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NOVEL VAGINAL ANTI-HIV DRUG DELIVERY SYSTEM OF TENOFOVIR DISOPROXIL FUMARATE

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ABSTRACT

The present research work aimed at development and optimization of niosome based gel (NBG) for the vaginal delivery of Tenofovir disoproxil fumarate (TDF). The TDF was incorporated into niosomes using span 60 and cholesterol. Box-Behnken statistical screening design with 3 factors, 3 levels, and 15 runs was selected to statistically optimize the formulation parameters. The independent variables selected were parts of cholesterol (X_1), surfactant loading (X_2), amount of stabilizer (X_3). Fifteen batches were prepared by thin film hydration method and evaluated for percentage drug entrapment (PDE) and vesicle size. The transformed values of the independent variables and the PDE (dependent variable) were subjected to multiple regressions to establish a full-model second-order polynomial equation. F value was calculated to confirm the omission of insignificant terms from the full-model equation to derive a reduced-model polynomial equation to predict the PDE of niosome. A model was validated for accurate prediction of the PDE by performing checkpoint analysis. The niosomal dispersion was incorporated in to Carbopol 940NF gel. The NGB was evaluated for drug content, pH, spreadability, consistency and texture analysis. The *in-vitro* drug release study shows sustained release gel effect whereas the *in-vivo study* shows no signs of irritation on the applied vaginal site in rat. The gel was kept for 6 weeks accelerated stability studies. The niosomes and niosomal gel showed maximum stability at 2 to 8 °C.

Key-words: Niosome, vaginal drug delivery, Tenofovir disoproxil fumarate, span 60, Box Behnken design

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INTRODUCTION

Heterosexual transmission of human immune deficiency virus (HIV-1) accounts for nearly 90% of all HIV-1 infections in women. [1] Currently, an estimated 19.2 million women worldwide are infected with HIV-1, accounting for ~50% of the 40 million adults living with HIV/AIDS. [2] The emergence of HIV/AIDS as a disease spread through sexual intercourse, combined with growing public awareness about the problems associated with other viral sexually transmitted infections (STIs), has prompted the search for new, cost-effective, and safe vaginal microbicides for curbing transmucosal viral transmission.[3,4] Microbicides would provide protection by inactivating viruses or preventing viruses from replicating either in semen or the infected host cells that line the vaginal wall. Microbicides that are currently being investigated are directed mainly at preventing pregnancy as well as protection against STIs.[5, 6]

In vaginal drug delivery, the physiological conditions imposed by the protective mechanisms of the vagina often lead to the limited contact time of administered drugs with vaginal mucosa and a short duration of therapeutic efficacy, making a frequent dosing regimen necessary. Moreover, conventional vaginal dosage forms such as inserts and ointments give discomfort to the patients. The patients are known to tolerate gels better than inserts or ointment. Vaginal therapy would be thus significantly improved if an intravaginally administered drug can retain at the site of administration for prolonged period after more convenient dosing. [7,8]

Tenofovir disoproxil fumarate (TDF) belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors (nRTIs), which block reverse transcriptase, an enzyme crucial to viral production in HIV-infected people. [9] It is frequently prescribed not only for its efficacy but also for its decreased side effect profile compared with other nucleoside analogs.[10] Tenofovir is used in treatment and prevention of HIV/AIDS at 0.5 and 1.0% concentrations. Tenofovir disoproxil fumarate can cause acute renal failure, Fanconi syndrome, proteinuria or tubular necrosis if given orally. These side effects are due to accumulation of the drug in proximal tubules. TDF can interact with didanosine by increasing didanosine's concentration. TDF is very effective in 1 % strength but it causes erythema, atrophy and leukocyte infiltration on the applied area.

The Niosomes seems to be promising drug delivery in modern drug delivery systems. Niosomes are unilamellar or multilamellar vesicles that are made up of nonionic surfactant and can entrap amphiphilic and hydrophobic solutes. [11, 12] Stability is a prime concern in the development of any formulation. Niosomes are osmotically active and stable, as well as they increase the stability of

entrapped drug. The main benefit over liposome is that the lipids are replaced by non-ionic vesicles and hence the preparation is totally non-antigenic.[13]

In the present study thin film hydration method was used for the preparation and optimization of TDF niosome as this method is simple and easy to scale up. Various process parameters were also optimized for the thin film hydration method. It is hypothesized that incorporation of TDF into niosomes will improve the amount and time of the drug retention within the vagina, so as to increase the therapeutic index of the drug.

Traditional experiments require more effort, time, and materials when a complex formulation needs to be developed. Various experimental designs [14, 15] are useful in developing a formulation requiring less experimentation and providing estimates of the relative significance of different variables. In the work reported here, a Box-Behnken design [16] was used to optimize niosomes containing TDF. Independent variables selected were molar ratio of Span 60: cholesterol (X_1), surfactant loading (X_2), and amount of drug (X_3) to evaluate their separate and combined effects on percentage drug entrapment (PDE) and vesicle size expressed as the mean volume diameter (MVD). Hence the present work was envisaged to develop a stable, novel, and aesthetic niosomal vaginal drug delivery system with improved efficacy.

MATERIALS AND METHODS

Tenofovir disoproxil fumarate was generous gift from Glied Sciences Pvt Ltd., (Belgium). Span 60 and Tocopherol acetate were purchased from Croda Chemicals Pvt Ltd., (India). Cholesterol was generous gift from Merck chemicals, Virginia, USA. Dichloromethane, Ammonium acetate Glacial acetic acid and Sodium hydroxide were purchased from Finar Chemicals Ltd, (Ahmedabad, India). Ethanol, Chloform and Potassium hydroxide were purchased from S.D. Fine Chemicals Ltd., (Mumbai, India). A dialysis tube (DM-70; capacity 2.41 mL/cm, width 29.31mm, average diameter 17.5mm, and molecular weighted cutoff 12 000 to 14 000) was purchased from Himedia Laboratories, (Mumbai).

EXPERIMENTAL

Box-Behnken Experimental Design

The traditional approach to develop a formulation is to change 1 variable at a time. By this method it is difficult to develop an optimized formulation, as the method reveals nothing about the interactions among the variables. Box-Behnken statistical screening design with 3 factors, 3 levels, and 15 runs was selected to statistically optimize the formulation parameters and evaluate main effects, interaction effects of the formulation ingredients on the TDF of niosomal vaginal

gel formulations. The experimental design consists of a set of points lying at the midpoint of each edge and the replicated center point of the multidimensional cube. The independent and dependent variables are listed in (Table 1). The polynomial equation generated by this experimental design (using Statistica Release 6, Statsoft Inc) is as follows:

$$Y_i = b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_{12} X_1 X_2 + b_{13} X_1 X_3 + b_{23} X_2 X_3 + b_{11} X_1^2 + b_{22} X_2^2 + b_{33} X_3^2 \quad (1)$$

Where Y_i is the measured response associated with each factor level combination; b_0 is an intercept; b_1 to b_{33} are regression coefficients computed from the observed experimental values of Y; and X_1, X_2 and X_3 are the coded levels of independent variables. The terms X_1X_2 and X^2_i (i = 1, 2 or 3) represent the interaction and quadratic terms, respectively.

Table- 1: Variables and their levels in Box-Behnken Design

Independent Variables	**Levels**		
	Low	**Medium**	**High**
X_1 = parts of cholesterol	0.5	1.0	1.5
X_2 = surfactant loading	15X	20X	25X
X_3 = amount of Stabilizer (ml)	0.25	0.5	1.0
Transformed values	–1	0	1
Dépendent variables: Y_1 = Percentage drug entrapment, Y_2 = Particle size			

*1X corresponds to multiply ratio of the amount of Cholesterol.

Preparation of niosomes

Niosomes were prepared by thin film hydration method. [17] Span 60: cholesterol at a ratio of 20:1 by weight and 50 mg of TDF were dissolved in 20 ml of Dichloromethane, in a 250 ml round-bottomed-flask. The flask was fitted onto a rotary flask evaporator (Buchi Rotavapor instruments Pvt Ltd., Mumbai, India) and connected to a vacuum pump. The solvent system was evaporated under vacuum at 50 °C using water bath for 10 minutes. The dried surfactant film was hydrated with 50 ml distilled water for 30 minutes at 50 °C using bath sonicator (PCI Pvt Ltd, U.K). The hydrated dispersion was kept in refrigerator for 2 hours for sealing of vesicles. All the batches were prepared according to the experimental design shown in (Table 2).

Vesicle Size Determination

The vesicle sizes of niosomes were determined using a particle size analyzer (Malvern Zetasizer, ZS 90, U.K). The apparatus consisted of a He-Ne laser beam of 632.8 nm focused with a minimum power of 5 mW using a Fourier lens (R-5) to a point at the center of a multi-element detector and a small-volume sample holding cell (Su cell) The sample was stirred before determining the particle size as Mean Vesicle Diameter (MVD). [18]

Table -2: Box-Behnken experimental design with measured responses*

Batch	Parts of Cholesterol	Parts of SPAN60	Parts of Tocopherol acetate	(Y_1)† PDE (%)*	PDE after one week (%)*	(Y_2)† Particle size (nm)
F1	0.5	15	0.5	**Flaking**		
F2	1.5	15	0.5	22.88±1.42	19.16±1.34	224.2±1.3
F3	0.5	25	0.5	30.94±1.26	25.68±1.26	124.7±3.2
F4	1.5	25	0.5	38.09±1.43	36.44±1.55	146.5±2.1
F5	0.5	20	0.25	44.02±1.13	41.86±1.41	264.1±0.9
F6	1.5	20	0.25	50.35±0.98	48.76±0.56	179.9±1.3
F7	0.5	20	1	51.41±1.43	50.94±2.04	268.3±2.9
F8	1.5	20	1	46.64±2.12	45.48±0.96	304.0±3.5
F9	1	15	0.25	48.81±1.38	46.54±2.22	209.09±1.8
F10	1	25	0.25	48.32±0.92	45.48±0.96	124.2±1.6
F11	1	15	1	54.13±0.87	52.01±0.67	134.7±2.8
F12	1	25	1	63.32±0.92	59.09±0.96	138.5±2.1
F13	**1**	**20**	**0.5**	**68.63±1.46**	**67.08±1.32**	**137.6±1.3**
F14	1	20	0.5	67.63±1.07	66.08±1.32	138.6±1.3
F15	1	20	0.5	67.63±1.68	65.08±1.32	136.6±1.3

* PDE indicates percentage drug entrapment; † n = 3.

PDE (percentage drug entrapped)

The PDE of TDF niosomes was calculated after determining the amount of unentrapped drug by dialysis. [19] The dialysis was performed by adding the niosomal dispersion to a dialysis tube (donor compartment) and then dipping the tube into a beaker containing 400 ml of phosphate buffer pH 4.5 (receptor compartment) on a magnetic stirrer, rotated at a speed of 80 to 120 rpm for 4 hours. After 4 hours, the solution in the receptor compartment was estimated for un entrapped drug at 265 nm using a UV spectrophotometer (UV 1601, Shimadzu, Kyoto, Japan). The PDE of the niosomes was calculated by:

$$PDE = \frac{Wt\text{-}We}{Wt} \qquad (2)$$

Where, Wt - total amount of drug added and We - amount of unentrapped drug.

Scanning Electron Microscopy

The surface characteristics of the niosome were studied by scanning electron microscopy (SEM). The samples were observed for morphological characterization using a gaseous secondary electron detector (working pressure: 0.8 torr, acceleration voltage: 30.00 kV) XL 30, Philips (Eindhoven, Netherlands). The particles were observed for surface characteristics.

Optimum Formula

After developing the polynomial equations for the responses PDE and MVD with the independent variables, the formulation was optimized for the response PDE. Optimization was performed to find out the level of independent variables (X_1, X_2, and X_3) that would yield a maximum value of PDE with constraints on MVD.

Checkpoint Analysis

A checkpoint analysis was performed to confirm the role of the derived polynomial equation and contour plots in predicting the responses. Values of independent variables were taken at 3 points, 1 from each contour plot, and the theoretical values of PDE were calculated by substituting the values in the polynomial equation. Niosomes were prepared experimentally at 3 checkpoints, transformed to niosomes, and evaluated for the responses.

Formulation of niosome based gel (NBG) of TDF

The niosomal dispersion was incorporated in to Carbopol 940NF gel. The gel was kept for 6 weeks accelerated stability studies.

CHARACTERIZATION OF THE TDF-NBG

Determination of drug content, pH, Spreadability and Consistency

For determination of drug content, about 1 g of the gel was weighed in a 100-ml volumetric flask and dissolved in methanol; it was diluted appropriately and analyzed by the UV spectroscopy method described earlier. The pH of the various gel formulations was determined using digital pH meter; pH was adjusted by means of 0.1 N NaoH.

Spreadability was determined by wooden block and glass slide apparatus. Weights about 20g were added to the pan and the time were noted for upper slide (movable) to separate completely from the fixed slides. Spreadability was then calculated using the formula:

$$S = M.L / T \qquad (3)$$

where, S – spreadability, M - weight tide to upper slide, L - length of glass slide, T - time taken to separate the slide completely from each other.

The measurement of consistency of the prepared gels was done by dropping a cone attached to a holding rod from a fix distance of 10cm in such a way that it should fall on the centre of the glass cup filled with the gel. The penetration by the cone was measured from the surface of the gel to the tip of the cone inside the gel. The distance traveled by cone was noted down after 60sec.The measurement set up for the determination of consistency is shown in (Figure 1).

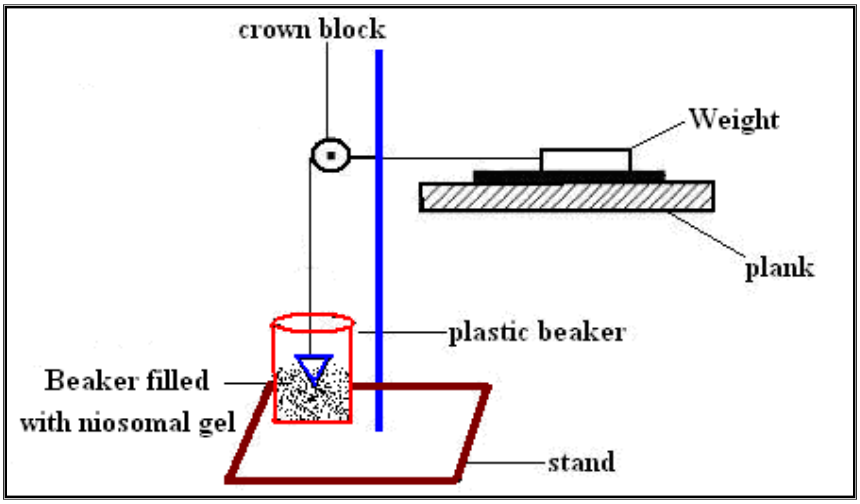


Figure- 1: Measurement set up for the determination of consistency.

Rheological studies

Brookfield (Brookfield Pro-V II Viscometer, Japan) with helipath stand was used for rheological studies. The sample (30 g) was placed in a beaker and was allowed to equilibrate for 5min before measuring the dial reading using a T-C spindle at 0.5, 1, 2.5, and 5 rpm. At each speed, the corresponding dial reading on the viscometer was noted.

Texture analysis

The texture analysis of NBG was conducted by Texture Analyser. The recorded forces represent responses to tension, compression, penetration or bending. The selected probe will move at a programmed speed and until specified force, distance or strain is reached, which will be indicated in the record. The sets of conditions for testing were found to be good, namely:

Pre-test speed: 1 mm/sec; test speed: 1 mm/sec; post-test speed: 1 mm/s; distance 10 mm; return to the start point.

The force and area were recorded for total back extrusion with respect to cohesiveness and adhesiveness. Standard deviations from 5 separate readings were all below 2%. The probe used was cylinder type.

***In-vitro* drug release study**

In-vitro methods are valuable as screening procedure and for deducing physicochemical parameters such as flux, partition co-efficient and diffusion co-efficient. Sigma dialysis membrane, 200 μm in thickness was used as an artificial membrane for preliminary in vitro studies because of simplicity, homogeneity and uniformity. This membrane was hydrated in pH 4.5 phosphate buffer saline for 24 hours prior to a permission run. The amount of Tenofovir present in the sample was determined by UV Spectrophotometer at 265 nm.

Accelerated stability study

The stability studies of niosomes and niosomal gel of TDF were carried out for six weeks at different accelerated conditions like 2-8 °C, 25 °C / 60 %RH and 40 °C / 75 %RH. The samples were analyzed for assay of the TDF. [20, 21]

***In-vivo* study**

The animal study was carried out by properly following CPCSEA guidelines and the project was approved by the Institutional Animal Ethical Committee (IAEC) of SKPCPER/2010-11.

Total 9 healthy female rats of average weight 250 gm were selected for study. The skin at the vaginal site was shaved carefully. The niosomes prepared with optimized parameters, were incorporated into Carbopol 940 NF gel base. Simultaneously plain drug was also incorporated into gel base as control. Solution of TDF had been prepared, too.

The present study evaluated the local tolerance of vaginal niosomal gel - planned for being tested by means of the rat vaginal irritation test, The vaginal formulations contained different types of TDF suits with the concentration of 1%.

The rats were treated once daily with 1 ml TDF solution, TDF gel and TDF niosomal gel. The formulations were inserted approximately 1.5 cm into the vagina via a soft rubber catheter that was wetted with water and attached to a syringe tube. For each formulation two rats were used. Prior to each administration, the animals were examined for clinical signs of vaginal or vulval irritation, discharge or bleeding from the vagina. The applied area was covered by cotton and bandage. The dosing procedure was repeated for 6 consecutive days. On day 7, the rats were killed by euthenesia and were necropsied. The vaginal and cervical tissues of each animal were examined as per C. Callens et al.[22] The vaginal and cervical tissues were examined microscopically by an experienced pathologist for the severity of epithelial loss and atrophy and the presence of leukocyte infiltration. The severity of the findings was described by the symptoms of atrophy, lesions and leukocyte infiltration were graded as: 5- severe; 4- marked; 3- moderate; 2- slight; 1- minimal; 0- absent.

RESULTS AND DISCUSSION

In preparation of niosomes, various ratios of cholesterol and surfactants were tried but due to homogeneity, drug entrapment and dispersion characteristics, some formulas were not selected. Span 60 is chemically sorbitan monostearate having HLB value 4.7. Span 60 has highest phase transition temperature as compare to other spans. So once after formation of vesicles there are

less chances of vesicle bursting. The phase transition temperature is around 50 ˚C for span 60 which is tolerable by the drug and hence the span 60 shows all the required characteristics.

The surfactant: cholesterol ratio is very important for drug entrapment because the cholesterol acts as stabilizer and itself is lipophilic in nature so increased concentration of cholesterol may cause reduction in drug entrapment. The optimum concentration is needed otherwise vesicle stability may decrease. Various ratios were tried for the preparation and finally 20:1 surfactant: cholesterol was selected to prepare further batches.

Data Analysis

A Box-Behnken experimental design with 3 independent variables at 3 different levels was used to study the effects on dependent variables. All the batches of TDF niosomes within the experimental design yielded niosomes on hydration, and these were evaluated for PDE and vesicle size. A Box-Behnken experimental design has the advantage of requiring fewer experiments (15 batches) than would a 3^3 full factorial design (27 batches). Transformed values of all the batches along with their results are shown in Table 3. The PDE (dependent variable) obtained at various levels of the 3 independent variables (X_1, X_2, and X_3) was subjected to multiple regression to yield a second-order polynomial equation (full model):

$$PDE = 79.31 - 4.87X_1 - 2.28X_2 - 3.52X_3 + 3.29\ X_1\ X_2 + 2.37\ X_1X_3 + 0.13X_2X_3 - 5.44X_{12} - 1.88X_{22} + 0.37X_{32} \quad (4)$$

The value of the correlation coefficient (r^2) of equation 4 was found to be 0.933, indicating good fit. The PDE values measured for the different batches showed wide variation (i.e., values ranged from a minimum of 22.68 to a maximum of 68.33). The results clearly indicate that the PDE value is strongly affected by the variables selected for the study. This is also reflected by the wide range of values for coefficients of the terms of Equation 1. The main effects of X_1, X_2, and X_3 represent the average result of changing 1 variable at a time from its low level to its high level. The interaction terms (X_1X_2, X_1X_3, X_2X_3, X_1^2, X_2^2, and X_3^2) show how the PDE changes when 2 variables are simultaneously changed. The negative coefficients for all 3 independent variables indicate an unfavorable effect on the PDE, while the positive coefficients for the interactions between 2 variables (X_1X_2, X_1X_3, and X_2X_3) indicate a favorable effect on the PDE. Among the 3 independent variables, the lowest coefficient value is for X_2 ($b_2 = -2.28$ and $P > 0.05$), indicating that this variable is insignificant in prediction of PDE.

Among the 3 independent variables, the lowest coefficient value is for X_2 ($b_2 = -2.28$ and $p > 0.05$), indicating that this variable is insignificant in prediction of PDE. The standardized effect

of the independent variables and their interaction on the dependent variable was investigated by preparing a Pareto chart (Figure 2), which depicts the main effect of the independent variables and interactions with their relative significance on the PDE. The length of each bar in the chart indicates the standardized effect of that factor on the response. The fact that the bar for X_2, X_1X_2, X_1X_3, X_2X_3, X_2^2, and X_3^2 remains inside the reference line in Pareto chart, and the small coefficients for these terms in equation 1, indicate that these terms contribute the least in prediction of PDE. Hence, these terms are omitted from the full model to obtain a reduced second-order polynomial equation (equation 5) by multiple regressions of the PDE and the significant terms ($P < 0.05$) of equation 1.

$$PDE = 78.44 - 4.87X_1 - 3.52X_3 - 5.33X_{12} \quad (5)$$

To confirm the omission of non significant terms, F statistic was calculated after applying analysis of variance for the full model and the reduced model. The F calculated value (2.74) is less than the tabled value of F (4.95) at a 0.05 confidence interval, $v_1 = 6$ and $v_2 = 5$. Hence it is concluded that the omitted terms do not significantly contribute for predicting the PDE. This implies that the main effect of the ratio of span 60:cholesterol and the amount of tocopherol acetate added is significant, as is evident from their high coefficients and the fact that the bars corresponding to variables X_1, X_3, and X_1^2 extend beyond the reference line in figure 2.

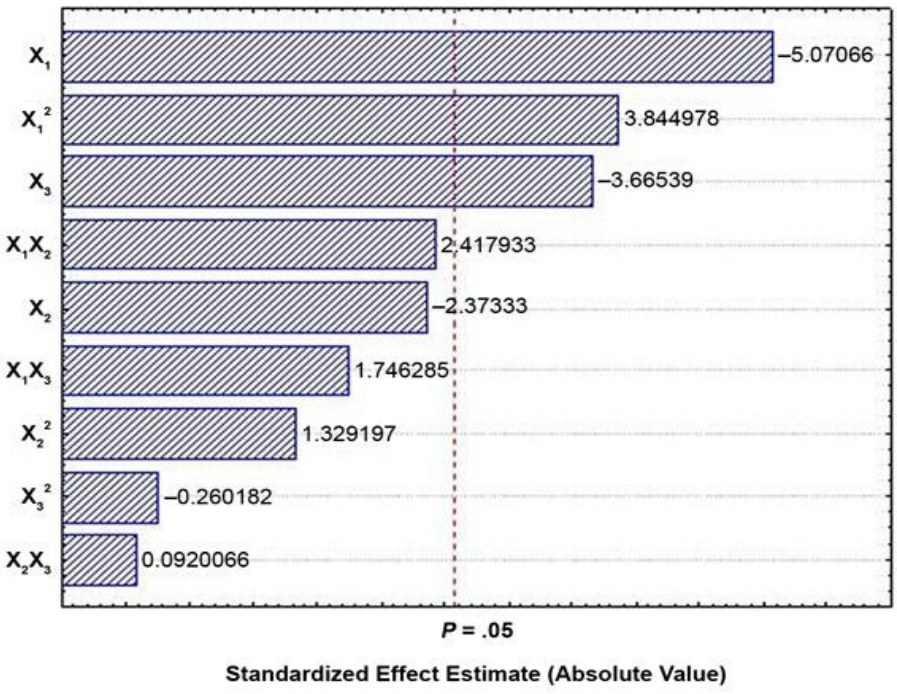


Figure -2: Pareto chart showing the standardized effect of independent variables and their interaction on the percentage drug entrapment of niosomes.

Checkpoint analysis

Three checkpoint batches were prepared and evaluated for PDE. Results indicate that the measured PDE values were as expected. When measured PDE values were compared with predicted PDE values using student *t* test, the differences were found to be insignificant ($p > 0.05$). Thus, we can conclude that the obtained mathematical equation is valid for predicting the PDE.

Optimization

After studying the effect of the independent variables on the responses, the levels of these variables that give the optimum response were determined. It is evident from the polynomial equation that cholesterol decreases the PDE within niosomes. Also, cholesterol is known to abolish the gel-to-liquid phase transition of niosomes, and the resulting niosomes are known to be less leaky. Hence, the medium level was selected as optimum for the ratio of span 60: cholesterol (X_1), as up to this level a high value of PDE can be obtained. The optimum formulation is one that gives a high value of PDE and a low MVD (≤5 μm) along with a high total amount of drug entrapped and a low amount of carrier in the resultant niosomes. Below the selected (optimum) level of X_2, decreases in surfactant loading result in a significant increase in the amount of carrier and an insignificant increase in the PDE. However, a decrease in the amount of drug (X_3) below the selected level leads to a decrease in the total amount of entrapped drug. Hence, a 0 level (1:1) for the molar ratio of span 60: cholesterol (X_1), a –0.158 level of surfactant loading (X_2), and a –0.158 level of amount of tocopherol acetate (X_3) were selected as optimum. For confirmation, a fresh formulation was prepared at the optimum levels of the independent variables, and the resultant niosomes were evaluated for the responses. The observed values of PDE and MVD were found to be 68.78% and 576.7 nm, respectively, which were in close agreement with the predicted values of batch F13 shown in the (Table 3).

The present study conclusively demonstrates the use of a Box-Behnken design in optimization of niosomal formulations. The derived polynomial equations aid in predicting the values of selected independent variables for preparation of optimum niosomal formulations with desired properties.

Particle size analysis

The average particle size was determined for the various batches having the different ratios of span 60: cholesterol by SEM analysis. However, the particular interest is to find the formulation which is having the least particle size at the same time negative repultative zeta potential, which

is the good sign of stability of colloidal system which is in the desired range for the niosomal formulation, too.

Table - 3: The observed and predicted values after by Box Behnken design.

Batch code	Observed PDE	Predicted PDE	Residuals	% Error
F1	NA			
F2	22.88±1.42	24.02	-1.51	2.18
F3	30.94±1.26	29.23	1.51	1.72
F4	38.09±1.43	40.09	-2.00	2.67
F5	44.02±1.13	45.38	-1.36	1.21
F6	50.35±0.98	48.35	2.14	0.30
F7	51.41±1.43	53.55	-2.14	6.61
F8	46.64±2.12	45.28	1.36	5.37
F9	48.81±1.38	49.15	0.64	3.24
F10	48.32±0.92	48.47	-0.15	4.82
F11	54.13±0.87	53.98	0.15	4.74
F12	63.32±0.92	62.64	0.64	5.70
F13	**68.63±1.46**	**69.34**	**-0.71**	**0.90**
F14	67.63±1.07	66.00	1.63	1.64
F15	67.63±1.68	67.10	0.53	0.89
Checkpoint batches				
Batch code	**X_1**	**X_2**	**X_3**	**PDE Measured† Predicted**
C_1	0	–0.5	0.5	46.64±2.15 ,44.28
C_2	0.5	0	–0.5	48.81±0.38,43.15
C_3	–0.5	0.5	0	45.32±2.91 45.47

* PDE indicates percentage drug entrapment, † n = 3.

The particle size range for the batch having span 60: cholesterol of 20:1 was found to be minimal, viz 576.2 nm. So the batch was considered as suitable for the formulation. However the batches 15:1 and 25:1 showed good results but considering the size and zeta potential deviation, the 20:1 was found to be most suitable. Zeta potential was found to be in the range of -11.3 mV to -23.3mV. Here in surfactant: cholesterol of 20:1 zeta potential was found to be -23.3mV which indicates high negative surface charge on niosomes indicate higher stability because of the anticipated surface repulsion between similar charged particles hence inhibiting aggregation of the colloidal niosomal particles.

Characterization of TDG-NBG

The TDF content of the NBG was found to be 97.8 ± 4.2 % of the theoretical value. The pH of TDF-NBG was 4.5 which is equivalent to the vaginal pH. Spreadability is an important property of topical formulation from patient compliance point of view. The diameter was found to be 6.9

g.cm/sec which is indicative of good spreadability. The TDF-NBG showed pseudo-plastic behavior and the viscosity of TDF-NBG at 5rpm was 62340 Cps.

Texture analysis

At the beginning of the optimization of the measurements, two types of gels were used, namely carbopol gel with span 60: cholesterol ratio of 15:1 and 20:1 to exclude the effect of the type of gel on the method set up. It was observed that the different beakers used in the measurement, varying the placement for the beaker and the surface of the gel (smoothness) had the direct effect on measurements.

Therefore, it was important to fix the beaker to a rack and mark the placement position. Under these more controllable conditions, characterization of Carbopol gels gave reproducible results with standard deviations below 2 %. They seem quite similar at first glance. But p-values reveal that the two batches are statistically different when comparing force 1, area 1 or area 2 with p-values between 0.00003-0.0014. Force 2 has a p-value of 0.241. *p*- values less than 0.05 indicate that there is less than 5% probability that the measured difference between the sample-sets are caused by coincidences. Even with small forces recorded, the method is capable of distinguishing between small variations within the tested gels. Data of p-value revealed that batch prepared with the ratio of span 60: cholesterol with 20:1 is the better one than the other compared.

***In-vitro* drug release study**

The figure 4 shows the comparative release profiles of all the formulations which makes easy for us to understand the release pattern. The release studies show that 99.12 % and 99.01 % drug diffusion occurred within 14 hours from drug solution and plain drug gel respectively, while 98.45 % and 99.15 % drug diffusion occurred at 15 and 20 hours from niosomal dispersion and niosomal gel respectively (Figure 3). The difference is significant between drug solution and niosomal dispersion as well as the plain drug gel and niosomal gel. This indicates the slow release properties of niosomal formulations. The release rate is retarded due to niosomal barrier.

The drug solution is highly diffusible by nature thus the curve shows slight decrease in release rate after some hours of release, whereas in niosomal dispersion, the curve shows slight increase in release rate as the saturation in receptor component has not been attained yet. The release rate is slow thus the drug concentration will not be too high in short time and thus this can prevent the irritation to epithelium of vagina.

The diffusion coefficients were found to be 1.68 x 10^{-09}, 1.26 x 10^{-09}, 9.07 x 10^{-10} and 5.94 x 10^{-10} for drug solution, drug gel, niosomal dispersion and niosomal gel respectively. The diffusion co-efficient is low for the niosomal formulations that indicates the slow release pattern for the niosomal formulations. The diffusion co-efficient is highest for the drug solution which indicates the faster release of the drug across the semi-permeable membrane.

Accelerated stability study

The result of stability studies showed steady decrease in percentage drug retained in samples of niosomal dispersion and gel. Percentage drug retained in niosomes stored at 2-8 °C showed 100.11% at first week which was reduced gradually to 93.26 % at sixth week, in niosomes stored at 25 °C / 60 % RH showed 99.31% at first week which was reduced gradually to 91.11% at sixth week, whereas, in niosomes stored at 40 °C / 75 %RH showed 96.94% at first week which was reduced gradually to 70.45 % at sixth week.

Percentage drug retained in niosomal gel stored at 2-8 °C showed 100.64% at first week which was reduced gradually to 92.12% at sixth week, in niosomes stored at 25 °C / 60 %RH showed 99.16% at first week which was reduced gradually to 90.94% at sixth week, whereas, in niosomes stored at 40 °C / 75 %RH showed 95.44% at first week which was reduced gradually to 62.54% at sixth week.

From above results, it can be concluded that the niosomes and niosomal gel showed maximum stability at 2 to 8 °C and hence such formulations should be stored at refrigerated conditions.

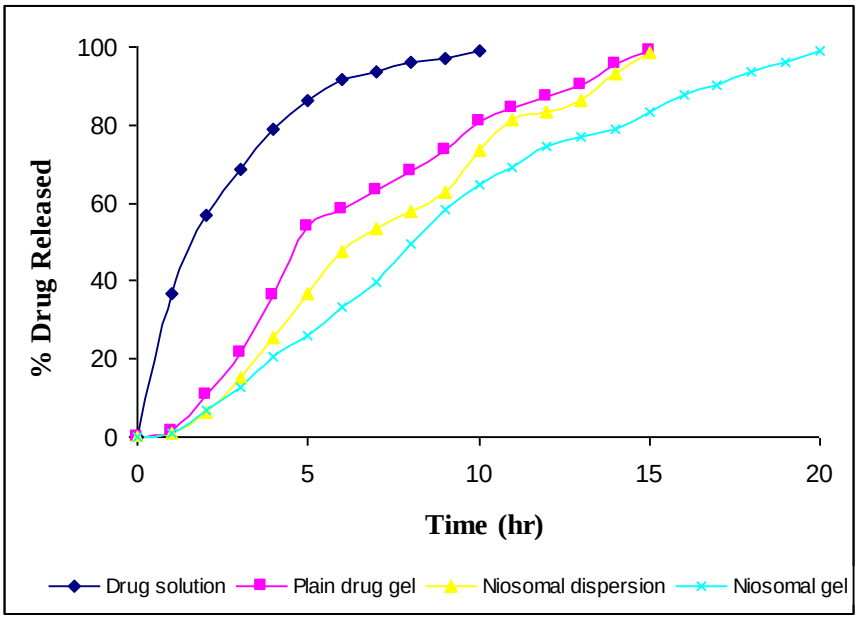
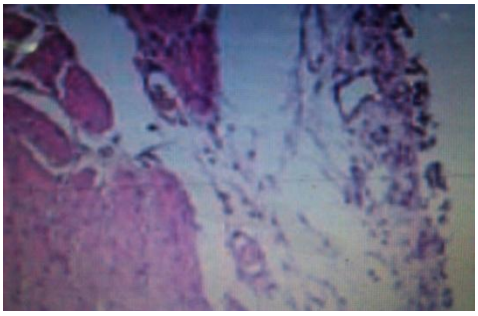
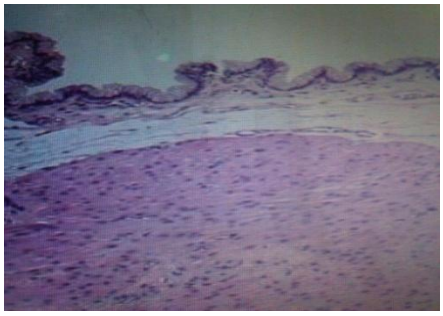


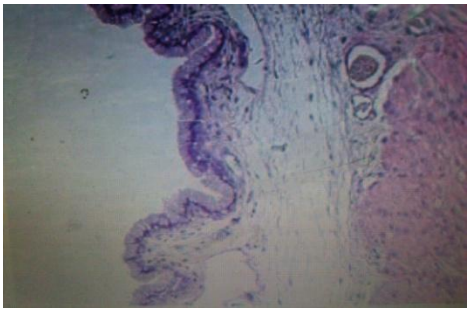
Figure- 3: Graphical representations of release profiles



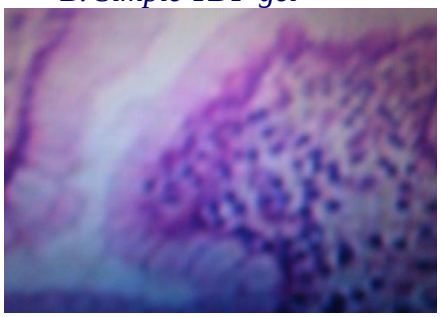
A. TDF solution



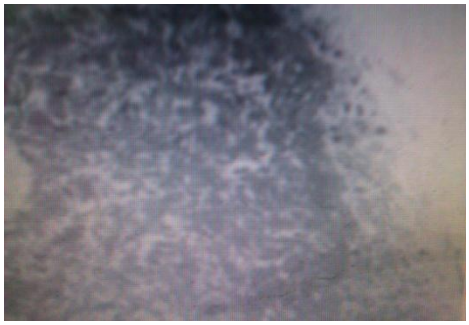
B. Simple TDF gel



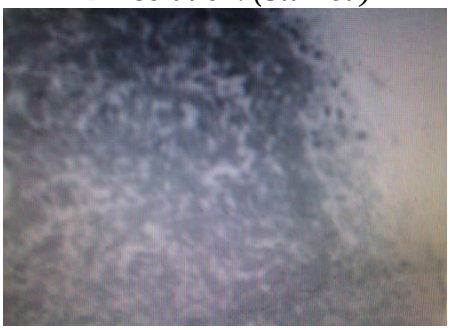
C. TDF Niosomal gel



D. Leukocyte infiltration seen after application of TDF solution. (Stained)



E . Leukocyte infiltration- TDF solution



F. Leukocyte infiltration- Simple TDF gel

G. Leukocyte infiltration – TDF niosomal gel

Figure -4: Representative rat vaginal sections following 6-day intravaginal administration of TDF solution, simple gel and niosomal gel of TDF respectively.

***In-vivo* study**

There were no mortalities in any of the groups during the 6-days application period. Clinical examination of the rats prior to each administration revealed no signs of vaginal or vulval irritation, discharge or bleeding from the vagina for all the rats.

Macroscopic examination of the vagina exposed to the simple gel, niosomal gel and TDF solution for 6 consecutive days revealed the presence of abnormal contents in 1-2 rats per group. The abnormal contents were considered likely to be retained gel.

The vagina of the rats treated with the niosomal gel, consisted of a single epithelial layer with many mucous cells, which is normal for the rat vagina. In contrast, all the rat vaginal tissues exposed to TDF solution and TDF gel showed marked to severe epithelial loss and atrophy accompanied by leukocyte infiltration as shown in (Figure 4).

The vaginal epithelial layer of the rats treated with niosomal gel appeared very thin without mucous cells. In the cervical tissues of rats treated with TDF solution and TDF gel, slight to marked epithelial loss, atrophy and leukocyte influx were seen.

The rats treated with TDF solution and plain gel are showing more irritation characteristics. The atrophy signs are more in the case of TDF solution; however it is moderate in plain drug gel. From the table 10.1, it is seen that the rats treated with niosomal gel are showing comparatively less irritation and atrophy characteristics. The leukocyte infiltration was at the least in the niosomal formulation as compared to the TDF solution and TDF gel.

From the result of *in-vivo* studies, the beneficial role of niosomal formulations over the plain drug formulations was indicated with regard to higher skin permeation and retention. Presenting these findings in more illustrative way, in terms of irritation and atrophy for the vaginal epithelium, the niosomal gel is less irritiative as compare to plain drug gel.

CONCLUSION

Niosome based gel (NBG) for the vaginal delivery of TDF using Carbopol 940NF was prepared and optimized using a three-factor, three-level Box-Behnken design. The quantitative effect of these factors at different levels on the maximum detachment force could be predicted by using polynomial equations. The thin film hydration technique using rotary flask evaporator shows good vesicle forming properties as well as better efficiency. TDF used in plain gel formation in strength of 1% showed higher release as compare to niosomal gel at the same time interval. The niosomal gel showed sustained release properties. Stability study of the optimized formulation proved the integrity of the developed gels. The niosomal gel applied on the rat vaginal epithelium showed no signs of atrophy whereas the plain drug gel showed it clearly. Thus, from this research work it can be concluded that the novel niosomal vaginal gel formulation is much better than the conventional oral dosage form and conventional vaginal gel as vaginal microbicide.

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