Preparation and Characterisation of Natamycin: γ-Cyclodextrin Inclusion Complex and its Evaluation in Vaginal Mucoadhesive Formulations

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ABSTRACT: Novel formulations of vaginal bioadhesive tablets were prepared where the natamycin was complexed with γ-cyclodextrin (NT-γCyD) to increase the solubility and stability of NT in aqueous solutions and reduce the side effects of the drug without decreasing antimycotic activity. Favourable interactions between the NT and γCyD and formation of the 1:1 inclusion complex were observed. The MIC_{90} of both NT alone and NT-γCyD complexes were below 0.0313 μg mL^{-1}, suggesting that complexation with γCyD has effectively increased the antimycotic activity of NT, thus indicating the clinical usefulness of NT-γCyD complexes. The sustained drug release of NT was achieved to over 8 h periods by altering the polymer component of formulations which was responsible for differences in water absorption and erosion behaviour of the tablets. Bioadhesion studies have clearly indicated that enhancement of mucoadhesion was achieved by inclusion of Carbopol®934P and by tailoring the ratio of Carbopol®934P in the formulation, a high mucoadhesion to vaginal mucosa can be achieved. Hence, the formation of complex between NT and γCyD and effective combination with polymers attain a bioadhesive and sustained release formulation of NT suitable for vaginal delivery and the effective treatment of *Candida* infections. © 2008 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 97:4319–4335, 2008

Keywords: natamycin; γ-cyclodextrin; inclusion complex; vaginal drug delivery; bioadhesion; hydroxypropyl methyl cellulose; xanthan gum; Carbopol®934P; *Candida* spp

INTRODUCTION

Candida infection is a common microbial problem in the vulvovaginal tract. Approximately 75% of women will have had a vaginal *Candida* infection during their lifetime, about 40–50% of them will suffer a relapse, and a small percentage will be affected chronically by this infection.[1]

Natamycin (NT) belongs to the family of polyene antifungal antibiotics. The antifungal activities of NT and other amphiphilic polyenes targets the cytoplasmic membrane by interacting primarily with ergosterol, resulting in enhanced leakiness of the fungal cell membrane and in turn cell death.[2]

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NT has been applied topically as a suspension or ointment for skin, ocular or oral candidiasis, as an ointment for dermatomycosis.[3] Importantly, Natamycin is also delivered as a vaginal tablet for the treatment of vaginitis/vaginosis caused by *Candida albicans* and *Trichomonas vaginalis*.[4] The vaginal tablet is usually administered as one vaginal tablet per day for 20 days or 2×1 vaginal tablets for 10 days after the menstrual cycle. Such conventional treatment for vaginitis/vaginosis is limited by poor patient compliance due to its high frequency of administration. NT is poorly soluble in water ($\sim$50 mg L^{-1}) and in acidic solutions, displaying rapid degradation under such conditions. Its poor solubility makes it largely unavailable for medical utility. Korteweg et al.[5] attempted to solubilise the drug by mixing it with a complex polysaccharide. Although the water solubility of the formulation increased dramatically, its antifungal activity decreased to about 1/3 of that of native NT and this formulation was more toxic than NT. The ability to increase the aqueous solubility and stability of NT by cholate formation has shown very limited success.[6] Koontz and Marcy[7] have already shown that the solubility of NT has been increased by complexation of NT and CyD.

Cyclodextrins (CyDs) and their chemically modified CyD derivatives are used in the pharmaceutical field to form inclusion complexes with drug molecules to improve the aqueous solubility of the encapsulated species, to improve their aqueous stability and photostability and to reduce side effects.[8–10] CyDs are cyclic oligosaccharides consisting of at least six D (+) glucopyranose unites covalently linked by α-(1,4) glucosidic bonds.[11,12] The natural CyDs, αCyD, βCyD and γCyD with 6, 7 or 8 glucose units, respectively, possess different cavity size and solubility. The unique molecular structure of CyDs, with hydrophobic internal cavities and a hydrophilic external surface, endows CyDs with the ability to form inclusion complex with various guest molecules.[13] The binding forces of the guest molecule within these inclusion complexes include hydrophobic, van der Waals, hydrogen bonding or dipole interaction.[11,14] CyD has also been reported to enhance the controlled release properties of certain active ingredients.[15] Importantly, while incomplete drug release may occur in cases whereby drug dissolution is limited by the solubility of the drug within the matrix tablets, incorporation of hydrophobic drug-CyD inclusion complexes into hydrophilic matrix tablets may provide a more controlled and complete *in vitro* drug release.[16]

Alternatively, the inclusion of mucoadhesive polymers into vaginal formulations (e.g., gels, tablets) has increased the residence time of the desired drug in the vagina, thereby boosting the efficacy of the treatment.[17,18] Apart from prolongation of drug release at the site of absorption, drug targeting to the affected site can also be realized.[19,20] Mucoadhesive drug delivery systems exploit the useful property of mucoadhesion of certain biopolymers on interaction with mucus that is present at the targeted physiological sites, for example, the vaginal mucosa. Mucus is a mixture of large glycoproteins (mucins), water, electrolytes, epithelial cells, enzymes, bacteria and various other materials depending on the targeted mucosal route. The vaginal epithelium is usually considered as a mucosal surface, albeit the absence of goblet cells and the lack of direct release of mucin, which often characterise mucosal surfaces.[21] Bioadhesive polymers with a high swelling index in aqueous environment are often used to produce controlled release formulations. Commonly employed bioadhesive polymers in pharmaceutical preparations can be derived from synthetic and from natural sources. Examples of bioadhesive polymers include cellulose derivatives such as hydroxypropyl cellulose, hydroxypropyl methylcellulose (HPMC), carboxymethyl cellulose; polyacrylic acid derivatives such as Carbopol®934P (C934P), polycarbophil; and natural gums such as xanthan gum (XG) and guar gum.[22–25]

Here, it is hypothesized that increased solubility of NT will lead to the reduction in NT dose sufficient for producing antimycotic activity may result in formulations with lower toxicity. The full characterisation of NT-γCyD confirmed that formation of inclusion complexes. The development of vaginal tablets containing NT with enhanced bioavailability is therefore warranted for treatment of vaginal *Candida* infections. By employing the NT-γCyD inclusion complexation approach, it is hoped that controlled release and bioadhesive vaginal tablets with improved NT solubility, stability, dissolution properties and reduced side effects can be realised. Accordingly, bioadhesive vaginal tablet containing NT-γCyD complexes present as an admixture with bioadhesive polymers such as XG, HPMC and C934P has been prepared.

MATERIALS AND METHODS

Materials

Natamycin (M_w: 666 Da) was obtained from Shenzhen SED Industry Co., Ltd., China.

γ-Cyclodextrin (M_w: 1297 Da) and xanthan gum (XG 200) was purchased from ISP, Wayne, New Jersey. Hydroxypropylmethyl cellulose K100M was purchased from Colorcon, Kent, UK. Carbopol®934 was purchased from BF Goodrich, Cleveland, Ohio. RPMI 1640 medium, L-glutamine, 3(N-morpholino) propanesulfonic acid and Methanol was purchased from Sigma-Aldrich, St. Louis, Missouri. Dimethyl sulfoxide was purchased from Aldrich, Munich, Germany. All other reagents were of analytical grade and used without further purification.

Phase Solubility Studies

Solubility studies were carried out according to the method reported by Higuchi and Connors.[26] Briefly, an excess of NT (20 mg) was added to 10 mL aqueous solution containing increasing amounts of γCyD (from 0 to 0.012 M). The suspensions were shaken at $25 \pm 1^{\circ}C$ for 7 days. After equilibrium was attained, the samples were assayed for the total dissolved NT content using high-performance liquid chromatography (HPLC). Each experiment was carried out in triplicate. The stability constant (K_s) according to the hypothesis of 1:1 stoichiometric ratio of NT-γCyD complex was calculated from the phase solubility diagram using the following equation:

$$K_s(1:1) = \frac{\text{Slope}}{\text{So}(1 - \text{Slope})} \quad (1)$$

where S_o is the equilibrium solubility of the NT in water.

High-Performance Liquid Chromatography (HPLC) Method

The HPLC method reported by Koontz et al.[27] was used with some modifications for solubility, stability and *in vitro* drug release studies.

A Shimadzu LC 10A liquid chromatography (Kyoto, Japan), consisting of a model LC 10 AT solvent delivery system and a Rheodyne injection system with a loop of 20 μL was used at 25°C. The components were detected at 304 nm by using model SPD 10A UV detector. Separation was performed on C18 column with particle size of 5 μm (Supelco, Discovery® C18, 250×4.6 mm). The mobile phase was methanol–water (65:35) at a flow rate 1.0 mL min^{-1}. The retention time of NT was 5.5 min. Under these conditions, the γCyD and polymers which were used to prepare inclusion complex and tablets, respectively, did not interfere with the NT peaks.

Preparation of NT-γCyD Inclusion Complex

A complex of NT and γCyD in the solid state was prepared by the lyophilisation method. The required 1:1 stoichiometric amount of NT was added to an aqueous solution of γCyD. As described previously, the suspensions were shaken at $25 \pm 1^{\circ}C$ for 7 days and finally filtered through 0.45 μm membrane filter. Filtrates were frozen at $-40^{\circ}C$ and lyophilised in a freeze dryer (Lyovac GT-2, Leybold-Heraeus, Cologne, Germany) to produce the NT-γCyD complexes. The NT content in the NT-γCyD complexes was determined by the HPLC method.

Nuclear Magnetic Resonance (NMR) Analysis

NMR spectra of NT, γCyD and their complex were acquired using Bruker Avance spectrometer operating at nominal ^1H frequency of 500 MHz and equipped with 5 mm BBO probe including *Z*-axis pulse field gradients. Spectra of samples in D_2O were acquired at 298 K. The partial assignments of the ^1H spectra were achieved using 2D [^1H,^1H] COSY and 2D [^1H,^1H] NOESY employing mixing times between 400 ms and 1200 ms.[28] All spectra were processed using TOPSIN 1.3. Chemical shifts were referenced to the residual HOD signal at 4.7 ppm (^1H).

Molecular Modelling Studies

All molecular modelling calculations were carried out using Macromodel 9.11[29] and MMFFs force field.[30] The implicit solvent representation was achieved using generalised Born/surface area continuum (GB/SA) method[31] with a constant dielectric function ($\varepsilon = 1$). An extended nonbonded cut off (van der Waals: 8 Å; electrostatics: 20 Å) was used. The complex modelling was carried out using conformational search and torsional sampling (MCMM). The NT was positioned in four different starting configurations in respect to the initial model of γCyD and 5000 steps of Monte Carlo conformational search were carried out for each configuration. The ensembles of generated structures were clustered and analysed using the cluster analysis program Xcluster.[32]

Differential Scanning Calorimetric (DSC) Analysis

Thermal analysis using a DSC method was carried out on NT, γCyD and NT-γCyD complex

employing differential scanning calorimeter (DSC823e, Mettler-Toledo, Columbus, Ohio). Samples (~5 mg) were run at a heating rate of $10^{\circ}C\,min^{-1}$ over a temperature range 25–360°C in a dynamic nitrogen atmosphere.

Fourier Transform Infrared (FTIR) Analysis

FTIR spectrum of the NT, γCyD and NT-γCyD complex were measured in potassium bromide disks using a Perkin Elmer Model 1600 FTIR spectrometer (Waltham, Massachusetts).

Powder X-Ray Diffraction (PXRD) Analysis

PXRD was used to assess the degree of crystallinity of NT, γCyD and NT-γCyD complex at an ambient temperature using a Philips PW 3830 X-ray diffractometer (Lelyweg, The Netherlands) using the Ni-filtered Cu Kα radiation in the following conditions: Cu Kα ($\lambda = 1.5418$ Å) radiation; angular range $2^{\circ} < 2\theta < 35^{\circ}$; step size: 0.02°; time per step: 2 s; scan speed: 0.01°.

Scanning Electron Microscopy (SEM) Analysis

SEM micrographs of the NT, γCyD and NT-γCyD complex were obtained using a scanning electron microscope (JXA 840A, JEOL Ltd., Tokyo, Japan). The samples were coated with gold under an argon atmosphere at room temperature yielding a film thickness of 5 nm.

Stability of NT-γCyD Complex

Stability studies of NT and NT-γCyD in distilled water were performed in a stability storage cabinet (5600-S, Vindon Scientific, Lancashire, UK) at $37 \pm 0.5^{\circ}C$ for 15 days. At predetermined time intervals from 0 to 15 days, samples were collected and analysed by the HPLC method. Each experiment was performed six times.

NT and NT-γCyD Susceptibility Testing of *Candida* Species

The broth microdilution method was performed according to the guidelines of National Committee for Clinical Laboratory Standards (NCCLS) document M27-A.[33] Stock solutions (16 $mg\,mL^{-1}$) of NT and NT-γCyD complex were prepared in dimethyl sulfoxide (DMSO) and in sterile distilled water respectively. Stock solutions were diluted with RPMI 1640 medium (with glutamine) supplemented with glucose (2% w/v) and buffered to pH 7.0 with 0.165 M 3(N-morpholino) propanesulfonic acid (MOPS). Various concentrations of NT and NT-γCyD (concentrations ranging from 16 to 0.0313 $\mu g\,mL^{-1}$) were added to yeast (*Candida* Spp.) suspensions prepared in RPMI 1640 medium (final inoculum concentration of 1.0×10^4 to 5.0×10^4 $CFU \times mL^{-1}$) in a 96-well round bottom microtitre plate. After incubation for 48 h at 35°C, the plates were read at 570 nm using Dynex MRX microplate reader (Dynex Technologies, Chantilly, Virginia). The minimum inhibitory concentration (MIC_{90}) of NT and NT-γCyD complex was defined as the lowest concentration at which there was 90% inhibition of growth compared with that of a control. Each experiment was carried out in triplicate.

Preparation of Vaginal Bioadhesive Tablets

Vaginal mucoadhesive tablet formulations containing NT-γCyD were prepared accordingly (Tab. 2). NT-γCyD and various bioadhesive polymers were separately passed through the 250 μm sieve and mixed in the cubic mixer (Erweka, Heusenstamm, Germany) for 20 min. Magnesium stearate as a lubricant (0.5% w/w) was added and additionally mixed for 5 min in the same mixer. Powder blend was compressed in a single punch tablet press (Korsch EK-0, Germany) using 0.55 cm flat faced punch applying 100 $kgf\,cm^{-2}$ compression forces.

Swelling and Matrix Erosion Studies

Water uptake of the tablets containing NT-γCyD was determined gravimetrically in a lactate buffer (pH 5). Each tablet was weighed (W_D) and immersed separately in the lactate buffer (pH 5) at $37 \pm 0.1^{\circ}C$. At predetermined time intervals from 0.25 to 24 h, the tablets were removed from the media, blotted with the filter paper to remove excess water and reweighed (W_S). The swollen tablets were dried at 60°C for 24 h in a ventilated oven, kept in a desiccator for 48 h and reweighed (W_E). Each experiment was performed in triplicate. The swelling index (SI) and matrix erosion (ME) were calculated as follows:

$$SI = \frac{(W_S - W_D)}{W_D} \tag{2}$$

$$ME(\%) = \frac{100(W_D - W_E)}{W_D} \tag{3}$$

Bioadhesion Studies

TA-XT*Plus* Texture analyser (Stable microsystems, Haslemere, UK) equipped with a 5 kg load cell was used for mucoadhesion test. Freshly excised cow vaginal mucosa was frozen at −30°C. A section that possessed 2 mm thickness was taken from the inner part of the surface of the frozen vaginal mucosa and fitted on the bioadhesion test rig and then 50 μL of cow vaginal mucus was applied on the surface of the tissue before the experiment. Mucus was collected from inner surface of the vagina using a spatula after excision, then frozen at −30°C and adjusted to 37°C during the experiment. The tablet was attached to the lower end of the cylindrical probe (P10 Perspex, *θ:* 10 mm) with double-sided adhesive tape. The tests were done at 37°C. The probe was lowered onto the surface of the tissue with a constant speed of 1 mm s^{-1} and contact force of 1 N applied. After remaining in contact for 30 s, the probe was then moved vertically upwards at a constant speed of 1 mm s^{-1}. Work of adhesion (mJ cm^{-2}) and peak detachment force (N cm^{-2}) were calculated from force-distance plot using *Texture Exponent 4.0.4.0* software package of the instrument. Each experiment was carried out in triplicate.

In Vitro Drug Release Studies

Drug release studies from mucoadhesive tablet formulations were performed using rotating basket method at 75 rpm.[34] A dissolution medium composed of 500 mL lactate buffer (pH 5) at 37 ± 0.5°C was used. An aliquot of sample was withdrawn at hourly intervals from 0 to 8 h and analysed by HPLC. Each experiment was performed in triplicate.

Mechanism of Drug Release

To evaluate the mechanism of drug release from mucoadhesive tablets, drug release data were plotted in Korsmeyer–Peppas semi-empirical model.[35]

$$\frac{M_t}{M_\infty} = k_{KP} t^n \quad (4)$$

where M_t/M_∞ is the fractional amount of drug release at time t, k_{KP} is the release rate constant and n is the diffusional exponent that characterises the type of the release mechanism during the dissolution process.[36] The values n and k_{KP} were estimated by linear regression of log (M_t/M_∞) versus log t. For the case of cylindrical tablets,[37] $n = 0.45$ corresponds to a Fickian diffusion release (Case-I diffusional), $0.45 < n < 0.89$ to a non-Fickian or anomalous diffusion, $n = 0.89$ to a Case-II transport or typical zero-order release and $n > 0.89$ to a super Case-II transport.

Statistical Analysis

Data obtained from each experiment were subjected to statistical analysis using one-way analysis of variance (ANOVA) followed by Newman–Keuls multiple comparisons test. $p < 0.05$ was considered to be indicative of significance.

RESULTS AND DISCUSSION

Studies on the NT- γCyD Complex

Phase Solubility Studies

The phase solubility diagram for the complex formation between NT and γCyD is presented in Figure 1. The correlation coefficient (r^2) of the phase solubility diagram was 0.999. It has revealed a linear relationship between the solubility of NT and concentration of γCyD and the solubility of NT increased significantly by 2.6 times due to the increase in γCyD/NT ratio (w/w) from 2.5 to 7.5 ($p < 0.05$). It is clearly observed that the solubility diagram of NT in the presence of γCyD can be classified as the A_L type,[26] generally ascribed to the formation of a soluble

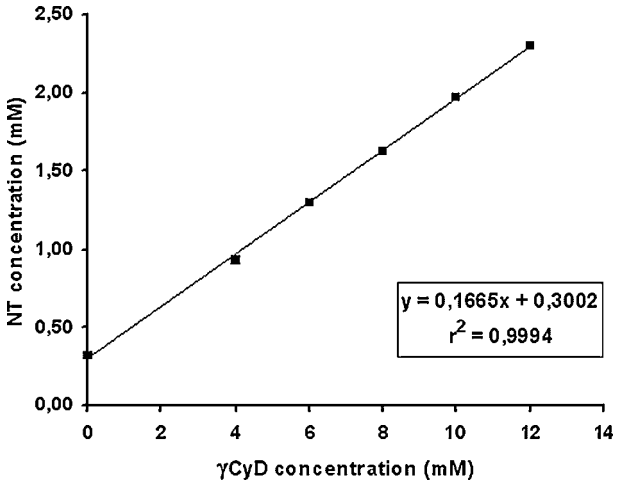

Figure 1. Dependence of NT solubility on the concentration of γCyD ($n = 3$).

NT-γCyD 1:1 complex.[27] The apparent 1:1 stability constant, K_s, was calculated based on the phase solubility diagram, using the equation described by Higuchi and Connors.[26] The apparent stability constant (K_s) of the complex formed can be obtained from the slope of the straight line and the K_s value was found to be 667 M^{-1}, indicating a stable association of NT and γCyD.[38] It was demonstrated that the slope of phase solubility diagram in a drug/cyclodextrin system could be linear in spite of enhanced solubility occurring through both inclusion and noninclusion process such as complex aggregates or micellar formation.[39]

Characterisation of the NT-γCyD Inclusion Complex

Characterisation of the solid binary system of NT and γCyD was carried out using ^{1}H-NMR, DSC, FTIR and PXRD studies.

A possibility of inclusion complex formation has been examined using a set of 1D and 2D NMR ^{1}H spectra of NT, γCyD and their inclusion complex prepared. Although the pure NT has the low solubility in aqueous media, all the spectra were acquired in D_2O to observe effects of mixing of NT with the γCyD. The 1D ^{1}H spectrum of pure NT is very weak and the spectrum was acquired using solvent suppression to remove the residual water peak at 4.7 ppm (Fig. 2a). Most of the peaks in the spectrum do not overlap with the peaks of γCyD (Fig. 2c), however, two main characteristic regions were used to prove the formation of the inclusion complex: 1.0 to 1.1 ppm (two methyl groups, C27-H_3 and C6′-H_3) and 5.7 to 6.35 (hydrogen atoms of CH groups on carbons 2, 3 and 16–23).

Partial assignment of ^{1}H spectra of the inclusion complex was achieved using a combination of COSY and NOESY peaks; γCyD [C*1-H 5.05, d, 1H; C*2-H 3.60, dd, 1H; C*3-H 3.87, t, 1H; C*4-H 3.53, t, 1H; C*5-H 3.78, overlap, 1H; C*6-H 3.81, overlap, 2H]. Partial assignment of NT [C2-H 5.96, d, 1H; C3-H 6.56, dd, 1H; C4-H 3.18, bd, 1H; C4-H 3.18, bd, 1H, C14-H 2.07, C27-H_3 1.29, d, 3H].

In the NMR spectrum of the inclusion complex, the signal of C27-H_3 of NT thus being in the

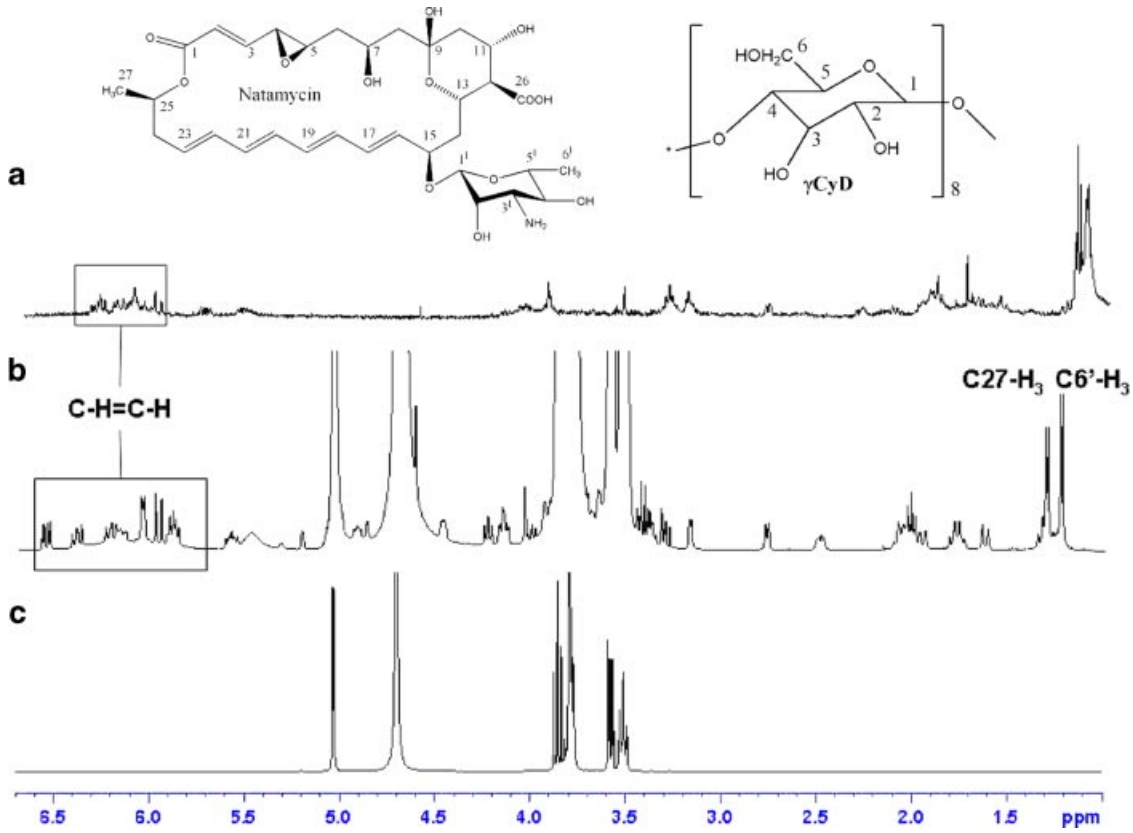

Figure 2. 1D—^{1}H spectra of (a) NT (b) NT-γCyD and (c) γCyD in D_2O at 298K. The spectrum of NT was acquired using solvent suppression to remove residual HOD peak at 4.7 ppm.

hydrophobic cavity of γCyD has the higher chemical shift (1.32 ppm) than corresponding peak in pure NT. Interestingly, the CH peaks have dispersed over the region between 5.7 and 6.6 ppm, indicating their exposure to different environments due to interactions of CH groups with the different parts of the γCyD cavity.

Further evidence of the complexation was observed in the NOESY spectra of the inclusion complex (Fig. 3). The most prominent crosspeak that indicate the complex formation was found at 1.30; 3.81 ppm (box A), which indicates the close proximity of C27-H_3 group of NT and C^*6-H_2 groups of γCyD. Additionally, there were further crosspeaks that indicate interaction between γCyD and NT CH groups of double bonds (box B) and aliphatic CH_2 (box C).

These findings were supported by molecular modelling of the inclusion complex. The conformational ensembles of NT and γCyD inclusion complexes that have been generated in this study were calculated using the MacroModel V9.11 suite software. Nonbonded interactions within 8 Å for van der Waals' and 20 Å for electrostatic interactions were included in all calculations. All the molecules were built in the builder subprogram of the Macromodel computational package. Monte Carlo Multiple Minimum (MCMM) conformational searching with 2000 steps was employed to find the lowest energy structures for the complex starting from four different configurations. The combination of MMFFs force field and GB/SA implicit representation of water as the solvent were used.

The lowest energy conformer of the NT-γCyD inclusion complex that supports NMR evidence is shown in Figure 4. The C27-H_3 is 2.97 Å away from the C^*6-H_2 of one of the monomers (Fig. 4a), which is within the range that could cause nOe effect responsible for the crosspeak in the NOESY spectrum assigned at 1.30; 3.81 ppm. A segment of the CH groups of double bonds are partially set in the cavity, while the other part is exposed to the solvent, and these two different environments are responsible for dispersion of corresponding signals when compared to free NT. In addition to the hydrophobic interaction that led to the formation of the inclusion complex, the intermolecular hydrogen bond could be formed between hydroxyl group on the C^*2 of the γCyD and the hydroxyl group on the C7 of NT.

Thermal analysis was carried out on NT, γCyD and NT-γCyD. Respective DSC thermogram in Figure 5 clearly indicated that NT showed two subsequent broad endothermic peaks at 97.8 and 132.1°C associated with water loss, sharp

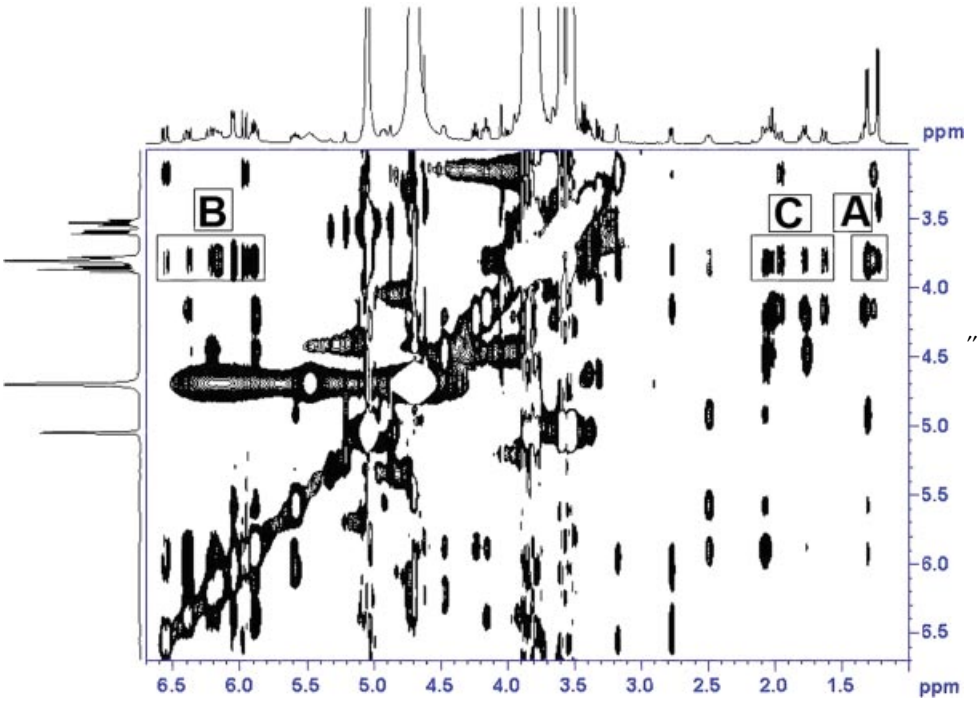

Figure 3. 2D [$^1H^1H$] NOESY spectrum of NT-γCyD in D_2O (intermolecular nOe crosspeak are framed). The spectrum was acquired at 298 K using mixing time of 400 ms.

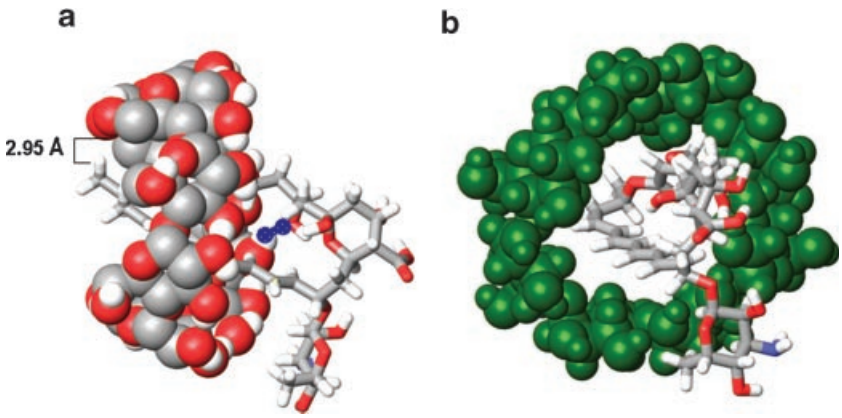

Figure 4. Lowest energy conformation of the NT-γCyD inclusion complex, modelled using MCMM conformational search. (a) side view of the complex with NT as sticks and γCyD in CPK representation (nonpolar hydrogens of γCyD omitted for clarity, blue line depicts intermolecular hydrogen bond). (b) Front view of the complex (γCyD is shown in green colour).

endothermic peak at 215.8°C which is due to the melting of NT and a characteristic sharp exothermic peak at 224.8°C which represents the crystallisation point of NT. On the other hand, thermal profile of γCyD showed endothermic peaks at 89.1°C and 295.7°C which were due to its dehydration and decomposition, respectively.[40] However, the exothermic and endothermic peaks of NT were not observed in the thermogram of NT-γCyD inclusion complex. The disappearance of these peaks in the thermogram of the complex can be indicative of a tight interaction between NT and γCyD, drug amorphization and inclusion complex formation.[40,41]

The FTIR spectra of NT-γCyD were compared to spectra of the NT and γCyD (Data is not shown). Characteristic bands of NT were observed at 3277 cm^{-1} ($-NH_3^+$ deformation), 3016 cm^{-1} (=CH vibration), 1715 cm^{-1} (−C=O vibration), 1677 cm^{-1} (C−C=C−C vibration, in the aromatic ring), 1571 cm^{-1} (CH=CH stretch; COO^-), 1266 cm^{-1} (C−O−C epoxy), 1142 cm^{-1} (=C−O−C= vibration), 1106 cm^{-1} (C–OH asymmetric vibration). The NT-γCyD showed only two NT characteristic peaks as 1677 cm^{-1} (C−C=C−C vibration, in the aromatic ring) and 1142 cm^{-1} (=C−O−C= vibration). The disappearance of the other NT characteristic peaks could be attributed to the formation of NT interaction with γCyD that result in the absence of these peaks.

^{1}H-NMR, FTIR and DSC results suggested that a strong interaction between the NT and γCyD occur in the inclusion complex, which may have been linked to the formation of NT-γCyD complex.

CyD complexation is known to alter the crystallinity of the drug, producing a drug with an amorphous nature.[42] PXRD was performed on NT, γCyD and NT-γCyD complex samples. It was found that the presence of several different peaks in the NT diffractogram indicated that NT and γCyD were in a crystalline and semi-crystalline form respectively (Fig. 6). In contrast, the diffraction pattern of the NT-γCyD differed considerably from that of NT or γCyD alone (Fig. 6). NT-γCyD complex exhibited considerable diminution of the diffraction peaks, thereby proving that NT-γCyD complex has an amorphous structure.

SEM photographs also revealed that the shape and size of the NT-γCyD inclusion complex was completely different from the NT alone and γCyD alone (Fig. 7). The commercial γCyD particles were tubular in shape with slightly diffused outline and cracks were observed on the surface. In contrast, NT-γCyD complexes were composed of flat and thin plate-like structures. The drastic change of the particles' shape and aspect in complex was indicative of the presence of a new solid phase, suggesting the existence of a single phase, supporting the results of the PXRD studies.[43]

Effect of γCyD Complexation to the Chemical Stability of NT in Aqueous Solution

CyDs improves the stability of labile drugs against dehydration, hydrolysis, oxidation and photodecomposition and thus CyDs increase the shelf life of drugs. CyD complexation encapsulates labile

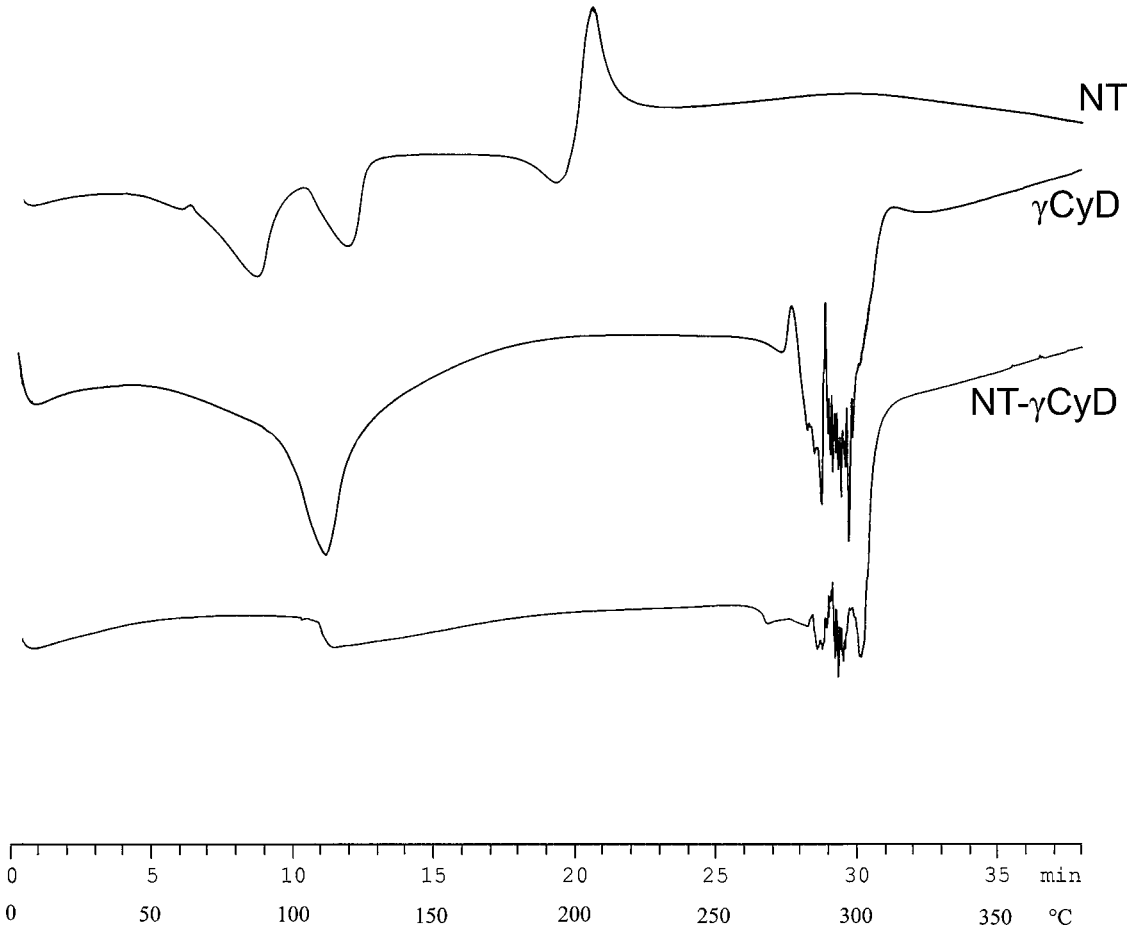

Figure 5. Differential scanning calorimetry thermograms of NT, γCyD and NT-γCyD inclusion complex. Analyses were carried out at a heating rate of $10^{\circ}C\,min^{-1}$ over a temperature range of 25–400°C under an atmosphere of nitrogen.

drug molecules at the molecular level, protecting the drug molecules from degradation.[44] NT is poorly soluble in water and in acidic solutions and the drug undergoes rapid degradation under these conditions.[27,45] The stability of NT was observed by incubating NT and NT-γCyD aqueous solutions in a stability cabinet at $37 \pm 0.5^{\circ}C$ for 15 days. The amounts of NT remaining in NT and NT-γCyD solutions after 15 days were determined as $60.5 \pm 4.2\%$ and $97.9 \pm 3.5\%$, respectively ($p < 0.01$) (Fig. 8). An apparent first-order degradation process was observed for both NT and NT-γCyD. Degradation rate constant (k_{obs}), estimated half life ($t_{1/2}$) and shelf life (t_{90}) of the NT and NT-γCyD were calculated from the slopes of the semi-log plots of concentration of drug remaining versus time by linear regression analysis (Data is not shown). The correlation coefficients (r) observed for both NT and NT-γCyD were greater than 0.99. k_{obs} for NT and NT-γCyD was determined to be $1.51 \times 10^{-3}\ h^{-1}$ and $1.59 \times 10^{-4}\ h^{-1}$, respectively. From these results, $t_{1/2}$ and t_{90} were determined to be 458 and 69 h for NT and 4358 and 524 h for NT-γCyD, respectively ($p < 0.01$). The results of the stability tests indicated that γCyD complexation has significantly increased the stability of NT ($p < 0.01$).

NT and NT-γCyD Complex Susceptibility Testing of Candida Species

CyDs have been used to ameliorate the toxicity caused by drugs.[15] By using CyDs as a drug solubiliser, the dose required for optimum therapeutic activity and hence its toxicity may be reduced. Importantly, the increase in drug efficacy and potency can improve the bioavailability and clinical usefulness of the solubilised drug.[46,47]

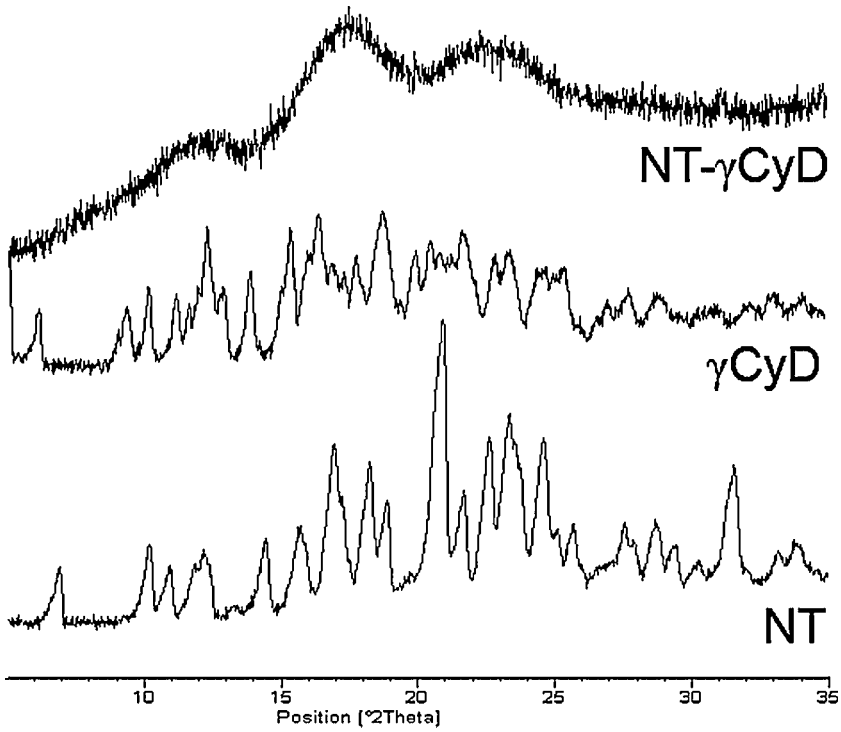

Figure 6. Powder X-ray diffractograms of NT, γCyD and NT-γCyD inclusion complex. Test conditions: Cu Kα ($\lambda = 1.5418$ Å) radiation; angular range $2^\circ < 2\theta < 35^\circ$; step size: 0.02°; time per step: 2 s; scan speed: 0.01°.

The biological activity of NT was compared to that of its NT-γCyD complex by performing MIC studies using the broth microdilution method. Interestingly, the standard ATTC bacterial strains (*Candida* spp.) showed similar susceptibility to free NT and its γCyD complex (MIC_{90NT} and $MIC_{90NT\text{-}\gamma CyD} \leq 0.0313$ μg mL^{-1}) ($p > 0.05$). By forming inclusion complex with γCyD, NT's efficacy as an antifungal agent was increased. The NT-γCyD complex, exhibiting the same MIC value as NT alone, with a lower NT amount (1.66% w/w), could reduce the side effects of NT.

Studies on the Vaginal Mucoadhesive Tablet Formulations

Vaginal mucoadhesive tablets containing NT-γCyD complex were prepared using XG, HPMC and C934P and the mixtures of these polymers with different ratios as bioadhesive polymers. All the formulations contained the same amount of the NT-γCyD (25 mg) as an active agent and magnesium stearate (0.5% w/w) as a lubricant (Tab. 2). Swelling, matrix erosion, bioadhesion and drug release studies were conducted on the mucoadhesive tablets.

Swelling and Matrix Erosion Studies

The rate of uptake of water by a given polymer in physiologically relevant fluids was critical in the interpretation of its bioadhesion property.[48] Fabregas and Garcia[49] found a relationship between the swelling rate and the *in vitro* bioadhesion force. The swelling of the polymer can increase the adhesive surface for maximum contact with mucosal surface and also induce the interpenetration of polymer into the mucus layer, resulting in the possibility of bioadhesion with the desired mucosal surface.[25] While some reports showed a direct relation between swelling and mucoadhesion, however, others did not.[50–52] Baloglu et al.[51] reported that excessive swelling of the bioadhesive polymer led to an abrupt drop in the adhesive strength.

The pH of the microenvironment of the vaginal mucosa has been reported to be of an acidic pH (pH 4.5–5.5).[17] Therefore, swelling studies were carried out in pH 5 lactate buffer. The results obtained from swelling studies are presented in Figure 9a and b. Visual observation denoted that the matrices appeared swollen from the initial stages of the swelling studies whereby a viscous

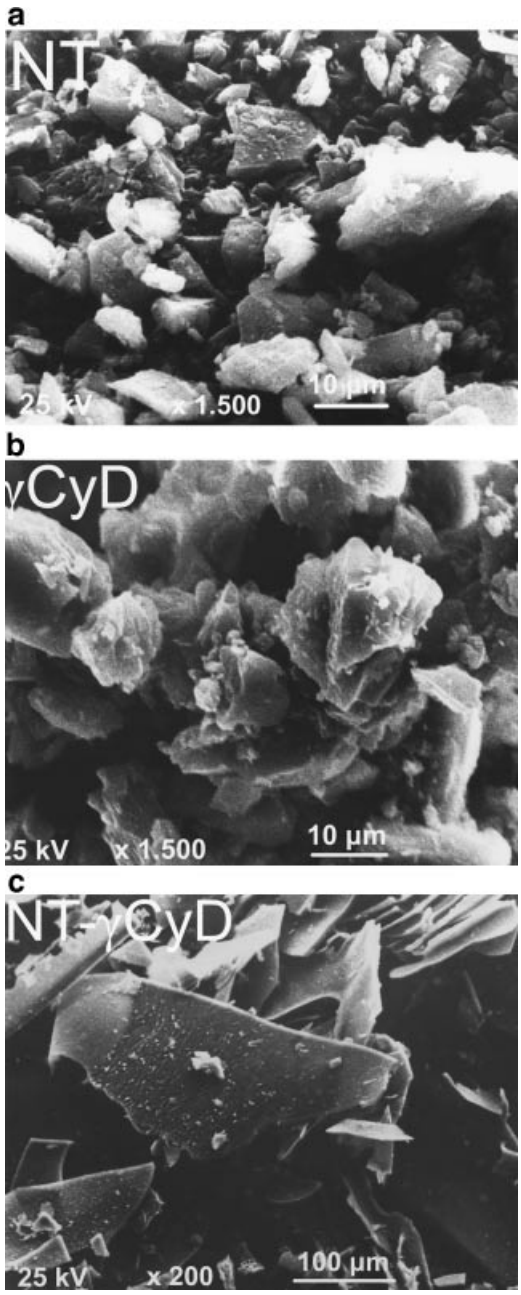

Figure 7. Scanning electron micrographs of (a) NT, (b) γCyD and (c) NT-γCyD inclusion complex.

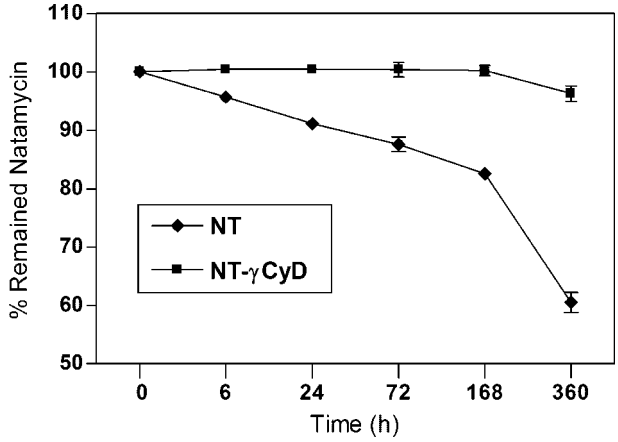

Figure 8. Stability of NT and NT-γCyD inclusion complex in distilled water ($n = 6$).

gel mass was created when they came into contact with the liquid. The initial swelling at 15 min of all mucoadhesive tablets varied between 1.2 (C) and 2.5 (X1). After 8 h incubation, the formulations containing HPMC alone (H1) or XG alone (X1) were found to have the highest swelling index of 14.9 ± 0.4 and 15.8 ± 0.6, respectively, compared to other tablet formulations. Upon addition of C934P at increasing amounts, the swelling index of the formulations containing HPMC and the formulations containing XG at 24 h also reduced from 21.6 ± 0.06 (H2) to 19.0 ± 0.4 (H6) ($p > 0.05$) and 22.4 ± 0.5 (X2) to 19.8 ± 0.4 (X6) ($p > 0.05$), respectively, albeit lower than that of HPMC alone (H1, SI_{H1}: 22.8 ± 0.9) ($p > 0.05$) and XG alone (X1, SI_{X1}: 23.7 ± 0.8) ($p > 0.05$) with the exception of C934P alone (C, SI_C: 18.6 ± 0.5) ($p > 0.05$) which has the lowest swelling index in all the formulations tested. HPMC and XG are neutral polymers and swelling in water. C934P is anionic and highly cross-linked polyacrylic acid derivative. The swelling index of C934P was reported to be less than that of neutral polymers.[53] Below pH 5, the carboxyl groups of C934P are in the unionised state, thereby it has poor water uptake ability justifying its low swelling index at such pH.[54]

Addition of HPMC and XG has altered the erosion of the matrix. After incubation of the mucoadhesive tablets for 24 h in a pH 5 lactate buffer, the formulation containing XG alone (X1, ME_{X1}: $66.00 \pm 5.43\%$) was found to have a higher matrix erosion than that of HPMC alone (H1, ME_{H1}: $58.96 \pm 9.63\%$) ($p > 0.05$) and that of C934P alone (C, ME_C: $10.56 \pm 3.23\%$) ($p < 0.05$) (Fig. 10). Upon addition of C934P at increasing amounts, the matrix erosion of the HPMC tablets and XG tablets were reduced from $34.76 \pm 6.59\%$ (H2) to $12.32 \pm 2.52\%$ (H6) ($p < 0.05$) and $42.24 \pm 6.72\%$ (X2) to $16.28 \pm 4.32\%$ (X6) ($p < 0.05$), respectively, albeit lower than that of HPMC alone (H1) or XG alone (X1) with the exception of C934P alone (C) with the lowest matrix erosion amongst all the formulations tested. However, when formulations prepared with the same amount of

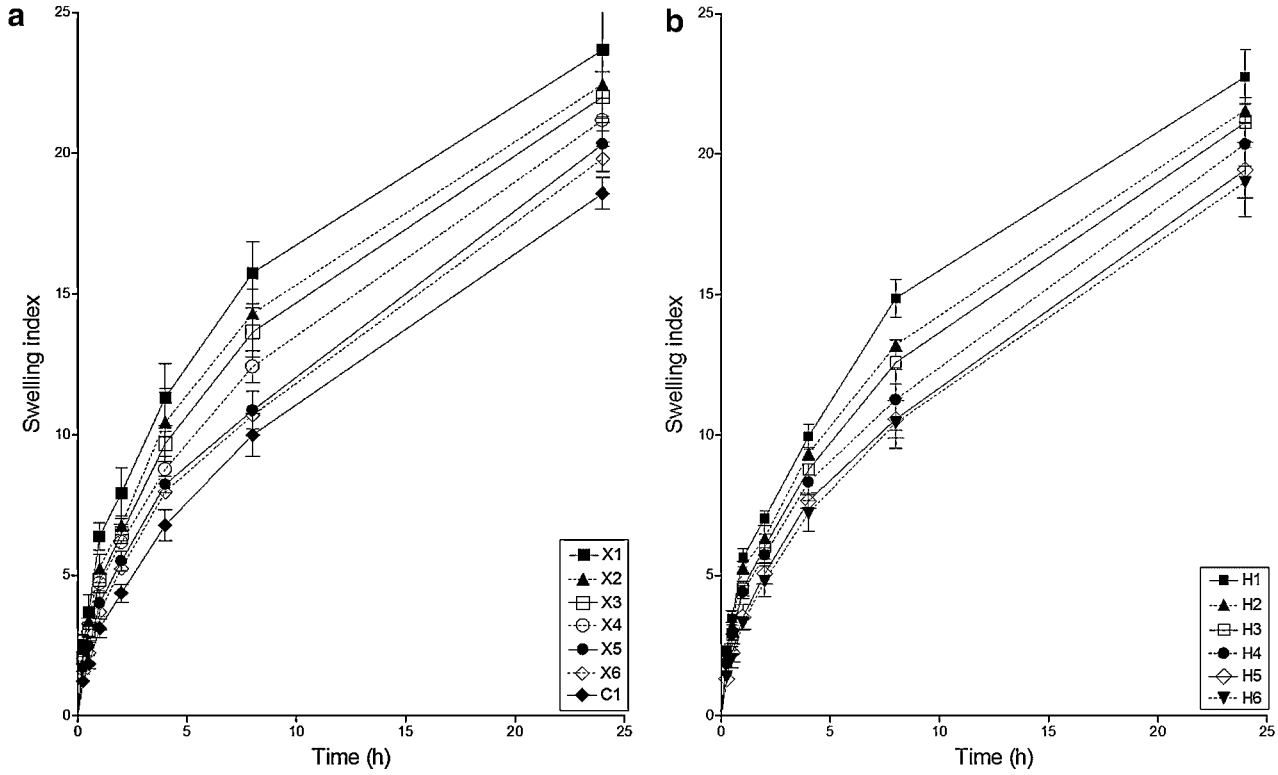

Figure 9. Swelling index of the mucoadhesive tablets prepared using XG, HPMC, C934P and the mixtures of these polymers at different ratios. Each tablet formulation contains same amount of NT-γCyD (25 mg) as an active and magnesium stearate (0.5% w/w) as a lubricant ($n = 3$).

HPMC and XG were compared, a slightly lower matrix erosion were observed for HPMC containing formulations ($p > 0.05$).

When the results of the swelling index and matrix erosion studies were compared, there seemed to be a relationship between swelling and erosion of the tablets whereby the formulations with the highest swelling index also have recorded the highest matrix erosion.

Bioadhesion Studies

The mucoadhesion test was performed to measure the adhesive strength of the formulated tablets to the vaginal mucosa. The mucoadhesion test results (work of adhesion and peak detachment force) of the formulations prepared were given in Table 1.

The mucoadhesion of the formulations were affected by the nature of the polymer. It was evident that the work of adhesion (WA) and the peak detachment force (PD) values increased in the presence of C934P in the formulations. The highest work of adhesion (2.900 ± 0.134 mJ cm^{-2}) and peak detachment force (2.471 ± 0.118 N cm^{-2}) were recorded in the formulation containing the highest amount of C934P (C), whereas the work of adhesion and the peak detachment force were gradually decreased with increasing amounts

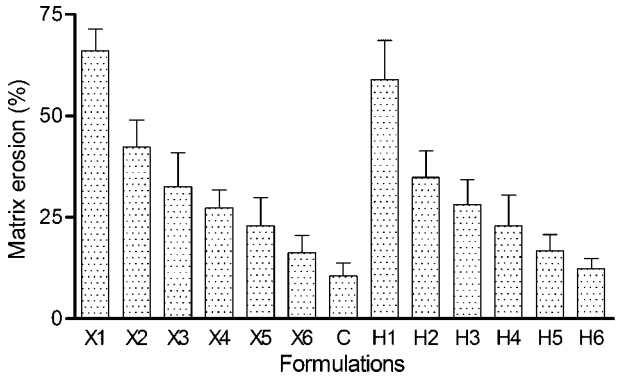

Figure 10. Matrix erosion of the mucoadhesive tablets prepared using XG, HPMC, C934P and the mixtures of these polymers at different ratios after 24 h. Each tablet formulation contains same amount of NT-γCyD (25 mg) as an active and magnesium stearate (0.5% w/w) as a lubricant ($n = 3$).

Table 1. Compositions and Bioadhesion Properties of Vaginal Mucoadhesive Tablet Formulations Containing NT-γCyD Inclusion Complex ($n = 3$)

Formulation	NT-γCyD (mg)	Xanthan Gum (mg)	HPMC (mg)	Carbopol®934P (mg)	Work of Adhesion ($mJ\,cm^{-2} \pm SD$)	Peak Detachment Force ($N\,cm^{-2} \pm SD$)
X1	25	25	—	—	0.598 ± 0.068	0.681 ± 0.089
X2	25	20	—	5	0.722 ± 0.162	0.698 ± 0.154
X3	25	15	—	10	0.984 ± 0.126	1.014 ± 0.162
X4	25	12.5	—	12.5	1.252 ± 0.195	1.103 ± 0.199
X5	25	10	—	15	1.527 ± 0.213	1.286 ± 0.364
X6	25	5	—	20	2.277 ± 0.098	1.933 ± 0.135
C	25	—	—	25	2.900 ± 0.134	2.471 ± 0.118
H1	25	—	25	—	0.705 ± 0.076	0.760 ± 0.068
H2	25	—	20	5	0.790 ± 0.087	0.810 ± 0.056
H3	25	—	15	10	1.249 ± 0.304	1.199 ± 0.341
H4	25	—	12.5	12.5	1.569 ± 0.167	1.441 ± 0.132
H5	25	—	10	15	1.865 ± 0.211	1.835 ± 0.264
H6	25	—	5	20	2.497 ± 0.121	2.288 ± 0.213

of XG (X1, WA_{X1}: 0.598 ± 0.068 $mJ\,cm^{-2}$, PD_{X1}: 0.681 ± 0.089 $N\,cm^{-2}$; X6, WA_{X6}: 2.277 ± 0.098 $mJ\,cm^{-2}$, PD_{X6}: 1.933 ± 0.135 $N\,cm^{-2}$) and HPMC (H1, WA_{H1}: 0.705 ± 0.076 $mJ\,cm^{-2}$, PD_{H1}: 0.760 ± 0.068 $N\,cm^{-2}$; H6, WA_{H6}: 2.497 ± 0.121 $mJ\,cm^{-2}$, PD_{H6}: 2.288 ± 0.213 $N\,cm^{-2}$) in the formulations ($p < 0.05$).

The work of adhesion of the formulations with highest C934P:HPMC (H6) and C934P:XG (X6) ratios was as 3.5- and 3.8-fold higher than the work of adhesion of the plain HPMC and XG tablets, respectively ($p < 0.05$). In accordance with the work of adhesion results, 3.5- and 2.8-fold increase was detected in the force required to detach the H6 and X6 formulations from the vaginal mucosa when compared to tablets prepared with HPMC and XG alone, respectively ($p < 0.05$) (Tab. 1).

In this study, no correlation was observed between swelling and mucoadhesion of tablet formulations. The comparatively weak mucoadhesion of the nonionic polymers HPMC and XG may be attributed to the absence of a proton-donating carboxyl groups which reduce its ability for the formation hydrogen bonds.[55] It has been reported that the polyanions such as C934P adhere strongly to the mucus compared to the nonionic polymers such as HPMC and XG.[54,56] At the high pH values, carboxylic acid groups of the C934P are ionized at a high percentage, this increases the swelling capability of C934P and decreases the hydrogen binding and mucoadhesion. At pH 5 and below, C934P cannot be charged electrostatically and exhibits lower swelling ability due to the existence of more than 90% of the carboxylic acid groups of the polymer in nonionized form.[57] Hence, the polymer binds directly to polysaccharides or proteins with hydrogen bonds and this increases the mucoadhesion. Thus, increasing amount of C934P in the formulation was significantly increased the mucoadhesion.

In Vitro Drug Release Studies

The release of NT as a function of time from different mucoadhesive tablet formulations is given in Figure 11. Drug release was recorded at predetermined hourly intervals up to 8 h. The differences observed in drug release as a function of the type of polymer were related to the differences in water absorption and erosion behaviour of the tablets in the dissolution medium. By reducing the swelling index and matrix erosion of the mucoadhesive tablets, the drug release was also reduced. The formulations containing the highest amount of XG (X1) and HPMC (H1) were found to have a higher drug release due to their high swelling index and matrix erosion than that of C934P alone (C) (Fig. 11). Upon addition of C934P at increasing amounts, the drug release rate was reduced accordingly. Previously, the mucoadhesive properties of polyacrylic acids have resulted in the prolongation of drug release in the vagina.[58] However, when formulations prepared with the same amount of HPMC and XG were compared, similar drug release rates were observed for HPMC containing formulations when compared to that of XG containing formulations.

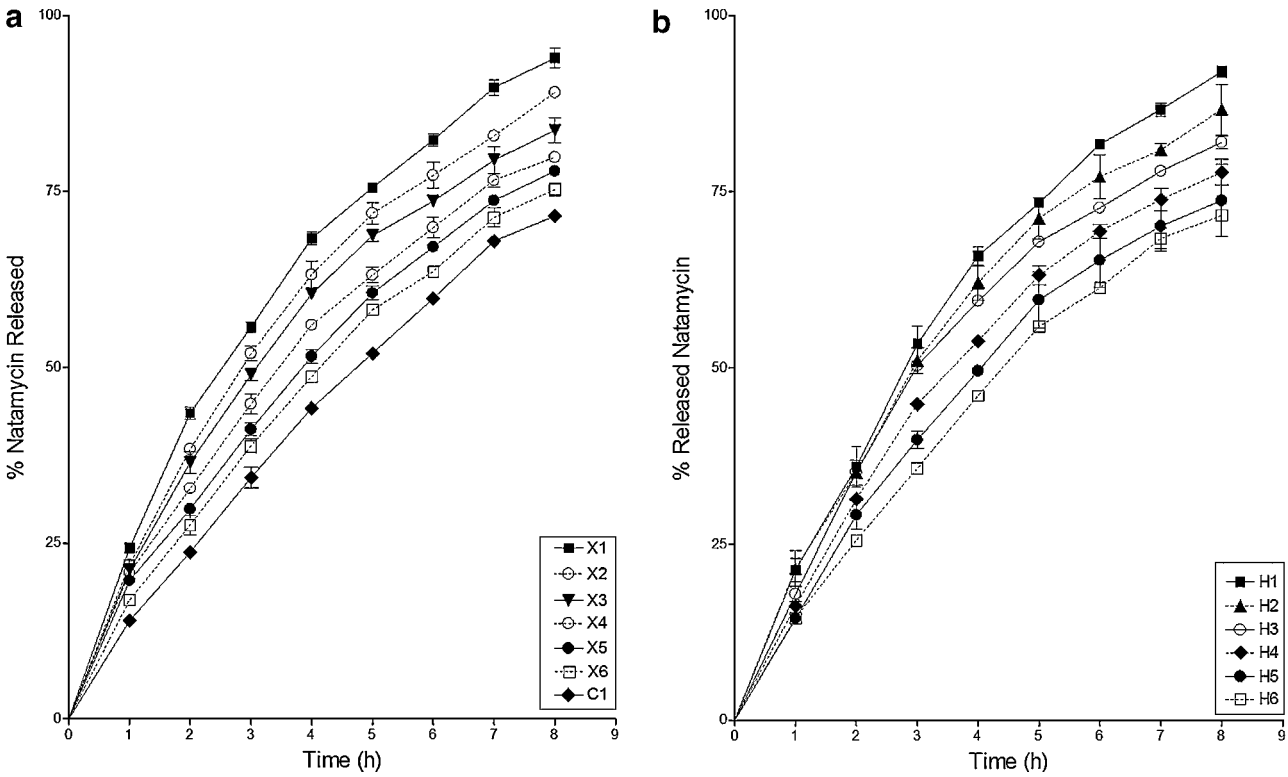

Figure 11. Natamycin release from mucoadhesive tablet formulations prepared using XG, HPMC, C934P and the mixtures of these polymers at different ratios after 24 h. Each tablet formulation contains same amount of NT-γCyD (25 mg) as an active and magnesium stearate (0.5% w/w) as a lubricant ($n = 3$).

Although release of the 94.0% of NT from X1 was achieved in 8 h, 89.1%, 83.7%, 79.8%, 77.9% and 75.3% drug release from X2, X3, X4, X5 and X6, respectively, were achieved in the same time (Fig. 11a). Similar results were obtained with formulations containing HPMC and the mixtures of HPMC and C934P. After 8 h, 92.0%, 86.7%, 82.0%, 77.7%, 73.8% and 71.6% of NT were released from H1, H2, H3, H4, H5 and H6 formulations, respectively (Fig. 11b).

The dissolution data obtained up to 8 h were fitted to Korsmeyer–Peppas semi-empirical equation (Eq. 4) and best-fit parameters were calculated. The values of k and r^2 are listed in Table 2. For all formulations, the value of diffusional exponent, n, was obtained between 0.582 and 0.778, indicating the mechanism of the NT release from all tablet formulations to be non-Fickian or anomalous diffusion which involves a combination of both diffusion and erosion mechanisms.[59] Similar findings by Fassihi and Ritschel[60] were observed with a matrix tablet formulations of theophylline. Baloglu et al.[61] reported that non-Fickian release behaviour from swelling-controlled vaginal tablet formulations containing high amount of ornidazole was observed. Our results were in accordance with previous data observed with swelling-controlled matrix systems.

Table 2. Release Parameters Obtained Following Modelling of Dissolution Data to Characterise the Type of Mechanism of Drug Release from Mucoadhesive Tablet Formulations

Formulation	k_{KP} (h^{-n})	n	r^2
X1	28.934	0.582	0.9883
X2	25.987	0.605	0.9876
X3	24.933	0.600	0.9860
X4	22.391	0.628	0.9924
X5	19.971	0.668	0.9950
X6	17.920	0.705	0.9945
C	14.655	0.778	0.9960
H1	25.392	0.639	0.9822
H2	25.073	0.614	0.9814
H3	24.075	0.611	0.9740
H4	20.920	0.654	0.9815
H5	18.549	0.687	0.9841
H6	16.208	0.734	0.9956

k_{KP} is the Korsmeyer–Peppas model release rate constant, n is the diffusional exponent and r^2 is the correlation coefficient.

CONCLUSION

Vaginal mucoadhesive tablets containing NT were successfully prepared by combining NT-γCyD complexes with various bioadhesive polymers such as XG, HPMC and C934P. Formation of an association complex between NT and γCyD was supported by results of various analytical studies, for example, ^{1}H NMR, DSC, FTIR and PXRD studies. The efficacy and potency of NT was also improved upon formation of inclusion complexation with CyDs, whereby similar MIC_{90} was recorded for both NT and its inclusion complex. Formulation variables, particularly the composition (type and ratio) of bioadhesive polymers were found to affect the swelling index, matrix erosion, bioadhesion and drug release rate. The hydrogen bonding between unionised anionic polymer, C934P, and mucus increases at pH 5 and below, thereby encouraging mucoadhesion at the intended vaginal site of administration. By reducing the swelling index and matrix erosion of the mucoadhesive tablets *via* addition of C934P in increasing amounts, prolonged drug release can be achieved. In conclusion, mucoadhesive vaginal tablets containing NT-γCyD inclusion complexes and optimised amounts of bioadhesive polymers have been achieved. The formation of CyD complexes has dramatically improved the aqueous solubility of NT without modifying its antimycotic activity, thereby enabling the production of mucoadhesive tablets with reduced side effects and prolonged residence time at the application site.

REFERENCES

1. Cast EK, Valenta C, Leopold M, Bernkop-Schnürch A. 2002. Design and in vitro evaluation of a novel bioadhesive vaginal drug delivery system for clotrimazole. J Control Release 81:347–354.
2. Mattia A, Cerniglia C, Baines J. Safety evaluation of certain food additives and contaminants natamycin (pimaricin), Who Food Additives Series: 48.
3. Committee for veterinary medicinal products, natamycin. The European agency for the evaluation of medicinal products veterinary medicines evaluation units. EMEA/MRL/342/98-FINAL, February 1998.
4. Czeizel AE, Kazy Z, Vargha P. 2003. A case-control teratological study of vaginal natamycin treatment during pregnancy. Reprod Toxicol 17:387–391.
5. Korteweg GC, Szabo KL, Rutten AM, Hoogerheide C. 1963. Some pharmacological properties of pimaricin and possible clinical applications of this antifungal antibiotic. Antibiot Chemother 11:261–272.
6. Brik H. Natamycin. In: Florey K, editor. Analytical profiles of drug substances and excipients. Vol. 10. New York: Academic Press, Inc. 1981. pp. 513–561.
7. Koontz JL, Marcy JE. 2003. Formation of Natamycin: Cyclodextrin inclusion complexes and their characterization. J Agric Food Chem 51:7106–7110.
8. Loftsson T, Brewster ME. 1996. Pharmaceutical applications of cyclodextrins, I: Drug solubilization and stabilization. J Pharm Sci 85:1017–1025.
9. Thompson DO. 1997. Cyclodextrins-enabling excipients: Their present and future use in pharmaceuticals. Crit Rev Ther Drug Carrier Syst 14:1–104.
10. Bilensoy E, Rouf MA, Vural I, Sen M, Hincal AA. 2006. Mucoadhesive, thermosensitive, prolonged-release vaginal gel for clotrimazole: beta-cyclodextrin complex. AAPS Pharm Sci Tech 7:E38.
11. Szejtli J. 1998. Introduction and general overview of cyclodextrin chemistry. Chem Rev 98:1743–1753.
12. Martin Del Valle EM. 2004. Cyclodextrins and their uses: A review. Process Biochem 39:1033–1046.
13. Munoz de la Pena A, Ndou TT, Zung JB, Greene KL, Live DH, Warner IM. 1991. Alcohol size as a factor in the ternary complexes formed with pyrene and beta cyclodextrin. J Am Chem Soc 113:1572–1577.
14. Hirsch W, Fries V, Altman L. 1985. Effect of cyclodextrins on sparingly soluble salts. J Pharm Sci 74:1123–1125.
15. Challa R, Alka A, Javed A, Khar RK. 2005. Cyclodextrins in drug delivery: An updated review. AAPS Pharm Sci Tech 6:E329–E357.
16. Lug M, Laćan MB. 2004. Influence of hydroxypropyl-β-cyclodextrin complexation on piroxicam release from bucoadhesive tablets. Eur J Pharm Sci 21:251–260.
17. Chang JY, Oh YK, Kong HS, Kim EJ, Jang DD, Nam KT, Kim CK. 2002. Prolonged antifungal effects of clotrimazole-containing mucoadhesive thermosensitive gels on vaginitis. J Control Release 82:39–50.
18. Vermani K, Garg S. 2000. The scope and potential of vaginal drug delivery. Pharm Sci Technol Today 3:359–364.
19. Duchene D, Ponchel G. 1992. Principle and investigations of the bioadhesion mechanism of solid dosage forms. Biomaterials 13:709–714.
20. Chowdary KPR, Rao YS. 2004. Mucoadhesive microspheres for controlled drug delivery. Biol Pharm Bull 27:1717–1734.
21. Valenta C. 2005. The use of mucoadhesive polymers in vaginal drug delivery. Adv Drug Del Rev 57: 1692–1712.
22. Smart JD. 1993. Drug delivery using buccal-adhesive systems. Adv Drug Del Rev 11:253–270.
23. Guggi D, Marschütz MK, Bernkop-Schnürch A. 2004. Matrix tablets based on thiolated poly(acrylic

acid): pH-dependent variation in disintegration and mucoadhesion. Int J Pharm 274:97–105.
24. Miyazaki S, Kawasaki N, Nakamura T, Iwatsu M, Hayashi T, Hou WM, Attwood D. 2000. Oral mucosal bioadhesive tablets of pectin and HPMC: In vitro and in vivo evaluation. Int J Pharm 204:127–132.
25. Jug M, Bećirević-Laćan M. 2004. Influence of hydroxypropyl-βcyclodextrin complaxation piroxicam release from buccoadhesive tablets. Eur J Pharm Sci 21:251–260.
26. Higuchi T, Connors KA. 1965. Phase-solubility techniques. Adv Anal Chem Instr 4:117–212.
27. Koontz JL, Marcy JE, Barbeau WE, Duncan SE. 2003. Stability of Natamycin and its cyclodextrin inclusion complexes in aqueous solution. J Agric Food Chem 51:7111–7114.
28. Jeener J, Meier BH, Ernst BP, Ernst RR. 1979. Investigation of exchange processes by two-dimensional NMR spectroscopy. J Chem Phys 71:4546–4553.
29. Mohamadi F, Richards N, Guida W, Liskamp R, Lipton M, Caulfield C, Chang G, Hendrickson T, Still W. 1990. MacroModel—An integrated software system for modeling organic and bioorganic molecules using molecular mechanics. J Comput Chem 11:440–467.
30. Cramer CJ, Truhlar DG. Continuum solution model: classical and quantum mechanical implementations. In: Lipkowitz KB, Boyd DB, editors. Reviews in computational chemistry. Vol. 6. New York: VCH. 1995. pp. 1–72.
31. Still WC, Tempczyk A, Hawley RC, Hendrickson T. 1990. Semianalytical treatment of solvation for molecular mechanics and dynamics. J Am Chem Soc 112:6127–6129.
32. Shenkin PS, McDonald DQ. 1994. Cluster analysis of molecular conformations. J Comput Chem 15: 899–916.
33. National Committee for Clinical Laboratory Standards. 1997. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved Standard. Document M27-A. National Committee for Clinical Laboratory Standards, Wayne, PA.
34. USP28/NF23. 2005. United States Pharmacopeia 28/National Formulary. 23. Pharmaceutical Convention Inc. Rockwell, MD, USA.
35. Korsmeyer RW, Gurny R, Doelker E, Buri P, Peppas NA. 1983. Mechanisms of potassium chloride release from compressed, hydrophilic, polymeric matrices: Effect of entrapped air. J Pharm Sci 72: 1189–1191.
36. Peppas NA. 1985. Analysis of Fickian and non-Fickian drug release from polymers. Pharm Acta Helv 60:110–111.
37. Ritger PL, Peppas NA. 1987. A simple equation for descriction of solute release. II Fickian and anomalous release from swellable devices. J Control Release 5:37–42.
38. Ahmed MO, El-Gibaly I, Ahmed SM. 1998. Effect of cyclodextrins on the physicochemical properties and antimycotic properties of clotrimazole. Int J Pharm 171:111–121.
39. Loftsson T, Magnusdottir A, Masson M, Sigurjonsdottir JF. 2002. Self-association and cyclodextin solubilization of drugs. J Pharm Sci 91:2307–2316.
40. Anselmi C, Centini M, Ricci M, Buonocore A, Granata P, Tsuno T, Facino RM. 2006. Analytical characterization of a ferulic acid/gamma-cyclodextrin inclusion complex. J Pharm Biomed Anal 40:875–881.
41. Pralhad T, Rajendrakumar K. 2004. Study of freeze-dried quercetin-cyclodextrin binary systems by DSC, FT-IR, X-ray diffraction and SEM analysis. J Pharm Biomed Anal 34:333–339.
42. Jambhekar S, Casella R, Maher T. 2004. The physicochemical characteristics and bioavailability of indomethacin from beta-cyclodextrin, hydroxyethyl-beta-cyclodextrin, and hydroxypropyl-beta-cyclodextrin complexes. Int J Pharm 270: 149–166.
43. Reddy MN, Rehana T, Ramakrishna S, Chowdhary KPR, Diwan PV. 2004. Beta-cyclodextrin complexes of celecoxib: Molecular-modeling, characterization, and dissolution studies. AAPS Pharm Sci Tech 5–6:E7.
44. Singla AK, Garg A, Aggarwal D. 2002. Paclitaxel and its formulations. Int J Pharm 235:179–192.
45. Brik H. 1976. New high-molecular decomposition products of natamycin (pimaricin) with intact lactone-ring. J Antibiot 29:632–637.
46. Tansho S, Abe S, Ishibashi H, Torii S, Otani H, Ono Y, Yamaguchi H. 2006. Efficacy of intravenous itraconazole against invasive pulmonary aspergillosis in neutropenic mice. J Infect Chemother 12: 355–362.
47. Hamada H, Ishihara K, Masuoka N, Mikuni K, Nakajima N. 2006. Enhancement of water-solubility and bioactivity of paclitaxel using modified cyclodextrins. J Biosci Bioeng 102:369–371.
48. Tur KM, Ching HS. 1998. Evaluation of possible mechanism(s) of bioadhesion. Int J Pharm 160: 61–74.
49. Fabregas JL, Garcia N. 1995. In-vitro studies on bucoadhesive tablet formulations of hydrocortisone hemisuccinate. Drug Dev Ind Pharm 21:1689–1696.
50. Peppas NA, Bury PA. 1985. Surface interfacial and molecular aspects of polymer bioadhesion on soft tissues. J Control Release 2:257–275.
51. Baloglu E, Ozyazici M, Hizarcioglu SY, Karavana HA. 2003. An in vitro investigation for vaginal bioadhesive formulations: Bioadhesive properties and swelling states of polymer mixtures. Farmaco 58:391–396.

52. Nafee NA, Ismail FA, Boraie NA, Mortada LM. 2004. Mucoadhesive delivery systems. I. Evaluation of mucoadhesive polymers for buccal tablet formulation. Drug Dev Ind Pharm 30:985–993.
53. Sharma G, Jain S, Tiwary AK, Kaur G. 2006. Once daily bioadhesive vaginal clotrimazole tablet: Design and evaluation. Acta Pharm 56:337–345.
54. Noveon Pharmaceutical Bulletin 16: Bioadhesion, Noveon Inc., Cleveland, Ohio.
55. Mortazavi SA, Smart JD. 1994. Factors influencing gel strengthening at the mucoadhesive-mucus interface. J Pharm Pharmacol 48:285–287.
56. Ranga Rao KV, Buri P. 1989. A novel in situ method to test polymers and coated microparticles for bioadhesion. Int J Pharm 52:265–270.
57. Cevher E, Taha MAM, Orlu M, Araman A. 2008. Evaluation of mechanical and mucoadhesive properties of clomiphene citrate gel formulations containing carbomers and their thiolated derivatives. Drug Deliv 15:57–67.
58. Ceschel GC, Maffei P, Lombardi Borgia S, Ronchi C, Rossi S. 2001. Development of a mucoadhesive dosage form for vaginal administration. Drug Dev Ind Pharm 27:541–547.
59. Khamanga SM, Walker RB. 2006. Evaluation of rate of swelling and erosion of verapamil (VRP) sustained-release matrix tablets. Drug Dev Ind Pharm 32:1139–1148.
60. Fassihi RA, Ritschel WA. 1993. Multiple layer, direct compresiion controlled release systems: In vitro and in vivo evaluation. J Pharm Sci 82:750–754.
61. Baloglu E, Ozyazici M, Hızarcıoglu SY, Şenyigit T, Özyurt D, Pekcetin C. 2006. Bioadhesive controlled release systems of ornidazole for vaginal delivery. Pharm Dev Tech 11:477–484.