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CLOTRIMAZOLE ADORNED BETA-CYCLODEXTRIN BASED NANOSPONGES: FABRICATION AND *IN-VITRO* CHARACTERIZATION

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Abstract
This research was framed to formulate clotrimazole-loaded beta cyclodextrin-based nanosponges that seemed to be effective for treating fungal infections. Beta-cyclodextrin (rate-retarding polymer), polyvinyl alcohol (surfactant) and dimethylsulfoxide (aprotic solvent) were utilized in different concentrations, to prepare clotrimazole loaded nanosponges through emulsion solvent diffusion technology. Drug loaded nanosponges were evaluated for their physico-chemical parameters. Fourier Transform Infrared spectroscopy and scanning electron microscopy were used for structural analysis. Spherical, spongy, porous and nanosized three-dimensional structures of prepared nanosponges were obtained by SEM. Each formulation's particle size was in nanoscale range.

The percentage yield ranged from 77% to 87%. The entrapment efficiency and drug loading were in the range of 80 - 90% and 83 - 85%, respectively, for each formulation. The development of inclusion complexes with porous and spherical morphology was verified by FTIR without any chemical interaction between drug and polymer. These findings reinforced that developed nanosponge formulation serves as an effective nanocarrier, enhancing and regulating the delivery of clotrimazole.

Keywords: Nanosponges, clotrimazole, beta cyclodextrin-based nanosponges, anti-fungal, physicochemical analysis.

1. Introduction

Nano-pharmaceutics focuses on the concepts of nanotechnology that are applied to medications. This technology enables to make the size of drug particles at nano-level and hence can therefore change the pharmacokinetic properties and bioavailability of drugs. The formulation, development, and delivery features of nano-pharmaceuticals are all addressed by nanotechnology. Nano-pharmaceuticals hold great potential for upgrading the old, inadequate therapeutic system and introducing targeted drug delivery systems, providing better patient compliance and minimum toxic effects (1).

Traditional therapies have ended in failure because they do not deliver the site-specific targeting of drug. Using nano-pharmaceuticals for such specific targeting will eliminate undesirable systemic side effects and improving patient care compliance (2). Due to their unique physico-chemical, biological, and chemical features, chemical content, and distinctive size, they can interact with biological molecules and cells (3).

Nanotechnology can create biocompatible and biodegradable therapeutic nano-carriers with minimal pharmacological adverse effects and maximum therapeutic efficacy (4). This technique provides novel properties at nanoscale level in nano-sized items such as dendrimers, micelles, organic, inorganic, metallic, lipid, and polymeric nanostructures (liposomes, nano particles, nano capsules, nano spheres, nano suspensions, and nanosponges) (5,6).

Targeted drug delivery is now a major issue in drug delivery systems. Target oriented drug delivery system (also referred as"smart drug delivery system") minimize toxic effect by restricting the access of drug to normal or non-target cells and maximize the therapeutic index of drug by selective and effective release of drug at preselected target site in predictable and controllable manner (7).

Nanosponges is an emerging and innovative technology for targeted drug delivery system, which is the major problem with conventional dosage form. It is a polymeric drug delivery technique that allows predictable and controlled drug release. They have spherical, porous, and spongy structure (8). Nanosponges are self-sterilizing products because bacteria cannot penetrate their nanoscale porous structure. These are highly porous structures with three times more drug entrapment capacity due to their porous structure (9). Nanosponges are useful for a number of purposes including, masking the taste of drugs, enhancing the solubility of insoluble drugs, increasing their bioavailability, improving drug stability, lessening adverse effects, and delivering drugs to particular drug delivery sites. Due to the creation of inclusion and non-inclusion complexes, they can transport both hydrophilic and lipophilic medicinal molecules owning to their outer hydrophilic branching and inner lipophilic chambers (10). The developed NS formulations include parenteral, topical, and oral applications (11). Nanosponges can be blended with various excipients, diluents, colorants, flavorants, binders, and lubricants for tablet formation. Hydrogel is made by dispersing nanosponges in it and using it as a topical formulation vehicle (12). To date, almost four generations of nanosponges have been introduced by the researchers. The first-generation nanosponges were synthesized by using starch derivative polymer that is cyclodextrin along with organic solvent ether and carbonates as prominent cross linkers (13). In second generation, few chemical properties were engineered to enhance the potential of their polymeric structure, there were group of radicals. The third-generation manufacturing had the ability to undergo modification according to the variation in pH, temperature and redox environment. The latest nanosponges also called fourth generation nanosponges are highly selective in action of binding with molecules due the presence of specific functional groups responsible to make bonds with specific molecules and increase the uptake of drug (14).

By altering the cross linker to polymer ratio, different particular sizes of nanosponges can be produced. Nanosponges are stable at pH values between 1 and 11 and up to 300°C. They have multiple loading mechanisms, high entrapment efficiency, diverse synthetic materials, predictable release pattern, extended-release pattern, and have a biodegradable nature (15).

Fungal infections are a leading cause of morbidity and mortality in immunocompromised individuals. Dermatophyte infections are brought on by mycosis and affect human skin. Fungal infections might be superficial or invasive. Invasive fungal infections are especially common in immunosuppressed individuals. Systemic fungal infections include inhaled fungal infections and opportunistic infections (16). *Histoplasma capsulatum*, *Candida*, *Aspergillus*, *Cryptococcus* species, and *mucoromycetes* are

examples of pathogenic fungi. Mucosal infections are brought on by *Candida albicans*. *Aspergillus fumigates* are responsible for allergic fungal infections. Treatment of fungal infections is a serious problem nowadays. Poor therapeutic efficacy of anti-fungal drugs are caused by limited bioavailability, toxicity, and resistance (17).

Clotrimazole is BCS class II drug with strong antifungal effects and is available in several different formulations, including creams, tablets and gels. Its solubility and release profile can be improved by forming complex with beta-cyclodextrin (18). It is used to treat trichomoniasis that is resistant to metronidazole. For the topical treatment of oropharyngeal and vulvovaginal candidiasis, it is frequently used (19). Taking altogether, the current study was designed to develop and optimize clotrimazole loaded β-cyclodextrin nanosponges formulation for enhanced topical bioavailability.

2. Material and Methods

2.1 Materials

The following materials were used with the best possible grades available, supplied by the manufacturer. Clotrimazole and polyvinyl alcohol (PVA) were obtained as kind gift sample from Punjab University College of Pharmacy, University of the Punjab, Lahore, Pakistan. Dimethyl sulfoxide (DMSO), dichloromethane (DCM) and beta-cyclodextrin were procured from Punjab University College of Pharmacy, University of the Punjab, Lahore, Pakistan. All the materials used were of analytical grades.

2.2. Methods

2.2.1. Calibration Curve of Clotrimazole

To construct the calibration curve, dilutions of clotrimazole were prepared in DMSO. Using UV spectrophotometer, by measuring and plotting absorbance of known concentration from 10 to 100ug/ml the calibration curve was prepared at λ_{max} of 263 nm, and a curve between concentration and absorbance was plotted. The average of triplicate was taken.

2.2.2. Development of β-cyclodextrin based Clotrimazole Loaded Nanosponges

Clotrimazole-loaded beta-cyclodextrin-based nanosponges were manufactured by using emulsion solvent evaporation technology. Beta-cyclodextrin (100-400 mg) and clotrimazole (100 mg) were dissolved in 10 mL of DMSO to prepare the organic phase. For the production of the aqueous phase, water was heated at 60°C with continuous stirring to dissolve PVA (0.3% w/v-3% w/v) in 100 mL of deionized water. The organic phase was gradually emulsified into the aqueous phase dropwise. The dispersion was then stirred for 24 hours on a thermostatically controlled magnetic stirrer at constant stirring of 1000 rpm at room temperature. To remove the adsorbed PVA, clotrimazole was rinsed three times with ultrapure water. After the organic solvent had completely been evaporated, ultracentrifugation at 17,000 rpm and 4°C for 30 minutes was used to collect nanosponges (20).

Table 1. Composition of different formulations of clotrimazole loaded beta-cyclodextrin based nanosponges

Formulation Code	Drug (mg)	Beta-cyclodextrin (mg)	Polyvinyl Alcohol (w/v)	Dimethylsulfoxide (ml)	Distilled Water (ml)
F1	100mg	100mg	0.3%	10ml	90ml
F2	100mg	200mg	3%	10ml	90ml
F3	100mg	300mg	2 %	10ml	90ml
F4	100mg	400mg	0.3%	10ml	90ml

2.2.3. Characterization of Beta-Cyclodextrin Based Nanosponges of Clotrimazole

2.2.3.1 Percent Drug Loading

Accurately weighed NS (10mg) and 5ml of 0.1 N NaOH) was taken in vortex tube (15ml) and shaken for 1 minute. With further addition of (0.1 N NaOH), volume was made upto 10 ml after shaking. 1ml of filtered solution was taken in volumetric flask and volume was made up to 10ml with 0.1 N NaOH.

In order to calculate the concentration of clotrimazole, absorbance was determined at λ_{max} 263 nm (21).

$$\text{Drug Loading (\%)} = \frac{\text{Drug content of NS}}{\text{Weight of NS recovered}} \times 100$$

2.2.3.2. Percent Yield

The actual yield of nanosponges was determined by weighing dried nanosponges. The weight of each solid ingredient was utilized to formulate the nanosponges, including the drug, beta-cyclodextrin NS. Percent yield was calculated by the following formula (22).

$$\text{Percent Yield} = \frac{\text{Actual yield}}{\text{Theoretical yield}} \times 100$$

2.2.3.3. Entrapment Efficiency

An accurately weighed NS (10mg) was added to 5ml of 0.1N NaOH in the vortex tube (15ml) and shaken for 1 minute. After that, the volume was made up to 10ml with 0.1N NaOH. 1ml of filtered solution was taken and made volume up to 10ml with 0.1N NaOH. At λ_{max} of 263 nm, absorbance was determined which was used to calculate the concentration of clotrimazole. Entrapment efficiency was calculated by following formula (22).

$$\text{Entrapment efficiency} = \frac{\text{Drug content of NS}}{\text{Initial weight of the drug}} \times 100$$

2.2.3.4 Particle Size & PDI

Using a Malvern Zeta Sizer, the particle size as well as particle size distribution of clotrimazole nanosponges were determined and mean diameter was calculated. The samples were appropriately diluted with distilled water for each analysis. All samples were analyzed using the same fixed angle of 90°. Particle size against time was maintained on a cumulative graph to assess the impact of particle size on drug release. Aqueous dispersion was diluted up to specified scattering at 25°C. Dynamic light scattering with zeta sizer was used to determine the diameter of nanosponges (23).

2.2.3.5. Zeta Potential

This method makes use of particle-size equipment with an extra electrode for measuring surface charge. After diluting the sample containing nanosponges with 0.1mol/l KCl, A 15V/cm electric field was applied to nanosponges in an electrophoretic cell. Surface charge was measured by zeta potential. Using a zeta sizer, it was possible to detect the surface charge of nanosponges (24).

2.2.3.6. Scanning Electron Microscopy

SEM analysis was used to determine the surface morphology of nanosponges. Following a random scan of the samples, photomicrographs were taken at 20KV acceleration voltage. SEM was used to evaluate particle size and shape morphology (25).

2.2.3.7. Fourier Transform Infrared (FTIR) Spectroscopy

To assess compatibility or interaction of clotrimazole with polymer, FTIR spectroscopy was used. Sample FTIR analysis was completed by ATR-FTIR spectrophotometer. Frequency range for verification of spectra was (4000-600 cm^{-1}) (26).

2.2.3.8. *In vitro* Drug Release Studies & Kinetic Modeling

A section of dialysis membrane weighing 12,000–14,000 Da was cut prior to release study. A dialysis membrane was dipped in 2% sulfuric acid for 3-5 minutes, washed with distilled water, and then soaked in phosphate buffer solution overnight. The receptor compartment was filled with phosphate buffer solution (PBS 7.4) and fresh formulation was placed in the donor compartment. Pre-soaked dialysis membrane (DM) in diffusion media was placed over-night (PBS 7.4). The dialysis membrane in contact with the receptor medium was filled with 3ml of the formulation. A 250 ml vessel

containing 100 ml of PBS pH 7.4 and the dialysis membrane was used. The complete assembly was placed on a magnetic stirrer that was thermostatically controlled at a speed of 1000 rpm and it was continuously stirred. The temperature of the medium was kept constant at 37±0.5°. 1 ml of the sample was removed from the receptor compartment at pre-determined intervals and replaced with an equivalent volume of PBS 7.4. UV-visible spectrophotometer was used to measure the sample's absorption/drug content at 263 nm after appropriate dilution (27).

Later, the data about the release of drug was fitted into zero-order, first order, Higuchi and Korsemeyer Peppas kinetics models followed by regression analysis. The equation for each model is depicted as follows:

Zero Order; $Q_T = Q° + K°t$

First order; $\log Q_t = \log Q^o - \frac{k_1 t}{2.303}$

Higuchi; $Q_t = k_H t^{1/2}$

Korsmeyer-Peppas; $\frac{M_t}{M_\infty} = kt^n$

Whereas, "Q_t" is drug dissolved over time t, "Q°" is initial amount of drug dissolved in diffusion medium i.e., equal to zero. "K°" is zero order kinetics constant, k_1 is first order rate constant, "$k_H t$" is Higuchi model constant. "M_t" and "M_∞" is cumulative drug release at time t and infinite time respectively. "k" is rate constant of drug, "t" is release-time and "n" indicates diffusional exponent denoting release mechanism (28).

2.3. Statistical Analysis

The data was statistically analyzed by one way ANOVA, using SPSS software (version 18.0) for the determination of statistical significant difference between the groups. P value < 0.05 was deemed statistically significant.

3. Results

3.1. Calibration Curve of Clotrimazole

Using UV spectrophotometer, by measuring and plotting absorbance of known concentration from 10 to 100ug/ml, the calibration curve was prepared at λ_{max} of 263 nm and plotted. The average of triplicate was taken and shown in Figure 1. A linear relationship between the known concentration was studied and resultant absorbance was shown by a straight line with a regression equation.

$$\boldsymbol{y = 0.0192x - 0.0048, R2 - 0.979}$$

For the determination of concentration in unknown samples this curve was used.

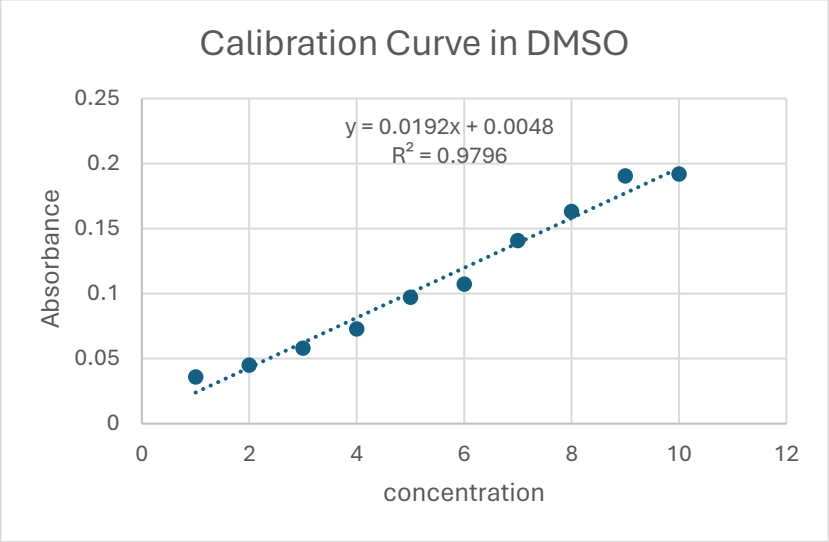


Figure 1. Calibration curve of clotrimazole in DMSO

3.2. Formulation of Nanosponges

Different combinations of nanosponges loaded with drugs were prepared in different proportions via emulsion solvent evaporation technique in which various trials were performed before finalizing the desired nanosponges. Multiple trials were conducted to find out the appropriate ratio between drug and polymer. After passing series of formulations, 1:3 (drug: beta-cyclodextrin) was found to be best suited for nanosponges formulation along with loaded drug in polymer in order to achieve maximum entrapment and ideal particle size with less polydispersity index i.e., < 0.20. The concentration of the polymer beta-cyclodextrin and the stabilizer PVA (3% w/v) is crucial to the formulation's optimization. With variable polymer and stabilizing agent concentrations, the drug's particle size and EE percentage varied.

The production yield of batches from F1 to F4 ranged from 77% to 87%. Increase in drug polymer ratio increased the production yield. It was found that the entrapment efficiency of the batches from F1 to F4 ranged from 80% to 90%. Entrapment efficiency was calculated to evaluate the percentage of drug being successfully placed inside the voids of nanosponges (Table 2).

Table 2. Percentage yield, drug-loading, particle size and entrapment efficiency

Formulation Code	Drug/beta-CDs ratio	Particle Size	Percent Yield (%)	Entrapment Efficiency (%)
F1	1:1	310nm	77%	80%
F2	1:2	473.2nm	80%	83%
F3	1:3	495nm	83%	85%
F4	1:4	550nm	87%	90%

3.3. Particle Size & Zeta Potential Analysis

The particle size of formulations (F1-F4), as shown in Table 2, depicted that the formulated nanosponges were nano-sized with a polydispersity index of < 1. Surface charge is measured by zeta potential. Zeta potential of all the formulations (F1-F4) ranged between -6 ± 2.4 mV to -11 ± 4.39 mV, indicating stability of the developed nanosponge formulations.

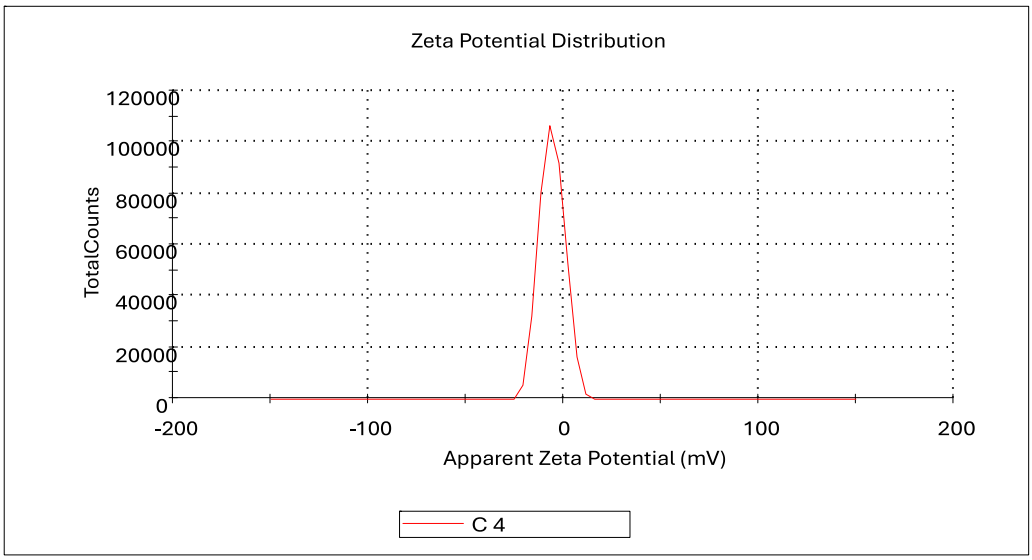


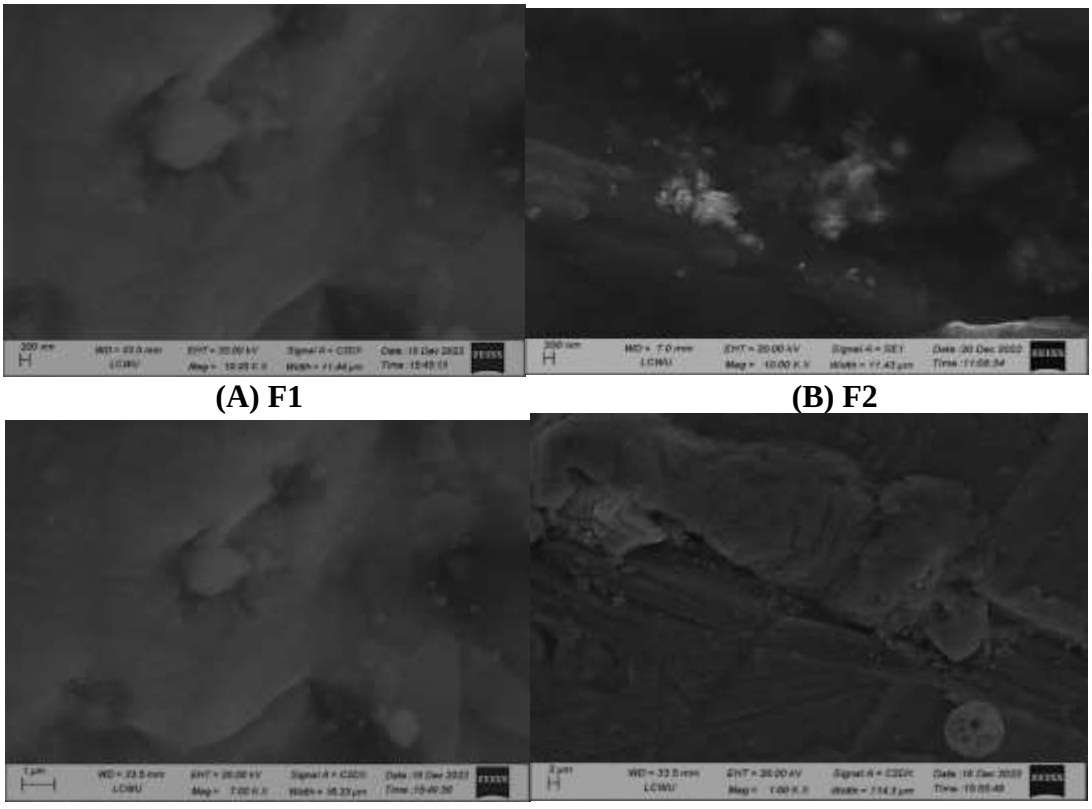
Figure 2. Zeta potential of F4 formulation

3.4. Structural Analysis

For the analysis of structure and morphology of prepared NS formulations, two approaches SEM, and FTIR were used. Higher drug loading and entrapment efficiency formulations were selected for structural analysis. Nanosponges' surface morphology was analyzed using SEM while FTIR was done for pure drug.

3.4.1 SEM Analysis of Clotrimazole Loaded Nanosponges

SEM performed for the surface morphology of formulations and confirmed the formation of orange peel like shape with porous and spongy nature which shows that the polymer based nanosponges had a porous three-dimensional surface with some variations. For comprehensive SEM analysis, diluted samples of nanosponges were prepared after washing nanosponges. Nanosponges were found having spherically discrete and porous surface. Moreover, SEM analysis also brought the confirmation about nano size range of particles which also supported the findings of particle size analysis (Figure 3).



(A) F1 **(B) F2**

(C) F3 **(D) F4**

Figure 3. SEM images of formulations

3.4.2 Fourier Transform Infrared Spectroscopy

Clotrimazole's spectrum displayed remarkable absorption peaks at 3057 cm^{-1} (for C-H stretching vibrations), 1585 cm^{-1} (for C=N stretching vibrations), 1481 and 1438 cm^{-1} (for C=C stretching vibrations), 1082 and 1039 cm^{-1} (for C-N stretching vibrations), 1202 cm^{-1}, and 1313 cm^{-1} (for C-H bending vibrations) as well as 702 cm^{-1}, 902, 823, 756 (for C-H bending vibrations) (29). According to prior research, beta-cyclodextrin had a high adsorption band at 3394 cm^{-1} for O-H stretching vibrations, 2928 cm^{-1} for C-H stretching vibrations, 1157 cm^{-1} for C-H, C-O stretching, and 1033 cm^{-1} for C-H, C-O stretching (11).

FTIR spectra of clotrimazole drug, beta-cyclodextrin and clotrimazole loaded NS formulations demonstrated in Figure 4 (a), (b) and (c), respectively, showed the broadening and masking of drug peaks, which depicted that encapsulation of clotrimazole in nanosponges core. Comparison was made between the spectra of the pure drug and the physical mixture of the drug and excipients. When compared to the spectra of the pure drug, spectra showed no evidence of drug-excipient interaction because all functional groups were present. The results were presented in the form of peaks.

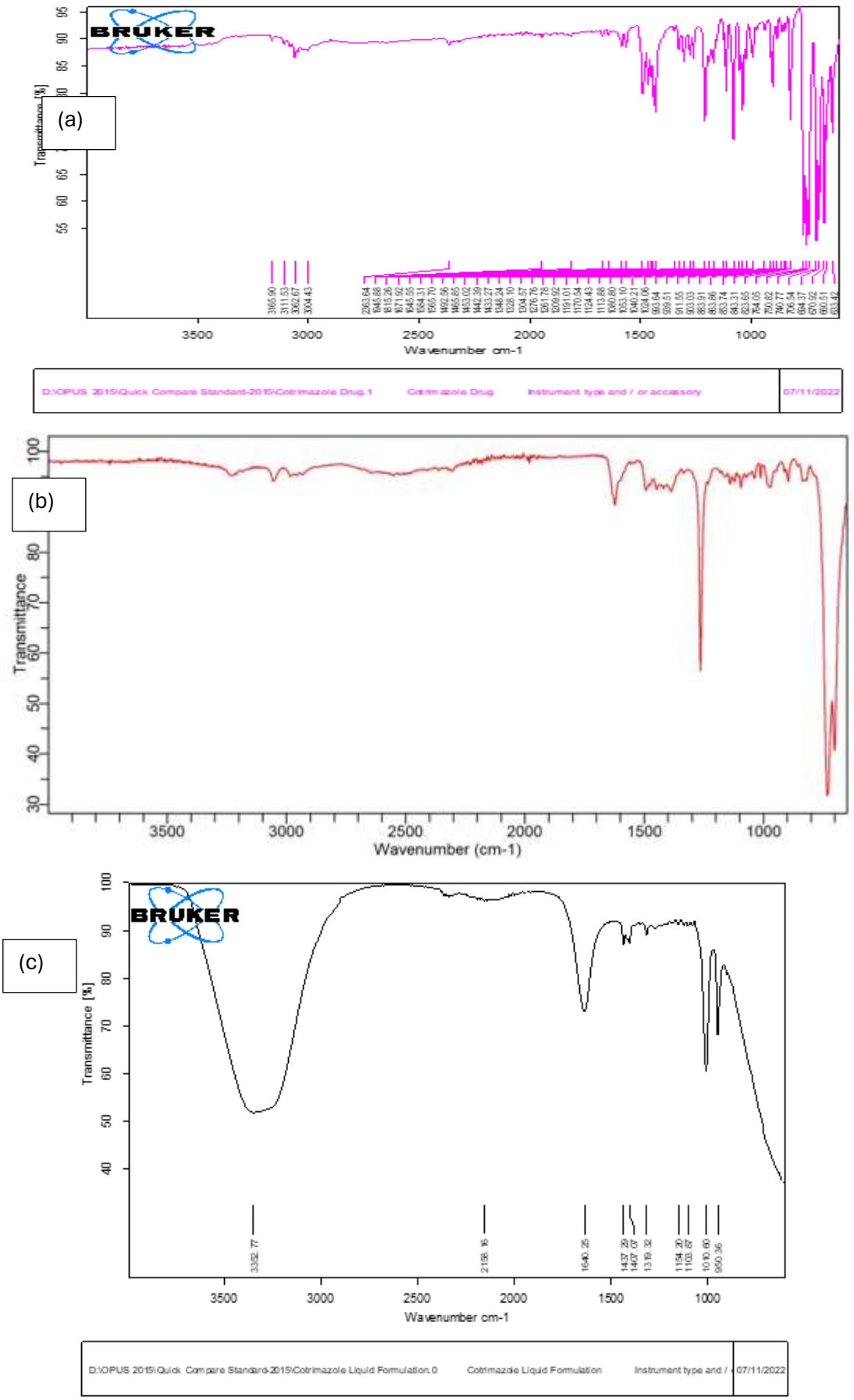


Figure 4. FTIR spectra of (a) clotrimazole (b) beta-cyclodextrin and (c) beta-cyclodextrin based NS of clotrimazole (liquid formulation)

3.5. *In vitro* Drug Release &Kinetic Modeling

During *in vitro* studies, two patterns of drug release were observed. Initially, it was comparatively fast and burst release i.e., 80% release, which was later followed by sustained release for at least 24 hours (Figure 5). The designed formulation retarded the release of clotrimazole in a controlled manner over span of time. When the release data was fitted in various kinetic models, Korsemeyer Peppas model was found to be best fit model with R^2 value of 0.996 and n value of 0.678, indicating non-Fickian anomalous diffusion.

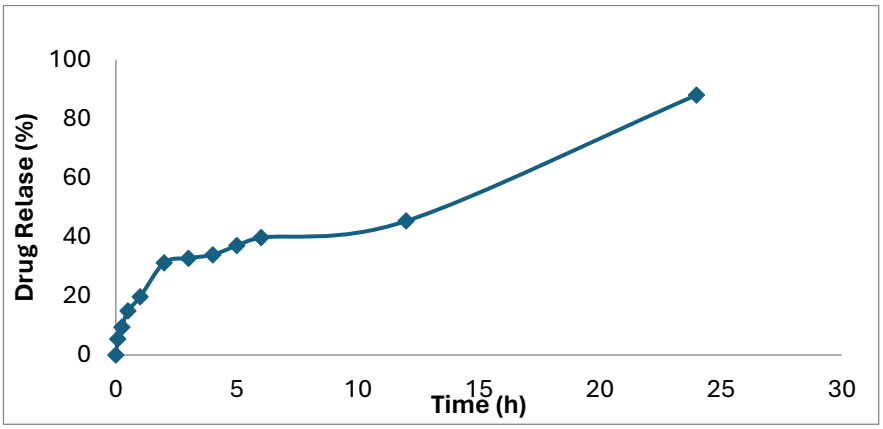


Figure 5. *In vitro* drug release profile of F4 nanosponge formulation

4. Discussion

Clotrimazole nanosponges were successfully synthesized by the process of emulsion solvent evaporation in which organic solvent was allowed to evaporate from the formulation by vigorous stirring overnight (30). The concentration of polymer for the development of nanosponges was adjusted to obtain desired attributes related to characterization of nanosponges including particle size, zeta potential and *in-vitro* release studies. The solubility of clotrimazole was determined in different organic solvents including methanol, ethanol, dichloromethane, and DMSO. Clotrimazole showed optimum solubility in DMSO being single organic solvent (31). Stirring speed was found an important parameter for desired size and entrapment of drug into nanosponges. Optimal stirring speed was observed between 1000-3000 rpm which was used for further formulations. Another critical parameter for the formulation was stirring time, the observed irregularity in the particles was found due to incomplete evaporation of organic solvent from the formulation and time period of 1-3 hours was found insufficient for this purpose. So overnight stirring was preferred to enable fully evaporation of solvent (32). Although, different polymers were under consideration for formulation: such as Eudragit L 1000, beta cyclodextrin and ethyl cellulose. Among them, beta-cyclodextrin was found appropriate with the selected method of preparation and showed encouraging results related to drug entrapment, therefore finalized for further processing.

According to observations, as the ratio of drugs to polymers increased, entrapment efficiency of clotrimazole loaded nanosponges also increased. The entrapment efficiency of clotrimazole loaded nanosponges showed high loading compared with other batches may be as a result of a high cross-linking ratio (33).

The average particle size of nanosponge formulations was in direct correlation to the drug : β-cyclodextrin concentration. It was found that as the drug**:** polymer ratio increased, the mean particle size also increased. More likely, at high drug**:** polymer ratios, more drug was encapsulated, resulting in a thinner polymer wall and the formation of nanoscale sponges. This can be attributed to the higher drug concentration and the reduced amount of polymer available per nanosponge for encapsulation. As a result, the polymer wall becomes thinner, and the nanosponges are smaller in size (34). Increasing the polymer ratio by four times in formulation F4 led to a notable rise in particle size, which can be explained by the thicker polymer structure resulting from the increased polymer proportion. The high viscosity inhibits the emulsion from breaking into smaller droplets, leading to the formation of larger nanosponge particles (35). Conversely, a low polymeric concentration

enhances the diffusion of DMSO (internal phase) into the aqueous solution (external phase), reducing the time required to form droplets and thereby decreasing the particle size (36).

Surface charge is a variable that influences body distribution and interactions with the biological environment. Diffusion coefficient, electrophoretic mobility, electrolyte concentration, and pH are taken into consideration while measuring zeta potential. Zeta potential analysis can be used to evaluate the stability of the manufactured nanosponges. The zeta potential in a water should be around 30 mV in order to create stable nanosponges that do not aggregate over time (37).

The surface of the produced nanosponges was spherical in shape, uniform in size, and porous in character, as evidenced by the SEM data. While beta-cyclodextrin showed amorphous spheres, clotrimazole exhibited a crystal-like structure. The physical mixture of clotrimazole and beta-cyclodextrin showed the characteristic clotrimazole crystals that appeared to be mixed with cyclodextrin particles. The clotrimazole beta-cyclodextrin inclusion complex, in contrast, the original morphology of both components dissipated and appeared as irregular particles, indicating the aggregation of particles into amorphous deposits with irregular forms (38). These SEM images showed that, the complex was structurally unique from the physical mixture and the isolated components. Clotrimazole and beta-cyclodextrin particles were distinct in size and shape from inclusion complex particles, which indicated the development of the inclusion complex. These results indicate that the inclusion complex between clotrimazole and beta-cyclodextrin was sufficiently developed and that it was partially responsible for the drug's increased solubility (39).

The presence of characteristic peaks of clotrimazole in the prepared formulation of nanosponges showed no significant change in their positions which ruled out the chances of any unwanted chemical interaction between clotrimazole and polymer. The main peaks of clotrimazole in the drug polymer physical mixture were revealed to be unaltered, it was determined from the data that there was no interference with the functional group (40).

As the drug to polymer ratio increased, it was observed that the drug release also enhanced. The thickness of the polymer layer enclosing the nanosponges was lowered when the drug molecule concentration was increased. All batches of nanosponges had a biphasic release with an initial burst impact, according to the release profile. The burst effect is caused by two factors: initially, the drug's availability close to or on the surface of the nanosponges; and second, the porous nature of the nanosponges, which allowed the drug to be released via the pores (41). The *in vitro* release kinetic data indicated anomalous non-Fickian diffusion suggesting that release was controlled by a combination of diffusion and polymer relaxation. These results clarified that the drug release was controlled by the rate of solvent penetration into a non- swellable water-insoluble polymer such as β-cyclodextrin, which controls drug release through the micropores present in their framework structure (42).

5. Conclusion

Beta-cyclodextrin-based nanosized sustained release nanosponges of clotrimazole were successfully formulated with the help of the emulsion solvent diffusion approach using PVA as surfactant. Beta-cyclodextrin was used as release retarding polymer in emulsion solvent diffusion method, all of the formulations depicted size in nano range. Polymer ratio affects the physiochemical parameters of NS like production yield, entrapment efficiency zeta potential and particle size. Spherical and spongy structure was observed by morphological analysis. *In vitro* release study showed Beta cyclodextrin prolonged the release rate of clotrimazole that was not very soluble. These nanosponges were characterized and results encouraged this approach for modification of clotrimazole from conventional to sustained release novel dosage form.

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