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RESEARCH PAPER

Cyclodextrin Inclusion Complexes of Miconazole and Econazole—Isolation, Toxicity on Human Cells, and Confirmation of a New Interpretation of the Drug Supersaturation Phenomenon

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ABSTRACT

Parameters that influence the precipitation of the β-cyclodextrin (β-CD) inclusion complexes of the antimycotics miconazole and econazole were investigated. The mechanistic reason for the superior antimycotic activity of the miconazole inclusion complex was studied. The toxicity of the complex was estimated. The temperature, the buffer strength, and the effect of the addition of hydrotropic agents on the CD solubility diagrams for the antimycotics were estimated. The miconazole and the CD dissolution rate for the complex was measured. The hemolytic activity of the miconazole inclusion complex, the physical mixture, miconazole, and the nitrate salt were compared. The toxicity on TR146 oral cell layers was measured. Lowering the temperature meant that both complexes precipitated at lower CD concentrations. Addition of hydrotropic agents and variation of the buffer strength affected the solubility diagrams. The dissolution medium was supersaturated with miconazole. The supersaturation was not disclosed by the traditional method to analyze for drug

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supersaturation. The miconazole complex was more toxic to erythrocytes than the physical mixture. On the other hand, the toxic effects of the two products on the TR146 cell layers were similar. Lowering the temperature eased the isolation of genuine CD inclusion complexes of miconazole and econazole. The miconazole supersaturation is likely to be the reason for the superior antimycotic activity of the complex. The complex and the physical mixture had about the same toxicity on TR146 cell layers.

INTRODUCTION

Cyclodextrins (CDs) are cyclic oligosaccharides that are able to complex lipophilic drugs or lipophilic parts of drugs. The complexation can change the physicochemical and biopharmaceutical properties of the drugs in a desirable way (1).

The isolation of genuine or true β-cyclodextrin (β-CD) inclusion complexes of the antimycotic drugs econazole and miconazole has been described previously (2,3). The antimycotic effect of the inclusion complexes was superior to the effect of both the antimycotics individually and the physical mixtures of β-CD and the antimycotics.

The aim of the present work was to study the effect of temperature, hydrotropic modifiers, and buffer strength on the crystallization of miconazole and econazole β-CD inclusion complexes from buffer solutions. The toxicity of the miconazole β-CD complex on erythrocytes and on a human oral cell culture TR146 was evaluated. The applied TR146 cell culture model was described by Jacobsen et al. (4).

Dissolution rate studies were carried out for the miconazole inclusion complex. A new method to disclose drug supersaturation during dissolution rate testing was applied (5). The results were correlated with previously published in vitro antimycotic activity results (3).

EXPERIMENTAL

Materials

Miconazole nitrate, econazole nitrate, and β-CD were purchased from Sigma Chemical Company (St. Louis, MO). Miconazole base was a generous gift from Janssenpharma (DK). Dulbecco's modified Eagle medium, gentamicin, and other human cell culture media were obtained from Gibco BRL (Paisley, UK). Heat-inactivated fetal calf serum was purchased from Sera-Lab (Sussex, UK). The continuous cell line TR146, derived from a human neck mode metastasis originating from a buccal carcinoma (6), was kindly provided by the Imperial Cancer Research Technology (London, UK). Falcon cell culture inserts (polyethylene terephthalate, 1.6×10^6 pores/cm^2, pore diameter 0.45 μm, growth area 4.6 cm^2) and Falcon six-well culture plates (tissue culture treated polystyrene) were from Becton Dickinson Labware (NJ).

Methods

Differential Scanning Calorimetry

A Perkin Elmer DSC7 was used. It was equipped with a Perkin Elmer TAC/PC Instrument Controller and Perkin Elmer multitasking software. Closed aluminum pans were applied. The scan speed was 10°C/min, and nitrogen was used as the carrier gas. The sample size was in the range 2–5 mg.

X-ray Powder Diffraction Analysis

X-ray powder diffraction patterns were recorded with a Guinier XDC 700 IRDAB powder diffraction camera using a Philips PW 1720 X-ray generator. CuKα radiation was applied.

Solubility Diagrams

Solubility measurements were carried out as described by Higuchi and Connors (7). To 10 ml of phosphate buffer adjusted to pH 7.1 or 10.0 and containing various concentrations of β-CD, 10 mg of econazole nitrate or miconazole were added. After approximately 2 weeks, the suspensions were filtered through 0.2-μm Sartorius cellulose acetate membrane filters. The concentration of drug in the filtered samples was analyzed by a high-performance liquid chromatography (HPLC) method.

High-Performance Liquid Chromatography Methods

Econazole and miconazole concentrations were measured by reversed-phase HPLC methods (2,3). Solid miconazole β-CD preparations were dissolved in dimethyl sulfoxide before the analysis. The concentration of β-CD in the dissolution medium and in the dimethyl sulfoxide solutions was determined by a reversed-phase HPLC method. The eluent was composed of 15% methanol and 85% deionized water, the column was a Merck Lichro-

sorb 100 18RP (4 ± 125 mm) equipped with a Lichrosorb 100 RP18 guard column. A Merck refractive index detector was applied. The quantitative detection limit for a 20-µl loop was 0.5 mg/ml. The correlation coefficient was 1.000 for the concentration range 0.5–16 mg/ml.

Determination of Water Content

About 25.0 mg solid miconazole β-CD complex was dissolved in methanol:dimethyl formamide 1:1. Afterward, the solution was titrated automatically with Hydranal composite 5 (Riedel de Haen) on a Methrom 701KF Titrino (Karl Fischer titration).

Dissolution Testing

The dissolution testing of miconazole, physical mixture, and inclusion complex was carried out using the same experimental conditions as during the test of the antimycotic effect (3). Physical mixture and complex 16 mg/ml and neat miconazole 2.4 mg/ml were added (i.e., the concentration of miconazole was the same for the three samples). In comparison with the solubility of miconazole in the 0.05 M ammonium phosphate buffer, pH 7.1 dissolution medium, excessive amounts of miconazole were added. That is, possible drug supersaturation phenomena would be disclosed. Samples were taken from the dissolution medium and immediately filtered through Sartorius cellulose acetate 0.2-µm membrane filters. The filtrates were immediately analyzed for miconazole and β-CD.

Hemolysis

Erythrocytes were separated by centrifugation of citrated human blood at 1500 xg for 10 min and washed three times with isotonic phosphate buffer, 154 mM sodium chloride, 10 mM phosphate, pH 7.4. The human erythrocytes were resuspended in the buffer to give a hemotocrit value of 5% (8). Buffer (3.8 ml) and 0.2 ml erythrocyte suspension (5%) were mixed. The test substances, miconazole 1.4 mg/ml, miconazole nitrate 1.6 mg/ml, CD inclusion complex (1.4 mg/ml miconazole and 7.8 mg/ml CD), physical mixture of CD 7.8 mg/ml and miconazole 1.4 mg/ml or CD 7.8 mg/ml alone, were added as solid compounds at time zero. The samples were incubated in a 37°C water bath for 15 min. Afterward, the samples were centifuged for 3 min at 2000 xg. The absorbance of the supernatant was measured at 543 nm. A 100% hemolysis value was obtained by mixing 0.2 ml erythrocyte suspension and 3.8 ml distilled water, instead of buffer, and incubating the sample at 37°C for 15 min and measuring the 543-nm absorbance of the supernatant (8).

TR146 Cell Culture

The TR146 cells were incubated and maintained in 25 cm^2 T-flasks at 37°C in a 98% relative humidity atmosphere of 5% CO_2/95% air. The culture medium consisted of Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum, 50 µg/ml gentamicin, and 0.2 µg/ml *p*-hydroxybenzoic acid *n*-butyl ester. Further details concerning maintenance and seeding of TR146 cells were as described previously (4).

The cell-seeding density on the Falcon filters was 2.4 ± $(10)^4$ cells/cm^2. Of the culture medium mentioned above, 2.5 ml and 2.0 ml were added to the apical and the basolateral side, respectively. The culture medium was changed three times a week. The cells were grown for 35 days on the filters at the temperature and atmosphere mentioned above before they were used for toxicity (transepithelial electrical resistance [TEER]) studies.

Transepithelial Electrical Resistance

The integrity of the TR146 cell layers on Falcon filters was evaluated before and during the cell culture toxicity study by measuring the electrical resistance (R, Ω) of the layers. Afterward, the TEER was calculated (Ω cm^2).

The measurement of R was carried out at 24°C by applying an Endohm-24 connected to an epithelial volt-ohmmeter (EVOM), World Precision Instruments (FL). The Falcon filter inserts, covered with cells and containing 2.5 ml Dulbecco's modified Eagle medium and the test substance, were transferred to the Endohm-24, which contained 4.5 ml medium. R was recorded and TEER calculated according to the equation

$$\text{TEER} = (R_{(\text{insert with TR146})} - R_{(\text{insert without TR146})}) \times A$$

where A is the surface area of the filters, 4.6 cm^2.

The test concentration for both the inclusion complex and the physical mixture corresponded to 22.7 mg/ml β-CD and 3.6 mg/ml miconazole.

Protein Determination and TR146 Mortality

The TR146 cells were grown to confluence in 25 cm^2 T-flasks as described by Jacobsen et al. (4). The growth medium was removed, and the flasks were rinsed with 5 × 9 ml Hanks' buffer. Afterward, 10 ml Hanks' buffer and the test substance were added, that is, inclusion complex and physical mixture corresponding to 22.7 mg/ml β-CD and 3.6 mg/ml miconazole, miconazole nitrate 4.2 mg/ml, and miconazole 3.6 mg/ml. The flasks were placed on an orbital plate shaker at room temperature, and 2-ml samples were withdrawn after 30 and 240 min

and frozen until the protein determination was carried out. After the last sampling, the flasks were rinsed with 2 × 5 ml Hanks' buffer, and the cells were exposed to 3 ml staining solution, trypan blue solution 0.4% diluted 1:1 with water (Sigma Catalogue 1530-1531, 1992). The TR146 mortality was calculated by counting dead cells (stained blue) and the total number of cells in three areas of the flasks using an inverted light microscope and a graticule (4).

The protein concentration analysis was performed according to Lowry et al. (9) by measuring the absorbance at 280 nm and using alkaline cupper reagent and Folin and Ciocalteu's phenol reagent. Bovine serum albumin was applied as a standard.

RESULTS AND DISCUSSION

Solubility Diagrams

The temperature affected the miconazole and econazole β-CD solubility diagram considerably (Figs. 1a, 1b). Regarding the isolation of a solid inclusion complex, it is noteworthy that the crystallization of the complexes (i.e., the start of the plateau) took place at lower β-CD concentrations when the temperature during equilibration was decreased from 24°C to 16°C to 6°C. Especially during isolation of a miconazole β-CD complex, it is of value to decrease the temperature to 6°C because, at higher temperatures (e.g., 24°C), the plateau region was quite narrow, that is, there was only a small difference between the minimum β-CD concentration at which crystallization of the complex took place and the β-CD solubility (18 mg/ml). At 6°C, the complex crystallization started at 10 mg/ml β-CD, and the β-CD solubility was between 14 and 16 mg/ml (Fig. 1a). Descending parts of the Bs diagrams were hardly present for miconazole and β-CD. A discussion of the lack of descending parts was presented previously (3). On the contrary, a descending part was present for econazole and β-CD at both 24°C, 16°C, and 6°C (Fig. 1b). According to Higuchi and Connors (7), the descending part of the diagram asymptotically approaches the solubility of the econazole β-CD complex. The solubility of the complex at 24°C corresponded to 21 μg/ml econazole, at 16°C it corresponded to about 10 μg/ml, and finally at 6°C it corresponded to 3 μg/ml econazole.

Addition of 0.5 M urea, which has a positive hydrotropic effect, was previously shown to increase the apparent solubility of the imidazole antimycotic clotrimazole and to lower the size of the clotrimazole β-CD stability constant (10). According to Fig. 2b, the crystallization of the econazole β-CD complex was shifted to a higher

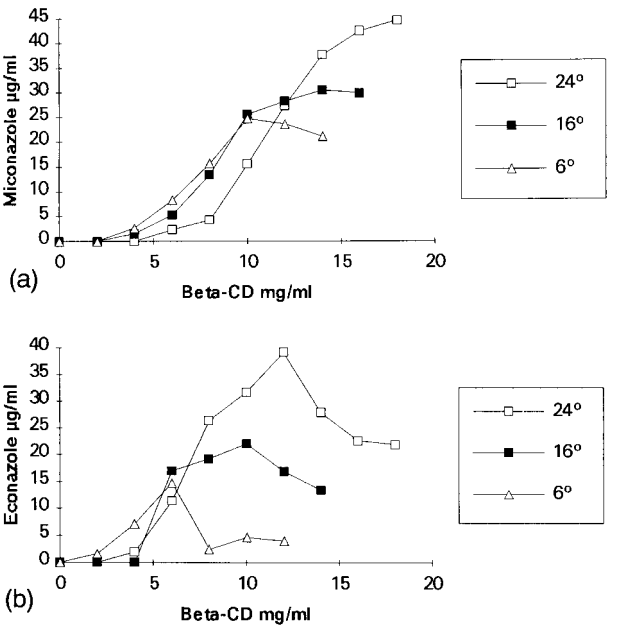

Figure 1. Effect of temperature on the β-CD solubility diagrams for (a) miconazole and (b) econazole (medium: phosphate buffer 0.05 M, pH 10.0 and 7.1, respectively).

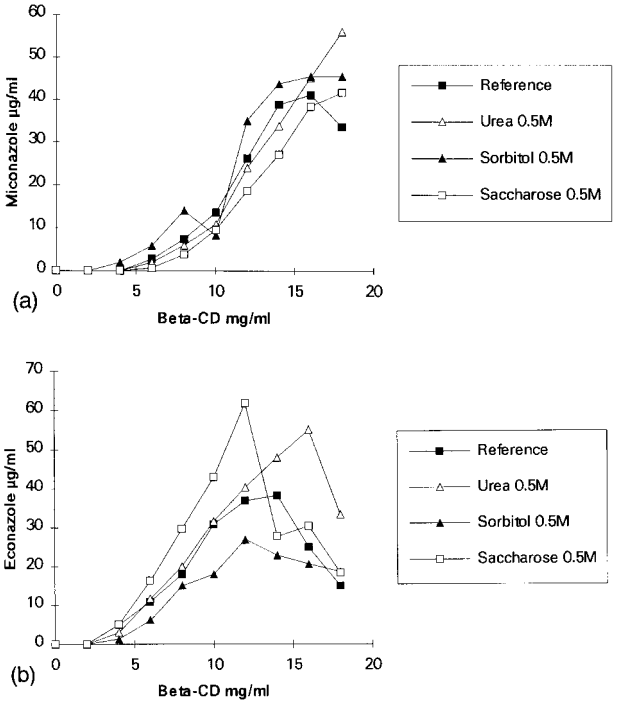

Figure 2. Influence of hydrotropic agents on the β-CD solubility diagrams for (a) miconazole and (b) econazole (medium: phosphate buffer 0.05 M, pH 10.0 and 7.1, respectively).

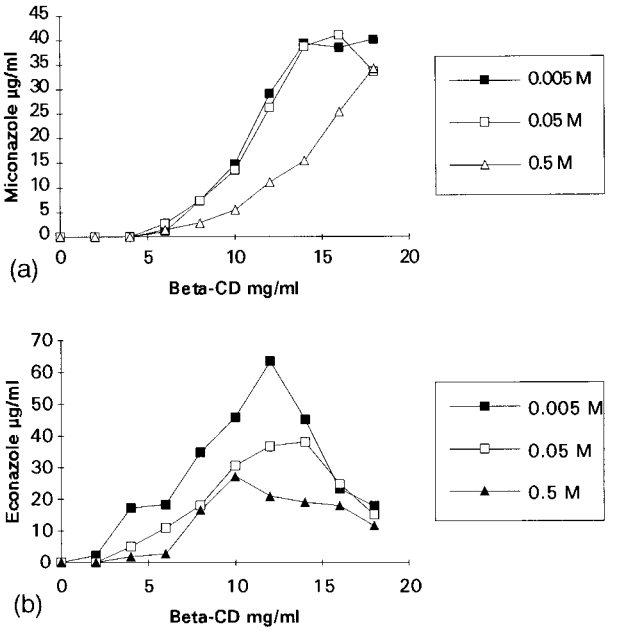

Figure 3. Effect of phosphate buffer strength on the β-CD solubility diagrams for (a) miconazole and (b) econazole (pH in medium 10.0 and 7.1, respectively).

CD concentration when 0.5 M urea was added. Regarding miconazole, the addition of 0.5 M urea inhibited the crystallization of the miconazole CD complex and an Ap-type solubility diagram was the result. The two additives, saccharose and sorbitol, with negative hydrotropic effect did not lower, as expected, the β-CD concentration at which crystallization of the inclusion complexes took place (Figs. 2a, 2b). On the contrary, saccharose 0.5 M moved the crystallization of the miconazole complex to a higher CD concentration.

Increasing the phosphate buffer concentration (i.e., increasing the ion strength) lowered the solubilizing capacity of β-CD on miconazole and econazole (Figs. 3a, 3b). The lower capacity may be caused by a lowering of the intrinsic solubility of econazole and miconazole when the phosphate buffer concentration was increased. Increasing the buffer concentration corresponded to a decrease of the temperature for the econazole β-CD system (Figs. 1b and 3b). For miconazole, this correspondence was not present. Throughout the studies, it was checked that the buffer solutions were able to keep the pH at the desired value (i.e., 7.1 and 10.0 for the econazole and miconazole solubility diagrams, respectively).

Solid Miconazole β-Cyclodextrin Inclusion Complex

The miconazole β-CD inclusion complex was isolated and dried as described previously (3). The X-ray powder diffraction patterns for miconazole, β-CD, the inclusion complex, and a physical mixture of β-CD and miconazole are depicted in Fig. 4. The pattern for the inclusion complex differed significantly from the other patterns.

Karl Fischer titration of the water content and HPLC analysis of miconazole and β-CD showed that the solid inclusion complex had the following composition: miconazole 15.1% ± 0.8%, β-CD 82% ± 3%, and water 5.0% ± 0.1% (n = 3, ± SEM), that is, the molar ratio

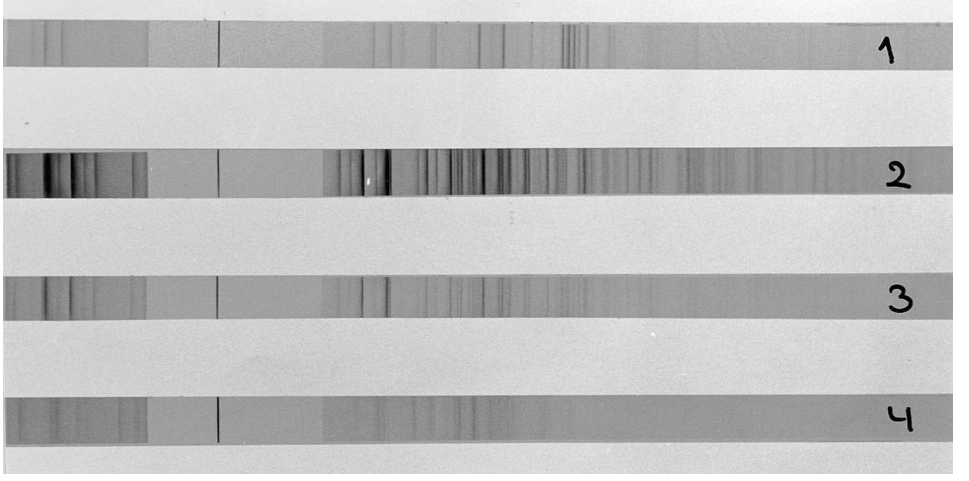

Figure 4. X-ray diffraction patterns: (1) miconazole, Janssenpharma; (2) β-CD; (3) physical mixture of miconazole (Janssenpharma) and β-CD, molar ratio 1:2; and (4) miconazole β-CD inclusion complex, molar ratio 1:2.

between miconazole and β-CD was 1:2.0. It was confirmed by DSC analysis that the inclusion complex did not contain a traceable amount of the miconazole (data not shown).

Dissolution Rate and Supersaturation

The miconazole β-CD inclusion complex, compared with neat miconazole and the physical mixture of miconazole and β-CD, had superior activity on *Candida albicans* (3). The miconazole dissolution rate for the inclusion complex and the physical mixture is depicted in Fig. 5. The dissolution rate for neat miconazole was measured, too, but throughout the study, the miconazole concentration was below the detection limit of 0.5 μg/ml. According to Fig. 5, the drug dissolution rate did not correlate with the antimycotic activity. Although the physical mixture had the faster miconazole dissolution rate, the inclusion complex was superior in respect to antimycotic activity. The drug dissolution rate curves for the inclusion complex and the physical mixture in Fig. 5 did not have the profile that, according to Frömming and Szejtli (1), is characteristic for products that give rise to drug supersaturation of the dissolution medium. Previous in vitro studies of the miconazole β-CD complex indicated that the complex only had a minor, if any, antimycotic activity in its own right (11,12), that is, the complex's own activity can hardly explain the above-mentioned lack of correlation between activity and drug dissolution rate.

The β-CD dissolution rate for the inclusion complex and the physical mixture is depicted in Fig. 6. The

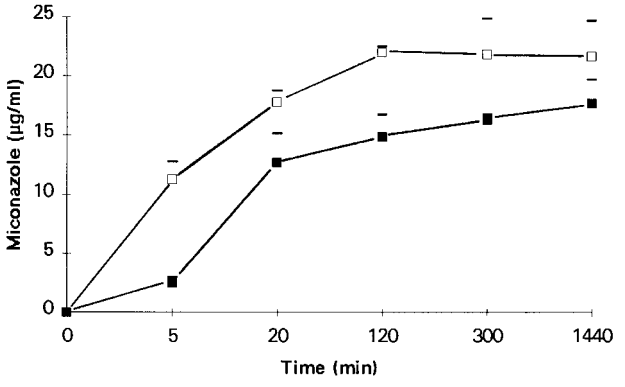

Figure 5. Miconazole dissolution rate from ■ complex of miconazole and β-CD, molar ratio 1:2.0, 16 mg/ml; □ physical mixture of miconazole and β-CD, molar ratio 1:2.0, 16 mg/ml, $n = 3$, SEM. Dissolution medium was phosphate buffer 0.05 M at pH 7.5.

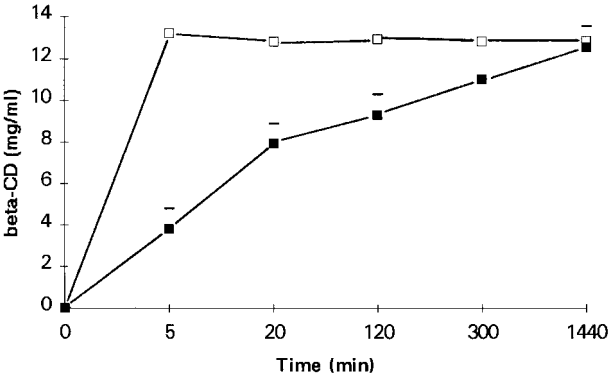

Figure 6. β-CD dissolution rate from ■ complex of miconazole and β-CD, molar ratio 1:2.0, 16 mg/ml; □ physical mixture of miconazole and β-CD, molar ratio 1:2.0, 16 mg/ml, $n = 3$, SEM. Dissolution medium was 0.05 M phosphate buffer at pH 7.5.

CD dissolution rate from the physical mixture was much faster than the dissolution rate from the inclusion complex. The reason may be that the binding of β-CD in the inclusion complex crystals lowered the dissolution rate.

The corresponding miconazole and β-CD concentrations for both the physical mixture and the inclusion complex dissolution rate study (Figs. 5 and 6) were plotted in Fig. 7. The miconazole β-CD solubility diagram in

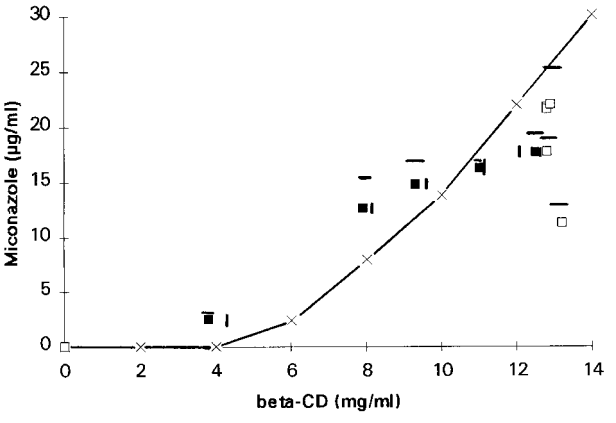

Figure 7. Corresponding miconazole and β-CD concentrations from the dissolution rate study: ■ complex of miconazole and β-CD, molar ratio 1:2.0, 16 mg/ml; □ physical mixture of miconazole and β-CD, molar ratio 1:2.0, 16 mg/ml; × solubility diagram for miconazole and β-CD in the dissolution medium, $n = 3$, SEM. Dissolution medium was 0.05 M phosphate buffer at pH 7.5.

the dissolution medium also was plotted in Fig. 7. The corresponding miconazole and β-CD concentration points from the initial part of the inclusion complex dissolution study were placed above the solubility diagram curve, that is, the inclusion complex gave rise to miconazole supersaturation of the dissolution medium (5), although the supersaturation was not visible by the traditional method of disclosing supersaturation (Fig. 5). The physical mixture did not give miconazole supersaturation of the dissolution medium (Fig. 7), although the miconazole dissolution rate was quite high (Fig. 5). The reason for the lack of drug supersaturation was that the high β-CD dissolution rate hindered it.

The difference in ability to cause miconazole supersaturation of the dissolution medium may be the reason for the superior in vitro antimycotic activity of the inclusion complex (3). The study indicates that the traditional way to evaluate drug dissolution rate and drug supersaturation from CD inclusion complexes (Fig. 5) is insufficient. By applying the procedure outlined in Fig. 7, the miconazole supersaturation was disclosed. Using the new procedure to disclose drug supersaturation episodes during CD inclusion complex dissolution studies may show that drug supersaturation plays a more important role than hitherto assumed in the ability of inclusion complexes to improve the bioavailability of drugs.

Toxicity to Human Erythrocytes

According to Fig. 8, the inclusion complex of miconazole was much more hemolytic than the other samples tested (i.e., miconazole base, the nitrate salt, β-CD, and the physical mixture of miconazole and β-CD). The high hemolytic activity of the complex was probably due to an improved availability of miconazole. It is plausible that the improved availability was caused by the ability of the inclusion complex to cause drug supersaturation (Fig. 7). The miconazole nitrate was expected to have higher hemolytic activity than the miconazole base because the nitrate salt is assumed to be the more bioavailable of the two (3). But, the hemolytic activities of the base and the nitrate salt were about the same (Fig. 8). Neat β-CD and the physical mixture of CD and miconazole were equally hemolytic.

Toxicity to TR146 Cell Culture

The TEER of TR146 human oral cell layers is a measure of the integrity of the layers (4). The inclusion complex, the physical mixture, and neat β-CD decreased the TEER value to some extent during a 4-hr exposure period

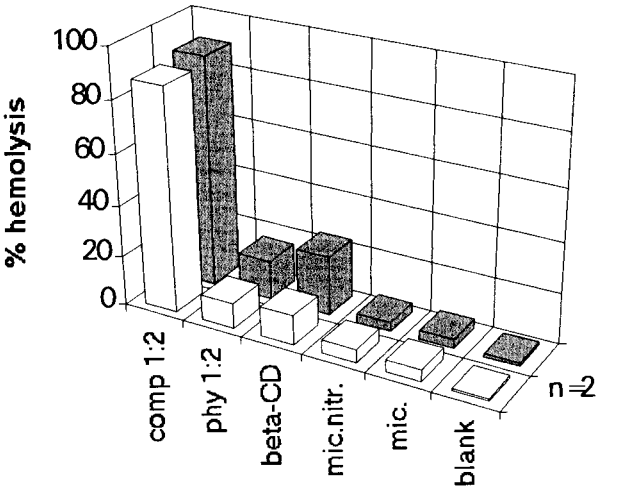

Figure 8. Hemolysis (%) of various miconazole compositions (comp = inclusion complex of miconazole 1.4 mg/ml and β-CD 7.8 mg/ml, molar ratio 1:2.0; phy = physical mixture of miconazole 1.4 mg/ml and β-CD 7.8 mg/ml, molar ratio 1:2.0). Miconazole, β-CD, and miconazole nitrate, 1.4, 7.8, and 1.6 mg/ml, respectively, were added.

(Fig. 9). Pure Hank's buffer (i.e., the blank) and miconazole did not affect the TEER value to the same extent as the three previously mentioned samples.

Determination of the TR146 cell viability by trypan blue staining indicated that the physical mixture was as

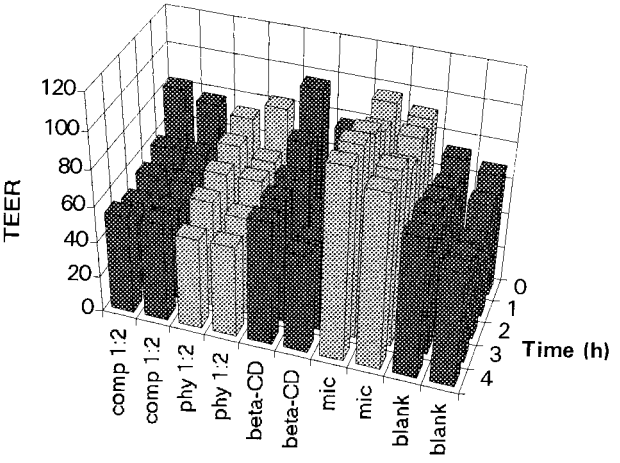

Figure 9. TEER (Ω cm^2) of TR146 cell layers during exposure to comp/phy (miconazole β-CD complex/physical mixture, molar ratio 1:2; 22.7 mg/ml β-CD and 3.6 mg/ml miconazole), to β-CD 22.7 mg/ml and to miconazole 3.6 mg/ml.

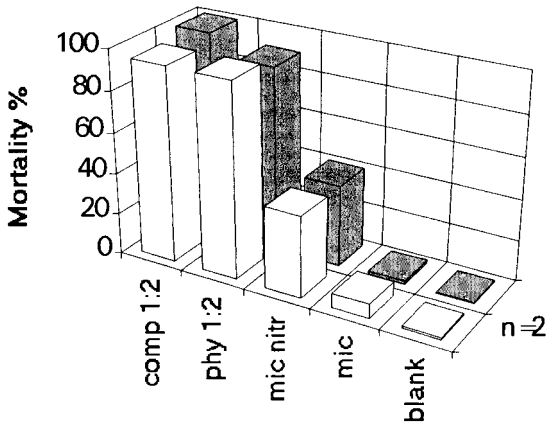

Figure 10. Mortality of TR146 cells after 240 min exposure to comp/phy (miconazole β-CD complex/physical mixture, molar ratio 1:2; 22.7 mg/ml β-CD and 3.6 mg/ml miconazole to miconazole nitrate 4.2 mg/ml and to miconazole 3.6 mg/ml).

toxic as the inclusion complex (Fig. 10). The physical mixture and the inclusion complex caused significantly more TR146 mortality than miconazole base, miconazole nitrate, and the Hank's buffer in its own right. The protein concentration in the buffer was measured after the TR146 cells had been exposed to the above-mentioned entities for 30 and 240 min. No correlation between protein concentration and mortality of TR146 cells was found (data not shown).

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