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Clotrimazole-cyclodextrin based approach for the management and treatment of *Candidiasis*- A formulation and chemistry based evaluation

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Abstract

Clotrimazole (CT) is a poorly soluble antifungal drug that is most commonly employed as a topical treatment in the management of vaginal candidiasis (VCD). The present work focuses on a formulation approach to enhance the solubility of CT using cyclodextrin (CD) complexation. A CT-CD complex was prepared by a co-precipitation method. Various characterization techniques such as differential scanning calorimetry (DSC), infrared (IR) and X-ray spectroscopy, scanning electron microscopy and nuclear magnetic resonance (NMR) spectroscopy were performed to evaluate the complex formation and to understand the stereochemistry between CT and CD. Computational molecular modeling was performed using the Schrödinger suite and Gaussian 09 program to understand structural conformations of the complex. The phase solubility curve followed an A_L -type curve, indicating formation of a 1:1 complex. Molecular docking studies supported the data obtained through NMR and IR studies. Enthalpy changes confirmed that complexation was an exothermic and enthalpically favorable phenomenon. The CT-CD complexes were formulated in a gel and evaluated for release and antifungal activity. The in-vitro release studies performed using gels demonstrated a sustained release of CT from the CT-CD complex with the complex exhibiting improved release relative to the un-complexed CT. Complexed CT-CD exhibited better fungistatic activity towards different *Candida* species than un-complexed CT

Keywords

complexation; cyclodextrin; computational molecular modeling; candidiasis; solubility enhancement

Introduction

Vulvovaginal candidiasis (VCD) or vulvovaginitis is a common vaginal infection affecting 75% of women at least once in their lifetime. VCD is most commonly caused by *Candida* species and hence has been given the name “candidiasis”. VCD is characterized by inflammation of the vagina or the vulva and is most often caused by bacterial, fungal, or parasitic agents. VCD can be treated through systemic and topical applications of azole antifungal agents of which clotrimazole (CT) is the most common one. All azoles given orally or topically are associated with a dramatic symptom relief within 24-48 hours (1). CT is an imidazole derivative having broad-spectrum antifungal activity and has been extensively used in topical therapy as a first line, broad-spectrum antifungal agent for prophylaxis as well as treatment of candidiasis (2). A seven-day topical azole therapy is more effective than single dose oral therapy in treating severe infections (3).

Clotrimazole is highly lipophilic in nature (logP 4.1) and has poor aqueous solubility (solubility=0.49 mg/L) (4, 5) requiring repeated applications to maintain a therapeutic concentration. The requirement for multiple daily doses of conventional formulations for an extended period of time results in poor patient compliance. Conventional drug delivery systems (exemplified by tablets, creams, pessaries, and foams) also suffer from poor retention in the vaginal tract, since they are rapidly dislodged by the self-cleansing action of the vaginal tract (6). These two problems i) delivery of effective amounts and ii) patient compliance are compounded and impair the therapeutic efficacy of the formulation in the treatment of vulvovaginitis. We suggest that vaginal therapy can be significantly improved if a delivery system is used that can maintain high concentration of the drug at the site of administration for a prolonged period of time. Various methods have been described to improve the solubility of CT such as using microcapsules (7), thermosensitive gels(8), liposomes(9), suspension with hydroxyl-propyl-methylcellulose in nanospheres (10) and hot melt extrusion (11). Most of these techniques utilize multiple excipients and processes that are difficult to scale up. Hot melt extrusion has been able to overcome these disadvantages by employing a continuous manufacturing process using thermoplastic polymers (12-14). However, it cannot be effectively utilized for liquid/semisolid formulations.

Complexation with cyclodextrins and/or their derivatives is one of the techniques that can be utilized to increase intrinsic solubility of poorly soluble compounds in liquid formulations. Investigators have reported complexation of clotrimazole with beta-cyclodextrins (15, 16) and gamma-cyclodextrin (17) which resulted in increased solubility. Modified CDs such as 2-hydroxypropyl- β -cyclodextrin (HPCD) are considered as Inactive Pharmaceutical Ingredients and have been used in oral as well as parenteral formulations in high concentrations. CD derivatives have been effectively utilized to increase the solubility of less water-soluble azoles such as miconazole (18), itraconazole (19) and sertaconazole (20) and

have been found to be very successful. The other important factor that can increase the efficacy of a formulation approach is a correct delivery system that favors patient compliance and at the same time does not hinder the release or absorption of the active component. Gels can present several advantages over alternative formulations, such as increased bioavailability, safety, versatility, bio-adhesiveness, and cost-effectiveness and are preferred over other vaginal dosage forms (21-23). The present study details a formulation approach utilizing the complexation of CT with 2-hydroxypropyl β -cyclodextrin to increase CT's solubility and hence its efficacy. A CT-HPCD complex was prepared and characterized using various techniques to confirm the type of association between HPCD and CT. In addition, computational molecular modelling was performed using the Schrödinger suite and Gaussian program to further support the experimental data and better understand the inclusion phenomena. The formed CT-HPCD complex was formulated as a gel and evaluated for *in vitro* release and antifungal activity.

Materials

Clotrimazole (CT) was purchased from Spectrum Chemicals (Gardena, CA), 2-hydroxypropyl- β -cyclodextrin (HPCD) was purchased from Sigma-Aldrich (St. Louis, MO, USA), and (hydroxypropyl)methyl cellulose (HPMC) was received as a sample from Dow Chemicals. Other materials included Tween-80, Pierce pleated SnakeSkin dialysis membrane (Thermo Fisher Scientific, Inc., Rockford, IL USA), distilled water, acetonitrile. Purified water was prepared in the laboratory using Nanopure systems (Barnstead International, Inc., Dubuque, IA, USA). Chemical and reagents needed were purchased from Fisher Scientific (Fair Lawn, NJ) or Spectrum Chemicals.

Methods

HPLC method

A modified HPLC method was used for the analytical testing of clotrimazole in the formulation and complexes. A Waters 600 pump with a dual wavelength Waters 2489 UV detector was used for the HPLC analysis. A Phenomenex Luna C18 column, 150×4.60 mm, with an inner particle size of 5 μ m was used as a stationary phase. The mobile phase constituted a mixture of acetonitrile (ACN) and 50 mM strength pH 5.5 buffer at a ratio of 65:35 (% v/v). UV detector set at 210 nm wavelength and Empower software was used to analyze the chromatograms. The gels were analyzed by dissolving in 50:50 ACN:Water mixture and then filtered using a 0.45 μ m filter. The dissolution samples were centrifuged to remove any particulate matter, filtered using a 0.45 μ m filter and injected into the column. All standards and samples were injected at a 20 μ l injection volume.

Phase Solubility Studies

Clotrimazole-CD phase solubility studies were performed using the shake flask method as described by Higuchi and Connors (24). An excess amount of drug was added to increasing, known concentrations of HPCD solutions and allowed to equilibrate. Concentrations ranging from 0 to 50 mM, in increments of 5 mM, were prepared in glass vials using double distilled water. The vials were kept under constant shaking at 70 rpm for 7 days at 25 °C. Excess

drug allowed for saturation equilibrium to be reached. After 7 days, drug solutions were collected and filtered, and the supernatant was analyzed using HPLC to measure the amount of CT dissolved. A graph was generated using the concentration of CT dissolved against the concentration of HPCD used. The K constant was calculated using the following Higuchi and Connors equation from the slope.

$$K_{1:1} = \frac{\text{Slope}}{S_0(1 - \text{Slope})}$$

where S_0 stands for the intrinsic solubility of the drug.

The complexation efficiency (CE) was calculated as per the following equation.

$$CE = \frac{\text{Slope}}{(1 - \text{Slope})}$$

Characterization of Complexes

A 25 mM solution of CT and HPCD was prepared by dissolution in ethanol and allowed to equilibrate overnight under constant stirring. The obtained solution was filtered and then dried using a vacuum dryer at 30-40 °C for 24 h. The complex which formed was characterized using different techniques such as Differential Scanning Calorimetry (DSC), Nuclear Magnetic Resonance Spectroscopy (NMR), Fourier Transform Infra-Red Spectroscopy (FTIR), X-ray Diffraction (XRD) and Scanning Electron Microscopy (SEM).

Complexes were also prepared by physical mixing, by kneading methods which employed small amounts of solvent to make a slurry followed by solvent evaporation, or by a co-precipitation method.

Differential Scanning Calorimetry

A Perkin Elmer Hyper Differential Scanning Calorimeter was used to analyze the CT, HPCD and CT-HPCD complexes. The samples (n=3) were hermetically sealed in aluminum pans and heated over a range from 25-250 °C, incremented at 10 °C min⁻¹. The obtained spectra were analyzed using Pyris manager software.

Fourier Transform Infrared Spectroscopy (FTIR)

Infrared spectroscopy was performed using a Perkin Elmer FTIR Paragon 500 spectrometer and the scans were analyzed using Spectrum Ver. 5.0.2 software. The CT, HPCD, and CT-HPCD complex were analyzed by incorporating them with KBr in a ratio of 1:100 and compressing them as pellets. The pellets were scanned from 600 to 4000 cm⁻¹ eight times and the spectrum was averaged to reduce the noise. Samples were prepared by kneading and solvent evaporation methods.

Nuclear Magnetic Resonance Spectroscopy (NMR)

A Bruker AV 400 MHz nuclear magnetic spectrometer having a 3 mm natorac dual proton optimized probe and running Topspin 2.0 software was used to perform NMR analysis. Pure CT, HPCD and the mixtures of the two in different mole fractions of 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 0.9 were dissolved in deuterated dimethyl sulfoxide (DMSO) and left to equilibrate overnight at room temperature. Samples of 600 μ l volume were then taken in 3 mm NMR glass tubes and analyzed. The DMSO peak was utilized as a reference peak. MestReNova (MNOVA) software was utilized to analyze the data.

Powder X-ray Diffractions studies

Powder X-ray diffraction (XRD) studies were performed on a D-8 Advance X-ray diffractometer (Bruker AXS) equipped with a SolX detector and running DiffractionPlus[®] software. The generator voltage and current were set to 40 kV and 30 mA, respectively. The samples were scanned from 15-40 °C, 2 θ values with 0.05° ramp at 3 sec/step. Pure components as well as complexes manufactured using different methods were evaluated.

Scanning Electron microscopy

Scanning Electron Microscope (SEM) was used to study the surface morphology of CT, HPCD and that of complexes. Samples were sputter coated with gold using a Hummer[®] 6.2 sputtering system (Anatech LTD, Springfield, VA) in a high vacuum evaporator. A JEOL JSM-5600 scanning electron microscope operating at an accelerating voltage of 10 kV was used for imaging. Samples were mounted on adhesive carbon pads placed on aluminum stubs prior to sputter coating.

Computational Modeling

The 3D X-ray crystal structure of methyl β -cyclodextrin was taken from the protein complex of alpha-amylase II (PDB id: 1jl8).(25) The 2-hydroxypropyl group was added at position 6 of the methyl β -cyclodextrin X-ray structure to prepare 2-hydroxypropyl- β -cyclodextrin (HPCD). This structure was energy minimized using MacroModel (26) software to avoid any steric clashes. The energetically favorable conformers of HPCD were searched using the Macrocycle Conformational Sampling (MCS) tool from the Schrödinger suite (26). During the Macrocycle Conformational Sampling, the large-scale low mode (LLMOD) method was used with the Generalized Born model (GB/SA) solvent treatment. The resulting conformers were then minimized in the gas phase using the Polak-Ribière Conjugate Gradient (PRCG) method with gradient convergence required to be $< 0.001 \text{ kJ (mol} \cdot \text{\AA)}^{-1}$ with the OPLS2005 force field. The energy window for saving structure was adjusted to 10 kcal/mol with the intermediate torsional sampling options. The structure of clotrimazole (Figure 1) was sketched in Maestro from the Schrödinger software suite (27) and prepared using LigPrep (28) at pH 7.4.

The receptor grids were generated for each conformer of HPCD keeping in view that the generated receptor grids covered the whole HPCD structure as an active site. The docking of HPCD was done using the Glide (29) standard precision (SP) method and flexible ligand sampling. During docking, no additional constraints were used. Emodel docking scores [a mathematical combination of the Glide-score, the ligand strain (E_{internal}), and the Coulomb

and van der Waals energies] and post-docking calculations of energy differences (using the Embrace module of the Schrödinger suite (26) were used to select the best docked (binding) pose of CT with HPCD.

The best docked complex was further optimized using hybrid density functional theory (DFT) with B3LYP and the 6-31G(d,p) basis set using the Gaussian 09 program (30). The input Cartesian coordinates of the complex for the Gaussian 09 job were prepared using Avogadro graphics software (31). The results of the Gaussian 09 job were viewed in Gaussview 3.0 (32) and Avogadro. Frequency calculations were performed at the above level to confirm that the obtained structures are minima on the potential energy surface and to obtain the thermodynamic parameters. Single point energy calculations were performed at the B3LYP/6-311+G(d,p) level to obtain accurate energies based on the above optimized geometries. The same geometry optimization and single point calculation methods were also used for the individual molecules such as CT and HPCD.

Preparation of Cyclodextrin Complexes

A 25 mM concentration of HPCD was prepared in deionized water and allowed to equilibrate with excess drug at 25 °C at 75 rpm for 24 hr. A clear solution that formed was then freeze dried to obtain the CT:HPCD complex, which was utilized to prepare gel formulations.

Formulation of Gel

The gel was prepared by dissolving (hydroxypropyl)methyl cellulose (HPMC) in double-distilled water to achieve a targeted concentration of 1%. The polymer was allowed to swell and hydrate overnight and the swollen matrix was utilized to formulate CT and CT-HPCD complex gels, Gel A and Gel B, respectively. A targeted drug concentration of 10% CT was utilized for both the gels. CT, due to its low solubility, was first dissolved in a 50:50 mixture of polyethylene glycol 400 and ethanol, which was then dispersed in HPMC gel using a mechanical homogenizer. An Unguator® EWR electronic mortar and pestle was utilized until all of the drug or of the complex had blended in. The pH of the gels was adjusted to 5.5 using phosphoric acid. Care was taken to avoid formation of any air bubbles in the gels. The so-formed gels were analyzed for content and stored at 4 °C until further use.

The assay was performed using an HPLC method. The extraction procedure was optimized and a 50:50 mixture of acetonitrile and water was used to extract the drug from the gels. Pre-weighed gel samples were dissolved and filtered using a 0.45 µm filter. A uniform measure of content was established by averaging assay results obtained from samples collected from different locations within the gel.

In vitro Release studies

In vitro release studies were performed using a SR8 Plus Dissolution apparatus from Hanson Research using a modified Type II basket apparatus. Snakeskin Pleated Dialysis tubing with a cutoff molecular weight of 3500 Daltons was used to contain 5 g of Gel A or B. The gel bags were lowered into the basket and fixed to the dissolution apparatus. The release studies were performed in replicates of three each. The baskets were rotated at 50 rpm. The

dissolution media consisted of 900 ml of degassed pH 5.5 buffer maintained at 37 °C with 1% Tween 80 added to maintain sink conditions. Samples were collected at intervals of 0.5, 1, 2, 3, 4, 5, 6, 8, 12, 24, 48, 72 and 96 hr and analyzed for percent drug release. Samples were filtered through a 0.45 µm filter before injecting into the HPLC. The dissolution data was fitted to various release models to help understand the mechanisms involved.

Antifungal Testing of the Gels

Comparative antifungal evaluation of CT and complexed gels was performed utilizing cultures of *Candida albicans*, *C. glabrata* and *C. krusei*. Three to five colonies of each *Candida sp.* were suspended in 0.9% sterile saline solution and the number of colonies formed was estimated by measuring the optical densities at 630 nm. The samples were then diluted appropriately to yield 1×10^6 Colony Forming Units per ml (CFU/ml) in a Saboraud Dextrose Broth (SD). 500 µl of cell suspensions of different species of the *Candida* were uniformly spread on SD agar petri plates with a sterile swab, covered and left to dry under the hood. Cylindrical wells of ~75 µl were made into the cultures and equal amounts of gel were added into them. The cultures were then incubated in an incubator for 72 hr at 37 °C. Appropriate controls were used to assess any antifungal activity due to the excipients used. The zone of inhibition, marked by lack of any fungal growth around the wells, was used as a measure to compare fungistatic action of the gels.

Results and Discussion

Phase Solubility

Phase solubility studies were performed using the shake flask method as described by Higuchi and Connors (24). The concentration of CT dissolved was plotted against the concentration of HPCD used. Addition of HPCD resulted in a significant improvement of solubility of CT. The graph was an A_L type curve with the linear plot having a R^2 value of 0.99 (Fig.2) (33). This suggested formation of a 1:1 inclusion complex of CT and HPCD. The stability constant obtained from the intrinsic solubility value S_0 and the slope $K_{1:1}$ was 446.5 M^{-1} using the Higuchi and Connors equation.

For drugs with very low water solubility, complexation efficiency (CE) is a better standard of stability due to apparent difficulty in estimating S_0 . The CE obtained was 0.01, suggesting 1 in 100 molecules of CT undergo complexation. CE values can be effectively utilized to estimate the final formulation weight for oral formulations, an approach that is far more accurate than S_0 for understanding the efficiency of a complexation process for sparingly soluble molecules.

Thermal studies

CT is a crystalline drug with a sharp melting point at 145 °C which can be observed as a melting endotherm in the DSC spectra (Fig. 3). The physical mixture had the characteristic CT peak at 145 °C and a broad peak for HPCD at around 100 °C due to loss of bound water. Since HPCD is amorphous in nature, no sharp melting peaks were obtained in neat or in physical mixture samples. Thermogravimetric studies (not shown) confirmed water loss and a degradation above 240 °C, suggesting thermal degradation of HPCD, which was also

observed in the DSC thermograms. The DSC spectrum of the complex was void of any peaks or broad endotherms, signaling formation of a thermodynamic equilibrium between the drug and the cyclodextrin. CT formed an amorphous complex with HPCD (34).

Fourier Transform Infra-Red spectroscopic analysis (FTIR)

The FTIR spectra of drug, HPCD, physical mixture and the complex are presented in Figure 4. The kneaded product exhibited slight difference in the peaks, a feature which was more prominent in complexes obtained through solvent evaporation. The peaks corresponding to aromatic C-H stretching above 3000 cm^{-1} for pure CT disappeared in the complex. The aromatic ring structure characterized by peaks at 1465 cm^{-1} and 1457 cm^{-1} disappeared and reappeared as a single broad peak at 1457 cm^{-1} , similar to an earlier reported spectral change observed in a complex of CT (34). The influence on the central π -bonded moiety could be seen by the disappearance of peaks at 1327 cm^{-1} , 1305.46 cm^{-1} and 1262.56 cm^{-1} . The aromatic regions corresponding to CT, prominent in the $600\text{--}800\text{ cm}^{-1}$ region, were slightly influenced in the kneaded product and disappeared completely in the complex. This indicates inclusion of the aromatic ring in the cavity of the HPCD and possible hydrogen bonding (16). C=N and C=C stretching vibrations were observed at 1650 cm^{-1} and 1450 cm^{-1} and C-Cl stretching was found at 750 cm^{-1} in pure CT, but these disappeared in the complex, suggesting the involvement of aromatic rings 'A' and 'B' in the complexation process. Similar results were observed from NMR spectroscopy.

Nuclear Magnetic Resonance Spectroscopic analysis

A continuous variation method was employed to establish the stoichiometry of the complex using NMR. DMSO solutions with increasing mole fraction of CT in HPCD from 0.1 to 1 were utilized to generate the NMR spectra. A physical parameter in this case, proton signals, can be directly related to change in concentration of guest and host molecule. However, because of the nature of complexation behavior, NMR signals pertaining to the CT molecule could not be identified accurately and assignment could not be performed for all the clotrimazole protons involved. Hence, only those protons that exhibited maximum deviation have been reported. As shown in Fig. 5, H2, H4, H8 and H10 protons suggest inclusion of CT's A and B aromatic rings in the CD cavity as these exhibit maximum deviation at a 1:1 ratio of CT and HPCD. H2 and H4 imidazole protons, corresponding to the aromatic ring 'A', exhibited an upward shift, suggesting their involvement in forming complexes. Then upward shifts for H8 and H10 suggest the inclusion of the aromatic ring 'B' as well in the cyclodextrin cavity. It can be assumed from the NMR data that clotrimazole is forming a complex by the inclusion in the CD cavity of these aromatic rings which are held in place by vander Waals forces and hydrogen bonding. Similar observations have been reported by Navarro *et al* (35) and from the FTIR analysis reported herein. The observed downward shift for some of the protons could possibly be due to interactions with the solvent. However, relatively small δ values suggest a relatively weak association, which is also influenced by the solvent used to prepare the samples.

X-Ray diffraction studies

Powder X-ray diffraction studies were performed on pure components as well as on complexes to evaluate changes in crystallinity. Complexes manufactured from various

methods such as kneading, physical mixing and freeze drying were evaluated. Characteristic crystalline peaks corresponding to CT were observed at 18, 21, 23, 25.2 and 28 2 θ values (36, 37) in physical mixtures and in pure CT (Fig. 6). In vacuum-dried complexes and, to a lesser extent, in kneaded complexes, peaks corresponding to CT were either missing or decreased in intensity, suggesting inclusion of CT in the complex. A decreased intensity in physical mixtures can suggest complexation.

Scanning Electron Microscopy (SEM)

SEM studies were performed to study the morphology of components before and after complexation. HPCD exhibited globular structure and lacked sharp features characteristic of a crystalline nature (Fig. 7). DSC results also suggested an amorphous nature. CT is a crystalline compound and was characterized by sharp needle-like crystals. Complexes formed from the freeze drying process were cubic in nature and exhibited a structure different from either of the components, suggesting formation of a complex.

In conclusion, data collected from NMR and FTIR suggests that aromatic rings A & B are most probably involved in forming complexes. The hydrophobic moiety of CD has affinity towards the hydrophobic aromatic rings and forms a complex due to possible CH- π interactions or hydrogen bonding of the imidazole nitrogen and the secondary hydroxyl group of HPCD as well as van der Waals attraction. Since these interactions are part of a dynamic process, complexes are formed and dissociated constantly and hence it takes time to reach equilibrium. This equilibrium and the affinity of the CD molecule towards the guest defines whether a 1:1 or 1:2 or 2:1 association is formed. However the type of complex formed cannot be confirmed using XRD, DSC, FTIR and SEM studies alone and hence a plethora of tests need to be performed to verify that there is complexation.

Computational modeling

The computational modeling studies aid and support the experimental observations and also present possible conformers involved in complex formation. The molecular modeling studies provided understanding of the binding mode geometry, energetics and stability of the inclusion complex of CT in HPCD. The observed ¹H-NMR shifts of the H2 and H4 protons of CT can be attributed to the predicted hydrogen bonding of N3 with the secondary hydroxyl group of HPCD and this interaction may be stabilized by hydrophobic interaction of the O-chlorophenyl group of CT with the apolar cavity of HPCD. Rings A and B of CT were involved in inclusion complex formation with HPCD. This was in excellent agreement with the experimental IR and NMR data (Fig. 4-5). The energetic profile of the most favorable complex of CT with HPCD from the molecular mechanics calculations is given in Table 1. Figure 9 shows the energetically most favorable inclusion complex of CT with the lowest energy conformer of HPCD from the molecular mechanics (MM) calculation.

Out of the 750 conformers of HPCD generated using the MCS tool, a total of 11 conformers (Fig. 8) within an energy cutoff of 7.5 kcal/mol were selected on the basis of the Boltzmann population analysis. The docking and scoring of CT into the generated grids of 11 conformers of HPCD resulted in the most favorable HPCD:CT complex, which was further optimized using hybrid density functional theory (DFT) B3LYP/6-31G (d,p) followed by

single point energy calculation at the level of B3LYP/6-311G+(d, p) using the Gaussian 09 program.

The Statistical thermodynamics calculations for the MM-based favorable inclusion complex of CT and HPCD were carried out at 1 atm and 298.15 K using the quantum mechanical method B3LYP/6-31G(d,p). The calculated thermodynamic parameters, the enthalpy change (ΔH°), the thermal Gibbs free energy (ΔG°), and the entropy contribution (ΔS°) are given in Table 2 and Table S1. As listed a negative value of the enthalpy change ΔH° (−21.957 kcal/mol) upon the formation of the inclusion complex suggested that the complexation process is exothermic in nature and is an enthalpically driven process. Similarly, the negative values of the Gibbs free energy ΔG° (−4.392 kcal/mol) also confirmed that the inclusion complex formation is a spontaneous and favorable process. It is well understood that the apolar cavity of the CD molecule results in water molecules, which are “enthalpy rich” and their displacement can be the driving force in the complexation process (38). From the HOMO and LUMO values (shown in Table 3) it can be interpreted that the complex has a large HOMO-LUMO gap (=5.44 eV), thus indicating its high stability index (39). The negative binding energy values from the single point energy calculation [B3LYP/6-311G+(d,p)] and smaller basis set [B3LYP/6-31G(d,p)], further confirmed the stability and formation of the inclusion complex of CT with HPCD. Finally, the optimized structures of HPCD alone, of CT alone, as well as of the HPCD–CT complex, using the B3LYP/6-31G(d,p) method, revealed that the dipole moment of the inclusion complex was higher than that of CT alone, but lower than that of HPCD alone. This indicated a decrease in the total polarity of the HPCD cavity after CT binds to it. These results in turn supported the formation of the inclusion complex of CT with HPCD. Figure 10 shows the optimized geometry of the substrates and complex determined using the B3LYP/6-31G(d,p) method.

***In Vitro* Release Profile**

The CT-HPCD based complex (Gel B) exhibited an improved release in comparison to uncomplexed CT gel (Gel A). An incomplete drug release of 75% was obtained for Gel A in comparison to a complete release from Gel B (Fig. 11). The increased release can be attributed to the solubilization effect of the cyclodextrins. HPCD assists in solubilization of CT by incorporating CT into the lipophilic cavity of the CD and imparting a hydrophilic character to the drug molecule. The dissolution data was fit to various kinetic models to understand the release mechanism involved. The drug release from Gel A followed zero-order kinetics with a 24 hr release constant of 0.008. Gel B followed zero-order kinetics during the first 24 hours, but then deviated from zero order. A 24 hr release constant of 0.02 was obtained, suggesting a faster release from the complex. The dissolution data for the complex gel gave the best fit to the Higuchi model of release, suggesting a controlled release from HPCD. Cyclodextrins have been reported to result in controlled release behavior of the guest molecule if the association is strong. The HPMC utilized was a lower molecular weight polymer and may also have affected the release. However, it can be assumed that the effect would be similar in both the gels and hence the slower release is indeed due to CD complexation. In order to understand the type of diffusion mechanism involved, the release profiles were fit to the Korsmeyer–Peppas equation. A diffusion release exponent, n , can be used to understand whether the diffusion is Fickian or non-Fickian. An n value of 0.5

confirms a Fickian diffusion and a value between $0.5 < n < 1$ supports a non-Fickian or anomalous diffusion (40). A diffusional release exponent of 0.6 was obtained for both the gels, confirming anomalous (non-Fickian) diffusion of the drug. The hydrophilic polymer surrounds the drug molecule and the release occurs when the drug molecule dissolves in the media and diffuses out of the matrix. The solubilized drug is thus removed from the external layer by the movement of fresh solvent layer resulting in a zero order release. HPCD results in increased solubilization of drug and creation of a higher concentration gradient which results in complete and faster drug release. The precipitation method employed to form complexes also converted the drug to an amorphous form. CDs have been known to aid the stabilization of amorphous systems in solid dosage forms, resulting in increased solubility (33). Characterization studies performed on the CT-CD complexes also support an amorphous CT. However in the present work CD contributed to increased solubilization not because of a decrease in solubilization energy but rather due to conveying a hydrophilic character to a lipophilic drug.

Antifungal Testing

The gels were tested for antifungal activity against *Candida* cultures. Size of the zone of inhibition (Z_i) was utilized as a measure of efficacy of the gels. The antimycotic activity of CT could be seen by the marked absence of fungal growth due to the fungistatic activity of CT in the Z_i . A considerable decrease in the growth of *Candida spp.* was observed in all three species of *Candida* tested—*C. albicans*, *C. glabrata* and *C. krusei* (5). Increased solubility in turn allowed for an increase in concentration in the agar matrix, which contributed towards a larger area of growth inhibition (Fig. 12). The appropriate controls utilized confirmed that antifungal activity was indeed due to enhanced solubility of CT and not due to presence of other excipients. These tests confirmed that complexation of CT with HPCD was indeed beneficial and can be effectively utilized in the treatment of *Candidiasis*. Gels complexed using cyclodextrin were found to be efficient in treatment and management of VCD due to maintenance of an increased localized concentration.

Conclusion

CT-HPCD complexation was confirmed using various analytical characterization techniques such as phase solubility, DSC, FTIR, XRD, NMR and SEM studies. Molecular modelling studies further supported the experimental data and provided putative bioactive structural conformations. The complexes exhibited a significant improvement in solubility and dissolution profiles. The CT-HPCD complex gel underwent a complete release whereas incomplete release was obtained from un-complexed CT gel in the same amount of time. Antifungal testing supports a better fungistatic activity for the CT-HPCD gels than for pure CT. Cyclodextrins can be effectively utilized to maintain therapeutic concentrations for an extended period of time, allowing for fewer applications, which will encourage patient compliance and thereby lead to better management of *Candidiasis*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Clotrimazole structure and ring assignment

Figure 2. Phase solubility curve of CT-HPCD in water

Figure 3. DSC thermograms of complex, HPCD, physical mixture, and clotrimazole

Figure 4. FTIR spectra of pure HPCD, CT, physical mixture and complex

Figure 5. Continuous variance plots of complexed to un-complexed CT

Figure 6. Powder XRD images of pure drug, HPCD, physical mixture, freeze dried and kneaded complex

Figure 7. SEM images of HPCD, CT and complex

Figure 8. Side (A) and top (B) views of an overlay of all of the 11 lowest energy conformations of HPCD that were used as inputs in docking. The lowest energy conformer is shown in stick form with green carbons.

Figure 9.

(A) The two-dimensional (2D) interaction diagram of CT with HPCD (shown as BCD601); and (B) putative binding mode of CT (shown as ball and stick with green carbons) with HPCD (shown as CPK).

Figure 10.

Optimized geometries of CT (A), HPCD (B), and CT-HPCD complex (C). Only polar hydrogens are shown in figures, for clarity. Carbons of CT in cyan and carbons of HPCD in yellow. The black dashed line indicates hydrogen bond.

Figure 11. *In vitro* release studies from gels of complexed CT and pure drug

Figure 12. Antifungal activity of CT in pure and complexed form

Table 1
The energetic profile of the most favorable inclusion complex of CT with HPCD

Complex	HPCD conformer energy (kcal/mol)	Docking Score	E _{model}	E [#] (kJ/mol)	vdW (kJ/mol)	Electrostatic energy (kJ/mol)
Clotrimazole + HPCD	7.372	-4.151	-29.701	-100.148	-100.020	-5.390

[#] E = E_{Complex} - E_{clotrimazole} - E_{HPCD}

Table 2
Energies, thermodynamics and electronic properties of the optimized geometries of free clotrimazole, HPCD and clotrimazole–HPCD using B3LYP/6-31G(d,p) and single point energy (SPE) using B3LYP/6-311G+(d,p)

Properties	Energy Difference between CT-HPCD Complex and Sum of the CT and HPCD
E° (kcal/mol)	–22.595
H° (kcal/mol)	–21.957
G° (kcal/mol)	–4.392
S° (cal mol ^{–1} K ^{–1})	–54.022
E° (SPE)(kcal/mol)	–9.409

Table 3
The energy levels of the frontier molecular orbitals, and dipole moments of the optimized geometry of free clotrimazole-HPCD using B3LYP/6-31G(d, p)

Properties	Clotrimazole (CT)	HPCD (Lowest Energy Conformer)	CT-HPCD (Inclusion Complex)
E_{HOMO} (eV)	-5.940	-6.532	-6.422
E_{LUMO} (eV)	-0.728	1.214	-0.979
$E_{\text{HOMO}}-E_{\text{LUMO}}$ gap (eV)	5.212	7.746	5.443
Dipole moment [field-independent basis, Debye (D)]	2.977	4.197	3.419