International Journal of Pharmaceutics, 90 (1993) 247–254

IJP 03077

Formation and antimycotic effect of cyclodextrin inclusion complexes of econazole and miconazole

M. Pedersen, M. Edelsten, V.F. Nielsen, A. Scarpellini, S. Skytte and C. Slot

Department of Pharmaceutics, Royal Danish School of Pharmacy, 2 Universitetsparken, DK 2100 Copenhagen O (Denmark)

(Received 25 August 1992)
(Accepted 9 October 1992)

Key words: β-Cyclodextrin; Hydroxypropyl-β-cyclodextrin; Miconazole; Econazole; Antimycotic effect; Stability constant; Solubility diagram; Imidazole derivative

Summary

The stability constants between β-cyclodextrin (β-CD) and the two antimycotic imidazole derivatives, miconazole and econazole were measured. Increased ionization of the imidazole derivatives decreased the size of the stability constants. The same phenomenon was observed for miconazole and hydroxypropyl-β-cyclodextrin. In addition, the type of solubility diagram obtained was dependent on the degree of ionization of the imidazole derivatives. A type Bs solubility diagram was obtained for econazole and β-CD in buffer solution, pH 7.1. An econazole β-CD complex with a molar ratio of 1:1 was isolated. In a fluid medium the antimycotic effect of the econazole β-CD complex against a strain of *Candida albicans* was superior to the effect of a physical mixture of the two compounds. A small inhibitory effect of β-CD on the growth of the test organism was observed.

Introduction

Cyclodextrins are cyclic oligosaccharides which are known to form inclusion complexes with many lipophilic drugs, thus changing the physicochemical and biopharmaceutical properties of the drugs. Imidazole derivatives with antimycotic activity are lipophilic drugs. The formation and antimycotic activity of inclusion complexes of cyclodextrins and the above-mentioned imidazole derivatives were studied by Van Doorne et al. (1988a,b), and Bononi (1988).

Correspondence to: M. Pedersen, Department of Pharmaceutics, Royal Danish School of Pharmacy, 2 Universitetsparken, DK 2100 Copenhagen O, Denmark.

Phase solubility diagrams in water containing different concentrations of β-CD were established for some imidazole derivatives (Van Doorne et al., 1988a), using the method described by Higuchi and Connors (1965). The calculated stability constant, assuming a 1:1 stoichiometric ratio for miconazole and β-CD, was surprisingly small, 808 M^{-1}. In addition, the intrinsic solubility of miconazole in water was on average 500-times higher than those of structurally related imidazole derivatives (Van Doorne et al., 1988a). The high solubility and the small stability constant found by Van Doorne et al. could be due to the fact that the nitrate salt of miconazole was used, while the base form of the other imidazole derivatives was used in the study. The acidic nitrate salt presumably lowered pH in the water,

increasing the solubility of miconazole and decreasing the apparent stability constant due to ionization.

An econazole β-CD and a miconazole β-CD preparation, isolated by freeze-drying, have been patented (Bononi, 1988). According to the patent the preparations containing β-CD were superior to the pure drugs with respect to effectiveness on, e.g. ,vulvovaginal candidosis (Bononi, 1988). Econazole was not included in the investigations performed by Van Doorne et al. (1988a,b).

The present study was carried out to examine the effect of pH on the complex formation between miconazole and cyclodextrins respectively econazole and cyclodextrins. In addition, the antimycotic activity of the complexes was evaluated using both inhibition zone measurements and estimations of the growth rate of *Candida albicans* in a fluid medium.

Materials and Methods

Materials

Econazole nitrate and β-CD were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). The other sample of β-CD used in the study was a generous gift from AVEBE (Foxhold, The Netherlands). Miconazole and miconazole nitrate were kindly donated by Janssenpharma (Birkerod, Denmark) and Medio-blast (Milano, Italy), respectively. Hydroxypropyl-β-cyclodextrin (HP-β-CD), average molar substition 0.9, was from Aldrich (Steinheim, Germany). Peptone and yeast extract were purchased from Difco (Detroit, U.S.A.). All other chemicals were of analytical grade, except for *n*-nonylamine from Sigma Chemical Co. (St. Louis, U.S.A.).

C. albicans PF 1383 88 from Statens Seruminstitut (Copenhagen, Denmark) and *Saccharomyces cerevisiae* NCYC 87 from the National Collection of Yeast Cultures (Norwich, U.K.) were used as test organisms.

Phase diagrams

Solubility measurements were carried out according to the method described by Higuchi and Connors (1965). To 10 ml water or ammonium phosphate 0.05 M buffer solution, pH 7.1, containing various concentrations of β-CD or HP-β-CD, 10 mg of the antimicrobial agent were added. The suspensions were rotated until equilibrium was reached after approx. 10 days. When the solubilizing capacity of HP-β-CD was estimated it was often necessary to add additional amounts of the antimicrobial agent to ensure a solid phase in the samples.

The suspensions were filtered through 0.2 μm Sartorius cellulose acetate membrane filters. The concentration of the antimicrobial agent in the filtered solutions was measured by the HPLC method described below.

Solubility determination

Buffer solution, pH 7.1, was shaken with an excess of econazole nitrate, miconazole nitrate or miconazole while water was shaken with an excess of miconazole for at least 48 h. The suspensions were filtered through 0.2 μm Sartorius cellulose acetate membrane filters and 100 ml of the filtrate were transferred to a separator funnel. The filtrate was extracted twice with 10 ml dichloromethane which was evaporated on a rotary evaporator. The residue was dissolved in 1 ml of methanol and the concentration of the imidazole derivative was determined by the HPLC method described below.

Isolation of econazole β-CD complex

About 4 l of phosphate buffer solution, pH 7.1, containing 54 g β-CD (18 mg/ml) and 4 g econazole nitrate were stirred for 10 days. The precipitate was isolated by paper filtration. The precipitate was washed with a few milliliters of water, followed by drying in a vacuum over phosphorus pentoxide for 24 h.

HPLC method

The concentrations of miconazole and econazole in the filtered solutions were estimated by an HPLC method using a Merck/Hitachi model 655A-11 pump or a Waters Associates model 6000A Chromatography Pump, a Merck/Hitachi model 655A-22 UV-monitor or a Waters Associates model 450 UV-monitor, a Rheodyne model 1725 injection valve fitted with a 20 μl sample

loop, a Merck 2000 chromatointegrator or a BBCX, Goertz Metrawatt SE 120 recorder, a Merck Lichrospher 100 RP18 reverse-phase column (4 mm × 125 mm), and a Merck Lichrosorb RP 18 guard column (4 mm × 10 mm). The detection wavelength was 230 nm. When the miconazole analyses were performed the mobile phase consisted of 82% methanol, 18% aqueous 0.01 M diammonium hydrogen phosphate/0.02 M ammonium dihydrogen phosphate buffer, and 0.005 M *n*-nonylamine to avoid tailing. During the econazole analyses the composition of the mobile phase was 75% methanol, 25% buffer, composed as mentioned above, and 0.005 M *n*-nonylamine. The retention times for miconazole and econazole were 3.7 and 6.6 min, respectively.

The content of econazole in a solid econazole β-CD complex was measured by the HPLC method. The complex was dissolved in dimethyl sulfoxide before the analysis.

Inhibition zone measurements

The antimycotic activity of some of the solutions used to construct the phase diagrams was measured by a plate microbioassay. The indicator strains of *C. albicans* PF 1383 88 and *S. cerevisiae* NCYC 87 were grown for approx. 48 h at 32°C on a dish of agar. The strain was seeded to a concentration of 10^5 yeast cells per ml in agar at 48–50°C. The composition of the agar was as described previously (Pedersen and Rassing, 1990). The seeded agar was poured in lots of 35 ml into 14 cm Petri dishes; six wells were cut, each 6 mm in diameter. 25 μl samples of the phase diagram solutions were placed in the wells according to randomisation schemes devised in advance. Each solution was applied twice. The dishes were incubated at 32°C for 18 h before the zone diameters were measured. The results reported are inhibition zone diameter minus well diameter (6 mm).

Antimycotic activity in a fluid medium

Cultures of *C. albicans* PF 1383 88 were grown at 37°C in sterile glucose, peptone, yeast extract broth (Pedersen and Rassing, 1990), adjusted to pH 7.8 with sterile 0.5 M disodium hydrogen phosphate buffer. At zero time 50.0 ml of the broth, pH 7.8, were inoculated with 0.5 ml of a suspension of *C. albicans* in sterile water, containing approx. 10^8 viable counts/ml. A 1 ml sample was taken from each flask containing inoculated broth, subsequently 764.3 mg econazole β-CD complex (molar ratio 1 : 1), 764.3 mg econazole β-CD physical mixture (molar ratio 1 : 1), prepared by mixing in a mortar for a few minutes, 164.3 mg econazole base, and 191.4 mg econazole nitrate or 600.0 mg β-CD being added to the flasks. The compounds were sieved through a 300 μm sieve before being added. A control containing neither econazole nor β-CD was also included. Samples were taken during a 24 hour experimental period. The fungal viability was estimated by plating 100 μl of appropriate dilutions of the samples on agar plates (Pedersen and Rassing, 1990). Colony counting was performed after incubation at 33°C for 48 h.

Differential scanning calorimetry (DSC)

DSC analysis was carried out using a Perkin Elmer DSC 7 equipped with a Perkin Elmer TAC 7/PC Instrument Controller and Perkin Elmer multitasking software. Nitrogen was used as carrier gas, and the scan speed was 10°C/min. The sample size was in the range 1–5 mg.

Scanning electron microscopy (SEM)

SEM photographs were recorded on a JEOL JSM-5200 Scanning Microscope. The samples were coated on a Bio-Rad Polaron Division E 5200 Auto Spotter prior to photography.

Results and Discussion

According to the classification introduced by Higuchi and Connors (1965), phase diagrams, i.e., solubility curves, are divided into two major categories. Type A solubility curves are obtained when the apparent solubility of the substrate increases with increasing ligand concentration over the entire concentration range. A linear relationship is designated as an A_L type, A_P and A_N curves exhibiting positive and negative curvature, respectively. If a plateau region in the solubility curve of the substrate is observed, it is a type B

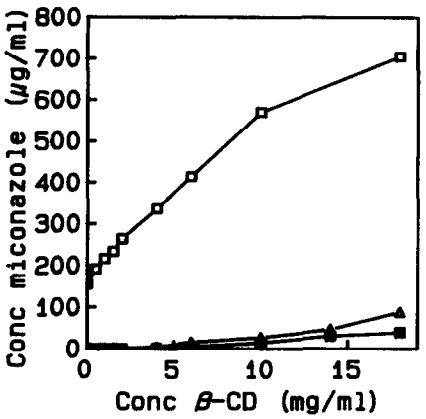

Fig. 1. Solubility of miconazole and miconazole nitrate in the presence of β-CD at 23°C. (□) Miconazole nitrate in water, (△) miconazole in water, (■) miconazole and miconazole nitrate in buffer, pH 7.1.

diagram. The curve is designated B_S if an initial increase in the apparent solubility is observed before the plateau region is reached (Van Doorne et al., 1988a).

The solubility curves for miconazole nitrate and miconazole in water and buffer solution, pH 7.1, are depicted in Fig. 1. Miconazole nitrate gave an A_N diagram with β-CD in water which is in agreement with that reported by Van Doorne et al. (1988b), whereas in another paper the latter authors claimed that a B_S diagram was obtained (Van Doorne et al., 1988a). In order to determine whether the discrepancy between the diagrams was due to the fact that Van Doorne et al. used β-CD from AVEBE, whereas β-CD from Sigma Chemical Co. was employed in the present study, β-CD from AVEBE was obtained and a new solubility curve was constructed. β-CD from AVEBE also yielded an A_N diagram (unpublished data), which means that the discrepancy probably was not due to the use of different qualities of β-CD.

A_P diagrams were obtained for miconazole and β-CD both in water and in the buffer solution, pH 7.1. The nitrate salt also gave an A_P diagram in the buffer solution (Fig. 1). The intrinsic solubility of miconazole nitrate in water was 3.7×10^{-4} M, while those of miconazole nitrate and miconazole in buffer, pH 7.1 and of miconazole in water were less than 4.8×10^{-9} M which was the limit of detection for the HPLC method. The reason for the difference in intrinsic solubility was that miconazole nitrate lowered the pH in water to about 4.2 while it did not affect the pH value of the buffer solution. The pK_a for miconazole is 6.7 (Peeters, 1978), i.e., the drug is almost

TABLE 1

Physicochemical characteristics of miconazole and econazole complexes with cyclodextrins

Substrate	Ligand	Solvent	Solubility (S_0) (M)	Stability constant (M^{-1})	Type of diagram
Miconazole nitrate	β-cyclodextrin	water	3.7×10^{-4}	4.1×10^{2}	A_N
Miconazole	β-cyclodextrin	water	$< 4.8 \times 10^{-9}$	$> 4.0 \times 10^{5}$	A_P
Miconazole nitrate	β-cyclodextrin	buffer, pH 7.1	$< 4.8 \times 10^{-9}$	$> 1.9 \times 10^{5}$	A_P
Miconazole	β-cyclodextrin	buffer, pH 7.1	$< 4.8 \times 10^{-9}$	$> 2.2 \times 10^{5}$	A_P
Miconazole	hydroxypropyl-β-cyclodextrin	water	$< 4.8 \times 10^{-9}$	$> 9.1 \times 10^{5}$	A_P
Miconazole nitrate	hydroxypropyl-β-cyclodextrin	water	3.7×10^{-4}	2.8×10^{2}	A_N
Econazole nitrate	β-cyclodextrin	water	1.6×10^{-3}	4.1×10^{2}	A_N
Econazole nitrate	β-cyclodextrin	buffer, pH 7.1	2.9×10^{-8}	2.1×10^{5}	B_S

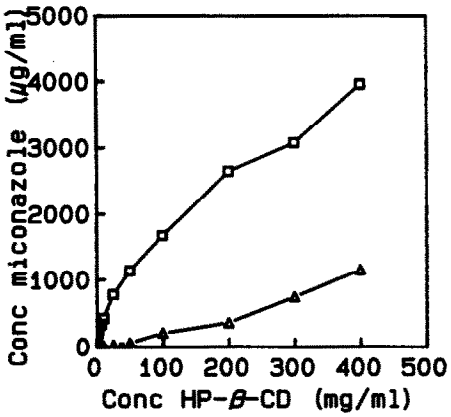

Fig. 2. Solubility of miconazole and miconazole nitrate in water in the presence of HP-β-CD at 23°C. (□) Miconazole nitrate, (△) miconazole.

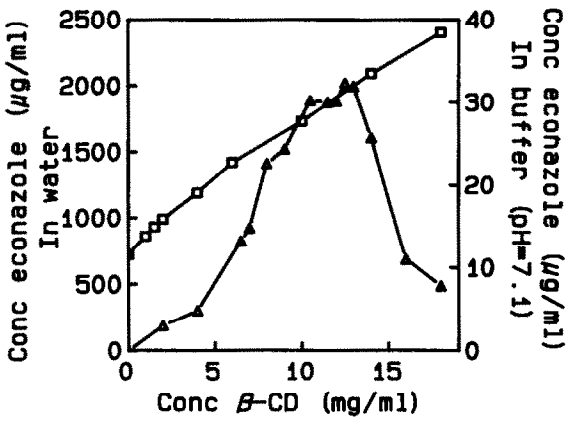

Fig. 3. Solubility of econazole nitrate in water and buffer in the presence of β-CD at 23°C. (□) Water, (△) buffer, pH 7.1.

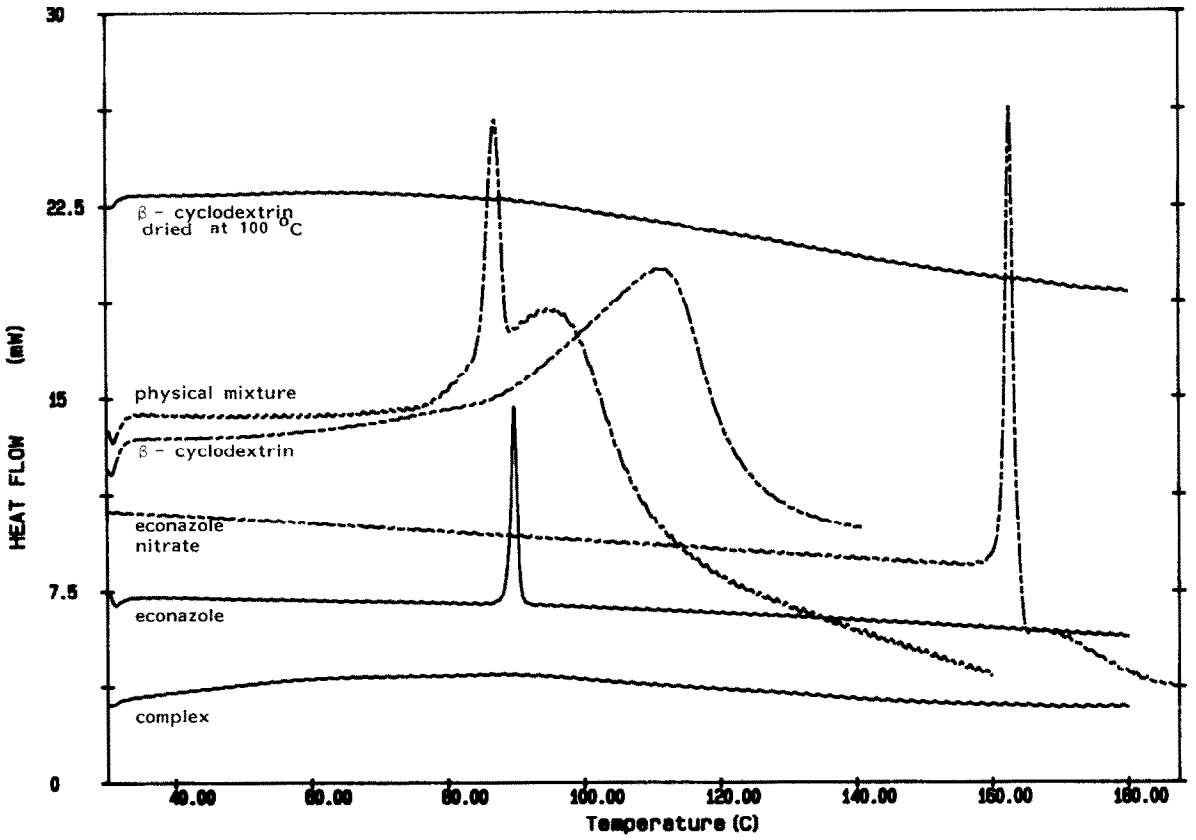

Fig. 4. DSC curves for econazole nitrate converted to econazole in buffer solution, pH 7.1, econazole nitrate, econazole-β-CD physical mixture, molar ratio 1 : 1, econazole-β-CD complex, molar ratio 1 : 1 and β-CD.

fully ionized at pH 4.2 while it is almost neutral at pH 7.1. The degree of ionization of the drug in solution coincides with the type of solubility curve obtained (Fig. 1). The ionized drug gave an A_N diagram while the neutral drug resulted in an A_P diagram, indicating that complexes of greater than 1:1 stoichiometry in β-CD are produced when the drug is in the neutral form (Higuchi and Connors, 1965). A similar pattern was observed when the ligand was HP-β-CD (Fig. 2).

The apparent stability constants ($K_{1:1}$) were calculated from the solubility data using the formula

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

where S_o denotes the intrinsic solubility of the substrate and slope is the slope of the linear initial part of the solubility curves (Higuchi and Connors, 1965). The limit of detection was used instead of the intrinsic solubility in the formula when the solubility was below the limit of detection.

The intrinsic solubilities, the type of diagram and the apparent complex constants are summarized in Table 1. It is obvious that the magnitude of the apparent stability constants is dependent on the degree of ionization of the imidazole derivatives. The value of the constant between miconazole nitrate and β-CD in water was 4.1×10^2 M^{-1} while that between miconazole and β-CD was at least 4.0×10^5 M^{-1}. A similar dependence on the degree of ionization was observed for the constants between miconazole (nitrate) and HP-β-CD and between econazole nitrate and β-CD (Table 1). The results are in agreement with the general observation that ionized species are weak complex forming agents, as far as cyclodextrins are concerned (Szejtli, 1988). The type of solubility diagram obtained for econazole nitrate was also dependent on the degree of ionization (Fig. 3). Econazole nitrate solubilized by β-CD in water and buffer, pH 7.1, resulted in A_N and B_S diagrams, respectively. This meant that a solid econazole β-CD complex could be isolated from the buffer solution. In order to obtain a pure complex the experimental procedure during the isolation of the complex corresponded to a point on the descending part of the solubility curve. The econazole content of the dried complex was $25.5 \pm 0.7\%$ (average ± SD, $n = 3$). The DSC curve for the dried complex indicated that it did not contain a detectable amount of water (Fig. 4). Therefore, the molar composition of the β-CD-econazole complex was $1:1.02 \approx 1:1$. In connection with the solubility determinations it was observed that econazole nitrate suspended in buffer solution was converted to the solid base (Fig. 4). This is why a physical mixture of β-CD and econazole, isolated

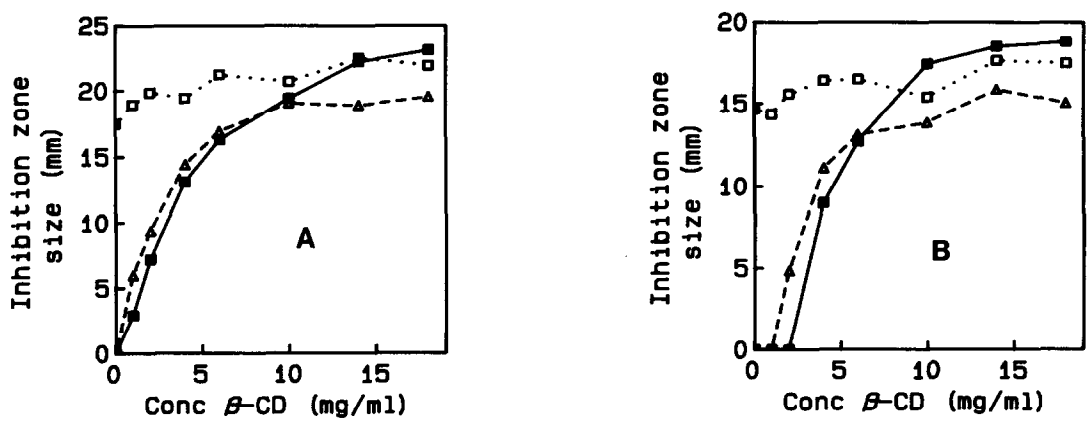

Fig. 5. Influence of β-CD concentration on inhibition zone size. (A) *C. albicans*. (B) *S. cerevisiae*. (□) Miconazole nitrate in water, (△) miconazole in water, (■) miconazole and miconazole nitrate in buffer, pH 7.1.

from buffer solution, was used as a DSC control of the solid complex. The DSC curve for the complex verified that a genuine complex had been isolated (Fig. 4).

The antimycotic effect of the saturated solutions of drug containing various cyclodextrin concentrations was estimated on the basis of inhibition zone size. The inhibition zone sizes minus well diameter were plotted vs cyclodextrin concentration (Figs 5–7). The shape of the curves depended on the degree of ionization of the drug. When the drug was almost totally unionized there was a sharp initial rise of the inhibition zone size after which the curves levelled off. The sharp initial rise was not seen when the drug was ionized, i.e., econazole nitrate or miconazole nitrate in water. The plots of inhibition zone size vs cyclodextrin concentration are consistent with the solubility diagrams, since the greatest solubility improvements were observed for the unionized drugs. β-CD (18 mg/ml) or HP-β-CD (300 mg/ml) did not affect the growth of the test organisms, however, HP-β-CD (500 mg/ml) gave rise to a small inhibition zone in its own right (8.47 mm) when *C. albicans* was used as test organism. Further discussion of the connection between inhibition zone size and cyclodextrin concentration was given by Van Doorne et al. (1988a).

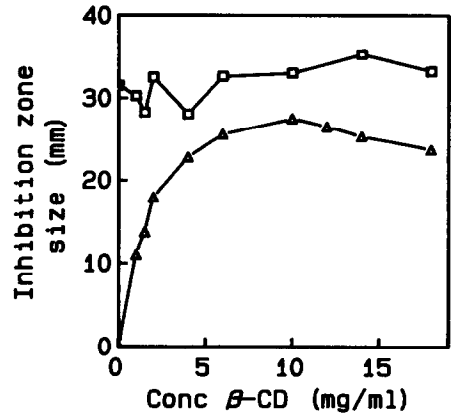

Fig. 7. Influence of β-CD concentration on inhibition zone size. (□) Econazole nitrate in water, *C. albicans*; (△) econazole nitrate in buffer, pH 7.1, *C. albicans*.

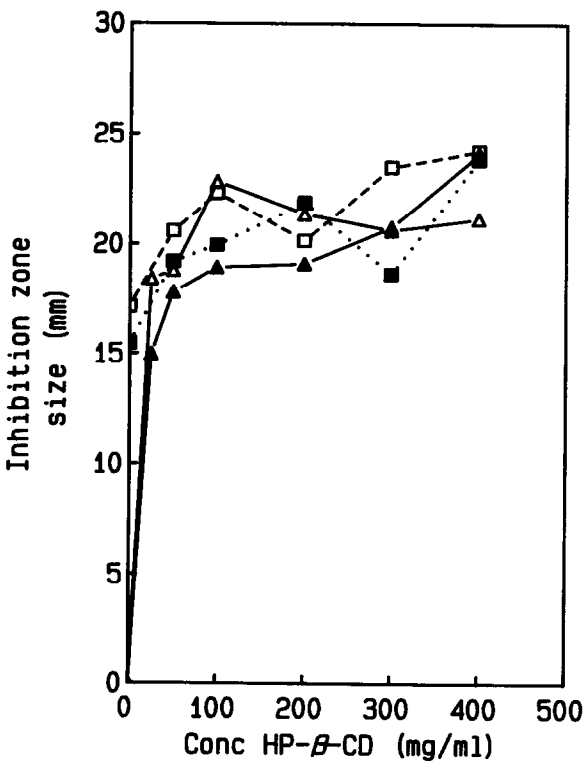

Fig. 6. Influence of HP-β-CD concentration on inhibition zone size. (□) Miconazole nitrate in water, *C. albicans*; (■) miconazole nitrate in water, *S. cerevisiae*; (△) miconazole in water, *C. albicans*; (▲) miconazole in water, *S. cerevisiae*.

In a fluid medium the antimycotic effect of the econazole β-CD complex was superior to the effect of econazole nitrate and econazole alone (Fig. 8). The *C. albicans* strain was to a large extent killed by the complex. Econazole nitrate was more active against the fungus than econazole, probably because the nitrate salt was more soluble than the base. β-CD had an inhibitory effect in its own right on the growth of the test organism. The reason could be that β-CD complexed essential nutrients in the medium or extracted compounds from the *C. albicans* cells. It is unlikely that the superior effect of the econazole β-CD complex was due to an additive or synergistic effect of the two compounds, since the physical mixture did not display the same effectiveness. An increased econazole solution rate or formation of a supersaturated solution can ex-

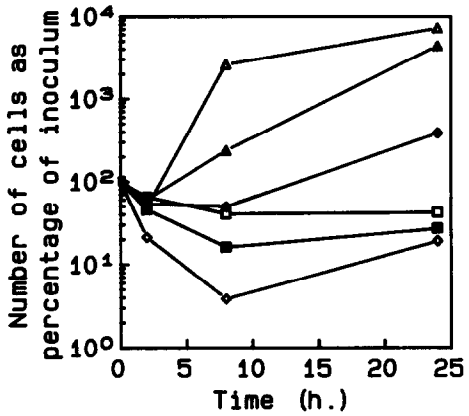

Fig. 8. Time-dependent changes of the viability of *C. albicans* cultures in nutrient broth, as influenced by econazole and β-CD. The following agents were added to the broth at zero time: (△) no addition; (▲) β-CD 12 mg/ml; (□) econazole 3.3 mg/ml; (■) econazole nitrate 3.8 mg/ml; (◆) econazole-β-CD physical mixture, molar ratio 1:1, 15.3 mg/ml; (◇) complex of econazole and β-CD, molar ratio 1:1, 15.3 mg/ml.

plain the antimycotic effect of the complex. In addition, it was observed that the complex was more readily wettable than the physical mixture and the pure drugs. During the experiment the complex formed a milky suspension in the growth medium while the physical mixture, econazole and econazole nitrate formed firm precipitates.

Scanning electron microscopy showed that the particles of the complex were larger than those of econazole and econazole nitrate. Hence, reduction of the particle size cannot explain the fungicidal effect of the complex.

Acknowledgements

Ms Jytte Davidsen and Ms Annelise Lisborg are acknowledged for their technical assistance, and Dr Steen Pedersen for performing the DSC analysis. This research was supported by grants from the PharmaBiotec Research Center, Fertin Laboratories A/S, Generalkonsul Ludvig Tegner og Hustrus Mindelegat and Erik Hoerslev og hustru Birgit Hoerslevs Fond.

References

Bononi, L.J., Beta-cyclodextrin complexes having anti-mycotic activity, *European Patent Application* 288,019 (1988).

Higuchi, T. and Connors K.A., Phase-solubility techniques. *Adv. Anal. Chem. Instrum.*,, 4 (1965) 117–122.

Pedersen, M. and Rassing M.R., Miconazole chewing gum as a drug delivery system – application of solid dispersion technique and lecithin. *Drug Dev. Ind. Pharm.*,, 16 (1990) 2015–2030.

Peeters, J., Determination of ionization constants in mixed aqueous solvents of varying composition by a single titration. *J. Pharm. Sci.*, 67 (1978) 127–129.

Szejtli, J., Cyclodextrin technology, *Topics In Inclusion Science,* Kluwer, Dordrecht, 1988, p. 260.

Van Doorne, H., Bosch, E.H. and Lerk, C.F., Formation and antimicrobial activity of complexes of β-cyclodextrin and some antimycotic imidazole derivatives. *Pharm. Weekbl. Sci. Ed.,* 10 (1988a) 80–85.

Van Doorne, H., Bosch, E.H. and Lerk, C.F., Interactions between cyclodextrins and some antimycotic imidazole derivatives: Studies on solubility and antimicrobial activity. In Huber, O. and Szejtli, J. (Eds), *Proceedings of the Fourth International Symposium on Cyclodextrins*, Kluwer, Dordrecht, 1988b, pp.285–291.