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Research paper

Cyclodextrin inclusion complexes of antimycotics intended to act in the oral cavity – drug supersaturation, toxicity on TR146 cells and release from a delivery system

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Abstract

The dissolution rate, the toxicity and the release from chewing gum of miconazole and econazole cyclodextrin products and complexes were investigated. The dissolution rate studies showed that an amorphous miconazole hydroxypropyl-β-cyclodextrin product gave drug supersaturation, whereas drug supersaturation was not present during dissolution rate testing of an econazole hydroxypropyl-β-cyclodextrin product. The miconazole hydroxypropyl-β-cyclodextrin product and genuine cyclodextrin inclusion complexes of miconazole, econazole and clotrimazole were toxic on a human TR146 buccal cell culture model. The toxicity was probably due to drug supersaturation, thereby increasing the bioavailability of the antimycotics. The econazole hydroxypropyl-β-cyclodextrin product and physical mixtures of miconazole or econazole and β-cyclodextrin did not give supersaturation and were not as toxic as the above-mentioned compounds. Neat econazole and miconazole, a genuine econazole β-cyclodextrin complex and the miconazole hydroxypropyl-β-cyclodextrin product were incorporated in chewing gum. The miconazole hydroxypropyl-β-cyclodextrin gum had a much higher drug release in vitro than the neat miconazole gum. The genuine econazole β-cyclodextrin complex only increased the drug release moderately when compared with the release from the neat econazole gum. The release studies were performed on a mastication device.

Keywords: Cyclodextrin inclusion complex; Hydroxypropyl-β-cyclodextrin; Econazole; Miconazole; Solubility diagram; Dissolution rate; Supersaturation; TR146 cell culture; Toxicity; Antimycotic effect

1. Introduction

In the pharmaceutical field, cyclodextrins are mainly used to increase the aqueous solubility and the dissolution rate of drugs, to improve the stability of drugs and to increase drug bioavailability [1–5]. The naturally occurring cyclodextrins composed of six, seven and eight D-glucose units are named α-, β-, and γ-cyclodextrin, respectively. Hydroxypropyl-derivatives of β-cyclodextrin have a much higher water solubility than the native β-cyclodextrin. In addition, the hydroxypropyl-β-cyclodextrin derivatives give rise to fewer concerns about safety than the native β-cyclodextrin with regard to parenteral administration [6].

In vivo application of cyclodextrins has been reviewed recently [7]. However, the use of cyclodextrins in the delivery of antimycotics to the oral cavity has received little attention [8–11].

Miconazole, econazole and clotrimazole are imidazole antimycotics with a broad spectrum of activity. Miconazole and clotrimazole are used extensively in the local treatment of fungal infections in the oral cavity. Cyclodextrin complexation of miconazole, econazole and clotrimazole has been studied in detail [8–10,12–24].

The aim of the present study was to investigate the physicochemical, toxicological and antimycotic properties of genuine cyclodextrin inclusion complexes of miconazole, econazole and clotrimazole, and of kneaded products of hydroxypropyl-β-cyclodextrin and econazole or miconazole. The physicochemical properties were investigated by a recently published method disclosing possible supersaturation episodes during dissolution rate testing of cyclodextrin inclusion complexes and cyclodextrin products [25]. The toxicological properties were examined by a MTS/PMS assay optimized for the cell line TR146 [26] originating from a human buccal carcinoma [27].

Chewing gum can be an attractive delivery system for antimycotics intended to act against fungal infections in

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the oral cavity [28]. Due to the low water solubility and the high lipid solubility of imidazole antimycotics, they are released extremely slowly from the lipophilic chewing gum base [29]. The effect of cyclodextrin inclusion complexation upon the release of imidazole antimycotics from chewing gum was measured on an in-vitro mastication device in the present study. The antimycotic activity of the released antimycotic was tested on a *Candida albicans* strain.

2. Materials and methods

2.1. Materials

Miconazole was a generous gift from Janssenpharma (Birkeroed, Denmark). Econazole nitrate, clotrimazole, β-cyclodextrin, and phenazine methosulphate (PMS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). α-Cyclodextrin was obtained from AVEBE (the Netherlands) and γ-cyclodextrin was purchased from Cyclolab (Hungary). Hydroxypropyl-β-cyclodextrin, average molar substitution 0.9, was obtained from Aldrich (Steinheim, Germany). Econazole base was prepared from econazole nitrate as described previously [22]. Peptone and yeast extract were purchased from Difco (Detroit, MI, USA). *C. albicans* PF 1383 88 was generously supplied by Statens Serum Institut (Copenhagen, Denmark). The *C. albicans* growth medium consisted of glucose, peptone, yeast extract, and distilled water, adjusted to pH 7.5 with 0.5 M phosphate buffer [29].

The TR146 cell line, derived from a human neck node metastasis originating from a buccal carcinoma [30], was kindly provided by Imperial Cancer Research Technology (London, UK). Heat-inactivated fetal calf serum (FCS) was obtained from Harlan Sera-Lab (Belton, UK). Dulbecco's modified Eagle medium (DMEM) was purchased from HyClone® Laboratories (Logan, UT, USA). Penicillin G/streptomycin sulphate solution was obtained from Gibco BRL (Paisley, UK). A kit CellTiter 96® AQ_{ueous} non-radioactive cell proliferation assay based on MTS ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was purchased from Promega (Madison, WI, USA).

All other chemicals were of analytical grade and used as received.

2.2. Preparation of miconazole, econazole and clotrimazole cyclodextrin complexes/products

Crystalline cyclodextrin inclusion complexes of miconazole, econazole and clotrimazole were prepared by precipitation from phosphate buffer solutions as described previously [10,22,24]. Miconazole hydroxypropyl-β-cyclodextrin product, molar ratio 1:2, and econazole hydroxypropyl-β-cyclodextrin product, molar ratio 1:4, were prepared by a kneading method which has also been described previously [23]. The respective physical mixtures of the antimycotics and the cyclodextrins were prepared by gently mixing the components in a mortar for a few minutes.

2.3. Solubility diagrams

Solubility measurements were carried out as described by Higuchi and Connors [31]. Ten milligrams of miconazole or 10 mg of econazole nitrate was added either to 5 ml of 0.05 M ammonium phosphate buffer, pH 7.1, at 23°C, containing various concentrations of α- or γ-cyclodextrin, or to 10 ml of sterile *C. albicans* growth medium at 37°C containing various concentrations of hydroxypropyl-β-cyclodextrin, The suspensions in phosphate buffer were rotated for 10 days, and those in growth medium for 2 days in order for equilibration to take place. Afterwards, the suspensions were filtered through 0.2 μm Sartorius cellulose acetate membrane filters, and the concentration of antimycotic in the filtrates was analyzed by the reversed phase HPLC methods described previously [22,24].

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

$$K_{1:1} = slope/S_0(1 - slope) \quad (1)$$

where S_0 denotes the intrinsic solubility of the drug, i.e. miconazole < 0.0020 μg/ml and econazole 0.011 μg/ml [22]. The slope is the slope of the initial part of the solubility curves [31].

2.4. Dissolution rate studies of kneaded miconazole and econazole hydroxypropyl-β-cyclodextrin products

The dissolution rate studies were made in 10 ml portions of *C. albicans* growth medium at 37 ± 1°C adding 160 mg miconazole hydroxypropyl-β-cyclodextrin product or 148 mg econazole hydroxypropyl-β-cyclodextrin product. Samples were taken from the dissolution medium over a 24-h experimental period. The samples were immediately filtered through Sartorius cellulose acetate 0.2 μm filters. The concentrations of miconazole and econazole in the filtrates were measured by the reversed phase HPLC methods described previously [22,24]. The hydroxypropyl-β-cyclodextrin concentration in the filtrates was measured by a spectrophotometric method. The method was based on measuring the decrease in the absorbance of a phenolphthalein solution at 550 nm and pH 10.5 as a function of the concentration of hydroxypropyl-β-cyclodextrin [32].

2.5. MTS/PMS toxicity assay with TR146 cells

The TR146 cells were grown in T-75 flasks at 37°C and in a 98% humidified atmosphere of 5% CO_2/95% air. The medium consisted of DMEM supplemented with 10% FCS, 4.5 mg/ml glucose, 100 IU/ml penicillin G, and 100 μg/ml streptomycin sulphate. The maintenance of TR146 cells has been described previously [26].

A MTS/PMS toxicity assay on TR146 cell monolayers grown for 24 h in a 96-well plate was carried out as described previously [10,27]. The MTS/PMS assay is a tetrazolium salt-based colorimetric assay measuring absorbance at 492 nm, determining the cellular viability by means of cellular dehydrogenase activity and hence reduction of the tetrazolium salt, MTS, to the corresponding colored MTS-formazan. Presence of the electron coupling reagent, PMS, speeds up the formation of MTS-formazan [27].

Genuine β-cyclodextrin inclusion complexes of econazole and miconazole, a genuine clotrimazole γ-cyclodextrin inclusion complex, neat hydroxypropyl-β-cyclodextrin, kneaded miconazole and econazole hydroxypropyl-β-cyclodextrin products, neat β-cyclodextrin, and finally physical mixtures of miconazole or econazole and β-cyclodextrin were studied. The test solutions/suspensions contained 1 or 4 mg/ml of the above-mentioned compounds and were prepared immediately before use. Sodium dodecylsulphate solution 10^{-2} M was included as a positive control of the MTS/PMS assay procedure.

2.6. Miconazole and econazole chewing gum

Chewing gum, 0.9 g a piece, containing 10 mg econazole or 30 mg miconazole was prepared. A conventional chewing gum base was applied. The chewing gum base was composed of Fertin gum base 35.0%, solid paraffin wax 0.8%, lycasin 10.0%, sorbitol 52.1%, menthol 1.3%, and peppermint oil 0.8%. The drugs were incorporated in the chewing gum base as the neat antimycotics, the econazole β-cyclodextrin inclusion complex and the miconazole hydroxypropyl-β-cyclodextrin kneaded product. The difference in amount of miconazole and econazole per piece reflects roughly the difference in antimycotic activity of the drugs, i.e. 30 mg miconazole was considered equal to 10 mg econazole as far as antimycotic activity is concerned [17].

The in vitro release of the antimycotics from chewing gum was measured on a mastication device [33]. The release medium consisted of 0.05 M phosphate buffer, pH 7.4. The volume of release medium was 10 ml, and this was replaced every 2 min. Application of 10 ml release medium per 2 min is assumed to reflect the saliva production during chewing [29]. The mastication period was 30 min. A homogenized part of the release medium samples was mixed with dimethyl formamide (1:1) to dissolve the antimycotic released as particles. Afterwards, the concentrations of econazole and miconazole were measured by the above-mentioned reversed phase HPLC methods. The percentage released of the initial content of antimycotics in the chewing gum was calculated.

The antimycotic activity in some of the miconazole release samples was measured by an agar diffusion method. Release samples without dimethyl formamide were applied. The test organism was a *C. albicans* strain. The agar diffusion method has been described in detail previously [34].

2.7. Scanning electron microscopy (SEM)

SEM photographs were recorded on a JEOL JMS-5200 scanning microscope. The samples were sieved through a 400-μm sieve and coated on a Bio-Rad Polaron Division E 5200 Auto Spotter prior to photography.

3. Results and discussion

3.1. Physicochemical properties of cyclodextrin complexes and products

The stability constant, $K_{1:1}$, and the type of solubility diagram for the various combinations of econazole and miconazole with α-, β- and γ-cyclodextrin are given in Table 1. The solubility medium was 0.05 M phosphate buffer, pH 7.1. For both econazole and miconazole, the size of the stability constants was in the range $\alpha- > \beta- > \gamma$-cyclodextrin. The stability constants for α-cyclodextrin were about 100 times higher than the stability constants for γ-cyclodextrin and 5–10 times higher than the β-cyclodextrin stability constants. For all three cyclodextrins, the miconazole stability constants were higher than the econazole stability constants. The stability constants between the antimycotics and α-cyclodextrin were unusually high when compared with stability constants for most drug cyclodextrin combinations. However, unusually high stability constants for miconazole/econazole and cyclodextrins have been reported previously [15,16,18,22]. Except for miconazole γ-cyclodextrin and econazole β-cyclodextrin, the solubility diagrams were of the A_p type. The miconazole γ-cyclodextrin B_s solubility diagram is depicted in Fig. 1. The diagram did not have a descending part although only 2 mg miconazole was present per milliliter. The reason for the lack of a descending part was probably that equilibrium was not reached within the experimental period, i.e. within 10 days. Differential scanning calorimetry analysis of precipitates from sample tubes corresponding to the plateau region showed that solid miconazole was still present after 10 days of equilibration. Mico-

Table 1
Physicochemical characteristics of miconazole and econazole cyclodextrin inclusion complexes in 0.05 M phosphate buffer pH 7.1

Substrate	Ligand	Apparent stability constant ($K_{1:1}$) (M^{-1})[b]	Type of solubility diagram
Miconazole	α-cyclodextrin	$>2.23 \times 10^6$	A_p
	β-cyclodextrin	$>2.20 \times 10^5$[a]	A_p[a]
	γ-cyclodextrin	$>4.30 \times 10^4$	B_s
Econazole nitrate	α-cyclodextrin	9.40×10^5	A_p
	β-cyclodextrin	2.10×10^5[a]	B_s[a]
	γ-cyclodextrin	1.88×10^4	A_p

[b] Calculated by means of Eq. (1).
[a] Data are given in [22].

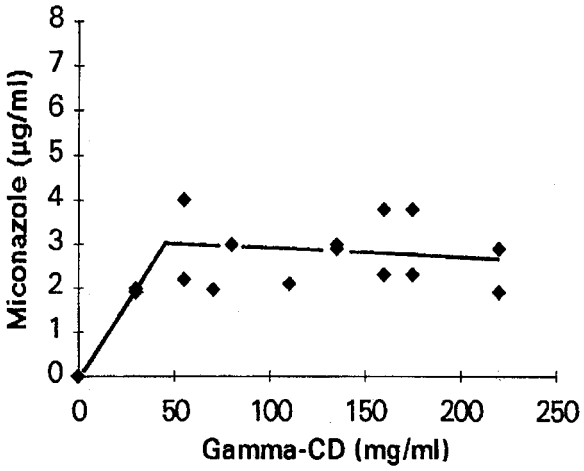

Fig. 1. Miconazole γ-cyclodextrin solubility diagram at 23°C in 0.05 M phosphate buffer, pH 7.1.

nazole melting peaks were present on the differential scanning calorimetry curves (data not shown). That equilibrium was not reached within the 10 days experimental period was probably the reason for the scattered solubility data depicted in Fig. 1. The equilibrium was not reached because the apparent miconazole solubility in the medium was low even when γ-cyclodextrin was present. The low apparent solubility made the miconazole γ-cyclodextrin complexation and precipitation slow. The complexation is assumed to take place in solution. Due to the lack of purity, the miconazole γ-cyclodextrin complex was not included in subsequent experiments. Differential scanning calorimetry analysis of the kneaded miconazole hydroxypropyl-β-cyclodextrin, molar ratio 1:2, and econazole hydroxypropyl-β-cyclodextrin, molar ratio 1:4, products showed that melting peaks corresponding to the neat antimycotics were absent (data not shown). In addition, powder X-ray diffraction studies showed that the products were amorphous. SEM micrographs of the econazole and miconazole hydroxypropyl-β-cyclodextrin products are shown in Fig. 2. On the other hand, the miconazole β-cyclodextrin complex, molar ratio 1:2.0, and the clotrimazole γ-cyclodextrin complex, molar ratio 1:1.0, appeared crystalline (Fig. 2). The crystalline appearance of the two complexes has been confirmed by powder X-ray diffraction studies [9,10]. The econazole β-cyclodextrin complex, molar ratio 2:3.0, formed agglomerates (Fig. 2). According to Pedersen et al. [8], the econazole β-cyclodextrin complex showed distinct lines on an X-ray powder diffraction spectrum, i.e. the complex is crystalline although it is difficult to recognize the crystalline structure of this complex in Fig. 2.

The above-mentioned genuine and crystalline complexes had quite modest antimycotic and cyclodextrin dissolution rates [8–10], whereas both the drug and the hydroxypropyl-β-cyclodextrin dissolution rate from the kneaded amorphous miconazole and econazole hydroxypropyl-β-cyclodextrin products was quite fast (Figs. 3 and 4). The dissolution studies were performed under non-sink conditions, i.e. possible drug supersaturation episodes taking place during the studies could be observed. Within a few minutes, all of the hydroxypropyl-β-cyclodextrin was

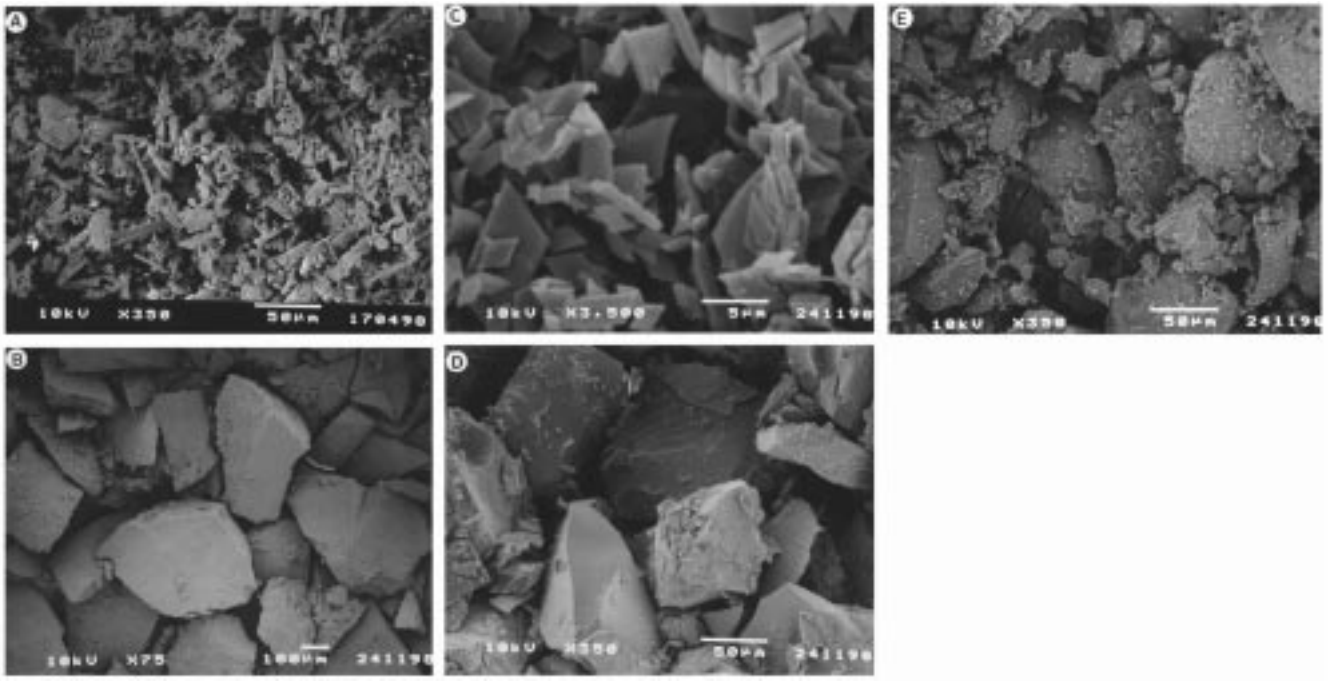

Fig. 2. Scanning electron micrographs of imidazole antimycotic cyclodextrin complexes and products. (A) Clotrimazol γ-cyclodextrin inclusion complex, molar ratio 1:1.0. (B) Econazole β-cyclodextrin inclusion complex, molar ratio 2:3.0. (C) Miconazole β-cyclodextrin inclusion complex, molar ratio 1:2.0. (D) Econazole hydroxypropyl-β-cyclodextrin product, molar ratio 1:4.0. (E) Miconazole hydroxypropyl-β-cyclodextrin product, molar ratio 1:2.0.

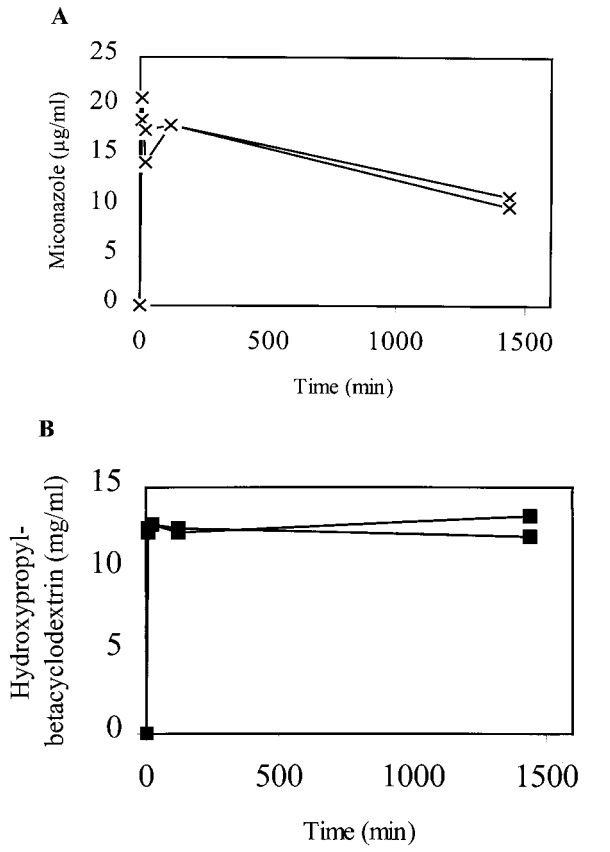

Fig. 3. (A,B) Dissolution rate curves at 37°C in *C. albicans* growth medium for miconazole hydroxypropyl-β-cyclodextrin kneaded product, molar ratio 1:2 containing 12.4% drug, $n = 2$. Initially, 16 mg miconazole hydroxypropyl-β-cyclodextrin product was added per milliliter.

dissolved (Figs. 3 and 4). Regarding the miconazole hydroxypropyl-β-cyclodextrin product, a typical drug supersaturation episode was present during the dissolution rate test (Fig. 3). Econazole supersaturation was not present during the dissolution rate testing of the econazole hydroxypropyl-β-cyclodextrin product (Fig. 4). Despite the slow drug dissolution rate from the above-mentioned genuine and crystalline cyclodextrin inclusion complexes, these complexes did indeed give rise to a profound drug supersaturation of the dissolution medium. These supersaturation phenomena or episodes were not disclosed by the classical approach to disclose drug supersaturation, but by a newly developed method which takes in consideration both the drug dissolution rate, the cyclodextrin dissolution rate and the drug cyclodextrin solubility diagram in the dissolution medium [8–10,25]. This new procedure was applied on the econazole hydroxypropyl-β-cyclodextrin case. As per Fig. 4c, all the corresponding econazole hydroxypropyl-β-cyclodextrin concentrations obtained during the dissolution rate test were placed at or below the solubility diagram curve. This indicates that drug supersaturation was not present even when this new procedure was applied [25]. The theoretical background for the new procedure was discussed in detail recently [25]. Regarding the miconazole hydroxypropyl-β-cyclodextrin dissolution rate case, it was not necessary to apply the new approach to disclose the miconazole supersaturation episode. As mentioned above Fig. 3(B) clearly indicates that miconazole supersaturation was present initially.

In conclusion, the drug and the cyclodextrin dissolution rate from the above-mentioned cyclodextrin complexes and hydroxypropyl-β-cyclodextrin products depended on the physical state of the complexes and products. The crystalline complexes had a slow dissolution rate, whereas the amorphous hydroxypropyl-β-cyclodextrin products had a fast dissolution rate.

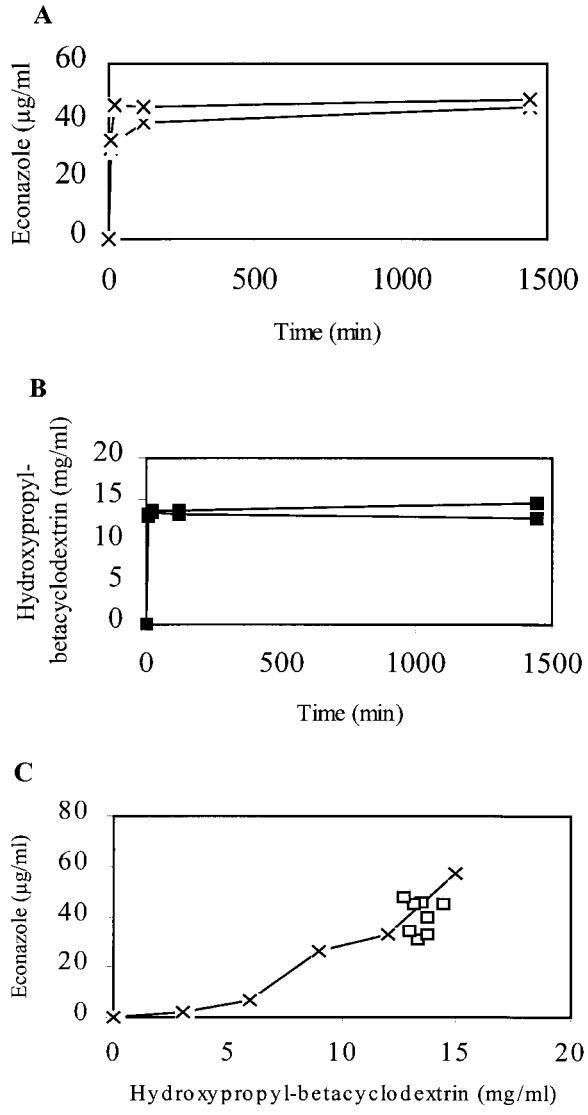

Fig. 4. (A,B) Dissolution rate curves at 37°C in *C. albicans* growth medium for econazole hydroxypropyl-β-cyclodextrin kneaded product, molar ratio 1:4 containing 6.0% drug, $n = 2$. Initially, 14.8 mg econazole hydroxypropyl-β-cyclodextrin product was added per milliliter. (C) (×) Econazole hydroxypropyl-β-cyclodextrin solubility diagram in *C. albicans* growth medium at 37°C. (□) Corresponding econazole and hydroxypropyl-β-cyclodextrin concentrations during dissolution rate testing in *C. albicans* growth medium.

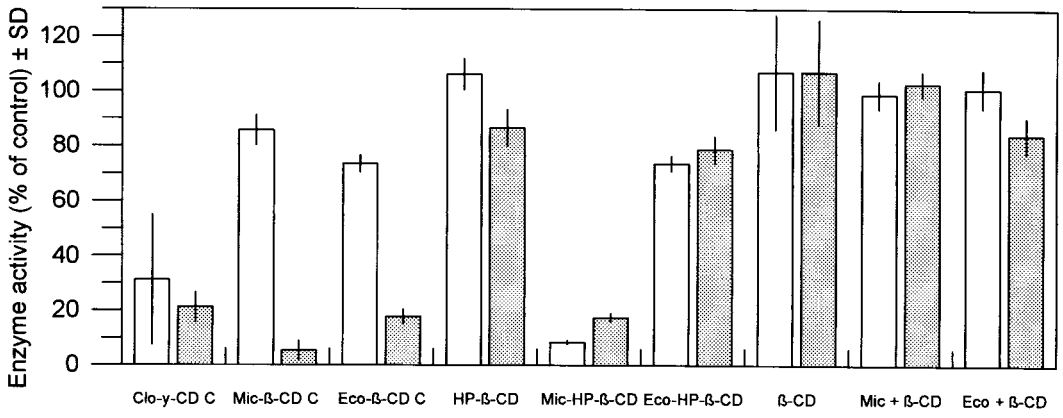

Fig. 5. MTS/PMS assay applying TR146 cells. Effect of various clotrimazole (clo), econazole (eco), and miconazole (mic) compositions on dehydrogenase activity after 4 h exposure, expressed as percentage of mean control values corrected for background values, absorbance was recorded at 492 nm. Specification of compositions: (C) Inclusion complex of antimycotic and γ-cyclodextrin or β-cyclodextrin; (-HP-β-CD) antimycotic and hydroxypropyl-β-cyclodextrin product, (+) physical mixture of antimycotic and β-cyclodextrin. Initial concentrations: Open bar is 1 mg/ml, dotted bar is 4 mg/ml (mean ± SD, $n = 5$).

3.2. Toxicity on TR146 cell culture

The toxicity to TR146 cells applying a MTS/PMS assay is depicted in Fig. 5. The positive control, i.e. sodium dodecylsulphate10^{-2} M, resulted in 100% toxicity to TR146 cells (data not shown). The genuine and crystalline cyclodextrin inclusion complexes of miconazole, econazole, and clotrimazole and the miconazole hydroxypropyl-β-cyclodextrin product exerted a toxic action, whereas the econazole hydroxypropyl-β-cyclodextrin product and the physical mixtures of miconazole or econazole and β-cyclodextrin showed none or little toxicity to TR146 cells (Fig. 5). Similarly, the neat β-cyclodextrin and the neat hydroxypropyl-β-cyclodextrin only showed a minor if any toxic effect on the TR146 cells. In addition, neat γ-cyclodextrin was reported to be non-toxic to TR146 cells [10].

The toxicity exerted by genuine antimycotic cyclodextrin inclusion complexes and the kneaded miconazole hydroxypropyl-β-cyclodextrin product might possibly be due to an increased bioavailability of the antimycotics as a result of the drug supersaturation displayed by these compounds. The other compounds tested did not give rise to supersaturation (Fig. 4 and [8–10]). In addition, the toxicity exerted by the different cyclodextrin complexes, products and physical mixtures did not correlate with the drug or the cyclodextrin dissolution rate of the compounds. If there were a correlation between drug or cyclodextrin dissolution rate and the MTS/PMS toxicity on TR146 cells the physical mixtures of econazole or miconazole and β-cyclodextrin should be much more toxic than they proved to be (Fig. 5 and [8,9]).

Although the complexes and products having the ability to give drug supersaturation were toxic to the TR146 cells in the present study, it may not be of clinical relevance. Only one layer of non-differentiated TR146 cells was used in the present toxicity study. This is assumed to make the test very sensitive. On multilayers of differentiated TR146 cells the antimycotic cyclodextrin inclusion complexes hardly showed any toxic effects [8,9,10].

3.3. In vitro release of miconazole and econazole from chewing gum

Chewing gum as a drug delivery system has been reviewed by Rassing [35]. Medicated chewing gum is a convenient and acceptable mode of treating local diseases in the oral cavity. The release rate of a drug from a chewing gum is dependent on the water solubility of the drug. The results of the in vitro release of miconazole and econazole from chewing gum are depicted in Table 2. The kneaded miconazole hydroxypropyl-β-cyclodextrin product-chewing gum showed a large

Table 2
In vitro release of miconazole and econazole from chewing gum over a 30-min experimental period[a]

Release	Formulations			
	Neat miconazole	Kneaded miconazole hydroxypropyl-β-cyclodextrin product	Neat econazole	Genuine econazole β-cyclodextrin complex
Percentage of initial content	0.7 0.7	23 27	0.7 1.2	2.0 2.6

[a] The release medium consisted of 0.05 M phosphate buffer pH 7.4, $n = 2$.

Table 3
Inhibition zone diameters (mm) caused by in vitro release samples of miconazole from chewing gum over a 30-min mastication period[a]

Formulations	Release time (min)		
	2	14	30
Neat miconazole	6.2	5.4	3.5
	7.3	5.8	–
Kneaded miconazole hydroxypropyl-β-cyclodextrin product, molar ratio 1:2	10.7	7.9	7.8
	10.7	7.5	5.7

[a] *C. albicans PF 1383 88* was applied as test organism using an agar diffusion method, $n = 2$. Chewing gum without miconazole did not give any inhibition zone. The results reported are inhibition zone diameter − well diameter.

miconazole release, about 25% was released during the 30 min mastication period, whereas only 0.7% miconazole was released from the neat miconazole-chewing gum. The genuine econazole β-cylodextrin inclusion complex-chewing gum showed a modest release, about 2.3% of the initial content was released within the 30 min period compared to 0.1% from the neat econazole-chewing gum.

The chewing gum release data are in perfect agreement with the dissolution rate data for the neat antimycotics, the econazole β-cyclodextrin inclusion complex, and the miconazole hydroxypropyl-β-cyclodextrin product (Fig. 4 and [8,9]).

The antimycotic activity of some of the release medium samples is shown in Table 3. The results showed that the miconazole released from chewing gum had antimycotic activity. The release samples from the miconazole hydroxypropyl-β-cyclodextrin-chewing gum experiment showed higher antimycotic activity than the samples from the neat miconazole-chewing gum experiment. This is in agreement with the release data based on chemical analysis (Table 2).

It could be worthwhile to test the release properties of the clotrimazole γ-cyclodextrin inclusion complex from chewing gum. However, so far the clotrimazole inclusion complex has not been isolated in sufficient quantities to be incorporated in a chewing gum batch.

In conclusion, the chewing gum experiment showed that both incorporation of the econazole β-cyclodextrin inclusion complex and the miconazole hydroxypropyl-β-cyclodextrin kneaded product improved the antimycotic release from the chewing gum. The higher effect on the release was achieved using the kneaded miconazole cyclodextrin product.

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