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# A genuine clotrimazole γ-cyclodextrin inclusion complex-isolation, antimycotic activity, toxicity and an unusual dissolution rate

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**Abstract**

A crystalline clotrimazole γ-cyclodextrin inclusion complex, molar ratio 1:1.0, was isolated from phosphate buffer 0.05 M, pH 7.1. Due to the low water solubility of the inclusion complex, the clotrimazole dissolution rate from the complex was very low. However, application of a new method to disclose supersaturation phenomena showed that the complex gave rise to a profound clotrimazole supersaturation during the dissolution rate test. Probably, the clotrimazole supersaturation was the reason why the inclusion complex had higher antimycotic activity than both clotrimazole and a physical mixture of clotrimazole and γ-cyclodextrin, molar ratio 1:1.0. In addition, the inclusion complex was the most toxic on human erythrocytes and on non-differentiated monolayers of epithelial like human TR146 cells. On the other hand, no difference in the toxicity of the inclusion complex, the physical mixture and clotrimazole was observable when robust multilayers of differentiated TR146 cells were exposed to the compounds.


*Keywords:* Antimycotic; Cell culture TR146; Cyclodextrin; Dissolution rate; Erythrocytes; Inclusion complex; Solubility diagram; Supersaturation; Toxicity

## 1. Introduction

Cyclodextrins are oligosaccharides which are able to form complexes with lipophilic drugs or lipophilic parts of drugs, thus changing their

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physicochemical and biopharmaceutical properties in a desirable manner.

Isolation of genuine $\beta$-cyclodextrin complexes of the antimycotic lipophilic drugs, econazole and miconazole has been reported previously (Pedersen et al., 1993a, 1998; Pedersen, 1994). Other studies not resulting in isolation of genuine complexes of cyclodextrins and imidazole antimycotics were carried out by Piel et al. (1997, 1998), Tenjarla et al. (1998), Okimoto et al. (1996), Esclusa-Diaz et al. (1996a,b), Gerloczy et al. (1995), Pedersen (1993), Pedersen et al. (1993b), Mura et al. (1992), Pearson and Salole (1989), Bononi et al. (1988) and Van Doorne et al. (1988a,b).

The main aim of the present study was to isolate a new genuine cyclodextrin inclusion complex of the antimycotic clotrimazole, and to study the antimycotic, toxicological, and physicochemical properties of the isolated inclusion complex. The toxicology of the complex was tested on human erythrocytes and on a newly established human buccal cell culture model based on the cell line TR146 (Jacobsen et al., 1995, 1996). Regarding the physicochemical properties, a recently published method was included to disclose possible clotrimazole supersaturation episodes during the dissolution rate testing of the cyclodextrin inclusion complex (Pedersen, 1997).

## 2. Experimental

### 2.1. Materials

Clotrimazole and $\beta$-cyclodextrin were purchased from Sigma (St. Louis, MO, USA) and $\gamma$-cyclodextrin was purchased from Cyclolab (Hungary). $\alpha$-Cyclodextrin was from AVEBE (The Netherlands). Candida albicans PF 1383 88 was generously supplied by Statens Seruminstitut (Copenhagen, Denmark). Dulbecco's modified Eagle medium (DMEM; Cat. No. B-1001) was purchased from HyClone® Laboratories (Logan, UT, USA), and penicillin ($10^4$ units/ml)/streptomycin ($10^4$ μg/ml) solution was obtained from Gibco BRL (Paisley, UK). Heat-inactivated fetal calf serum was purchased from Sera-Lab (Sussex, UK). The continous cell line TR146, derived from a human neck mode metastasis originating from a buccal carcinoma (Rupniak et al., 1985), was kindly provided by Imperial Cancer Research Technology (London, UK). Falcon cell culture inserts (polyethylene terephthalate, $1.6 \times 10^6$ pores/cm$^2$, pore diameter 0.45 $\mu$m, growth area 4.2 cm$^2$) and Falcon 6-well culture plates (tissue culture treated polystyrene) were from Becton Dickinson Labware (New Jersey, NJ, USA). Nunclon™ Delta Surface Microwell Plates 96-well flat bottomed (polystyrene, certified surface treatment) was obtained from NNI Nunc A/S (Roskilde, Denmark).

All other chemicals were of analytical grade.

### 2.2. Method

#### 2.2.1. Solubility diagrams and isolation of inclusion complex

Solubility measurements were carried out as described by Higuchi and Connors (1965). In 5 ml of distilled water up to 18 mg/ml $\beta$-cyclodextrin was dissolved and 10 mg clotrimazole was suspended. Similarly, the solubility diagram was determined for clotrimazole and $\beta$-cyclodextrin in ammonium phosphate buffer, 0.05 M, pH 7.1 or 10.0, and at various temperatures, 6 and 21°C.

Solubility diagrams of clotrimazole and $\alpha$-cyclodextrin up to 120 mg/ml, and $\gamma$-cyclodextrin up to 225 mg/ml were measured at 21°C in 0.05 M diammonium phosphate buffer, pH 7.1. As above, 10 mg clotrimazole and 5 ml buffer were applied.

Throughout the solubility studies, the samples were rotated at least 10 days for equilibration to take place. Afterwards, the samples were filtered through 0.2-$\mu$m Sartorius cellulose acetate membrane filters. The concentration of antimycotic in the filtrate was analysed by a HPLC method.

A solid clotrimazole $\gamma$-cyclodextrin inclusion complex was isolated at 21°C by shaking a 0.5 l screw cap bottle containing 0.5 l diammonium phosphate buffer 0.05 M, pH 7.1, in which 2 mg/ml clotrimazole was suspended and 60 mg/ml $\gamma$-cyclodextrin was dissolved initially. The isolation conditions corresponded to a point on the descending part of the Bs solubility diagram.

During the shaking period, homogeneuos samples were taken from the bottle and dried. The dried products were analysed by differential scan-

ning calorimetry to measure the residue of crystalline clotrimazole. After ten days of shaking, there was no longer crystalline clotrimazole in the dried product, i.e. the termodynamic equilibrium had been reached, and all clotrimazole had been converted to the solid clotrimazole γ-cycloddextrin complex, except for the insignificantly amount of clotrimazole dissolved in the solubility medium. After the equilibrium was reached, the solid complex was isolated by paper filtration. The complex was washed with a few millilitres of water and dried at 60°C for 3 days. The dried product was analysed for the content of clotrimazole, γ-cyclodextrin, and water.

A physical mixture of clotrimazole and γ-cyclodextrin, molar ratio 1:1.0, was prepared by gently mixing in a mortar.

*2.2.2. 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 aluminium pans were applied. The scan speed was 10°C/min and nitrogen was used as carrier gas. The sample size was in the range 2–5 mg.

*2.2.3. 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. Cr Kα radiation was applied.

*2.2.4. HPLC methods*

The concentration of clotrimazole was measured by a previously published reversed phase HPLC method (Pedersen, 1993). The dried (3 days at 60°C) clotrimazole γ-cyclodextrin inclusion complex was dissolved in dimethyl formamide before the clotrimazole analysis. The γ-cyclodextrin content of the dried complex was measured by the γ-cyclodextrin HPLC reversed phase method described below. Before the analysis the complex was dissolved in dimethyl formamide. The γ-cyclodextrin concentration in the growth medium/dissolution medium was measured by the same reversed phase HPLC method. The eluent was composed of 7% (v/v) methanol and 93% (v/v) deionised water, the column was a Merck Lichrosorb 100 RP18 ($4 \times 125$ mm) equipped with a Lichrosorb 100 RP18 guard column. A Merck refractive index detector was applied. The quantitation limit for a 20-μl loop was 0.5 mg/ml γ-cyclodextrin. The correlation coefficient was 0.999 for the concentration range 0.5–6 mg/ml.

*2.2.5. Determination of water content*

The water content of the dried (3 days at 60°C) clotrimazole γ-cyclodextrin inclusion complex was measured by a Karl Fischer titration procedure, previously described (Pedersen et al., 1998).

*2.2.6. Antimycotic activity*

Cultures of Candida albicans were grown for 2.5 h at $37 \pm 1$°C in 10 ml samples of sterile glucose, peptone, yeast extract growth medium (Pedersen and Rassing, 1990), which was adjusted to pH 7.5 with sterile 0.5 M disodium hydrogen phosphate buffer. The initial number of yeast cells was $5 \times 10^5$ per ml. After the 2.5 h growth period, clotrimazole 1.5 mg/ml, γ-cyclodextrin 5.7 mg/ml, clotrimazole γ-cyclodextrin inclusion complex 7.65 mg/ml, molar ratio 1:1.0 or clotrimazole γ-cyclodextrin physical mixture 7.65 mg/ml, molar ratio 1:1.0 were added. The compounds were sieved through a 300-μm sieve just before the addition. Samples of 100 μl were taken from the growth medium tubes during a 24 h experimental period. The samples were diluted appropriately and the dilutions were plated on agar plates. After incubation of the agar plates for 192 h at 33°C, colony counting was carried out.

*2.2.7. Dissolution rate studies in Candida growth medium*

The dissolution rate studies of clotrimazole 1.5 mg/ml, dried clotrimazole γ-cyclodextrin inclusion complex 7.65 mg/ml and physical mixture of clotrimazole and γ-cyclodextrin 7.65 mg/ml were carried out using the same experimental conditions as during the testing of the antimycotic activity. Samples were taking from the tubes and immediately filtered through Sartorius cellulose acetate 0.2-μm filters, and the concentration of clotrimazole and γ-cyclodextrin in the filtrates

was measured immediately by HPLC methods. Regarding the inclusion complex, the solid phase in one of the tubes after 24 h testing was isolated by paper filtration. The solid phase was washed with a few drops of water, dried at 60°C for 3 days and analysed for the clotrimazole content.

*2.2.8. Hemolysis*

Erythrocytes were separated by centrifugation of citrated human blood, washed three times with isotonic phosphate buffer and resuspended in the buffer to give a hematocrit value of 5% (Ohtani et al., 1989). Buffer (3.8 ml) and 0.2 ml erythrocyte suspension (5%) were mixed. Test substances, i.e. clotrimazole 3.1 mg/ml, $\gamma$-cyclodextrin 12.0 mg/ml, clotrimazole $\gamma$-cyclodextrin inclusion complex 16 mg/ml and physical mixture of clotrimazole and $\gamma$-cyclodextrin 16 mg/ml were added as solid compounds at time zero. The samples were incubated in a 37°C water bath for 15 min. Afterwards, the samples were centrifuged for 3 min at $2000 \times g$. The absorbance of the supernatant was measured at 543 nm. A 100% hemolysis absorbance value was obtained by mixing 0.2 ml erythrocyte suspension and 3.8 ml distilled water instead of isotonic buffer, and incubating it for 15 min at 37°C and measuring the absorbance at 543 nm (Ohtani et al., 1989).

*2.2.9. Toxicity on TR146 cell culture*

TR146 cells were incubated and maintained in 75-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 heat-inactivated fetal calf serum 10%, glucose 3.5 mg/ml and penicillin 100 units/ml/streptomycin 100 μg/ml. Further details concerning maintenance and seeding of TR146 cells were as described previously (Jacobsen et al., 1995). Regarding seeding and growth of TR146 cells on Falcon filters, the procedure described previously (Pedersen et al., 1998) was followed strictly. After 29 days' growth on the filters, the cell layers were applied for transepithelial electrical resistance measurements. The effect on the transepithelial electrical resistance of exposing the cell layers to clotrimazole 3.1 mg/ml, $\gamma$-cyclodextrin 12 mg/ml, clotrimazole $\gamma$-cyclodextrin inclusion complex 16 mg/ml and a physical mixture of clotrimazole and $\gamma$-cyclodextrin 16 mg/ml was estimated during a 4 h exposure period. The transepithelial electrical resistance measurement on TR146 cell layers was carried out as described by Pedersen et al. (1998).

Immediately after the 4 h period, the cell layers were removed by trypsination, and 500 $\mu$l cell suspension was mixed with 500 $\mu$l 0.4% Trypan Blue Solution (Sigma catalogue 1530-1531, 1992). The TR146 viability was calculated by counting dead cells (stained blue) and total number of cells using an inverted light microscope and a graticule.

A MTS/PMS toxicity assay on TR146 cell monolayers, grown for 24 h in a 96-well plate, was carried out as described by Jacobsen et al. (1996). The MTS/PMS assay is a tetrazolium salt-based colorimetric assay determining the cellular viability by means of cellular dehydrogenase activity and hence reduction of the tetrazolium salt, MTS, to the corresponding coloured MTS-formazan. Presence of the electron coupling reagent, PMS, speeds up the formation of MTS-formazan (Jacobsen et al., 1996).

The MTS/PMS toxicity of clotrimazole 0.19, 0.78, and 3.1 mg/ml, $\gamma$-cyclodextrin 0.75, 3.0, and 12 mg/ml, clotrimazole $\gamma$-cyclodextrin inclusion complex 1, 4, and 16 mg/ml and finally clotrimazole $\gamma$-cyclodextrin physical mixture 1, 4, and 16 mg/ml was measured. Sodium dodecylsulfate $10^{-2}$ M was used as a positive control of the MTS/PMS toxicity procedure.

## 3. Results and discussion

*3.1. Solubility diagrams*

Van Doorne et al. (1988b) reported that clotrimazole gave an An solubility diagram with $\alpha$-cyclodextrin in distilled water at room temperature. In the present study of $\alpha$-cyclodextrin and clotrimazole the solubility diagram in phosphate buffer 0.05 M, pH 7.1 at room temperature was of the Ap type, i.e. no sedimentation of $\alpha$-cyclodextrin inclusion complex took place (data not shown). Previously, it was shown that application of 0.05 M phosphate buffer instead of water caused an

econazole nitrate β-cyclodextrin solubility diagram to switch from the An to the Bs type (Pedersen et al., 1993a).

The solubility diagrams for clotrimazole and β-cyclodextrin in different media are depicted in Fig. 1. Each solubility diagram in Fig. 1 is based on one determination. According to the figure, distilled water at 21°C gave a Bs diagram. However, the β-cyclodextrin concentration at which formation of solid inclusion complex started was quite near β-cyclodextrin's own solubility. The formed inclusion complex was of a colloid like nature and it was not possible to isolate the complex by filtration or centrifugation. Application of 0.05 M phosphate buffer pH 7.1 instead of water resulted in an A type solubility diagram. A type solubility diagrams were also the result when distilled water at 6°C, Fig. 1 or phosphate buffer 0.05 M, pH 10 was applied as medium (data not shown). Lowering the temperature a few degrees or increasing the pH value from 7.1 to 10.0 were previously applied to get a Bs solubility diagram for β-cyclodextrin and the imidazole derivative miconazole (Pedersen, 1994). However, it did not work for clotrimazole and β-cyclodextrin.

γ-Cyclodextrin gave a Bs type solubility diagram with clotrimazole, Fig. 2. According to the equation,

$$K_{1,1} = \text{slope}/S_o(1 - \text{slope})$$

where $S_o$ is the intrinsic solubility of clotrimazole $0.61 \times 10^{-6}$ M (Pedersen, 1993), and the initial

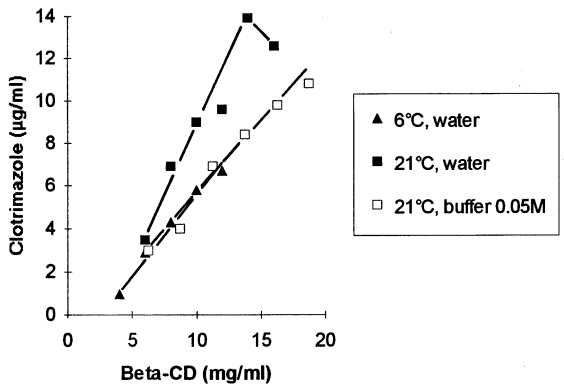


Fig. 1. Clotrimazole β-cyclodextrin solubility diagrams in different media and at different temperatures. pH in buffer 7.1.

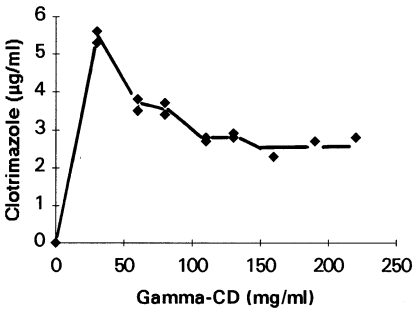


Fig. 2. Clotrimazole γ-cyclodextrin solubility diagram at 21°C in phosphate buffer 0.05 M, pH 7.1.

slope of the solubility diagram in Fig. 2 is $6.62 \times 10^{-4}$,

$K_{1,1} = 1086\ \text{M}^{-1}$.

The DSC results of the clotrimazole γ-cyclodextrin complex, a physical mixture of drug and γ-cyclodextrin, same ratio as for the complex (see below), clotrimazole and γ-cyclodextrin indicated that the isolated complex did not contain a residue of clotrimazole, Fig. 3. No clotrimazole melting peak was present during the DSC analysis of the inclusion complex. The content of clotrimazole, γ-cyclodextrin and water in the isolated, washed, and dried complex was $19.7 \pm 0.2$ ($n = 3$, ± S.E.M.), $77.7 \pm 1.2$ ($n = 3$, ± S.E.M.) and $5.6 \pm 0.2\%$ ($n = 3$, ± S.E.M), respectively. The results are the average of the content of three batches. The molar ratio of the complex was clotrimazole:γ-cyclodextrin:water, 1:1.0:5.4. According to the X-ray diffraction patterns, the inclusion complex was crystalline and the pattern for the complex differed from the patterns of the physical mixture, clotrimazole and γ-cyclodextrin, Fig. 4.

### 3.2. Antimycotic activity

The antimycotic effect of clotrimazole 1.5 mg/ml, physical mixture of clotrimazole and γ-cyclodextrin 7.65–1.5 mg/ml clotrimazole and 5.7 mg/ml γ-cyclodextrin, the inclusion complex 7.65–1.5 mg/ml clotrimazole and 5.7 mg/ml γ-cyclodextrin, and γ-cyclodextrin 5.7 mg/ml is depicted in Fig. 5. The inclusion complex was by far

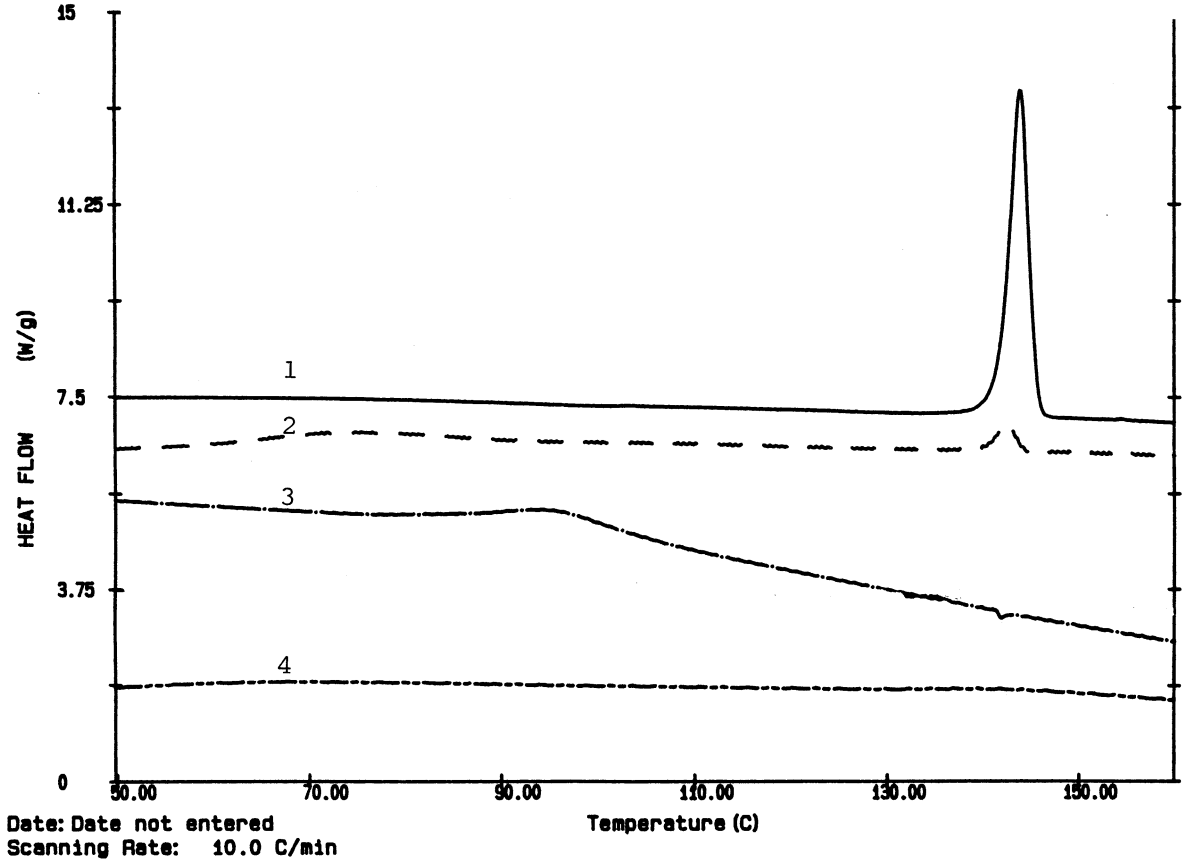


Fig. 3. Normalized DSC curves for (1) clotrimazole; (2) physical mixture of clotrimazole and $\gamma$-cyclodextrin, molar ratio 1:1.0; (3) $\gamma$-cyclodextrin; and (4) clotrimazole $\gamma$-cyclodextrin inclusion complex, molar ratio 1:1.0.

the most active of the tested compounds. The physical mixture and clotrimazole alone had about the same antimycotic activity, while $\gamma$-cyclodextrin was practically without antimycotic effect.

A comparison of the antimycotic effect, the clotrimazole dissolution rate and the clotrimazole supersaturation tendency indicated that the clotrimazole supersaturation, which is disclosed in Fig. 8, was of significant importance for the superior antimycotic activity of the inclusion complex (see Section 3.3).

### *3.3. Dissolution rate studies in Candida growth medium*

The clotrimazole dissolution rate from neat clotrimazole 1.5 mg/ml, the physical mixture of clotrimazole and $\gamma$-cyclodextrin and the inclusion complex both with the molar ratio 1:1.0, is depicted in Fig. 6. The dissolution medium was the Candida albicans growth medium and 7.65 mg/ml was applied of the complex and the physical mixture, corresponding to 1.5 mg/ml clotrimazole. The initial dissolution rate for the inclusion complex and the physical mixture, i.e. the rate within the first 5 min was about the same for the two compounds. Both had a faster dissolution rate than neat clotrimazole. Apparently, equilibrium was established for the physical mixture already 5 min after applying it to the dissolution medium. Regarding the inclusion complex, the dissolution rate curve was more complicated. The concentra-

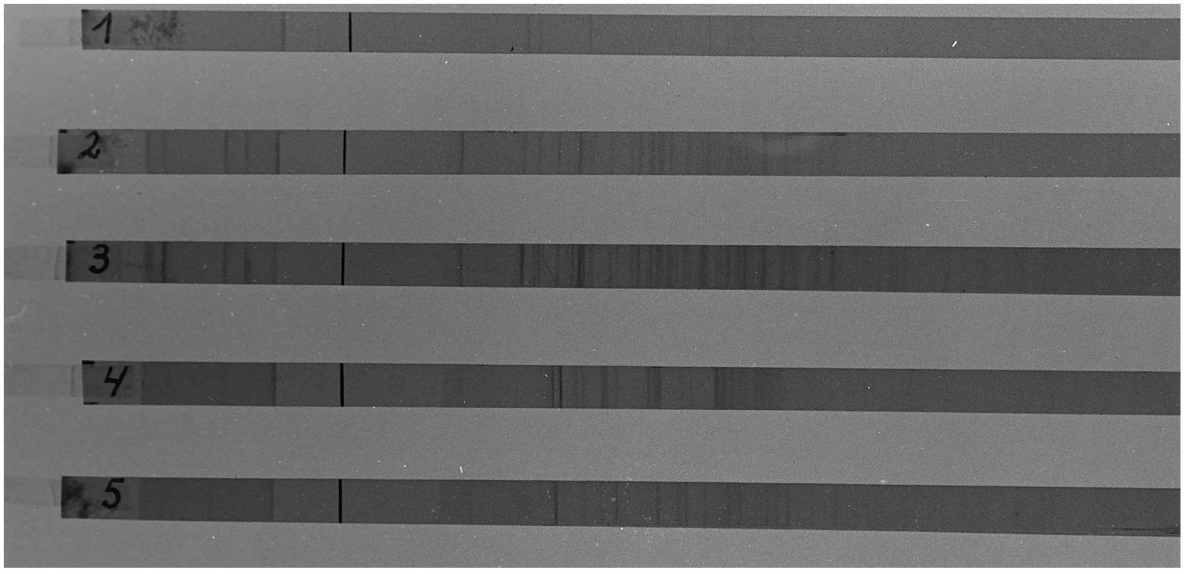


Fig. 4. X-ray diffraction patterns: (1) clotrimazole; (2) $\gamma$-cyclodextrin; (3) physical mixture of clotrimazole and $\gamma$-cyclodextrin, molar ratio 1:1.0; and (4) clotrimazole $\gamma$-cyclodextrin inclusion complex, molar ratio 1:1.0. (5) Precipitate isolated after 24 h. Dissolution rate experiment with the clotrimazole $\gamma$-cyclodextrin inclusion complex. Exposure time 1 h.

tion of dissolved clotrimazole reached its maximum after 5 min. Afterwards the clotrimazole concentration dropped throughout the rest of the test period (24 h). The drop in the concentration might indicate an initial clotrimazole supersaturation. However, it is unusual that the clotrimazole concentration during the complex dissolution testing dropped so much below the clotrimazole concentration which was obtained during the testing of the physical mixture. Fig. 7 shows the $\gamma$-cyclodextrin concentrations during the dissolution rate testing of the physical mixture and the inclusion complex. All the $\gamma$-cyclodextrin contained in the physical mixture was dissolved within 5 min, whereas the $\gamma$-cyclodextrin dissolution rate from the inclusion complex was much slower. Only 1.1 mg/ml $\gamma$-cyclodextrin was dissolved after 24 h. The initial amount of added inclusion complex 7.65 mg/ml contained 5.7 mg/ml $\gamma$-cyclodextrin. Analysis of the clotrimazole, $\gamma$-cyclodextrin and water content of the washed and dried cyclodextrin inclusion complex was actually carried out (see Section 3.1). That is the low $\gamma$-cyclodextrin dissolution rate from the inclusion complex was not due to a lower cyclodextrin content than expected. The slow dissolution rate for the inclusion complex was not due to a Bs solubility diagram for clotrimazole and $\gamma$-cyclodextrin in the dissolution medium. According to Fig. 8, the diagram was of the A type, at least up to 6 mg/ml. That is, the dissolution of the inclusion complex was not hindered by the complex becoming the stable solid phase during the dissolution rate study.

The initial slope of the solubility diagram of clotrimazole and $\gamma$-cyclodextrin in the growth medium (Fig. 8) was 0.0144 and clotrimazole's solubility in the medium was $< 0.5$ $\mu$g/ml$-1.5 \times 10^{-6}$ M. The stability constant $K_{1,1}$ was $> 10076$ $M^{-1}$, according to the $K_{1,1}$ equation shown previously. The stability constant in growth medium, pH 7.5, was about a factor of ten higher than the stability constant in 0.05 M phosphate buffer, pH 7.1. The higher pH value in the growth medium will probably increase the stability constant because clotrimazole is neutralized at higher pH, p$K_a$ in 50% aqueous ethanol was reported to be 4.7 (Buechel et al., 1972). However, a more important reason to the increased stability constant in the growth medium may be, that the medium

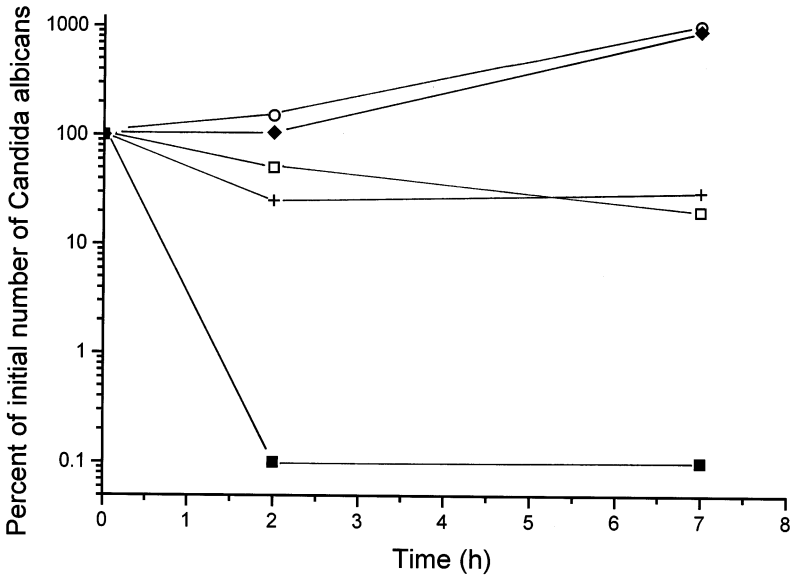


Fig. 5. Antimycotic effect of various compositions upon Candida albicans. ○, blank; ◆, 5.7 mg/ml γ-cyclodextrin; □, 7.65 mg/ml physical mixture clotrimazole γ-cyclodextrin, molar ratio 1:1.0; +, clotrimazole 1.5 mg/ml; ■, inclusion complex clotrimazole γ-cyclodextrin, molar ratio 1:1.0.

contained 4% w/w of the water structure forming agent glucose. Other water structure forming agents, i.e. sorbitol and fructose, were previously shown to increase the stability constant between clotrimazole and β-cyclodextrin (Pedersen, 1993).

Comparison of the solubility diagram and the paired or corresponding concentrations of clotrimazole and γ-cyclodextrin during the dissolution rate studies was done in Fig. 8 (Pedersen, 1997). According to the figure, the inclusion complex gave rise to significant clotrimazole supersaturation of the dissolution medium, whereas supersaturation did not take place when the physical mixture was tested. Supersaturation is present if the paired concentration points are placed above the solubility diagram curve (Pedersen, 1997). The clotrimazole supersaturation that the inclusion

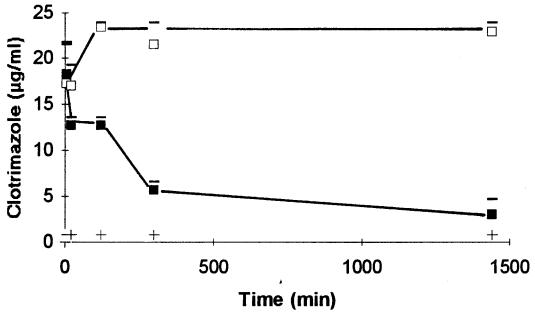


Fig. 6. Clotrimazole dissolution rate in microbiological growth medium, 37°C, average and S.E.M., $n = 3$. An amount corresponding to 1.5 mg/ml clotrimazole was added. □, 7.65 mg/ml physical mixture clotrimazole γ-cyclodextrin, molar ratio 1:1.0; ■, 7.65 mg/ml inclusion complex clotrimazole γ-cyclodextrin, molar ratio 1:1.0; +, 1.5 mg/ml clotrimazole.

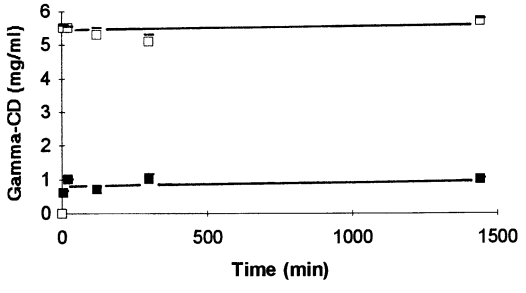


Fig. 7. γ-Cyclodextrin dissolution rate in microbiological growth medium, 37°C, average and S.E.M., $n = 3$. An amount corresponding to 1.5 mg/ml clotrimazole was added. □, 7.65 mg/ml physical mixture clotrimazole γ-cyclodextrin, molar ratio 1:1.0; ■, 7.65 mg/ml inclusion complex clotrimazole γ-cyclodextrin, molar ratio 1:1.0.

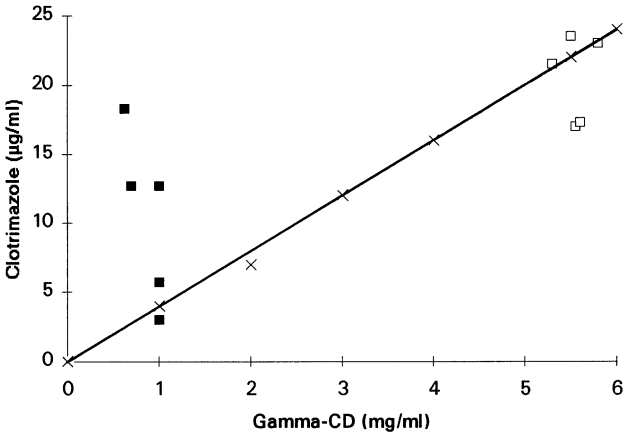


Fig. 8. ×, Clotrimazole γ-cyclodextrin solubility diagram in microbiological growth medium at 37°C. Corresponding clotrimazole and γ-cyclodextrin concentrations during dissolution rate testing in the medium. □, 7.65 mg/ml physical mixture clotrimazole γ-cyclodextrin, molar ratio 1:1.0; ■, 7.65 mg/ml inclusion complex clotrimazole γ-cyclodextrin, molar ratio 1:1.0.

complex gave rise to in the Candida albicans growth medium was probably the reason why the inclusion complex had superior antimycotic effect, Fig. 5.

After the 24 h dissolution test of the cyclodextrin inclusion complex, the solid phase in one of the tubes was isolated. After drying, HPLC analysis showed the content of clotrimazole was only 22.1%. This is in agreement with the fact that only a part of the γ-cyclodextrin dissolved during the dissolution testing, Fig. 7. According to Fig. 5, the precipitate's X-ray diffraction pattern differed from the pattern of the original clotrimazole γ-cyclodextrin inclusion complex.

### 3.4. Hemolysis and toxicity on TR146 cell culture

The toxicity of the clotrimazole inclusion complex, the physical mixture, clotrimazole and γ-cyclodextrin is compared in Figs. 9–11. The hemolytic activity of the compounds correlated with the antimycotic activity, Fig. 9. The inclusion complex had the highest hemolytic activity followed by clotrimazole and the physical mixture. γ-Cyclodextrin did not give hemolysis, Fig. 9. The MTS/PMS toxicity assay on a monolayer of TR146 cells (Jacobsen et al., 1996) showed that the inclusion complex was much more toxic than the other compounds tested, i.e. the physical mixture, clotrimazole and γ-cyclodextrin, Fig. 10. Due to the inclusion complex suspension's own absorbance of light at 492 nm, the testing was limited to 0.19 mg/ml. At 0.78 mg/ml and especially at 3.1 mg/ml the light absorbance of the suspended complex affected the results profoundly.

Measurement of the transepithelial electrical resistance of multilayers of TR146 cells before and after 4 h exposure to the compounds mentioned above, i.e. 16 mg/ml inclusion complex, 16 mg/ml physical mixture, 3.1 mg/ml clotrimazole and 12

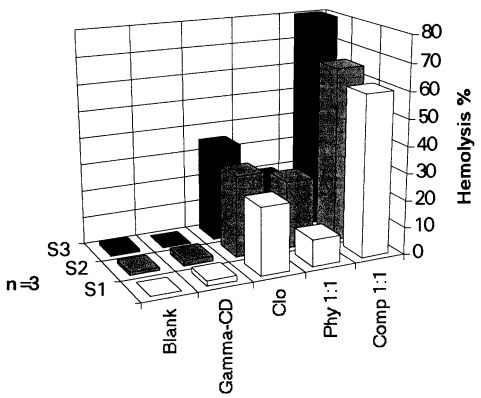


Fig. 9. Hemolysis (%) of γ-cyclodextrin 12 mg/ml; clotrimazole 3.1 mg/ml; 16 mg/ml physical mixture clotrimazole γ-cyclodextrin, molar ratio 1:1.0; 16 mg/ml inclusion complex clotrimazole γ-cyclodextrin, molar ratio 1:1.0. Incubation time 15 min.

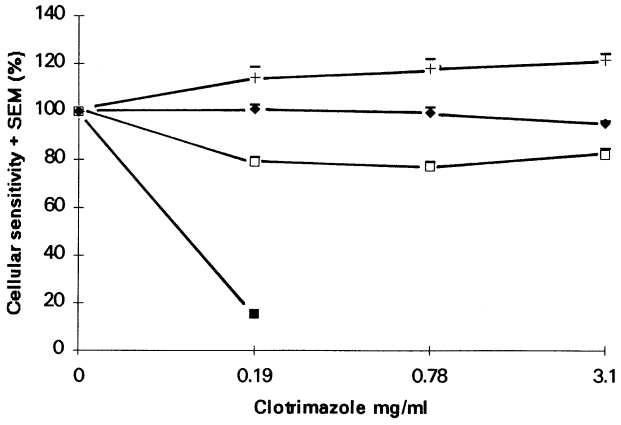


Fig. 10. MTS/PMS assay of TR146 cells testing of clotrimazole and γ-cyclodextrin compositions. The cellular sensitivity is expressed as a function of the clotrimazole concentration applied. Average and S.E.M., $n=8$. The absorbance was recorded at 492 nm. ■, inclusion complex clotrimazole γ-cyclodextrin, molar ratio 1:1.0; +, clotrimazole; □, physical mixture clotrimazole γ-cyclodextrin, molar ratio 1:1.0; ◆, neat γ-cyclodextrin applied in concentrations corresponding to the content of cyclodextrin in the inclusion complex.

mg/ml γ-cyclodextrin, did not give a significant difference between the compounds (data not shown). In addition, light microscopic inspection of the TR146 cells before and after exposure did not show the expected damaging effect of the inclusion complex. After the inspection, the TR146 cell layers were trypsinated and the viability of the suspended cells was tested by trypan blue staining, Fig. 11. According to the results, the inclusion complex was not more toxic than the other compounds.

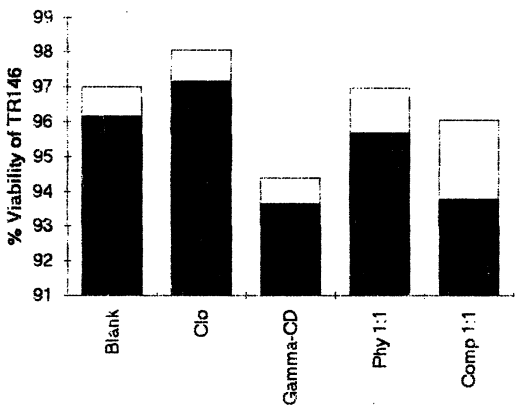


Fig. 11. Viability (trypan blue staining after trypsination) of TR146 cells after 4 h exposure to clotrimazole 3.1 mg/ml; γ-cyclodextrin 12 mg/ml; physical mixture clotrimazole γ-cyclodextrin, molar ratio 1:1.0, 16 mg/ml; inclusion complex clotrimazole γ-cyclodextrin, molar ratio 1:1.0, 16 mg/ml. Average (■) and S.E.M. (□), $n=6$.

The results of the yeast and toxicity studies indicate that the ability of the inclusion complex to cause clotrimazole supersaturation gave high activity on fragile cells, i.e. Candida albicans cells, human erythrocytes and monolayers of not differentiated TR146 cells. The toxicity of the complex was not detectable when a more robust cell system, i.e. multilayers of differentiated TR146 cells, was applied.

## 4. Conclusion

A genuine γ-cyclodextrin inclusion complex of clotrimazole, molar ratio 1:1.0, was isolated by crystallization in phosphate buffer 0.05 M, pH 7.1. The inclusion complex had superior in vitro antimycotic effect compared with clotrimazole and a physical mixture of clotrimazole and γ-cyclodextrin. Probably, the superior effect was caused by the complex's ability to cause clotrimazole supersaturation of the growth medium. The clotrimazole supersaturation was probably also the reason why the complex had higher toxicity than clotrimazole and the physical mixture on erythrocytes and on human TR146 cells.

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