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Stability and in vitro activity of nystatin and its γ-cyclodextrin complex against *Candida albicans*

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Summary

γ-Cyclodextrin (γ-CD) was found to form an inclusion complex with nystatin. In the presence of γ-CD, cells of *Candida albicans* absorbed less nystatin than in its absence. The γ-CD/nystatin complex had no or insignificant antimicrobial activity. Upon diffusion into a γ-CD-free medium the antibiotic was released from the complex and inhibited growth of the test organism. The stability of nystatin and the γ-CD complex was studied using both a microbiological and a spectrophotometric assay. The stability of nystatin in aqueous preparations was improved by γ-CD complexation. Degradation of pure nystatin did not follow first-order kinetics. The loss of antimycotic potency was more rapid than the decrease in concentration calculated from the spectrophotometric data for the pure antibiotic as well as its γ-CD inclusion complex.

Introduction

γ-Cyclodextrin belongs to the group of cyclic oligosaccharides commonly known as cyclodextrins. The cyclodextrins are known for their ability to form inclusion complexes with numerous compounds. Inclusion complexation of pharmaceuticals may result in an increase in aqueous solubility, stability and/or bioavailability of the drug compounds (Szejtli, 1982). Numerous publications have appeared on the interaction between cyclodextrins and drugs such as corticosteroids, non-steroidal anti-inflammatory drugs and prostaglandins (Jones et al., 1984; Duchêne et al., 1985). Some attention has also been paid to antimicrobial drugs. β-Lactam antibiotics were studied by Hsuy et al. (1984). Previously, we (Van Doorne et al., 1988a,b) studied the complex formation of β-cyclodextrins (β-CD) with some antimycotic imidazole derivatives. The antifungal activity of these complexes was also studied. The mechanism of cyclodextrin complexation of amphotericin B, a polyene antibiotic was studied by Rajagopalan et al. (1986). The amphotericin/γ-CD complex was reported to have the same antimycotic activity as the pure antibiotic (Vikmon et al., 1985). Nystatin (Fig. 1), another polyene antibiotic with great structural resemblance to amphotericin, is widely used in the topical treatment of infections of the skin, nails and mucous membranes. Oral administration is limited to the eradication of *Candida* sp. from the gastro-intestinal tract of severely immunosuppressed leukaemia patients. Nystatin is

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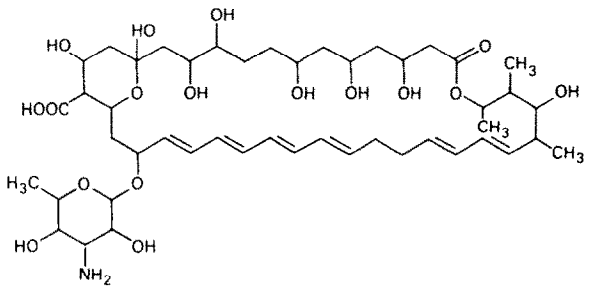

Fig. 1. Structure of nystatin.

practically insoluble in water and is sensitive to light, heat, oxygen and extreme pH values (Hammond, 1977). The stability and the antimicrobial activity of γ-CD/nystatin complexes were studied in some detail by Vikmon et al. (1988).

As there may be scope for stable aqueous nystatin preparations (eye drops, ear drops, creams and suspensions), we decided to make a more detailed study of the interaction between γ-CD and nystatin.

Materials and Methods

Materials

Nystatin (Albic B.V., Maassluis, The Netherlands) and γ-cyclodextrin (kindly donated by AVEBE, Foxhol, The Netherlands) were used without further preparation. All other chemicals were of a pharmaceutical grade. Throughout these studies McIlvaine phosphate/citrate buffers were used (Henry et al., 1974) Sabouraud Dextrose Agar (SDA) and Sabouraud Dextrose Broth (SDB) (Oxoid, Basingstoke, U.K.) was used for cultivation of the yeast and for the inhibition zone measurements. Where necessary, the required amounts of γ-CD were added to the medium before sterilization. *Candida albicans* DS 12559 was used for inhibition zone measurements because it was the most sensitive organism among our collection.

Methods

Solubility curve (dissolution technique)

Solubility measurements were carried out according to the method described by Higuchi and Connors (1965). 10 mg nystatin was mixed with 5 ml phosphate/citrate buffer pH 6 containing various amounts of γ-CD. The suspensions were shaken for 4 days at 20°C, filtered through a 0.2 μm membrane filter and the filtrate was analysed spectrophotometrically at 320 nm for total amount of nystatin dissolved.

Microbiological assay of nystatin

C. albicans DS 12559 was cultured for 48 h on SDA at 37°C. The cells were suspended in SDB (about 10^8 colony forming units per ml). 250 μl of the suspension were mixed with 18 ml melted (45°C) SDA and poured into petri dishes (ϕ 9 cm). In the centre of the plate a hole (ϕ 8 mm) was punched and filled with 50 μl of the solution to be tested. Inhibition zones were read after overnight incubation at 37°C. A calibration curve was constructed from the the inhibition zones of seven concentrations in the range between 20 and 200 μg/ml. Recorded values for zone sizes and concentrations of nystatin were computed from the average values of two independent runs each consisting of five measurements for every sample.

Preparation of solutions

A freshly prepared solution of nystatin in DMSO (10 mg/ml) was diluted with the phosphate/citrate buffer with the appropriate pH and finally the required amount of γ-CD was added.

Stability studies

For the stability studies the solutions were stored in glass, in the dark at 20°C. On the days of the analyses the relative intensities of the absorption peaks at 292, 306 and 320 nm were checked for confirmation with freshly prepared solutions of nystatin. The concentration of nystatin was calculated from the 320 nm readings.

Absorption of nystatin by cells of C. albicans

The absorption of nystatin by *C. albicans* was measured by means of a modification of the method originally described by Gosh and Gosh (1963). *C. albicans* was cultured overnight in SDB at 37°C with continuous stirring. The suspension was divided into 10 ml aliquots. The cells were washed once with sterile distilled water and the

supernatant was discarded. One fraction was dried at 110°C and the dry weight of the cells was assessed. 10 ml nystatin solution (100 μg/ml) in phosphate/citrate buffer pH 7 was added to the second fraction. To the third fraction 10 ml of a solution containing 100 μg/ml nystatin and 10 mg/ml γ-CD in phosphate/citrate buffer pH 7 was added. After 30 min both solutions were centrifuged. The concentration of nystatin in the supernatant was assayed spectrophotometrically at 320 nm. From these data the total amount of absorbed nystatin was calculated. The sediments were resuspended in phosphate/citrate buffer pH 7 and centrifuged. The concentration of nystatin was assessed in the supernatant. This procedure was repeated until the supernatant was free of nystatin. These data allowed calculation of loosely bonded nystatin. Thereafter the cells were treated with 50% isopropanol for 15 min at 80–85°C and the tubes were immediately cooled in ice water for 15 min and centrifuged. The concentration of nystatin in the supernatant was assayed spectrophotometrically. One fraction was treated identically, with the exception of the addition of nystatin. The isopropanol extract of these cells was used as the blank.

Results and Discussion

Complex formation of nystatin with γ-cyclodextrin

The solubility curve of nystatin in the presence of γ-CD is shown in Fig. 2. According to the Higuchi-Connors classification (1965), the solubility curve is of the A_1-type. A complex constant could not be calculated, because the intrinsic solubility of nystatin could not be determined, nor could reliable data be found in the literature. Vikmon and co-workers (1988) have also studied the solubility of nystatin in the presence of γ-CD; they reported solubilities which were a factor 10 higher than our results. An explanation for this discrepancy could not be found. Rajagopalan and colleagues (1986) studied the complexation of the closely related polyene antibiotic amphotericin B and cyclodextrins. They concluded that the antibiotic was too bulky to be contained in the small cavities of α-CD (six glucose units) or β-CD (seven glucose units). For this reason α-CD and β-CD were not included in our studies.

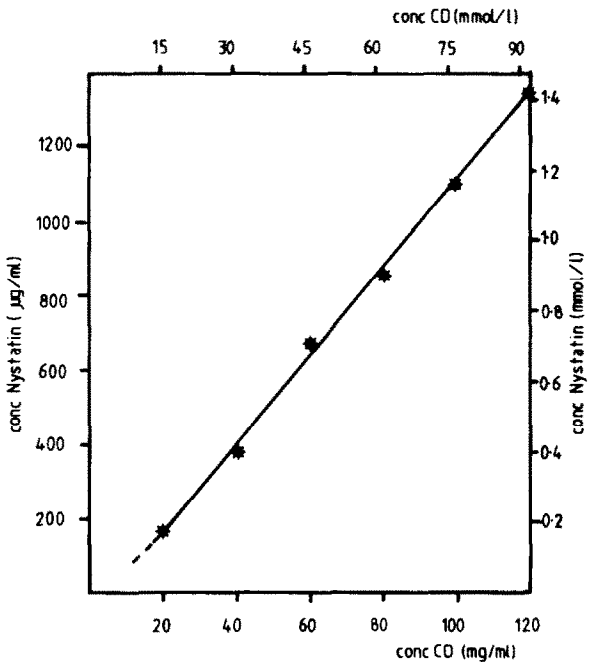

Fig. 2. Solubility of nystatin in the presence of γ-CD at 20°C.

Effect of complexation on the antifungal activity

Using the agar diffusion assay method, a linear relation between inhibition zone size and log concentration was observed for nystatin concentrations ranging from 20 to 200 μg/ml. When 100 mg/ml γ-CD was added to various solutions of nystatin, no inhibition zones were observed for solutions containing 20 or 40 μg/ml nystatin. Solutions containing 100 or 200 μg/ml in addition to 100 mg/ml γ-CD produced inhibition zones that were slightly smaller than those of corresponding cyclodextrin-free solutions.

In the presence of 10 mg/ml γ-CD, no differences in zone sizes were observed between these solutions and the γ-CD-free reference solutions. Therefore, a microbiological assay of nystatin can be performed in the presence of γ-CD provided that the relative concentration of γ-CD is not too high. These results indicate that either the complex is too large for sufficient diffusion through the agar, or that the complex has no antimicrobial activity. In order to test these two possibilities, we

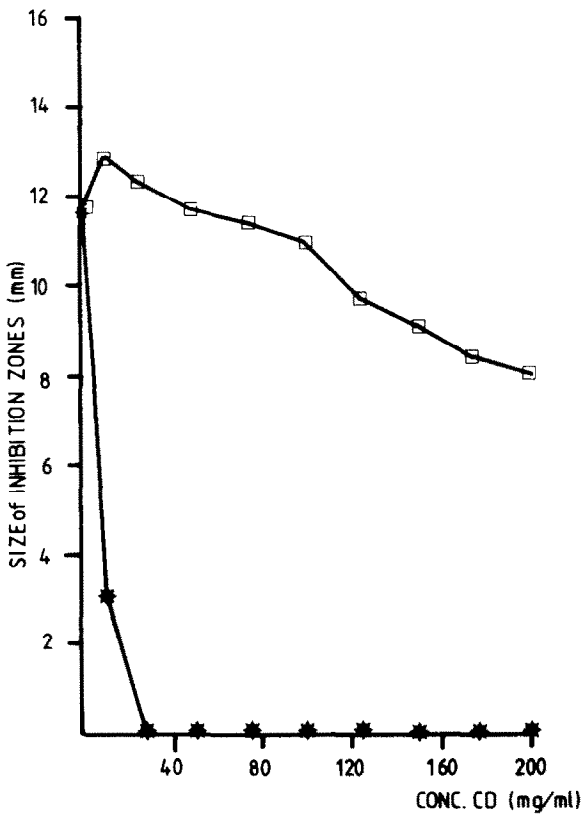

Fig. 3. (□) Antimicrobial activity of nystatin/γ-CD complexes, prepared with different concentration of γ-CD. (*) Antimicrobial activity of nystatin, measured with different concentrations of γ-CD in the growth medium.

prepared aqueous solutions of nystatin-γ-CD complex by means of the dissolution technique and measured their antimicrobial activity. The inhibition zone sizes are shown in Fig. 3. After a small initial increase there is a gradual decrease in activity when the amount of γ-CD present increases. Inhibition zones from solutions of nystatin only were also measured using agar media containing graded amounts of γ-CD. The results are also shown in Fig. 3. The zone sizes drop dramatically with increasing concentration of γ-CD in the growth medium. Beyond a concentration of 25 mg/ml no inhibition zones could be observed. These results can only be explained if it is assumed that the complex has no antifungal activity. If the complex were to possess a significant antimicrobial effect, complex formation, which inevitably occurs in the agar medium, would always lead to the formation of an active compound and hence to growth inhibition. These results are at variance with those of Vikmon et al. (1985), who reported that no loss of potency occurred after complexation, as measured by agar diffusion assay. Although no experimental details were given, probably no cyclodextrin had been incorporated into the medium. Thus, the measured antifungal activity was most likely due to free amphotericin and not to the complex.

Effect of complexation on absorption of nystatin by cells of C. albicans

It has been suggested (Hammond and Kliger, 1976) that yeast cells present two types of binding sites to polyene antibiotics. The first type is located on the cell wall and is presumed to be non-steroid. These sites should be almost saturated before the antibiotic can reach the second type of sites, which are located in the membrane. In the presence of γ-CD the cellular binding sites must compete with the binding sites of the cyclodextrin for the available nystatin. It was therefore expected that the absorption of nystatin to both binding sites would decrease when γ-CD was simultaneously present. The results (cf. Table 1) are in agreement with the theory of the two cellular binding sites. Most of the bonded nystatin could easily be removed from the cells by washing with phosphate/citrate buffer. A small fraction, however, could only be removed after isopropanol extraction. This fraction probably represents the antibiotic tightly bonded to the sterols in the cell membrane. In the presence of γ-CD the total amount of absorbed nystatin was

TABLE 1

Absorption of nystatin by cells of C. albicans

[γ-CD] (mg/ml)	Amount of nystatin recovered after washing with buffer (μg/mg dry weight cells)	Amount of nystatin recovered after isopropanol extraction (μg/ml dry weight cells)
0	13.7 [a]	3.2
2	0.8	0.3

[a] Average of triplicate experiments.

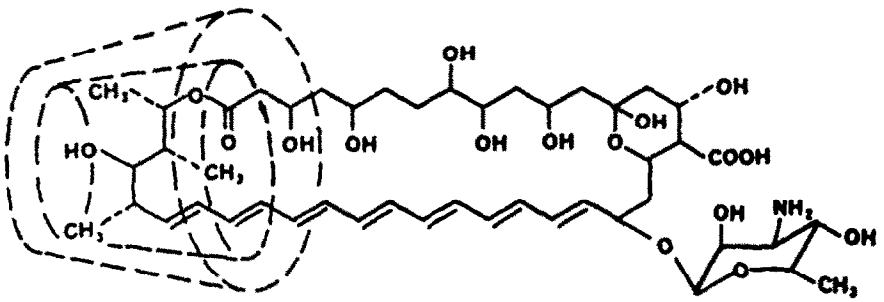

Fig. 4. Proposed mechanism for the formation of amphotericin B γ-CD 1 : 1 inclusion complex (reprinted from Rajagopalan, 1986, with permission of the copyright holder).

indeed lower than in its absence. The amount of nystatin that was recovered only after isopropanol extraction also decreased when γ-CD was present. Therefore, we concluded that γ-CD successfully competed with the cellular binding sites for nystatin. This decreased absorption, in combination with the absence of anti-mycotic activity of the complex, fully explains the decreased anti-mycotic activity of nystatin in the presence of γ-CD.

Stability of nystatin

Degradation of nystatin is associated with the reduction of the UV absorption maxima, meaning that the polyene part of the molecule is primarily

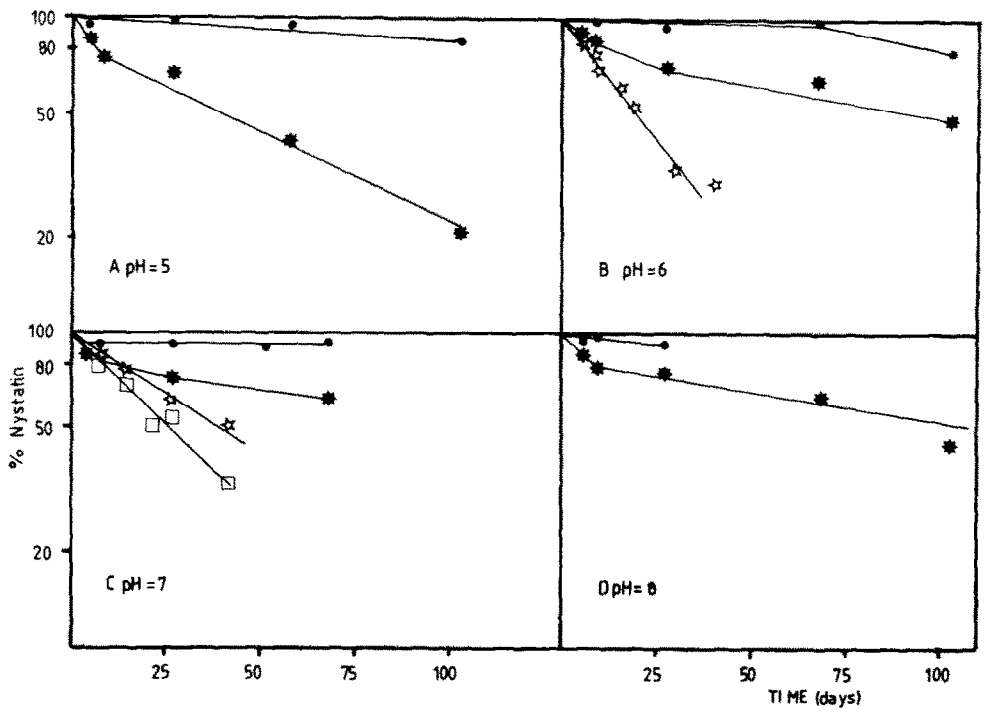

Fig. 5. Degradation of nystatin in aqueous solution. Effect of pH, initial concentration and γ-CD: (*) initial concentration 7 μg/ml, no γ-CD; (●) initial concentration 7 μg/ml, 100 mg/ml γ-CD; (☆) initial concentration 100 μg/ml, no γ-CD; (□) initial concentration 200 μg/ml, no γ-CD.

attacked. Rajagopalan and colleagues (1986) in their studies on the interaction of amphotericin B and γ-CD proposed a mechanism for the formation of the inclusion complex which is shown in Fig. 4. As nystatin is structurally very similar to amphotericin B (cf. Fig. 1), an analogous mechanism can be expected for the formation of a nystatin-γ-CD complex. As the polyene part of the molecule is partly included in the γ-CD cavity it may be effectively protected from oxidative or photolytic degradation.

The results of our studies of the stability at four different values of pH are shown in Fig. 5A–D. The degradation of solutions initially containing 7 μg/ml nystatin did not follow first-order kinetics. The degradation rate of all four solutions decreased upon the addition of 100 mg/ml γ-CD. The degradation rate was dependent on pH. The highest rate was observed at pH 5, the differences between the other three solutions being negligible. These results are at variance with those of Hamilton-Miller (1973), who observed optimum stability of nystatin at pH 7, for solutions initially containing about 2 mg/ml. The effect of the initial concentration of nystatin on the degradation rate was studied at pH 6 and 7. When the initial concentration at pH 6 was increased from 7 to 100 μg/ml the degradation rate increased (Fig. 5B). At pH 7 (Fig. 5C) there was a gradual increase in degradation rate when the initial concentration was increased from 7 to 200 μg/ml. The degradation rate of a solution containing initially 100 μg/ml is lower than at pH 6, which agrees with the results of Hamilton-Miller (1973). The explanation of the observed effect of the initial concentration on the rate of degradation would require detailed kinetic studies, which were beyond the scope of this investigation. The reaction mechanisms involved may be very complicated, as there are six double bonds in the nystatin molecule. Moreover, in aqueous systems the nystatin molecules form micelles (Hammond, 1977); dissociation or association of micelles during the degradation reaction will bring further complications.

A solution containing 200 μg/ml nystatin and 20 mg/ml γ-CD was prepared and used to compare spectrophotometric data with microbiological assay during storage. The results are shown in Fig. 6. The UV-spectrophotometric data do not correlate with the results of the microbiological assay. The antimicrobial potency of the γ-CD stabilized solution decreased to 90% in about 8 days, whereas after 28 days a 10% decrease in concentration was observed based on spectrophotometric determinations. Similar observations have been reported earlier by Hamilton-Miller (1973).

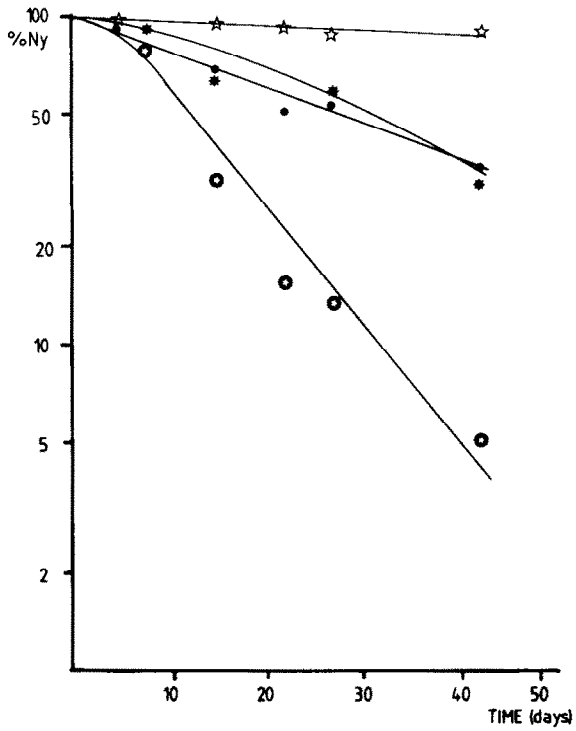

Fig. 6. Stability of nystatin/γ-CD complex. Comparison of microbiological and spectrophotometric assay: (●) nystatin; spectrophotometric assay; (○) nystatin; microbiological assay; (☆) nystatin/γ-CD complex; spectrophotometric assay; (*) nystatin/G-CD complex; microbiological assay.

It is concluded that the antimycotic antibiotic nystatin can be solubilized and stabilized by means of γ-CD. Although the complex itself has no or negligible activity, it will dissociate when it is present in a γ-CD free medium, thereby releasing nystatin. It is possible to prepare solutions which at room temperature do not lose more than 10% of their potency in 1 week. Such solutions may be sterilized by means of membrane filtration. Optimization of the formulation and in vivo studies

are required before such preparations can be used clinically.

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