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Antimycotic influence of β-cyclodextrin complexes—In vitro measurements using laser nephelometry in microtiter plates

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Abstract

To determine the in vitro susceptibility of fungal organisms to β-cyclodextrin (CD) complexes with the antifungal agents econazole-nitrate (EC) and ciclopirox-olamine (CI), a fast, rapid and simple method using laser nephelometry in 96-microtiter plate is used. The antimycotic influence of the complexes against *Candida albicans* DSM 11225 and *Candida krusei* ATCC 6258 species was determined using this method. A rapid inhibition and even killing of both fungi was observed only above certain concentrations of complex ranged between 12.5 and 100 μg/ml for β-CD–econazole complex (CD–EC), while for the complex with ciclopirox-olamine (CD–CI) the range was between 150 and 400 μg/ml. The stability constants of the CD complexes with the two antimycotic derivatives are given. In addition, the nephelometric method allows the determination of solubilities of active agents. Thus, the improvement of solubility of both antimycotic agents in PBS buffer solution was observed by complexation with CD.

Keywords: β-Cyclodextrin; Econazole; Ciclopirox; Antimycotic; Laser nephelometry

1. Introduction

Cyclodextrins, which are cyclic oligosaccharides consisting of six or more α-(1,4)-linked D-glucopyranose units have recently recognized as useful pharmaceutical excipients, due to their potential to form inclusion complexes with appropriately sized drug molecules (Szejtli, 1988). The resulting complexes generally offer a variety of physicochemical advantages over the free drug, including increased water solubility, enhanced bioavailability, improved stability, reduced side effects, etc. (Duchêne et al., 1987). EC and CI (Fig. 1) are antifungal agents suitable for the treatment of many mycotic infections. They are applied topically in the treatment of infections of the skin, hair and mucous membranes, and are given orally mainly for the treatment of candidacies, or intravenously in the treatment of systematic fungal infections (Sawyer et al., 1975; Heel et al., 1978). Previous studies showed that both dissolution properties and consequently microbiological activities of econazole, with very low water solubility (about 3 μg/ml at 25 °C), can be improved by complexation with natural cyclodextrins, particularly with β-CD (Bononi, 1988; Mura et al., 1992; Pedersen et al., 1993). However, alkylated and particularly methylated cyclodextrins demonstrated to be often more effective as solubilizing and complexing agents than parent cyclodextrins (Uekama and Irie, 1987). By increasing the water solubility of the drug it should be possible to improve its bioavailability, thus enabling improved oral or topical formulations. 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). Pedersen studied the formation and antimycotic effect of cyclodextrin inclusion complexes of EC and miconazole (Pedersen et al., 1993). They found that the antimycotic effect of CD–EC against a strain of *Candida*

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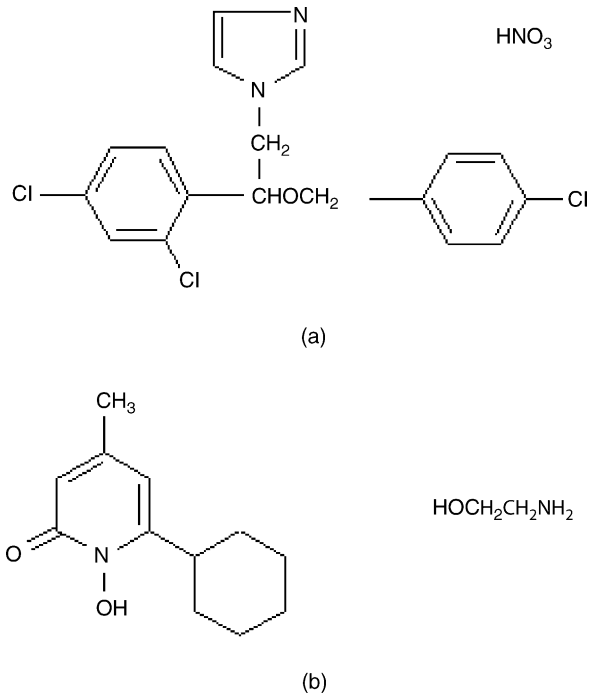

Fig. 1. Chemical structure of: (a) econazole nitrate, (b) ciclopirox olamine.

albicans was superior to the effect of a physical mixture of the two compounds. Laser nephelometry has been shown to be a reliable technique for the measurement of drug solubility in 96-well plate format (Bevan and Lloyd, 2000). Laser nephelometry is the measurement of forward scattered light. When a laser beam is directed through a clear solution, the more particles or turbid suspensions (fungi in this study) in the solution, the greater the amount of forward scattered light (measured as units). The energy of the scattered light is directly proportional to the particle concentration in the suspension for up to three orders of magnitude (Bevan and Lloyd, 2000). The overall aim of the present paper is to use the laser naphelometry, as a new technique, to investigate the effects of complexation on the drug antimycotic activity, in addition to the measurement of phase solubility diagram of both drugs alone and as complexes with CD.

2. Experimental section

2.1. Chemicals and materials

Sterile 96-well microtiter plates were kindly supplied by greiner bio-one GmbH, Germany. Breathe-Easy Gas Permeable Sealing Membrane for Microtiter Plates 6 in. × 3.25 in. were obtained from Carl Roth GmbH, Germany. CASY® cups, CASY® ton (isotonic dilution liquid for cell cultures), CASY® clean were supplied by Schärfe System GmbH, Germany. McFarland standard Kit-0.5,1,2,3,4,5 and Sabouraud-Glucose-Agar with gentamycin-chloramphenicol (SGA) were from bioMerieux, Germany. β-Cyclodextrin was purchased from Wacker-Chemie GmbH, Germany. Sabouraud-Glucose-Bouillon (SGB) was from Oxoid Ltd., England. Phosphate buffered saline tablets (PBS), econazole nitrate, ciclopiroxolamine were from Sigma–Aldrich Chemical Company, Germany. Fluorescent fungal surface labelling reagent FUN®-1 cell strain (F-7030) (300 μl of 10 mM solution in anhydrous dimethylsulfoxide) and GH solution (sterile 2% D-(+) glucose containing 10 mM Na-HEPES, pH 7.2) were from MoBiTec, Germany. All other chemicals and solvents are of analytical grade.

2.2. Cell cultures

Two types of microorganisms were used in this study: *C. albicans* DSM 11225 and *Candida krusei* ATCC 6258.

2.3. Preparation of cell cultures

Both of *C. albicans* and *C. krusei* were grown on (SGA) at 30 °C for 24–48 h. Three to five well-isolated colonies of the same morphological type were selected from an overnight culture using a sterile wire loop and inoculated in 20 ml (SGB). The suspensions were incubated with shaking at 250 rpm/30 °C for 24 h. Then the overnight cell cultures were counted using CASY® 1 and adjusted to a final working concentration of 6×10^5 cells/ml in (SGB).

2.4. Preparation of antifungal agents

Both EC and CI were independently dissolved in a mixture of chloroform/methanol 1:1 to achieve a final stock solution containing 20 mg/ml of antifungal agent. The stock solution of EC was diluted with SGB and adjusted to be 1.25–100 μg/ml while as for CI; it was in the range of 1.25–10 μg/ml. All solutions were stored at −80 °C until used.

2.5. Preparation of the inclusion and antifungal complexes

A solution of EC was prepared by dissolving it in a chloroform/methanol mixture 1:1. CD was dissolved in hot water at 85–90 °C. Equimolar amounts (1:1 molar ratio) of EC and CD solutions were mixed together with stirring for 30 min at 85–90 °C (Buschmann et al., 2001). By cooling, crystallization of the complex was obtained. The complex was filtered using G3 filter and kept in a desiccator overnight. On the other hand, the second complex between CI and CD was also prepared according to the previously mentioned method (Buschmann et al., 2001), in which methanol was used as a proper solvent for CI. Moreover, a molar ratio of 1:2 of CI:CD was also used. The antifungal complex of CD–EC was prepared in a concentration range of 12.5–100 μg/ml using DMSO as a solvent, while the CD–CI was prepared in a concentration range of 150–400 μg/ml using distilled water.

2.6. Phase solubility studies

In this experiment, both of drugs and complexes were diluted in DMSO. Then the drug and complex solutions were