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# An econazole β-cyclodextrin inclusion complex: an unusual dissolution rate, supersaturation, and biological efficacy example

Morten Pedersen [a,*], Simon Bjerregaard [b], Jette Jacobsen [b], Annette Rômmelmayer Larsen [b], Alex Mehlsen Sørensen [c]

[a] *PEDCY, Frejasvej 125, DK-7620 Lemvig, Denmark*
[b] *Department of Pharmaceutics, The Royal Danish School of Pharmacy, 2 Universitetsparken, DK-2100 Copenhagen O, Denmark*
[c] *Department of Analytical and Pharmaceutical Chemistry, The Royal Danish School of Pharmacy, 2 Universitetsparken, DK-2100 Copenhagen O, Denmark*

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**Abstract**

Genuine cyclodextrin inclusion complexes of the antimycotic econazole and β-cyclodextrin had higher antimycotic activity than a physical mixture of econazole and β-cyclodextrin. Surprisingly, the econazole dissolution rate from the physical mixture was higher than the dissolution rate from the inclusion complex. The improved antimycotic activity of the inclusion complex might be due to the superior ability of the complex to cause econazole supersaturation. A new procedure was applied to disclose the drug supersaturation. The genuine inclusion complex molar ratio econazole:β-cyclodextrin 2:3 gave rise to more hemolysis than the corresponding physical mixture. Toxicity testing on a human buccal epithelium in vitro model — based on TR146 cells — showed that the physical mixture was more toxic than the inclusion complex when TR146 cell mortality was evaluated. Neither measurement of the transepithelial electrical resistance of TR146 cell layers exposed to either the physical mixture or the inclusion complex nor analysis of the protein liberation from the TR146 cells during exposure revealed any differences between the two compositions. 

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

## 1. Introduction

Cyclodextrins are cyclic oligosaccharides which are able to form complexes with lipophilic drugs, thus changing their physicochemical and biopharmaceutical properties.

* Corresponding author. Tel.: +45 97821321.

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Isolation of genuine β-cyclodextrin complexes of the lipophilic antimycotic drugs, econazole and miconazole has been reported (Pedersen et al., 1993a; Pedersen, 1994). Compared with the pure drugs and with physical mixtures of drug and β-cyclodextrin, the genuine complexes showed improved antimycotic activity in vitro.

The overall aim of the present paper was to study the antimycotic effect of the econazole β-cyclodextrin complex in more detail. A newly reported method was included to disclose possible supersaturation episodes during dissolution rate testing of the econazole cyclodextrin inclusion complex (Pedersen, 1997).

In addition, a preliminary toxicity testing of the inclusion complex was carried out. Erythrocytes and a TR146 cell culture, a human buccal cell line, were employed.

## 2. Experimental

### 2.1. Materials

Econazole nitrate and β-cyclodextrin were purchased from Sigma (St. Louis, MO). Econazole base was prepared from econazole nitrate as described previously (Pedersen et al., 1993a). Peptone and yeast extract were purchased from Difco (Detroit, MI). *Candida albicans* PF 1383 88 from Statens Seruminstitut (Copenhagen, Denmark) was applied as test organism.

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 (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 μm, growth area 4.6 cm$^2$) and Falcon 6-well culture plates (tissue culture treated polystyrene) were from Becton Dickinson Labware (NJ).

### 2.2. Methods

#### 2.2.1. Antimycotic activity in a fluid medium

Cultures of *Candida albicans* PF 1383 88 were grown at $37 \pm 1$°C in sterile glucose, peptone, yeast extract growth medium (Pedersen and Rassing, 1990), adjusted to pH 7.5 with sterile 0.5 M disodium hydrogen phosphate buffer. At time zero 50 ml medium was inoculated with approximately $5 \times 10^5$ *Candida albicans* cells per ml. A 1-ml sample was taken from each flask containing inoculated broth to count the initial number of cells. Pure drug, β-cyclodextrin, inclusion complex or physical mixtures of drug and β-cyclodextrin were added to the broth. The compounds were sieved through a 180-μm sieve just before the addition.

Samples were taken during a 24-h experimental period. The fungal viability was estimated by plating 100 μl of appropriate dilutions of the samples on agar plates. Colony counting was performed after incubation at 33°C for various time periods.

#### 2.2.2. Solubility diagrams

Solubility measurements were carried out as described by Higuchi and Connors (1965). To 10 ml of sterile *Candida albicans* microbial growth medium, pH 7.5, temperature $37 \pm 1$°C, containing various concentrations of β-cyclodextrin, 10 mg of econazole nitrate were added. After approximately 2 weeks, the suspensions were filtered through 0.2-μm Sartorius cellulose acetate membrane filters. The concentration of econazole in the filtered samples was analyzed by an HPLC method.

#### 2.2.3. Econazole β-cyclodextrin preparations

About 1 l ammonium phosphate buffer solution 0.05 M pH 7.1 containing 18.0 g β-cyclodextrin and 1.00 g econazole nitrate was shaken at 22°C for 10 days in a bottle with a screw cap. During the period samples were taken from the bottle and the solid phase was analyzed by differential scanning calorimetry to ensure that the econazole nitrate was completely transformed to the econazole β-cyclodextrin inclusion complex. After the transformation was complete, the solid complex

was isolated by paper filtration. The complex was washed with a few millilitres of water followed by drying at 60°C for 3 days.

Physical mixtures of $\beta$-cyclodextrin and econazole or econazole nitrate were prepared by gently mixing in a mortar.

*2.2.4. 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 carrier gas. The sample size was in the range 2–5 mg.

*2.2.5. X-ray powder diffraction analysis*

X-ray powder diffraction patterns were recorded with a Guinier, XDC 700 IRDAB powder diffractometer using a Philips PW 1720 X-ray generator. Cr K$\alpha$ radiation was applied.

*2.2.6. HPLC methods*

Econazole concentrations were measured by a reversed phase HPLC method (Pedersen et al., 1993a). Solid econazole preparations were dissolved in dimethyl sulfoxide before the analysis.

The concentration of $\beta$-cyclodextrin in the microbiological growth medium was determined by a reversed phase HPLC method. The eluent was composed of 15% methanol and 85% deionized water, the column was a Merck Lichrosorb 100 18RP ($4 \times 125$ mm) equipped with a Lichrosorb 100 RP18 guard column. A Merck refractive index detector was applied. The detection limit for a 20-$\mu$l loop was 0.5 mg/ml. The correlation coefficient was 1.000 for the concentration range 0.5–16 mg/ml.

*2.2.7. Determination of water content*

About 25.0 mg solid econazole $\beta$-cyclodextrin complex was dissolved in methanol:dimethyl formamide 1:1. Afterwards the solution was titrated automatically with Hydranal composite 5 (Riedel de Haen) on a Methrom 701KF Titrino (Karl Fischer titration).

*2.2.8. Dissolution testing*

The dissolution testing of the drugs, physical mixtures and inclusion complex was carried out using the same experimental conditions as during the test of the antimycotic effect. In comparison with the solubility of econazole in the microbiological growth medium, excessive amounts of econazole 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-$\mu$m membrane filters. To avoid a possible precipitation of econazole after the filtration, one part of the filtrate was mixed with three parts of dimethyl sulfoxide. The mixtures were analyzed for econazole, while the neat filtrates were analyzed for $\beta$-cyclodextrin. Regarding the dissolution medium not filtered through Sartorius filters, precipitates were in some cases isolated by filtration through paper filters. The precipitates were washed with a few drops of water, dried and analyzed.

*2.2.9. Hemolysis*

Erythrocytes were separated by centrifugation of citrated human blood at $1500 \times g$ for 10 min and washed three times with isotonic phosphate buffer, 154 mM sodium chloride, 10 mM phosphate, pH 7.4. The erythrocytes were resuspended in the buffer to give a hemotocrit value of 5% (Ohtani et al., 1989). Buffer 3.8 ml and 0.2 ml erythrocyte suspension (5%) were mixed. The test substances, econazole, econazole nitrate, cyclodextrin inclusion complexes, physical mixture of cyclodextrin and econazole or cyclodextrin alone were added as solid compounds at time zero. The samples were incubated in a 37°C water bath. 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 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 and measuring the 543 nm absorbance of the supernatant (Ohtani et al., 1989).

*2.2.10. TR146 cell culture*

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 $\mu$g/ml gentamicin, and 0.2 $\mu$g/ml *p*-hydroxybenzoic acid *n*-butyl ester. Further details concerning maintenance and seeding of TR146 cells were as described previously (Jacobsen et al., 1995).

The cell seeding density on the Falcon filters was $2.4 \times 10^4$ cells/$cm^2$. Of the culture medium mentioned above, 2.5 ml and 2.0 ml were added to the apical and 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 (trans-epithelial electrical resistance) studies.

*2.2.11. 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. Afterwards the transepithelial electrical resistance was calculated (TEER; Ω $cm^2$).

The measurement of R was carried out at 24°C applying an Endohm-24 connected to an epithelial voltohmmeter (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 $cm^2$.

*2.2.12. Protein determination and TR146 mortality*

TR146 cells were grown to confluence in 25-$cm^2$ T-flasks as described by Jacobsen et al. (1995). The growth medium was removed, and the flasks were rinsed with $5 \times 9$ ml Hanks' buffer. Afterwards 10 ml Hanks' buffer and the test substance were added. 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 $2 \times 5$ 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 total number of cells in three areas of the flasks using an inverted light microscope and a graticule (Jacobsen et al., 1995).

The protein concentration analysis was performed according to Lowry et al. (1951) measuring the absorbance at 280 nm and using alkaline copper reagent and Folin and Ciocalteu's phenol reagent. Bovine serum albumin was applied as standard.

## 3. Results and discussion

### *3.1. Composition of inclusion complex*

The econazole $\beta$-cyclodextrin complex was prepared, isolated and dried as described ($n = 3$). The differential scanning calorimetry indicated that the drug was completely converted to the complex (Fig. 1). That is, no peak from melting of free econazole was present (melting point 87°C). Before equilibration was established, samples taken from the preparation bottles probably contained some free econazole because a differential scanning calorimetry peak just below 87°C was present. Regarding the complex isolated after equilibration, the X-ray powder diffraction pattern did not contain peaks from either free econazole or free econazole nitrate (Fig. 2).

According to the Karl Fischer titration, the water content of the inclusion complex after drying was 6.2% ($n = 3$, S.D. = 0.17%). Surprisingly, the presence of water (6.2%) did not give rise to a detectable water evaporation endothermic peak on the differential scanning calorimetry curve.

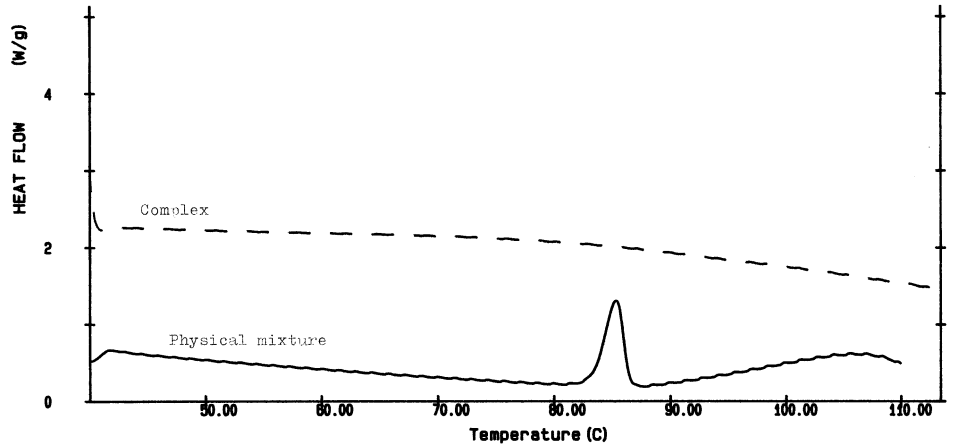


Fig. 1. Normalized differential scanning calorimetry curves for econazole $\beta$-cyclodextrin complex, molar ratio 2:3, and the corresponding physical mixture. Econazole melting point 87°C.

The cut-off temperature in Fig. 1 is 110°C. Other curves were run to 160°C without endothermic or exothermic peaks being present.

According to Szejtli (1988), evaporation of the water content from a cyclodextrin inclusion complex will often take place below 100°C whereas evaporation of crystal water from neat $\beta$-cyclodextrin is seen just above 100°C. The difference in evaporation temperature is caused by different strengths of the bonds between water molecules and cyclodextrin molecules. The reason why water evaporation was not detectable in the present case could be that the evaporation took place over a broad temperature interval or the water molecules were bound so efficiently that evaporation did not take place at all.

Three batches of complex were analyzed for econazole, $\beta$-cyclodextrin and water. The complex composition was econazole $17.0 \pm 0.3\%$, $\beta$-cyclodextrin $76 \pm 3\%$ and water $6.2 \pm 0{,}17\%$; $n = 3$, $\pm$S.D. That is, the molar ratio was (2:3:15.5). An econazole $\beta$-cyclodextrin inclusion complex with a 1:1 molar ratio of econazole and $\beta$-cyclodextrin has been reported previously (Pedersen et al., 1993a). The ratio determination was based solely on an econazole analysis, i.e. analysis of $\beta$-cyclodextrin and water was not carried out.

In the present study, the isolation conditions, i.e. temperature and pH were varied in the ranges 20–26°C and 6.0–10.7 to see if the difference between the present and previously reported molar ratio was due to a variation of the isolation conditions. It did not seem to be the case. The molar ratio econazole:$\beta$-cyclodextrin was 2:3 and it did not vary with temperature and pH.

X-ray powder diffraction analysis results for the two complexes, molar ratio 1:1 and 2:3 are depicted in Fig. 2. Minor differences between the two X-ray diffraction patterns are visible.

As a part of the biological experiments physical mixtures of econazole and $\beta$-cyclodextrin were prepared by gentle mixing in mortars. Differential scanning calorimetry curves of physical mixture samples mixed for various times, 1–10 min, showed that the area of the endothermic peak corresponding to the melting of econazole decreased with increasing mixing time. A physical mixture of econazole and $\beta$-cyclodextrin was prepared by vigorous mixing in a mortar for 10 min. A small amount of water was added during the mixing to make the product paste like. The final product composition, after drying at 60°C for 3 days, was 16.0% econazole, 74–80% $\beta$-cyclodextrin and 7.0% water. An econazole melting peak was not present on the differential scanning curve (data not shown). Due to the risk of changing econazole's properties, the physical mixtures applied in the present studies were prepared by gentle mixing in a mortar for 1 min.

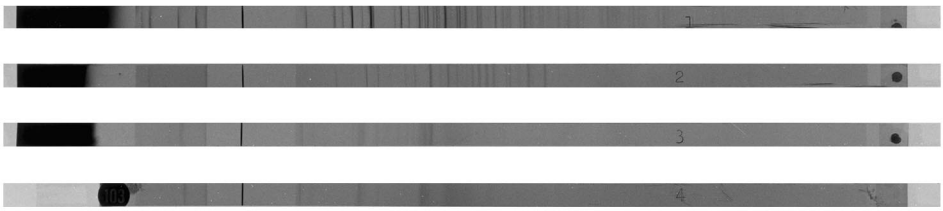


Fig. 2. X-ray diffraction patterns: (1) $\beta$-cyclodextrin; (2) econazole; (3) econazole:$\beta$-cyclodextrin, molar ratio 1:1; and (4) econazole:$\beta$-cyclodextrin, molar ratio 2:3. Exposure time 1 h.

### 3.2. Antimycotic activity in a fluid medium

The effect of the econazole $\beta$-cyclodextrin complexes, molar ratio 1:1 and 2:3 upon *Candida albicans*, PF 1383 88, in vitro is depicted in Fig. 3. The effect of econazole, econazole nitrate and physical mixtures of econazole/econazole nitrate and $\beta$-cyclodextrin, molar ratio 1:1 and 2:3 is included too. The genuine complexes were much more active than the physical mixtures, econazole alone and econazole nitrate alone. The 2:3 complex was the more active of the two complexes. After 2-h exposure to the complexes, the surviving *Candida albicans* did not recover and make colonies within a 48-h incubation period. After 96-h incubation colonies were present. Regarding the other treatments, i.e. physical mixtures, econazole and econazole nitrate, the surviving *Candida albicans* organisms recovered and grew to visible colonies within a 48-h incubation period (Fig. 3a). After 8- and 24-h exposure of the *Candida albicans*, the recovery rate for the different treatments was more equal (Fig. 3b and c).

### 3.3. Dissolution rate and supersaturation

The solubility diagram for econazole nitrate and $\beta$-cyclodextrin in the microbiological growth medium at $37 \pm 1$°C is depicted in Fig. 6. The diagram might be of the Ap type. All over the $\beta$-cyclodextrin concentration range, the solid phase in the tubes was econazole base. The identity of the solid phase was confirmed by HPLC and differential scanning calorimetry (data not shown). Regarding the diagram type, an alternative interpretation of the solubility diagram, Fig. 6, may be that proteins and other components from the growth medium competed with econazole for the cyclodextrin cavities. Up to 4 mg/ml $\beta$-cyclodextrin, almost no cavities were available for econazole. Above 4 mg/ml some of cavities were available for econazole. If this is the case, the diagram is of the Al type above 4 mg/ml $\beta$-cyclodextrin.

The results of the dissolution tests — performed in microbiological growth medium, pH 7.5, $37 \pm 1$°C — for econazole, econazole nitrate, the physical mixtures of econazole or econazole nitrate and $\beta$-cyclodextrin and the inclusion complex 2:3 are depicted in Fig. 4. Throughout the dissolution studies it was ensured by measurement that the pH was kept at 7.5. Due to the extremely low solubility of econazole and econazole nitrate, the concentration of antimycotic during the dissolution rate testing of the drugs alone was hardly detectable, $< 0.5$ $\mu$g/ml. On the contrary, the physical mixture of econazole nitrate and $\beta$-cyclodextrin gave rise to a fast drug dissolution rate. This dissolution rate curve is typical for a system causing drug supersaturation of the dissolution medium (Szejtli, 1988). Probably, the supersaturation was caused by conversion of the nitrate salt to econazole base. The drug supersaturation might be the reason why the econazole nitrate $\beta$-cyclodextrin physical mixture had a quite significant effect upon the *Candida albicans* (Fig. 3).

Regarding the inclusion complex and the physical mixture, both with the molar composition econazole:$\beta$-cyclodextrin 2:3, the drug dissolution rate from the physical mixture was faster than the dissolution rate from the inclusion complex (Fig. 4). The results indicate that the solubility of the econazole $\beta$-cyclodextrin complex in the medium was quite low. Neither the physical mixture nor

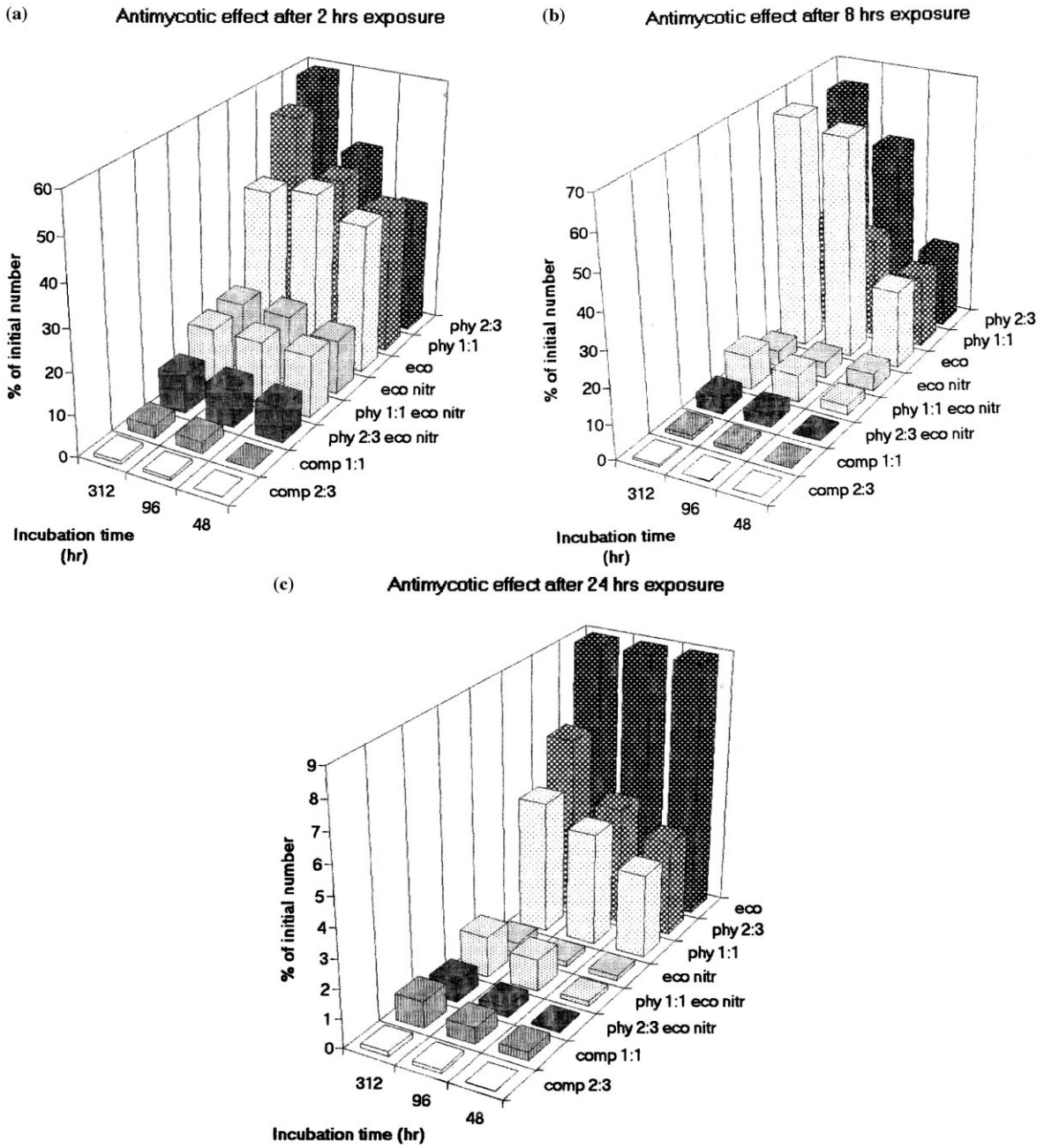


Fig. 3. Antimycotic effect of various econazole compositions upon *Candida albicans*. comp, inclusion complex of econazole and $\beta$-cyclodextrin; phy, physical mixture of econazole (nitrate) and $\beta$-cyclodextrin; 1:1 and 2:3, molar ratio of econazole and $\beta$-cyclodextrin.

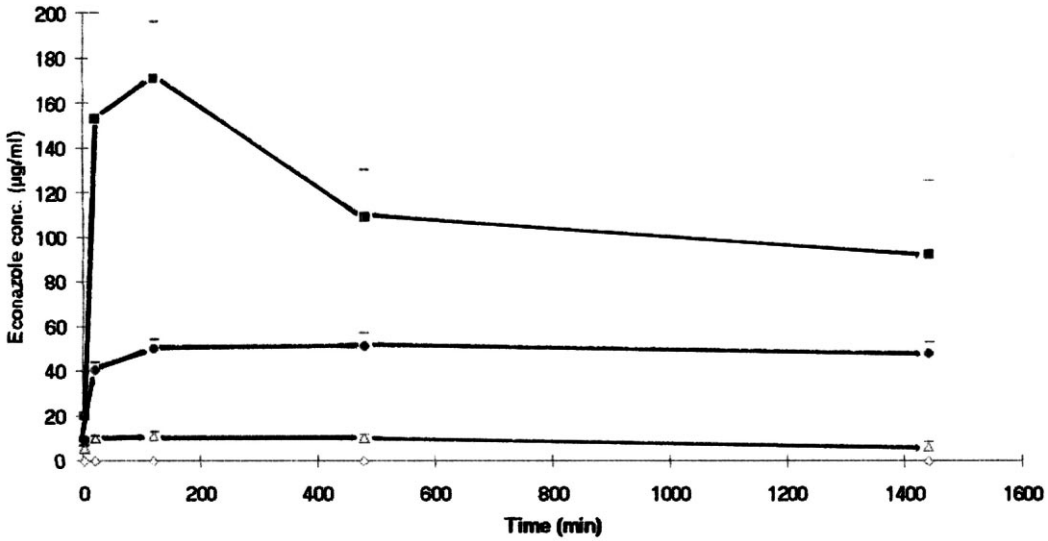


Fig. 4. ■, econazole β-cyclodextrin solubility diagram in microbiological growth medium at 37°C. Corresponding β-cyclodextrin and econazole concentrations during dissolution rate testing in the medium. △, econazole β-cyclodextrin inclusion complex, molar ratio 2:3; ●, econazole β-cyclodextrin physical mixture, molar ratio 2:3. An amount corresponding to 3.3 mg econazole per ml was added.

the inclusion complex gave a dissolution rate curve indicating that a drug supersaturation of the dissolution medium was present (Fig. 4). That is, the results of the dissolution rate study did not correlate with the microbiological results (Fig. 3). The antimycotic effect of the 2:3 inclusion complex was much more pronounced than the effect of the econazole β-cyclodextrin 2:3 physical mixture. Although the econazole nitrate β-cyclodextrin physical mixture gave rise to an extremely fast econazole dissolution rate and drug supersaturation, the antimycotic effect was not as pronounced as the effect of the inclusion complex.

Van Doorne et al. (1988) studied the influence of β-cyclodextrin upon the antimycotic activity of imidazole derivatives such as econazole. Complexation between antimycotic and β-cyclodextrin in a semi-solid medium decreased the antimycotic activity. Van Doorne et al. concluded that the dissolved antimycotic β-cyclodextrin complex only possessed a minor antimycotic activity if any. Recently, part inhibition of the antimycotic effect of dissolved miconazole and econazole by complexation with β-cyclodextrin was reported (Pedersen et al., 1993b). The previous studies indicate that the high antifungal activity of the present econazole β-cyclodextrin inclusion complexes hardly could be due to a high antimycotic effect of the complexes in their own right.

Based on the above, it is difficult to understand why the inclusion complex had higher antimycotic activity than the physical mixture of econazole and β-cyclodextrin. The reason for the lack of correlation between antimycotic effect and dissolution rate/supersaturation may be that tendency of the inclusion complex to supersaturate the growth medium was underestimated by the traditional way of disclosing drug supersaturation. The possible underestimation of the drug supersaturation could be due to the fact that the β-cyclodextrin concentration in the growth medium is not considered by the traditional method. The dissolution rate for β-cyclodextrin from the physical mixture and the inclusion complex of econazole and β-cyclodextrin, both with a molar ratio 2:3, is depicted in Fig. 5. The cyclodextrin dissolution rate was higher from the physical mixture than from the econazole β-cyclodextrin complex. Due to the complexation between econazole and β-cyclodextrin in the growth (dissolution) medium (Fig. 4), considering the β-cyclodextrin concentration may be of importance when the drug super-

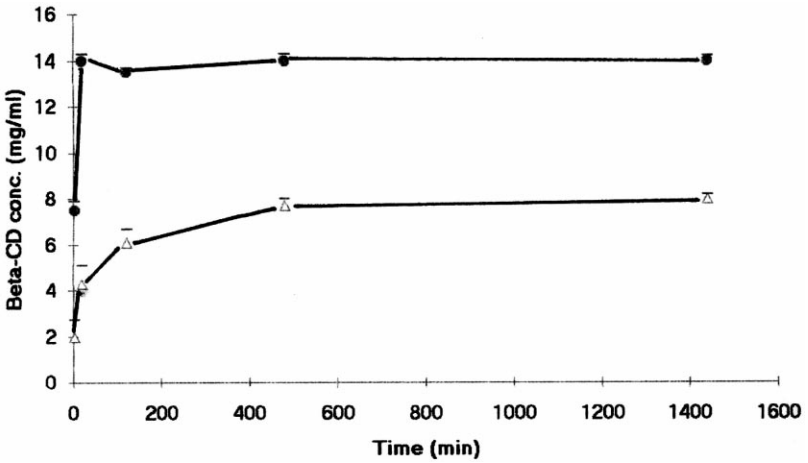


Fig. 5. $\beta$-cyclodextrin dissolution rate in microbiological growth medium, 37°C, average and S.E.M., $n = 3$. An amount corresponding to 3.3 mg econazole per ml was added. △, econazole:$\beta$-cyclodextrin inclusion complex, molar ratio 2:3; ●, econazole:$\beta$-cyclodextrin physical mixture, molar ratio 2:3.

saturation is estimated. The solubility diagram and the paired or corresponding concentrations of econazole and $\beta$-cyclodextrin from the dissolution rate studies (Figs. 4 and 5), are plotted in Fig. 6. Regarding the dissolution rate study of the physical mixture, all the points are placed below the solubility curve, whereas some of the points from the inclusion complex study are placed above or at the solubility curve. Other points are situated below the curve (Fig. 6). Points situated below the solubility curve correspond to the dissolution medium being unsaturated with econazole, while points placed above the curve correspond to the medium being supersaturated. Because the points are compared with the solubility curve, the complexation between econazole and $\beta$-cyclodextrin is taken into consideration when the drug supersaturation of the growth medium is evaluated by this way. According to Fig. 6, the inclusion complex gave rise to drug supersaturation whereas the physical mixture did not. In fact, the physical mixture was far away from just saturating the dissolution medium with econazole. That is, regarding drug supersaturation the picture presented in Fig. 6 is controversial when compared to the one in Fig. 4. The reason for the controversy is that the traditional method of disclosing supersaturation (Fig. 4) does not take into account the concentration of cyclodextrin in the dissolution medium and the complexation between drug and cyclodextrin.

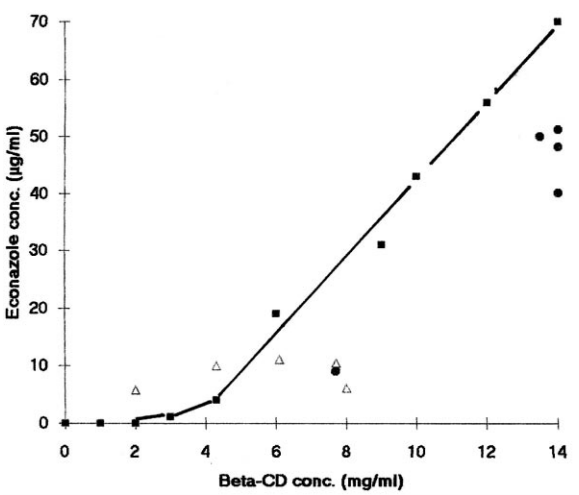


Fig. 6. ■, econazole $\beta$-cyclodextrin solubility diagram in microbiological growth medium at 37°C. Corresponding $\beta$-cyclodextrin and econazole concentrations during dissolution rate testing in the medium. △, econazole $\beta$-cyclodextrin inclusion complex, molar ratio 2:3; ●, econazole $\beta$-cyclodextrin physical mixture, molar ratio 2:3. An amount corresponding to 3.3 mg econazole per ml was added.

The reason why the econazole $\beta$-cyclodextrin inclusion complex worked better than the physical mixture with respect to antimycotic effect is likely to be due to the supersaturation phenomenon disclosed by the method illustrated in Fig. 6.

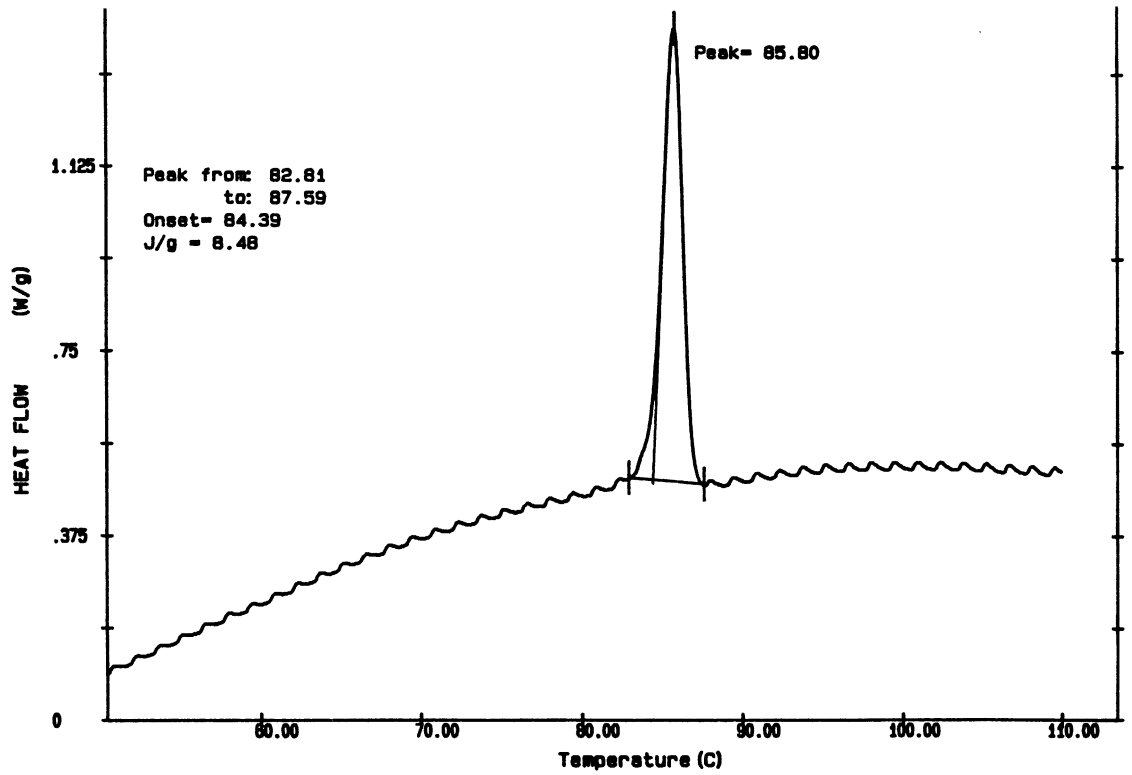


Fig. 7. Normalized differential scanning calorimetry curve of precipitate after 24-h dissolution rate testing of econazole $\beta$-cyclodextrin inclusion complex, molar ratio econazole:$\beta$-cyclodextrin 2:3. Econazole melting point 87°C.

In each dissolution rate experiment, econazole or econazole nitrate corresponding to 3.3 mg/ml econazole base was applied. For the 2:3 econazole:$\beta$-cyclodextrin physical mixture and complex 3.3 mg/ml econazole corresponded to 14.8 mg/ml $\beta$-cyclodextrin. According to Fig. 5, all the $\beta$-cyclodextrin from the physical mixture went into solution within 20 min, while, surprisingly, only 8 mg/ml of the cyclodextrin in the inclusion complex were dissolved after 24 h. Regarding the inclusion complex, the precipitate in two of the dissolution rate tubes was isolated after 24 h and the econazole content was measured by HPLC. The results were an econazole content of 27.1 and 28.9%, respectively. Taking into consideration the concentration of $\beta$-cyclodextrin in the 15 ml dissolution medium after 24 h, i.e. 8 mg/ml, the amount of inclusion complex added initially 289.5 mg, the percentage of econazole and water in the inclusion complex 17.0% and 6.2% respectively, and finally the percentage of econazole in the precipitate after 24-h dissolution experiment 28.0%, the precipitate consisted of 20 mg econazole and 134 mg inclusion complex with the molar composition econazole:$\beta$-cyclodextrin:water 2:3:15.5. That is, even after 24 h only about one half of the inclusion complex added initially was transformed to free econazole and $\beta$-cyclodextrin.

The presence of a neat econazole part in the precipitate after 24 h was detectable by differential scanning calorimetry (Fig. 7).

Applying the method illustrated in Fig. 6 may be worthwhile when studying other cyclodextrin inclusion complexes.

### 3.4. Hemolysis

The effect of $\beta$-cyclodextrin on the hemolysis is depicted in Fig. 8. The dose response study was in agreement with results previously reported by

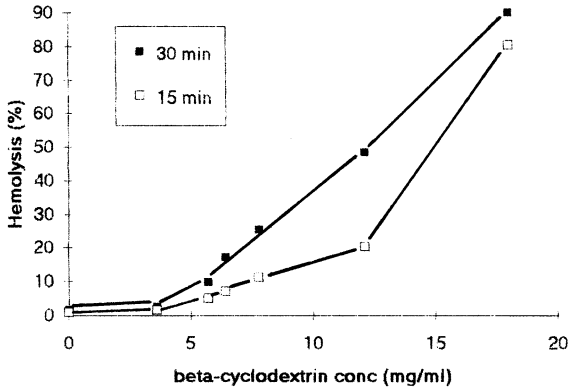


Fig. 8. Hemolysis (%) as a function of $\beta$-cyclodextrin concentration. Incubation time 15 and 30 min.

Ohtani et al. (1989). According to Fig. 9, the econazole $\beta$-cyclodextrin complexes, 1:1 or 2:3, caused significant hemolysis. The physical mixtures were considerably less hemolytic than the complexes. Probably, the higher hemolytic activity of the complexes was due to an improved availability of the antimycotic drug. Higher availability of econazole may also be the reason why econazole nitrate caused more hemolysis than the econazole base (Fig. 9). The lower $\beta$-cyclodextrin dissolution rate from the 2:3 inclusion complex compared with the corresponding physical mixture (Fig. 5) should decrease the hemolytic activity caused by the cyclodextrin part of the inclusion complex.

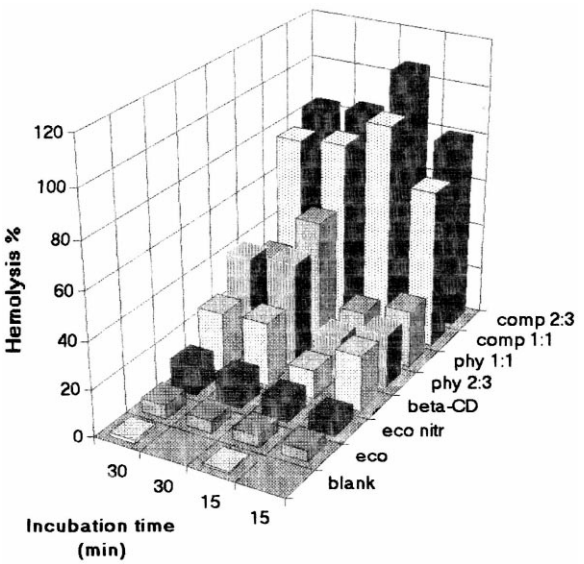


Fig. 9. Hemolysis (%) of various econazole compositions. comp, inclusion complex of econazole and $\beta$-cyclodextrin; phy, physical mixture of econazole (nitrate) and $\beta$-cyclodextrin; 1:1 and 2:3, molar ratio of econazole and $\beta$-cyclodextrin. For cyclodextrin containing samples, an amount corresponding to 7.8 mg $\beta$-cyclodextrin per ml was added; 1.6 and 1.9 mg per ml were added of econazole and econazole nitrate, respectively.

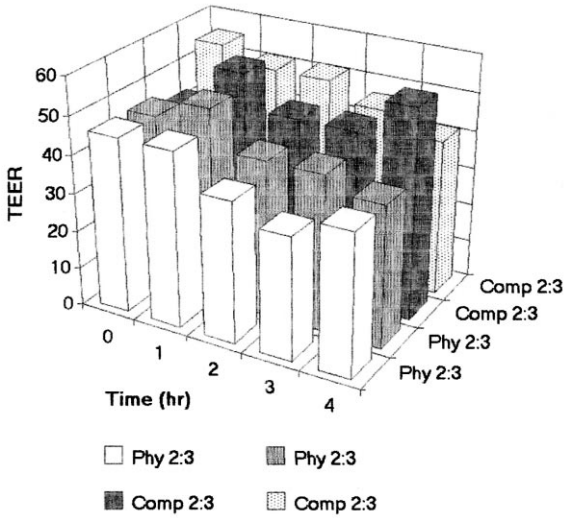


Fig. 10. TEER ($\Omega$ cm$^2$) of TR146 cell layers during exposure to 20.2 mg/ml Comp/Phy 2:3 (econazole $\beta$-cyclodextrin complex/physical mixture, molar ratio 2:3).

### 3.5. TR146 cell culture

Measurement of TEER is a way to evaluate the integrity of TR146 cell layers grown on filters (Jacobsen et al., 1995).

According to Fig. 10, neither the physical mixture nor the inclusion complex, both with the molar ratio econazole:$\beta$-cyclodextrin 2:3, affected the TEER value within the 4-h test period, although 20.2 mg/ml physical mixture or inclusion complex was applied on the apical side of the cell layer. The antimycotic alone 3.3 mg/ml, $\beta$-cyclodextrin alone 16.9 mg/ml or pure Hank's buffer did not affect the TEER value either (data not shown).

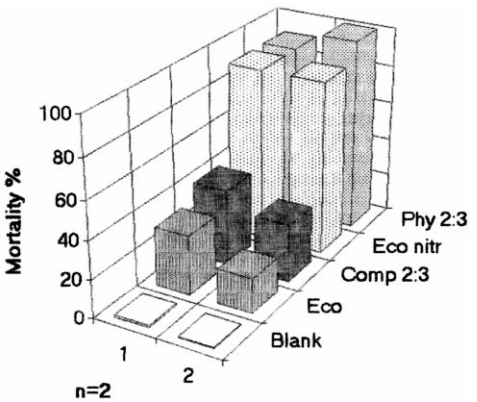


Fig. 11. Mortality of TR146 cells after 240-min exposure to 20.2 mg/ml Comp/Phy 2:3 (econazole $\beta$-cyclodextrin complex/physical mixture, molar ratio 2:3), 3.3 mg/ml econazole and 3.9 mg/ml econazole nitrate.

An analysis of the protein concentration in the buffer after TR146 cells were exposed to either inclusion complex, physical mixture, econazole, econazole nitrate or pure Hank's buffer for 30 or 240 min did not reveal a significant difference regarding the influence of the various treatments upon protein liberation or extraction from the cells (data not shown). Trypan blue determination of the TR146 cell viability after 240-min exposure indicated that the physical mixture and econazole nitrate were more toxic to TR146 than the econazole base and surprisingly also the inclusion complex (Fig. 11).

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