CHAPTER-3

EXPERIMENTAL
In this chapter, a detailed description of chemicals and reagents, apparatus/instruments and experimental procedure has been discussed.

3.1 Chemicals and Reagents

3.1.1 Water Water being one of the major solvent in the study which is also employed in calibration of instruments or apparatus was obtained by double distillation process. By volume, 1000 ml of pure water was collected from the double distillation unit (Harco & Co.) which was further subjected to distillation on acidified KMnO₄ over a 750 mm long fractionating column. Different fractions of distilled water were collected and their conductivity, κ (S cm⁻¹) and pH were determined. The sample of κ value ~ 1–3 x 10⁻⁶ S cm⁻¹ was collected for use. The pH of the sample collected in the range 6.75–6.95. Both of these parameters were measured at room temperature. The sample of purified water so obtained was not utilized after two days.

3.1.2 Solvents Absolute alcohols i.e. methanol, ethanol and 1–propanol were obtained from Merck Chemicals with purity ≥ 99.9 %. Other solvents in experimental and lab processes such as acetone, sulfuric acid and hydrochloric acid etc. for complete cleansing of glassware were also obtained from Merck Chemicals. Physico–chemical study of surfactants in presence of BHA/BHT was carried out in three different solvent compositions of alcohols i.e. 100, 70, 30% v/v methanol, ethanol and 1–propanol.

3.1.3 Pharmaceutical Ingredients Butylatedhydroxy anisole (BHA) and butylatedhydroxy toluene (BHT) were obtained from MERCK Chemicals and were used as received. Clotrimazole (CLZ) was obtained as gift sample from Glenmark Pharmaceuticals Pvt. Ltd. Carbopol 940 and triethanol amine were obtained from Himedia and were used as such for gel formulation.

3.1.4 Animals

In present study, male Sprague Dawley (SD) rats (160–180 g) were used. Animals were housed in plastic cages in a 12 h dark–light cycle, with controlled temperature (25 °C) and humidity (70%). Water and food were provided ad libitum throughout the study. The animals were housed in Central Animal Facility (CAF) of ASBASJSM college of Pharmacy, Bela, Ropar, India. All protocols were approved by Institutional Animal Ethics Committee (IAEC),
and experiments were performed in accordance with Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA).

3.1.5 Surfactants All the surfactants used in the study were of AR grade and purity > 99.0%. Anionic surfactant; sodium dodecyl sulfate (SDS) was obtained from Merck Chemicals, cationic surfactant; cetyltrimethylammonium bromide (CTAB) was obtained from Sigma, and nonionic surfactant; tert- octylphenol ethoxylated or Triton X–100 (TX100) containing 9 units ethylene oxide as the hydrophilic moiety was also obtained from Merck Chemicals.

3.2 Experimental Details (Apparatus and Methods)

3.2.1 pH Measurements

The pH of the purified water was tested using Cyber scan 2500 pH meter (Figure 3.1.). Also, the solutions prepared for investigation were monitored regularly while performing experiments using this apparatus.

![Figure 3.1: Cyber scan 2500 pH meter.](image)

3.2.2 Thermostat

A high precision water thermostat (supplied by Harco & Co.) fitted with a digital temperature controlled device was used for all experimental measurements. The temperature of thermostat was maintained within ± 0.1 °C over the entire temperature range studied. All precautions were taken to protect the heat loss due to convection, especially when the measurements were
carried out at higher temperatures. However, the temperature of bath was continuously monitored with the help of a calibrated thermometer.

3.2.3 Conductivity measurements

Conductivity measurements were carried out with digital conductivity meter Cyber scan CON–510 (Figure 3.2). It was calibrated at 25 °C by determining the limiting molar conductance, $A_0$ values of NaNO$_3$, AgNO$_3$, Bu$_4$NI, Bu$_4$NBr and NaBPh$_4$ in DMSO at 25 °C. $A_0$ values of these electrolytes were found to be 41.1, 44.0, 36.0 and 35.8 S cm$^2$ mol$^{-1}$, respectively which were in good agreement with those reported in literature [1]. The temperature of the solution was maintained ± 0.1 °C by circulating water from thermostat through a double walled vessel containing the solution. The circulation was done with the help of a high power digital water circulator supplied by Riviera Pvt. Ltd., Mumbai. The sample was allowed to attain the temperature of thermostat before taking the measurements.

![Figure 3.2: Cyber scan CON– 510 conductivity meter.](Image)

3.2.3.1 Determination of Critical Micelle Concentration (CMC)

The critical micelle concentration (CMC) was obtained at different temperatures (25–35) °C by measuring the specific conductance of SDS, CTAB and TX–100 at fixed concentrations of BHA and BHT i.e., (0.03 and 0.02) mol kg$^{-1}$, respectively. The choice of temperature was based on standard temperature, i.e. 25 °C, and relevance to body temperature, which remains at about 35 °C. The concentration of the solution was varied by adding aliquots of
concentrated stock solution of surfactants to the known volume of solution in the double walled vessel by means of a 10–100 μL eppendorf micropipette. The experiments were repeated twice with two different stock solutions of surfactants. The specific conductivity data were plotted against molar concentration of surfactants and CMC was estimated from the intersection point by drawing tangents in the resultant graph [2, 3]. The reproducibility of individual points was reasonably good and the standard deviation of the mean in the CMC was found to be $\pm 2\%$. This deviation was calculated from the CMC data obtained from two different runs.

3.2.3.2 Thermodynamics

The CMC values were thereafter utilized to evaluate and calculate the thermodynamic parameters. The $d \ln (X_{\text{cmc}})/dT$ was accounted as the slope of the straight line obtained by plotting $\ln X_{\text{cmc}}$ against temperature, $\alpha$ (degree of counter ion dissociation) was calculated from the relation, $\alpha = S_2/S_1$, where, $S_1$ and $S_2$ are the slopes in the pre micellar and post micellar regions. The relations [4, 5] used for standard enthalpy change, standard entropy ($\Delta S_m^\circ$) and Gibbs free energy change ($\Delta G_m^\circ$) for micellization are as follows.

$$\Delta H_m^\circ = -RT^2(2-\alpha)[d(\ln X_{\text{CMC}})/dT]$$  \hspace{1cm} (Eq. 3.1)

$$\Delta S_m^\circ = \frac{\Delta H_m^\circ - \Delta G_m^\circ}{T}$$  \hspace{1cm} (Eq. 3.2)

$$\Delta G_m^\circ = (2-\alpha)RT \ln(X_{\text{CMC}})$$  \hspace{1cm} (Eq. 3.3)

3.2.4 Density and ultrasonic sound velocity Measurements

Density ($\rho$) and ultrasonic sound velocity ($u$) measurements were performed on a high-precision digital Density and Sound Analyzer–5000 (DSA–5000) as shown in Figure 3.3, at different temperatures (25–35) °C. It was supplied by Anton Paar Gmbh, Graz, Austria. The instrument was calibrated periodically with distilled water over a temperature range (20–50 °C). The precision in the density data was found to be better than $\pm 2 \times 10^{-6}$ g·cm$^{-3}$ and that of velocity data, it was better than $\pm 0.20$ m s$^{-1}$. The precision in temperature of the DSA–5000 is $\pm 0.001$ °C.
Figure 3.3: Density and sound analyzer (DSA–5000).

3.2.4.1 Volumetric and compressibility parameters

The obtained data from density ($\rho$) and ultrasonic sound velocity ($u$) was utilized to calculate the apparent molar volume ($\phi_v$) and apparent molar adiabatic compression ($\phi_c$) values. These parameters were calculated using following relation [6, 7].

$$\phi_v = \frac{M}{\rho} + \left[\frac{\rho_o - \rho}{m \rho \rho_o}\right]$$  \hspace{1cm} (Eq. 3.4)

$$\phi_c = \phi_v \kappa_s + \left[\frac{\kappa_s - \kappa_o}{m \rho_o}\right]$$  \hspace{1cm} (Eq. 3.5)

where $m$ (mol kg$^{-1}$) is the molality of the solution calculated from the molar concentration data using $m = 1/[d/(C - M/1000)]$, here $m$ (mol kg$^{-1}$) stands for molal concentration and $M$ (g mol$^{-1}$) for relative molar mass of surfactant, $\rho$ (kg m$^{-3}$) is the density of the solution, $\rho_o$ (kg m$^{-3}$) is the density of the solvent system. $\kappa_s$ (TPa$^{-1}$) stands for isentropic compressibility of the solution and $\kappa_s$ was determined by using relation as $\kappa_s = 1/\rho u^2$. [6]

3.2.5 Viscosity Measurements

The viscosity ($\eta$) measurements [8] for various alcoholic/ hydroalcoholic solutions were carried out in a calibrated jacketed ubbelohde viscometer using calibrated stopwatch. The
viscosity ($\eta$) measurements for surfactants in presence of BHA and BHT at fixed concentration were determined at three temperatures (25–35) °C with an interval of 5 °C and accounted for 100%, 70% and 30% (v/v) alcohol (methanol, ethanol and 1-propanol) compositions with water. The ubbelohde viscometer was periodically cleaned by treating with chonic acid and distilled water and finally washed with alcohol and dried in oven for ~ 2 hrs. After drying, the ubbelohde viscometer was filled with fixed volume of the test solution. The approximate flow time of water was 460 sec at 25 °C. The viscometer was always placed vertically in a water thermostat having a digital temperature controller of accuracy ± 0.05 °C. The samples were kept imperturbable within viscometer for about 10 minutes before every measurement just to settle time dependent effect. The average deviation for three measurements of a single concentration of the solution did not exceed ± 0.03 sec. The precision achieved in viscosity measurement was well within ± 0.01%.

However, the viscometer was calibrated with DMSO and MeOH (both of A.R. Grade) at 298.15 K using viscosity coefficient, $n_r = 0.008903$ poise and density, $d = 0.99707$ g cm$^{-3}$ for water. The viscosity coefficients of DMSO and MeOH were found to be 0.02 and 0.0055 poise, respectively which were found in good agreement with the literature values [9]. The precision achieved in viscosity measurements in flow time was estimated to be better than ± 3 %. The entire work was concerned solely with relative viscosities which were determined by using the equation [10] (3.6).

$$\eta_r = \frac{\eta}{\eta_o} = \frac{(t \times d)}{(t_o \times d_o)} \quad \text{(Eq. 3.6)}$$

where, $t_o$, $d_o$ and $n_o$ refers to the flow time, density and viscosity of solvents and $t$, $d$ of solution, respectively.

3.3 Pre-formulation Drug analysis

3.3.1 Determination of melting point

Capillary fusion method was used to determine the melting point of clotrimazole using Remi’s melting point apparatus. The melting point was determined and compared with the literature value [11].
3.3.2 Determination of absorption maxima

A solution of clotrimazole (10μg/ml) in methanol was scanned between 200–400 nm, using Shimadzu 1700 spectrophotometer. The scanned λ_{max} was in good agreement with literature value [11].

3.3.3 Determination of solubility

Solubility studies of drug sample were carried out in pure methanol as well as optimized ratio of phosphate buffer (pH 7.4) and methanol i.e. 6:4. An excess amount of drug was added to screw capped vials containing 10 ml of solution, in each. The vials were kept in water bath shaker at 25 °C and shaken for 24 hours until the equilibrium was attained. The saturated solution then filtered through whatmann filter paper and was analyzed on UV Spectrophotometer at λ_{max} of each solvent.

3.4 Drug – excipient compatibility studies

While preparing a formulation for the development of final dosage form, it is mandatory to confirm the compatibility between the drug and polymer to be utilized and to ensure that the drug is not interacting with polymer. FTIR technique was been used to determine the interaction of drug with excipients.

3.4.1 Fourier Transform Infra Red spectral analysis

The Fourier Transform Infra – Red (FTIR) analysis of the drug and polymer were carried out for qualitative compound identification using Perkin Elmer 1600. The pellets were prepared on KBr – press (Spectra Lab., India). The spectra were scanned over wave number range of 4000 cm^{-1} – 400 cm^{-1}. Since FTIR is related to covalent bonds or hydrogen bonds, the spectra provide detailed information about the structural arrangements of molecular compounds. FTIR confirm the functional identity of the drug and to detect the interaction of the drug with excipients [12].

3.5 Preparation of standard plots

Standard plots of clotrimazole were prepared in:

1. Methanol
2. Methanol: Phosphate buffer (pH 7.4)
3.5.1 Standard plot of clotrimazole in methanol and methanol: phosphate buffer (pH 7.4)

50 mg of clotrimazole was dissolved in small volume of methanol in 100 ml volumetric flask and volume was made up to 100 ml with methanol to get a concentration of 500 µg/ml. From this stock solution, aliquots were withdrawn into a series of 10 ml volumetric flask and volume was made with methanol to get a concentration ranging from 10–500 µg/ml. Due to precipitation and hydrophobic nature of drug, the optimized concentration of buffer and methanol was defined in order to prepare standard calibration curve. Same amount of clotrimazole was dissolved in optimized methanol : phosphate buffer pH 7.4 (4 : 6) system in 100 ml volumetric flask and volume was made up to 100 ml with methanol: phosphate buffer 7.4 (4 : 6) to get a concentration of 500 µg/ml. Similarly, from this stock solution, aliquots were withdrawn into a series of 10 ml volumetric flask and volume was made with methanol to get a concentration ranging from 10–500 µg/ml. The absorbance of the resulting solutions was then measured at 261 nm using UV spectrophotometer against parent solvent as reference [13].

3.6 Formulation of gel

3.6.1 Preparation of micellar solution with SDS, CTAB and TX100

From our previous thermo–physical analysis studies, the critical micelle concentration (CMC) with most feasible and thermodynamic stable concentration was taken into consideration. Among them, 30% v/v EtOH was found to be the most feasible and thermodynamically controlled system which was lately utilized in the present formulation studies. It was found and reported that the presence of additives facilitated the micellization process, resulting early micelle formation. In context of this, three optimized surfactant’s (SDS, CTAB and TX100) concentrations near/ above CMCs were selected, respectively.

Likely, for SDS (6.0, 7.0 and 8.0 mmol kg\(^{-1}\)), CTAB (0.8, 0.9 and 1.0 mmol kg\(^{-1}\)) and TX100 (0.20, 0.22 and 0.24 mmol kg\(^{-1}\)) were the selected concentrations of surfactants. Optimized concentration was utilized for BHA and BHT with respect to obtained CMC values of surfactant in 30% v/v EtOH. All the calculate concentrations based on thermo–physical analysis are presented in Table 3.2. Accordingly, BHA (5 mg) and BHT (4 mg) were added to surfactant hydroethanolic solutions (30% v/v EtOH). The mixture was stirred at room
temperature at 700 rpm for 24 h. Afterward the mixture was centrifuged at 10000 rpm at 25 °C for 15 min and then upper solution was filtered through a nylon syringe filter (pore size 0.2 μm, Whatman Inc., USA). The prepared micellar solution was dispersed into the gel. The list of instruments used in present study has been provided in Table 3.1.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Equipments</th>
<th>Manufacturer/Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>U.V. Visible Equipments</td>
<td>Nanodrop, Schimadzu UV–1700</td>
</tr>
<tr>
<td>2</td>
<td>Diffusion cell apparatus</td>
<td>Orchid scientific &amp; innovative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>India Pvt. Ltd.</td>
</tr>
<tr>
<td>3</td>
<td>Humidity control chamber</td>
<td>Narang scientific works NSW–175</td>
</tr>
<tr>
<td>4</td>
<td>Electronic weighing balance</td>
<td>Shimadzu A x 200</td>
</tr>
<tr>
<td>5</td>
<td>Hot air oven</td>
<td>Narang scientific works NSW–143</td>
</tr>
<tr>
<td>6</td>
<td>Magnetic stirrer</td>
<td>Harco &amp; Co.</td>
</tr>
<tr>
<td>7</td>
<td>Melting point apparatus</td>
<td>Remi’s Equipment Pvt. Ltd.</td>
</tr>
</tbody>
</table>

3.6.2 Preparation of clotrimazole gel

The gel base was prepared by dispersing the polymer (carbopol 940) in distilled water. Carbopol 940 was chosen due to its hydrophilic nature and bioadhesive property, which may result in an increased residence time of a drug at the site of absorption by interacting with the topical membrane. The polymer was weighed accordingly for each formulation and then soaked in distilled water for 2 h prior to use. Afterward, it was dispersed in distilled water under magnetic stirring for 1 h so as to obtain a homogenous gel base of 1% w/w. Thereafter, SDS/CTAB/TX100 immobilized BHA, BHT and BHA + BHT were added to the gel base. Triethanolamine (TEA), pH = 7.0 was added drop-wise to obtain neutralized carbopol gels and were further subjected to constant stirring. The concentration of employed ingredients has been presented in Table 3.2.

3.7 Evaluation of gel

3.7.1 Homogeneity and grittiness

All the gel formulations were tested for homogeneity by visual inspection after the gels have been stabilized in the container. They were tested for their appearance and presence of any aggregates. All the formulations were evaluated microscopically for the presence of particles and no appreciable particulate matter was seen under light microscope. Hence the gel preparation fulfils the requirement as desired for any topical preparation.
3.7.2 pH measurement

Further, the pH of gel formulations was determined. The measurement of pH of each formulation was done in triplicate and average values were calculated.

**Table 3.2:** Formulations containing different concentration additives and excipients.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Codes</th>
<th>CLZ (%)</th>
<th>BHA (mg)</th>
<th>BHT (mg)</th>
<th>SDS (mg)</th>
<th>CTAB (mg)</th>
<th>TritonX–100 (mg)</th>
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<tr>
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<td>–</td>
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<tr>
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<td>4</td>
<td>17</td>
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<td>–</td>
</tr>
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<td>–</td>
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<td>–</td>
<td>–</td>
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</tbody>
</table>

CLZ = Clotrimazole; CA= CLZ+BHA; CT= CLZ+BHT; CAT= CLZ+BHA+BHT; S= SDS; C= CTAB; X= Triton X–100. In all the formulations ethanol absolute, triethanolamine and water were used as q.s.
3.7.3 Drug content

The drug content uniformity was determined for all the formulations by UV spectrophotometric method. A 500 mg of clotrimazole gel was taken and dissolved in 50 ml of methanol. The volumetric flask were kept for 2 hours and shaken well in a shaker to mix it properly and then filtered. The drug content was measured spectrophotometrically at 261 nm.

3.7.4 Viscosity study

The measurement of viscosity of the prepared gel was done with a Brookfield Viscometer. The gels were rotated at 20 rpm using spindle no. 64 and the corresponding dial reading was noted.

3.7.5 Spreadability

One of the criteria for a gel to meet the ideal quantities is that it should possess good spreadability. It is the term expressed to denote the extent of area to which gel readily spreads on application to skin. The therapeutic efficacy of a formulation also depends upon its spreading value. Spreadability is expressed in terms of time in seconds taken by two slides to slip off from gel which is placed in between the slides under the direction of certain load, lesser the time taken for separation of two slides, better the spreadability.

It is calculated by using the formula: \( S = \frac{M \times L}{T} \)  

(Eq. 3.7)

where, \( M \) = weight tied to upper slide

\( L \) = length of glass slides

\( T \) = time taken to separate the slides

3.7.6 In vitro release study

The diffusion studies of the prepared gels were carried out using Franz diffusion cell with the diameter of 0.50 cm. For studying the dissolution release profile of formulated gels, a cellulose membrane (pores size 0.22μm) was used which was hydrated in phosphate buffer pH 7.4 prior to use for 24 h before placing them between donor and receptor compartments. The receptor compartment contained 18 ml phosphate buffer at pH 7.4, under magnetic stirring. The temperature of the Franz diffusion cells was maintained at 37 ± 0.5 °C. Gel sample (0.5 g) was taken in cellophane membrane and the diffusion studies were carried out at
37 ± 1 °C. Samples were withdrawn periodically at 0.5, 1, 2, 3, 4, 5, and 6 h and then each sample was replaced with equal volume of fresh dissolution medium to maintain the sink conditions. Then the samples were analyzed spectrophotometrically by using phosphate buffer pH 7.4 as reference. The amount of drug permeated into the receptor solution was determined by removing 1 ml of sample hourly for 6 h. The withdrawn volume was replaced with an equal volume of buffer solution. The absorbance was measured at 261 nm. The result of in vitro permeation study can be represented by plotting graphs between:

i. cumulative percent permeated versus time
ii. cumulative percent permeated versus √time
iii. log cumulative percent permeated versus time
iv. log cumulative percent permeated versus log time

3.7.7 Mathematical modeling of release profile

Mathematical modeling [14], whose development requires the comprehension of all the phenomena affecting drug release kinetics, has a very important value in the process of optimization of such formulation. The use of mathematical modeling turns out to be very useful as this approach enables, the prediction of release kinetics before the release systems are realized. More often, it allows the measurement of some important physical parameters, such as the drug diffusion coefficient and resorting to model fitting on experimental release data.

The data from the in vitro study was fitted to the following kinetic models to determine the kinetics of drug release. The suitability of equation is judged on the basis of best fit to the equation using statistical indicators like $R^2$ [15].

3.7.7.1 Zero–order model

Drug dissolution from dosage forms that do not disaggregate and release the drug slowly can be represented by the equation [16]:

$$Q_t = Q_0 + K_0 t \quad \text{(Eq. 3.8)}$$

Where, $Q_t$ is the amount of drug dissolved in time t, $Q_0$ is the initial amount of drug in the solution (most times, $Q_0 = 0$), and $K_0$ is the zero order release constant expressed in units of concentration/time.
To study the release kinetics, data obtained from *in vitro* drug release studies were plotted as cumulative amount of drug released versus time. This relationship can be used to describe the drug dissolution of several types of modified release pharmaceutical dosage forms, as in the case of some transdermal systems, as well as matrix tablets with low soluble drugs in coated forms, osmotic systems, etc [17].

### 3.7.7.2 First order model

This model has also been used to describe absorption and/or elimination of some drugs, although it is difficult to conceptualize this mechanism on a theoretical basis. The release of the drug which followed first order kinetics can be expressed by the equation:

\[
\log C = \log C_0 - \frac{Kt}{2.303}
\]  

(Eq. 3.9)

Where, \(C_0\) is the initial concentration of drug, \(k\) is the first order rate constant, and \(t\) is the time.

The data obtained are plotted as log cumulative percentage of drug remaining vs. time which would yield a straight line with a slope of \(K/2.303\). This relationship can be used to describe the drug dissolution in pharmaceutical dosage forms such as those containing water-soluble drugs in porous matrices [18].

### 3.7.7.3 Higuchi model

The first example of a mathematical model aimed to describe drug release from a matrix system was proposed by Higuchi. Initially conceived for planer systems, it was then extend to different geometrics and porous systems.

This model is based on the hypothesis that

i. Initial drug concentration in the matrix is much higher than drug solubility

ii. Drug diffusion takes place only in one dimension (edge effect must be negligible)

iii. Drug particles are much smaller than system thickness

iv. Matrix swelling and dissolution are negligible

v. Drug diffusivity is constant

vi. Perfect sink conditions are always attained in the release environment.

Accordingly, model expression is given by the equation:
\[ F_t = Q = A \left( D (2C - C_s) C_s t \right)^{1/2} \quad \text{(Eq. 3.10)} \]

Where, \( Q \) is the amount of drug released in time \( t \) per unit area \( A \), \( C \) is the drug initial concentration, \( C_s \) is the drug solubility in the matrix media, and \( D \) is the diffusivity of the drug molecules (diffusion coefficient) in the matrix substance.

In a general way it is possible to simplify the Higuchi model as (generally known as the simplified Higuchi model):

\[ F_t = Q = K_H t^{1/2} \quad \text{(Eq. 3.11)} \]

where, \( K_H \) is the Higuchi dissolution constant. The data obtained were plotted as cumulative percentage drug release versus square root of time. This relationship can be used to describe the drug dissolution from several types of modified release pharmaceutical dosage forms, as in the case of some transdermal systems and matrix tablets with water soluble drugs [19].

### 3.7.7.4 Korsmeyer–Peppas model

Korsmeyer et al. (1983) [20] developed a simple, semi-empirical model, relating exponentially the drug release to the elapsed time (t). To find out the mechanism of drug release, first 60% drug release data were fitted in Korsmeyer Peppas model.

\[ \frac{M_t}{M_\infty} = K t^n \quad \text{(Eq. 3.12)} \]

Where, \( \frac{M_t}{M_\infty} \) is a fraction of drug released at time \( t \), \( K \) is the release rate constant, and \( n \) is used to characterize different release for cylindrical shaped matrices.

In this model, the value of \( n \) characterizes the release mechanism of drug as described in Table 3.3. For the case of cylindrical tablets, \( 0.5 \leq n \leq 1.0 \) corresponds to a Fickian diffusion mechanism, \( 0.5 < n < 1.0 \) to non-Fickian transport, \( n = 1.0 \) to Case II (relaxational) transport, and \( n > 1.0 \) to super case II transport. To find out the exponent of \( n \) the portion of the release curve, where \( \frac{M_t}{M_\infty} < 0.6 \) should only be used. To study the release kinetics, data obtained from in vitro drug release studies were plotted as log cumulative percentage drug release.
Table 3.3: Interpretation of diffusion release mechanism from polymeric films.

<table>
<thead>
<tr>
<th>Release exponent (n)</th>
<th>Drug release mechanism</th>
<th>Rate as a function time</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>Fickian diffusion</td>
<td>$t^{-0.5}$</td>
</tr>
<tr>
<td>0.5 &lt; n &gt; 1.0</td>
<td>Anomalous transport</td>
<td>$t^{0.1}$</td>
</tr>
<tr>
<td>1.0</td>
<td>Case II transport</td>
<td>Zero order release</td>
</tr>
<tr>
<td>Higher than 1.0</td>
<td>Super case II transport</td>
<td>$t^{0.1}$</td>
</tr>
</tbody>
</table>

3.8 In vitro antifungal activity against Candida isolates

3.8.1 Fungal strains

Fungal strains; Candida clinical isolates, used in the present study were collected from Department of Medical Microbiology, Postgraduate Institute of Medical Education and Research, Chandigarh, India and Indra Gandhi Medical College, Shimla, Himachal Pradesh, India. Among obtained 30 clinical isolates, 11 were fluconazole (FLZ) resistant C. albicans, 4 miconazole (MLZ) resistant C. albicans, 3 FLZ susceptible C. albicans, 8 FLZ susceptible C. tropicalis, and 4 FLZ susceptible C. glabrata clinical isolates.

3.8.2 In vitro antifungal activity

Standardized protocol M27–A2, CLSI (Clinical and Laboratory Standards Institute) was followed to perform the experiment [21]. The inocula were performed after growth (48 h/35 °C) on Sabouraud dextrose agar. The colonies were suspended in 0.85% sterile saline and this suspension was homogenized in a vortex mixer for 15 s; after that, cell density was set in a spectrophotometer and transmittance ($\lambda = 630$ nm) was adjusted to match standard 0.5 on the McFarland scale ($1 \times 10^6$ to $5 \times 10^6$ cells/ml). In the sequence, a 1:50 dilution in water was done, followed by a 1:20 dilution in RPMI 1640 medium, resulting in a final concentration of $1.5 \pm 1.0 \times 10^3$ cells/ml [22]. The micro–dilution technique [23] was performed in polystyrene sterile plates with flat–bottom, disposable, with 96 wells diluted in RPMI 1640 buffered broth. 100 µl of the standardized inoculum was added to each micro–dilution plate. The plates were incubated at 35 °C for 48 hrs and then 10 ml of 0.5% 2,3,5–triphenyltetrazolium chloride dye was added to all wells, and the plates were re–incubated at 35 °C for 20 min. Afterward, the minimum inhibitory concentration (MIC µg/ml) was determined.

To assess the interaction of drug combinations (BHA+BHT and CLZ) the obtained data was further analyzed using the Fractional Inhibitory Concentration Index (FICI), which is
based on the zero–interaction theory of Loewe additivity. The drug interactions were classified as synergic (FICI ≤ 0.5), additive (FICI 0.5 – 1.0), showing no interaction between FICI – >0.5 – 4, and antagonistic (FICI > 4) [24].

3.9 Morphological study

The surface morphology was characterized by scanning electron microscope (S–3400N, Hitachi, Japan). The gel sample was deposited on a glass cover slip previously adhered to a metallic stub by a bio–adhesive carbon tape. Afterward, the sample was air–dried before analysis and coated with gold to obtain a conducting surface. Finally, the sample was analyzed by scanning electron microscopy in vacuum. Transmission electron microscope (Hitachi H–7500 80 kV; Ibaraki, Japan) was further used to visualize the dispersed micellar structures within the gel.

The visualization of morphology of C. albicans was carried out by TEM [25, 26]. Initially untreated C. albicans was visualized and afterward, it was treated with the optimized best formulation at a concentration of 10 μg/ml. The images were taken at different interval of time likely; 15 min, 2 h and 6 h in order to gain information on the action of formulation on infection site.

3.10 Physical and photo–stability studies

The formulation was stored for 3 months after preparation in order to evaluate changes in drug content, pH, viscosity, and spreadability. Photo–stability study for CLZ was performed to quantify the drug after exposure to UV light for 14 h. Quartz cuvettes were filled with CLZ methanolic solution (C–1) and CAT–3S (CLZ with dispersed BHA + BHT micelles) and exposed to UV radiation (TUV lamp – 30 W) in a chamber at a fixed distance from each other for 14 h. In order to compare, the CLZ methanolic solution was covered with aluminium paper as dark control. The protection of CLZ against the UV radiations was evaluated by quantifying the remaining drug using HPLC.

3.11 Skin Irritation study and toxicity profiling

Healthy male Sprague Dawley (SD) rats with average weight of 160–180 g were selected. Initially, hair were removed from the dorsal side of rats (2 cm × 3 cm) with the help of electric clipper without damaging the skin. The control group was treated with normal saline and gel
was applied to the treatment group three times a day for three days consecutively \((n = 3)\). The visual observations were carried out at regular intervals of 12, 24, 48, and 72 h for various symptoms such as erythema, flakiness, dryness, erythema or edema. The irritation scores of the test area were obtained by judging the extent of erythema and edema according to the literature [27]. Erythema and edema were graded as follows: 0 for no visible reaction, 1 for just present reaction (barely perceptible—light pink), 2 for slight reaction (light red), 3 for moderate reaction (moderate red), and 4 for severe reaction (extreme redness).

\textit{In vivo} toxicity test was conducted to gain better perspective or perception. Twelve animals were randomly divided into two groups. The first group (untreated) served as control, whilst second group received the treatment (CAT–3S). The treatments were given via topical application. After 24 h of treatment, the remnants of the gels were gently washed away from the skin surface using adsorbent cotton dipped in physiological saline. Again the dorsal sites of treated animals were inspected for any erythema or edema. Thereafter, animals were sacrificed. Major organs likely, skin, liver, kidney, and intestine were taken out. The excised skin sample and isolated organs were presented in 10% formalin for histopathological examination. Sections were fixed and blocks were made following the procedure as reported in literature [28]. The sections were stained with eosin–hematoxylin (H and E) to determine gross histopathology.

3.12 Confocal laser scanning microscopy (CLSM)

The depth and mechanism of the skin permeation of Rhodamine B within prepared gel system was investigated in absence of drug using CLSM [29]. The formulation was applied to the dorsal skin of rats for 8 h. The rat was then sacrificed by heart puncture and dorsal area was excised and cleaned with a thin stream of water to remove any residual gel. Afterward, the skin was placed on aluminium foil and adhering fat was removed. The excised skin was sliced and examined with CLSM (FV fluoview 1000, Olympus, Tokyo, Japan).

3.13 \textit{In vivo} antifungal study

3.13.1 Preparation of Inoculum

Clinical isolate of \textit{Candida albicans} (PGI/DML54) was used to infect the animals. Cultures were revived from glycerol stock onto sabouraud dextrose agar (SDA) slants for 48 h at 35 °C before use. Cells were suspended by vortexing a single pure colony in pyrogen free normal
saline and subsequently diluted to a final concentration of $2 \times 10^6$ cells/ml. The colonies were pure as identified from the morphology, and none of the cell suspensions were contaminated with any other organism.

3.13.2 Cutaneous infection

Each animal’s back was shaved with an electric clipper and an approximately $3.0 \text{ cm}^2$ area was marked on each animal’s back. The marked area was infected with $10^7$ cfu/ml suspensions by gently rubbing onto the skin for 3 days with the help of a sterile, cotton–tipped swab until no more visible fluid was observed [30]. Infection was produced under an occlusive dressing and the infected area was covered with a sterile adhesive bandage, held in place with extra–adherent tape for 48 h before treatment began [31]. Control animals were infected in the same manner; however, they did not receive any formulation treatment.

3.13.3 In vivo efficacy

*In vivo* antifungal activity for the most potent formulation was carried out by using male SD rats (160–180 g). All animals were rendered immunosuppressed by injecting cyclophosphamide (150 mg/kg) intraperitoneally 4 days and 1 day before experimental infection. Treatment began 24 h after the infection was induced and test formulation was topically applied once daily for 3 consecutive days. The experimental animals were divided into four groups each containing 6 animals and the test animals were treated topically. Group 1 was treated with plain drug (1 mg/cm$^2$), group 2 with formulation CAT–3S at dose level of 1 mg/cm$^2$; group 3 with formulation CAT–3S at dose level of 5 mg/cm$^2$; and group 4 served as the control. All animals were sacrificed 48 h following the last treatment and 3.0 cm$^2$ of skin from the infected sites was excised. The infected skin samples were collected, washed and then plated into SDA culture media and incubated for 48 h at 37 ± 1 °C, and then viable CFUs were counted [32].

3.14 Statistics

The antifungal efficacy against *C. albicans* was analyzed with two–way ANOVA and followed by a Bonferroni test using graph pad prism software.
References


