking care to avoid spillage. The expulsion of gel from the lower end under the influence of gravity was then recorded as a function of time for 2 hr as a measure of retention.

Microbiological Studies

The microbiological studies were carried out on the optimized formulation and 2% w/v of plain drug solution for comprison against micro-organism. *Staphylococcus aureus* was used as the test micro-organism. A layer of nutrient agar (20 mL) seeded with the test micro-organism (0.2 mL) was allowed to solidify in the petriplate. Cups were made on the solidified agar layer with the help of sterile borer at 4 mm diameter. Then volume of the formulations (optimized formulation and plain drug solution) containing equivalent amount of drug was poured into the cups. After keeping petriplates at room temperature for 4 hr, the plates were incubated at 37◦C for 24 hr. The zone of inhibition was obtained. The diameter of zone of inhibition was measured by an antibiotic zone finder [13].

Irritation Test (HET-CAM Test)

For the present study, modified HET-CAM test as reported by (Velpandian et al, 2006) was carried out. [14] The HET-CAM has been shown to be a qualitative method of assessing the potential irritancy of chemicals. The potential irritancy of compounds may be detected by observing adverse changes that occur in the chorionallantoic membrane of the egg after exposure to test chemicals.[15] Briefly, fertilized hen`s eggs were obtained from poultry farm. Three eggs for each

formulation weighing between 50-60 g were selected and candled in order to discard the defective ones. These eggs were incubated in humidified incubator at a temperature of 37 ± 0.5°C for 3 days. The trays containing eggs were rotated manually in a gentle manner after every 12 hours. On the day three, egg albumin (3 ml) was removed by using sterile techniques from the pointed end of the egg. The hole was sealed by 70% alcohol sterilized parafilm (American Can Company, USA) with the help of heated spatula. The eggs were kept in the equatorial position for the development of Chorioallantoic membrane (CAM) away from the shell. The eggs were candled on the fifth day of incubation and everyday, thereafter non-viable embryos were removed. On the tenth day a window (2X2 cm) was made on the equator of the eggs through which formulations (0.5 ml) were instilled directly onto the Cam surface and left in contact for 5 minutes. The membrane is examined for vascular damage and the time taken for injury to occur is recorded.

A 0.9% NaCl solution was used as a control as it is reported to be practically non-irritant. The scores were recorded according to the scoring schemes as shown in Table 4 and score obtained was given in Table 5.

Stability Studies

Stability studies were carried out on optimized formulation according to ICH Guidelines. 3 packs of formulations were subjected to these stability studies. The packs were kept in Humidity and Temperature control cabinets (Topsun made) and maintained at 40^{o}C, 75% relative humidity. The samples were withdrawn at 0, 60, 120 and 180 days and analysed by HPLC. The degradation rate constant (K) was deduced with equation slope= K/2.303.

Results and Discussion

Interaction studies were carried out to check any interaction between formulation ingredients. UV spectra obtained before and after autoclaving were found to be identical. No additional peak or shift in peak reveals two facts. First, the ingredients were compatible to each other and no physicochemical reactions took place, and secondly it also shows that the formulation can be terminally sterilized by autoclaving.

Table 1: Combinations of chitosan/gellan gum studied

Formulation	Chitosan (% w/v)	Gellan Gum (% w/v)	Gelling Capacity
1	0.5	0.5	+
2	1.0	0.5	++
3	1.5	0.5	+++
4	0.5	1.0	++
5	**1.0**	**1.0**	**+++**
6	1.5	1.0	++++
7	0.5	1.5	+++
8	1.0	1.5	++++

+ Very weak gel, transparent, dissolves rapidly; ++ gel forms, transparent, dissolves after sometimes; +++ firm gel, transparent, do not dissolve on standing; ++++ Instant gel, turbid, do not dissolve

The placebo formulations were developed using different combination of chitosan and gellan gum which were evaluated for their physicochemical characteristics like physical appearance and viscosity It's prerequisite for an in situ gel system that it allows easy instillation into the cavity as liquid drops which undergoes sol-to-gel transition, triggered by presence of divalent cation ions of vaginal fluid. A concentration of 1% of both chitosan and gellan gum was selected as it gives a colorless and transparent formulation (Table 1) with good gelling capacity and taken further for evaluation. 2% of clindamycin phosphate is prescribed for antibacterial therapy for vaginal infections, hence we also used the same concentration to prepare medicated in situ gel formulation. 0.1% Methyl paraben was added as preservative and NaCl was added in calculated amount to maintain isotonicity of the formulation. Final formula was given in Table 2.

Table 2: Formula of the developed in situ formulation

Ingredients	Concentration (w/v)
Clindamycin phosphate	2%
Chitosan	1.0 %
Gellan Gum	1.0 %
NaCl	0.40%
Methyl Paraben	0.1%
Water (q.s.)	100%

Physicochemical characterization of formulation

The developed formulation was further characterized for various physiological parameters, like clarity, refractive index, viscosity, pH and osmolarity. The optimized chitosan/gellan gum vaginal gel is transparent in color. Refractive index of the gel is ranging from 1.335-1.337, proofing the transparency of gel. To avoid initial irritation due to difference in osmolarity of formulation and body fluid, the formulation was made iso-osmotic by addition of NaCl. The pH of the optimized formulations was found in the range of 5.0-5.5. (Table 3). Gellan gum converted into stiff gel in the presence of ions and results in sudden increase in the viscosity. The results were shown in Table 3.

Table 3: Physicochemical properties of the developed in situ gel formulation

Parameter	Inference
Clarity	Clear solution
pH	5.0-5.5
Osmolarity	299-302 mOsmol
Refractive Index	1.335-1.337
Viscosity (at formulation condition)	87±2 cps
Viscosity (in SVF)	438±5 cps
Mucoadhesive force	0.118 N
Retention time	98 minutes

Values are expressed as mean ± S.D. (n=5)

Rheological measurements

The evaluation of rheological properties for the gel type dosage forms is important for predicting their behavior in vivo. All prepared gels and Clindamycin phosphate was submitted to rheological tests in order to study their flow properties. It is important to analyze such data because these characteristics can influence the formulation stability during the storage and in case of temperature changes. The prepared formulations showed a pseudoplastic flow, guarantying an immediate flow after stress application. This behaviour is desirable because it can promote good storage at room temperature and easy application.

In vitro release kinetics

In vitro drug release kinetics was carried out by the use of Franz diffusion cells in order to evaluate clindamycin in situ gel release profile. Initially the formulation demonstrates rapid release (burst effect) followed by slow and constant release for the rest of time. Fig. 1.

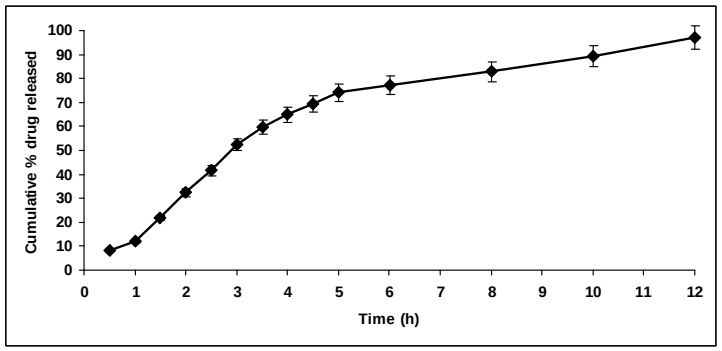

Fig 1: In vitro drug release profile of developed formulation. Values are expressed as mean±S.D. (n=5)

This pattern confirms the controlled release behavior of the formulation. The initial burst effect is beneficial for antibiotics as it help achieving the therapeutic concentration of drug in minimal time followed by constant release to maintain sustained and control release of the drug. Developed formulation displayed 32.3% cumulative drug release after 2 h. 77.4% after 6 h and 97.2% after 12 h. Burst effect might be due to initial migration of

the drug towards the surface of the matrix. Curve fitting of in vitro release data of optimized

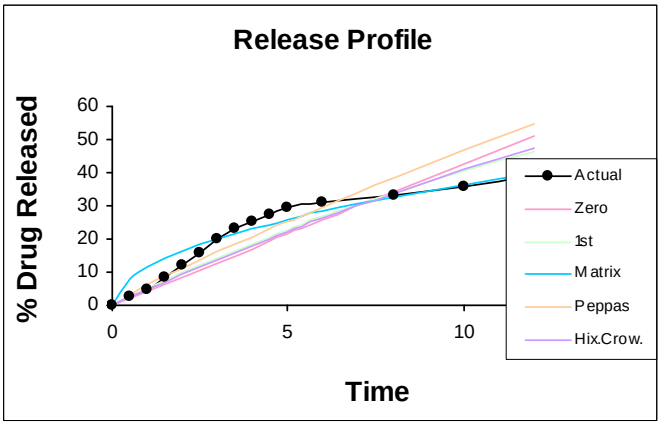

Fig. 2: Graph showing release kinetics of optimized formulation

formulation was compared with different release model to select best fitting model using PCP Disso V 3.0 software, confirming matrix release mechanism of drug release from in situ gel. The best fit kinetic model was matrix model (R = 0.9615, k = 11.5296, t-test = 12.608 (passes) (Fig.2).

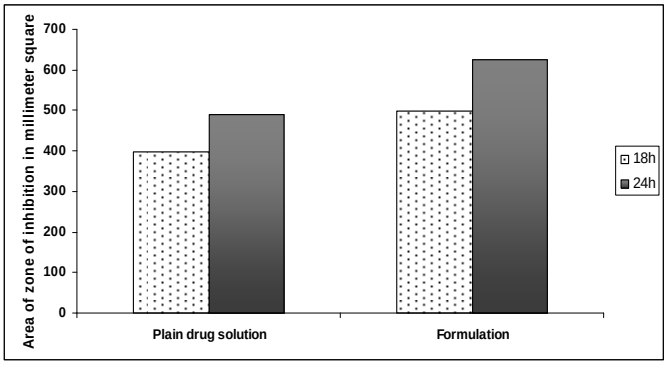

Fig 3: Area of zone of inhibition for the plain clindamycin phosphate drug solution and optimized formulation

Evaluation of mucoadhesive strength and retention time

Bioadhesion and long retention are desirable characteristics of a vaginal formulation. Chitosan act as mucoadhesive as well as permeation enhancer. Performed adhesion force studies and retention studies proves the mucoadhesive nature of chitosan. The optimized formulation shows a bioadhesion force and retention time of 0.118 N and 98 minutes respectively. Table 3, it was possible to note that chitosan possess good bioadhesive and retention properties. Chitosan presence improved gel performances and made them able to be employed in vaginal therapy.

Table 4: Scoring chart for HET-CAM Test

Effect	Scores	Inference
No visible hemorrhage	**0**	Non-irritant
Just visible membrane discoloration	**1**	Mild irritant
Structures are covered partially due to membrane discoloration or hemorrhage	**2**	Moderately irritant
Structures are covered totally due to membrane *discoloration or hemorrhages*	**3**	Severe irritant

Microbiological Studies

The optimized in situ gelling formulation showed antimicrobial activity when tested microbiologically by cup plate technique. Clear zone of inhibition were obtained. The diameter of zone of inhibition is shown in Figure 3.

Irritation Test (HET-CAM Test)

Irritation of the developed formulation was checked by Hen's egg chorioallantoic membrane test which is a rapid, sensitive and inexpensive test. Testing with incubated eggs is a borderline case between in vivo and in vitro systems and does not conflict with the ethical and legal obligations. The Chorioallantoic membrane of the chick embryo is a complete tissue including viens, artries and capillaries and is technically very easy to study. It responds to injury with a complete inflammatory process [15]. Developed formulation was tested by using this method and result was compared with those obtained using normal saline, which was used as control that is supposed to be practically non-irritant. A means score of 0 was obtained for normal

saline. Chitosan/gellan gum based formulation was non-irritant up to 2 h (mean score 0) while the mean score was found to be 0.33 up to 24 h (Table 5). The study shows that the formulation is non-irritant to mild irritant and is well tolerated.

Table 5: Scores obtained in HET-CAM Test

Formulations		Scores								
		Time (in minutes)								
		0	5	15	30	60	120	240	480	1440
Normal Saline as control	Egg1	0	0	0	0	0	0	0	0	0
	Egg2	0	0	0	0	0	0	0	0	0
	Egg3	0	0	0	0	0	0	0	0	0
	Mean	0	0	0	0	0	0	0	0	0
Developed Formulation	Egg1	0	0	0	0	0	0	0	0	0
	Egg2	0	0	0	0	0	0	0	0	1
	Egg3	0	0	0	0	0	0	1	1	1
	Mean	0	0	0	0	0	0	0.33	0.33	0.66

Stability studies

Stability study was carried out on the optimized formulation as per ICH guidelines for 6 months. The samples were analyzed for drug content by HPLC. The drug was degraded to a negligible extent and the degradation rate constant for optimized formulation was low (2.42 × 10−4). Because the overall degradation is <5%, a tentative shelf life of 2 years may be assigned to the optimized formulation.

Conclusion

Performed studies and obtained results prove the efficacy of chitosan/gellan gum based clindamycin in situ gel system for vaginal application. The formulation is non irritant, easy to administer along with good bioadhesive and retention properties. This formulation has potential for a better patient complaint vaginal formulation. The efficacy of the formulation can further be studied by in vivo and clinical experiments.

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