# Direct Formation of Nanospheres from Amphiphilic β-Cyclodextrin Inclusion Complexes

**Erem Memişoğlu,[1,2] Amélie Bochot,[2] Meral Özalp,[3] Murat Şen,[4] Dominique Duchêne,[2] and A. Atilla Hincal[1,5]**

*Received July 13, 2002; accepted October 1, 2002*

***Purpose.*** The aim of this work was to develop and characterize a highly loaded nanoparticulate system based on amphiphilic β-cyclodextrins (CDs) to facilitate the parenteral administration of poorly soluble antifungal model drugs bifonazole and clotrimazole.

***Methods.*** Inclusion complexes were characterized with spectroscopic techniques. Particle size distribution of nanospheres were determined by photon correlation spectroscopy (PCS). Nanospheres were assessed for hemolytic activity. Entrapped and released drug quantities were determined and minimum inhibitory concentration (MIC) values of drugs, amphiphilic β-CDs, and drug loaded nanospheres were evaluated.

***Results.*** 1:1 inclusion complexes of model drugs with amphiphilic β-CDs gave nanospheres <300 nm (polydispersity index < 0.15) by nanoprecipitation technique without using surfactants. By direct preparation from preformed inclusion complexes, loading was increased 2- to 8-fold depending on CD type and loading technique. Conventionally loaded CD nanospheres displayed immediate release whereas preloaded and highly loaded nanospheres liberated model drugs over a period of 1 h reducing the initial burst effect. MIC values of bifonazole and clotrimazole were lowered significantly when associated to amphiphilic β-CD nanospheres.

***Conclusion.*** Amphiphilic β-CDs form nonsurfactant, highly loaded nanospheres with lower hemolytic activity than that of natural CDs directly from inclusion complexes. They enhanced solubility and subsequently therapeutic efficacy of the model drugs.

**KEY WORDS:** amphiphilic β-cyclodextrins; inclusion complexes; nanospheres; bifonazole; clotrimazole; loading; release; hemolysis; minimum inhibitory concentration.

## INTRODUCTION

Amphiphilic cyclodextrins (CDs) are obtained by grafting of aliphatic chains of various length on either the primary or the secondary face of the glucopyranose units; they are designed to increase intimate contact of natural CDs with biologic membranes, which is limited by their external hydrophilicity (1). Amphiphilic properties of these derivatives were demonstrated at the air-water and oil-water interfaces. Nanocapsules and nanospheres were prepared using amphiphilic β- and γ-CDs modified on the secondary face by nanoprecipitation and emulsion/solvent evaporation techniques avoiding the use of additional surfactants. However, it was reported that the drug is mostly adsorbed on the particle surface rather than in CD cavity, thus leading to immediate release (2,3).

In this study, two derivatives that were synthesized by grafting of 6 C chains on either primary or secondary face of the CD glucose units were used. These amphiphilic β-CDs were reported previously to form nanocapsules (4–6) without using surfactants and their interfacial behavior was demonstrated (4) in air/water and oil/water interfaces. β-CDC6 modified on the secondary face and 6-N-CAPRO-β-CD modified on the primary face with 6C aliphatic chains (Fig. 1) were evaluated for their inclusion capabilities and subsequently for nanosphere loading and release characteristics.

It was assumed that by modification on the primary face, wider side of the cavity would be left open to facilitate drug inclusion since the presence of long substituents may prevent active ingredients from entering the cavity by steric hindrance (7).

Because loaded amphiphilic β-CD nanospheres consisted only of the drug and the amphiphilic β-CD, another factor affecting loading and release would be physicochemical properties of the entrapped drug. Bifonazole (BF) and clotrimazole (CL), two water-insoluble antimycotics from the imidazole group with extreme high and low association constants $K_{1:1}$ (11,000 $M^{-1}$ for BF and 500 $M^{-1}$ for CL; Ref. 8) were used as model drugs.

Loading and release properties of nanospheres prepared from β-CDC6 and 6-N-CAPRO-β-CD were evaluated regarding the influence of amphiphilic β-CD type and inclusion capability, $K_{1:1}$ association constant of drug toward the native β-CD, and loading technique. Hemolytic properties of amphiphilic β-CD nanospheres were evaluated in comparison with β-CD, which is known to cause considerable hemolysis properties upon parenteral administration arising from its capacity for inclusion of cell membrane components of erythrocytes. Finally, the minimum inhibitory concentrations of antifungal model drugs associated to nanospheres were compared to model drugs in solution.

## MATERIALS AND METHODS

Amphiphilic β-CDs represented in Fig. 1 are referred to as 6-N-CAPRO-β-CD (MW: 1813 g/mol) and β-CDC6 (MW: 2506 g/mol) according to nomenclature previously described (4–6). β-CD was modified on the primary face with 6C aliphatic chain with amide bond (6-N-CAPRO-β-CD) or on the secondary face with ester bonds and 6C aliphatic chains (C6). The structure and purity of both products were verified according to techniques such as proton nuclear magnetic resonance spectroscopy at 400 MHz, fast atom bombardment mass spectrometry, Fourier transform infrared spectroscopy (FT-IR), differential scanning calorimetry (DSC), and elemental analysis as reported previously (6). BF (MW: 310 g/mol) and CL (MW: 344 g/mol) were purchased by Sigma Chemicals, Steinheim, Germany, and used without any further purification. *Candida albicans* ATCC 90028 was prepared by Pharmaceutical Microbiology Laboratory in Hacettepe University. Polyethyleneglycol (PEG) 400 was purchased by Merck, Hohenbrunn, Germany. All organic solvents were of chemical grade and were used without purification.

[1] Hacettepe University, Faculty of Pharmacy, Department of Pharmaceutical Technology, 06100 Ankara, Turkey.
[2] Université de Paris-Sud, Faculté de Pharmacie, UMR CNRS 8612, 92296 Chatenay-Malabry, France.
[3] Hacettepe University. Faculty of Pharmacy, Department of Pharmaceutical Microbiology, 06100 Ankara, Turkey.
[4] Hacettepe University, Department of Chemistry, 06532, Beytepe-Ankara, Turkey.
[5] To whom correspondence should be addressed. (e-mail: hincal@tr.net)



0724-8741/03/0100-0117/0 © 2003 Plenum Publishing Corporation

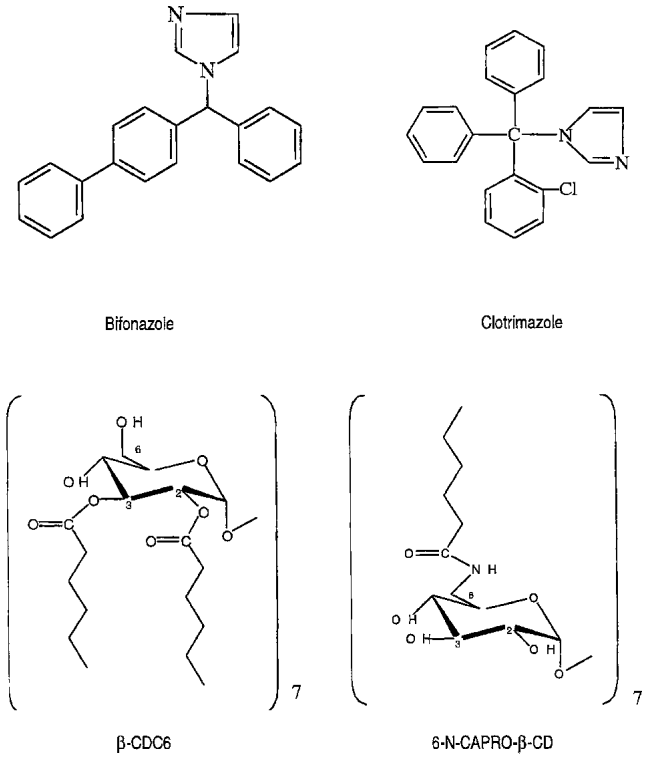


**Fig. 1.** Model drugs; bifonazole and clotrimazole and amphiphilic β-cyclodextrins modified on the primary or secondary face; β-CDC6 and 6-N-CAPRO-β-CD.

### Preparation of Inclusion Complexes

Inclusion complexes were prepared in a water-ethanol system using colyophilization technique (9). 1:1 molar ratio (drug:CD) complexes were studied. Fixed quantities of model drugs and amphiphilic β-CDs were dissolved in ethanol, mixed with an equal volume of water and left to equilibrate under constant stirring for 7 days. Ethanolic phase was evaporated under vacuum and the aqueous dispersion was then lyophilized to obtain the colyophilizate.

### Characterization of Inclusion Complexes

DSC was used to analyze thermal behavior of the model drugs, amphiphilic β-CDs, and 1:1 complexes. Thermograms were taken with a DuPont DSC 910 Instrument. The samples were heated in a hermetically sealed aluminum pan at a rate of 10°C/min from 25 to 200°C under nitrogen atmosphere. FT-IR spectra were recorded with a Nicolet 520 FTIR spectrophotometer using discs of 0.01 g sample in 0.1 g KBr between wavelengths 400 to 4000 $cm^{-1}$.

### Preparation and Drug Loading of Amphiphilic β-CD Nanospheres

Nanospheres were prepared according to the nanoprecipitation method introduced by Fessi et al. (10). Organic phase (1 mL) consisting of 1 mg of amphiphilic β-CD or amphiphilic β-CD:drug inclusion complex dissolved in acetone for β-CDC6 or ethanol for 6-N-CAPRO-β-CD, was added under constant stirring to 2 mL of aqueous phase consisting only of deionized water. After stirring for one hour at room temperature, organic solvent was evaporated under vacuum and the nanosphere dispersion was concentrated to the desired volume (2 mL).

Nanospheres of 6-N-CAPRO-β-CD and β-CDC6 were loaded with model drugs BF and CL according to the following methods;

**Preloaded Nanospheres**: Nanospheres were prepared directly from preformed drug:CD (1:1) complexes according to the above described nanoprecipitation technique. No further loading was performed.

**Conventionally Loaded Nanospheres**: Nanospheres were prepared from amphiphilic β-CDs only, according to nanoprecipitation technique as described before and were loaded by the addition of 200 μg of model drug to the organic phase during preparation.

**Highly Loaded Nanospheres**: Preloaded nanospheres described above were overloaded during preparation by dissolving an additional amount of drug (200 μg) in the organic phase.

After the formation of nanosphere dispersions, unbound water-insoluble antifungal drug in the nanosphere dispersions were separated by centrifugation at 30,000 g for 10 min and discarding of the precipitate. Supernatant was then ultracentrifuged at 120,000 g at 25°C for 1 h by a Sorvall RC28S with fix rotor type S20/20 (DuPont, USA) to precipitate the nanospheres and the encapsulated drug.

The precipitate was then lyophilized and the resulting powder containing the loaded nanospheres (≈300 mg) was dissolved in ethanol (1 mL) to obtain a clear solution, which was then analyzed spectrophotometrically for the encapsulated drug quantity using a Shimadzu 160A UV Spectrophotometer at wavelengths of 254 and 210 nm for BF and CL, respectively.

Loading capacity was expressed in terms of entrapped drug quantity, entrapment efficiency and associated drug percentage to give a better outline of the loading capacities of amphiphilic β-CD nanospheres.

Entrapped drug quantity is the total drug quantity determined in nanosphere dispersion after the elimination of unbound drug by centrifugation. Entrapment efficiency was calculated according to the following equation;

$$\text{Entrapment Efficiency} = \left[\frac{\text{determined drug quantity (μmol)}}{\text{Initial CD quantity (μmol)}}\right]$$

Associated drug percentage was also calculated according to the following equation;

$$\text{Associated drug \%} = 100 \times \left[\frac{\text{determined drug quantity (mg)}}{\text{Initial drug quantity (mg)}}\right]$$

### Characterization of Amphiphilic β-CD Nanospheres

Mean particle size (diameter, nm) and polydispersity index of amphiphilic β-CD nanospheres were determined by PCS using a Coulter Nanosizer N4 Plus apparatus (Coultronics, Margency, France). Measurements were realized as triplicate at a 90° angle at 20°C. A nanoparticle suspension of intensity between $10^4$ –$10^6$ cps was analyzed for 60 s freshly after the preparation of nanospheres.

### *In Vitro* Release of Model Drugs BF and CL from Amphiphilic β-CD Nanospheres

Release kinetics of model drugs from amphiphilic β-CD nanospheres (1 mg) were determined in 20 mL of Water:PEG 400 (60:40) providing sink conditions in a thermostated shaker bath system (Memmert, Schwabach, Germany) at 37°C. At given time intervals, samples were withdrawn from the system and replaced with equal volume of fresh release medium. Samples were ultracentrifuged at 120,000 g for 1 h (SORVALL RC 28S, DuPont, Newton USA) and the supernatant was analyzed for free drug with a spectrophotometric, analytically validated method (CV<2%) (Shimadzu 160A UV spectrophotometer, Bechman Instruments, Munchen, Germany) at 254 and 210 nm for BF and CL.

### Hemolytic Activity of Amphiphilic β-CD Nanospheres

Blood samples were withdrawn from healthy human subjects and separated by centrifugation into two parts to work on whole blood (WB) as well as erythrocytes (red blood cells, RBCs). For the latter, erythrocytes were separated by centrifugation, washed two times with isotonic phosphate buffer, pH 7,4 and then resuspended in this buffer in a ratio of 1 to 5.

One milliliter of unloaded amphiphilic β-CD nanosphere dispersion prepared in isotonic PBS was obtained from varying concentration range of amphiphilic β-CDs from 0.5 to 4 mM covering the actual amphiphilic β-CD concentration range used in nanosphere formulations (0.4 mM for β-CDC6 and 0.6 mM for 6-N-CAPRO-β-CD). Nanosphere dispersion was treated with 50-μL blood sample (WB or RBC) and vortexed.

Samples were incubated at 37°C for 30 min and centrifuged at 5000 rpm for 10 min. Supernatant was analyzed for hemoglobin content at 543 nm spectrophotometrically (Shimadzu UV 160-A, Japan). Total hemolysis (100%) was determined by UV absorbance of blood samples treated with distilled water leading to the total lysis of erythrocytes. Hemolysis percentage of each sample was then calculated according to the following equation.

$$\text{Hemolysis \%} = \left[\frac{\text{UV absorbance of sample treated with WB or RBC}}{\text{UV absorbance of sample treated with distilled water}}\right] \times 100$$

### MIC Determination for the System Constituents

Broth microdilution method was applied to determine the minimum inhibitory concentration (MIC) values for BF, CL, β-CDC6 and 6-N-CAPRO-β-CD and preloaded amphiphilic β-CD nanospheres. Procedures recommended by the National Committee for Clinical Laboratories were followed. In this method, 96-well microtiter plates (U-shaped) were used. All studies were conducted in RPMI 1640. *Candida albicans* ATCC 90028 was used as test organism. The final fungal inoculum was adjusted to $5 \times 10^5$ cfu/mL in the well. Microtiter plates were incubated for 24 h at 35°C. Minimum inhibitory concentrations were defined as the lowest concentration of antifungal agents that inhibited visible growth of the fungi using fluconazole as control (11).

### Statistical Analysis

Entrapment data were analyzed by Kruskall-Wallis analysis of variance for BF and CL. Differences between specific formulations at given time intervals were analyzed by Tukey test. Hemolysis of different CDs dependent on concentration were analyzed with Kruskall-Wallis analysis of variance and differences between groups were analyzed by Tukey test. In vitro release data were statistically analyzed using the two-way analysis of variance for repeated measures test.

## RESULTS AND DISCUSSION

### Characterization of Inclusion Complexes

Figure 2 represents the DSC thermograms of physical mixtures and co-lyophilizates of model drugs BF and CL with amphiphilic β-CDs. Melting endotherms of model drugs that appear around 130 and 140°C disappear totally in colyophilizates prepared with 1:1 molar ratio. This may be the result of two reasons; either model drug is completely included within the CD cavity or entangled within the "skirt" region comprising of long acyl chains or lyophilization process alters the crystalline properties of model drugs to amorphous structure and consequently hides the melting endotherm (12). However, the second hypothesis can be overruled since bifonazole and clotrimazole give clear melting endotherms after lyophilization as seen in Fig. 2.

Another technique to assess the formation of true inclusion complexes was FT IR spectroscopy. Figure 3 demonstrates the formation of inclusion complexes between model drugs and amphiphilic β-CDs. FT-IR spectra of model drugs are completely masked by CD spectra, suggesting the inclusion of model drugs by amphiphilic β-CD molecule. Characteristic aromatic band region of model drugs around 600–800 $cm^{-1}$ is not present in FT IR spectra of colyophilizates.

These findings are in accordance with previous studies demonstrating the existence of true inclusion complexes between amphiphilic CD derivatives and various substances (13–15). Complexation of BF and CL in β-CD also has been demonstrated in various studies. Generally, the preferred molar ratio of drug:CD in complexes were determined to be 1:1 which is used in the complexes in this study.

### Characterization of Amphiphilic β-CD Nanospheres

Mean diameter of nanospheres prepared directly from preformed inclusion complexes (1:1) sizes vary from 200 to 300 nm with polydispersity indices below 0.150 indicating a homogeneous dispersion of nanospheres. A slight increase in particle size can be observed for nanospheres of clotrimazole:amphiphilic β-CD inclusion complex.

### Loading Properties of Amphiphilic β-CD Nanospheres

Table I presents a summary of loading properties for amphiphilic β-CD nanospheres. Factors influencing drug loading were believed to be as follows;

1. Amphiphilic β-CD chemical structure.
2. Loading technique.
3. Affinity of model drug to mother β-CD.

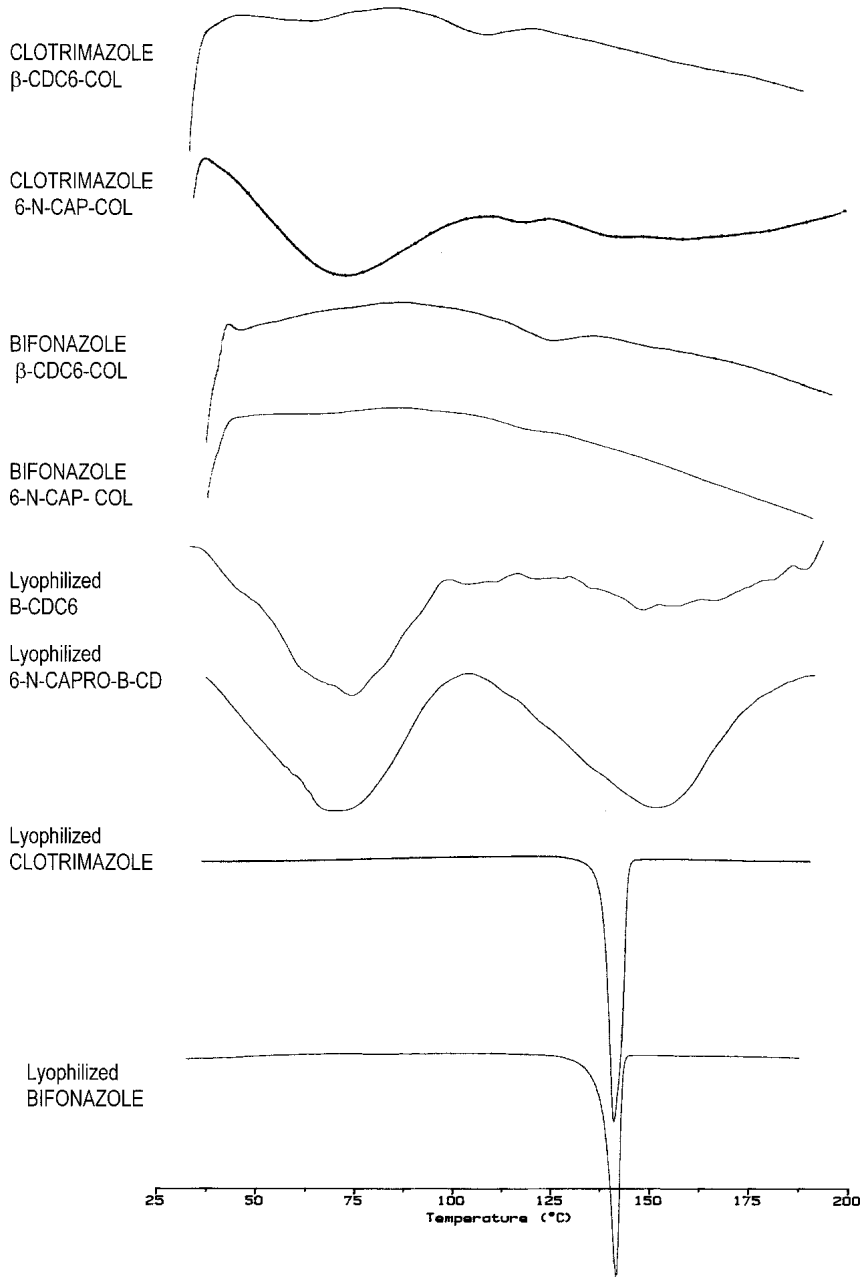


**Fig. 2.** DSC thermograms of physical mixtures and colyophilizates of bifonazole and clotrimazole.

Data are expressed in terms of entrapment efficiency and associated drug percentage in order to clarify the effect of the above listed parameters on the amount of drug associated in the nanospheres and the amount of drug entrapped per unit CD.

As far as the type of amphiphilic β-CD, which is the main constituent of the nanospheres, is concerned, entrapped drug quantity and associated drug percentage data suggest that 6-N-CAPRO-β-CD has a higher loading capacity than β-CDC6. Drug association is significantly higher ($p < 0.05$) for 6-N-CAPRO-β-CD modified on the primary face. This difference is even more pronounced in the case of bifonazole since it has a higher affinity to the CD cavity. On the other hand, when the drug (μmol) associated per unit CD (μmol) is evaluated, it is not possible to differentiate between the two amphiphilic CDs.

Nevertheless, these results in Table I confirm that drug inclusion may be facilitated by leaving the secondary face unmodified and thus reducing the steric hindrance and leaving the wider side of the cavity open for the entrance of active ingredients totally or partially.

It was observed that loading technique was the most influential parameter on drug entrapment to amphiphilic β-CD nanospheres. Preparation of nanospheres from preformed inclusion complexes of model drugs and amphiphilic β-CD proved to be an effective method to enhance drug loading to amphiphilic β-CD nanospheres.

Table I shows that it is possible to increase the entrap-

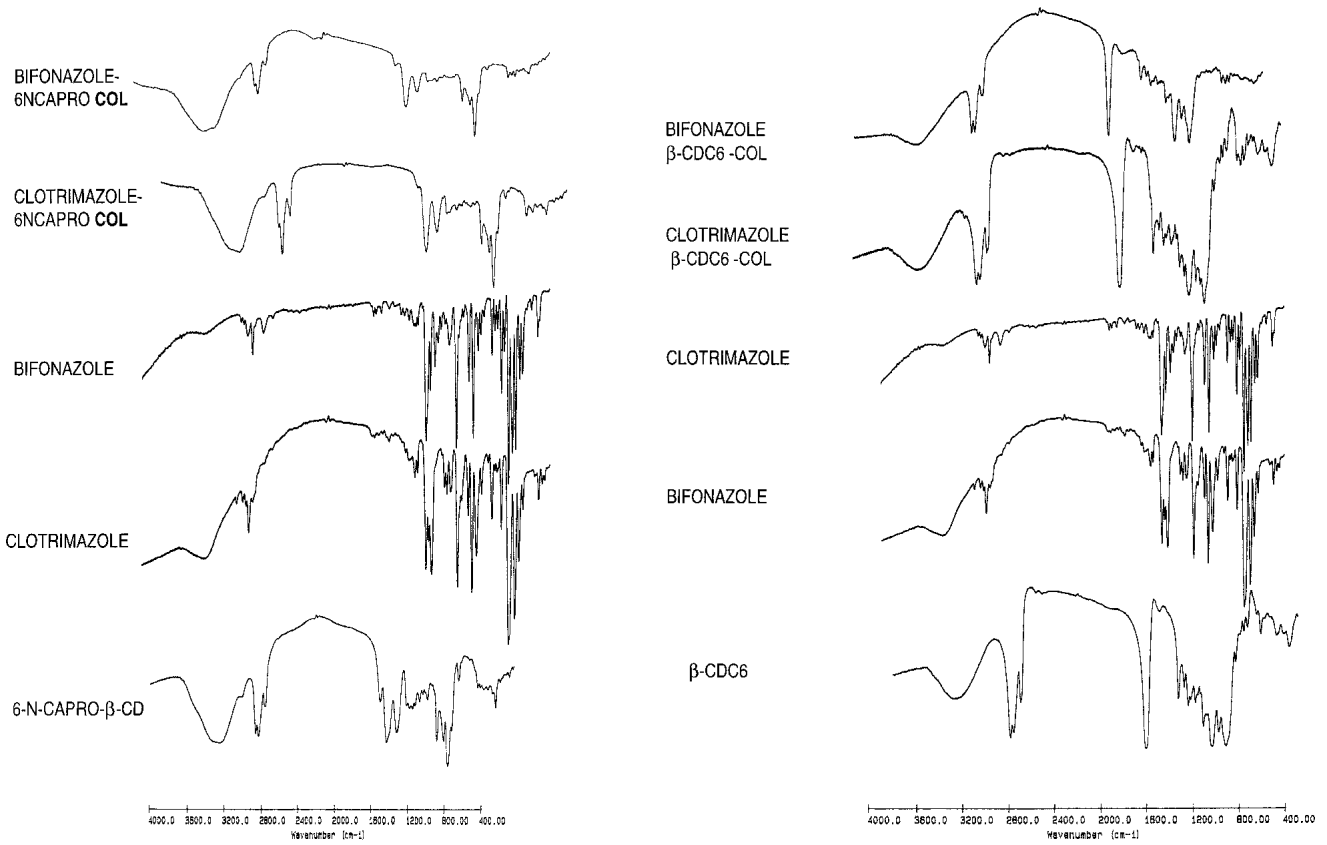


**Fig. 3.** FT-IR spectra of inclusion complexes of 6-N-CAPRO-β-CD and β-CDC6.

ment efficiency by 2-fold for both model drugs. There was a significant difference ($p < 0.05$) between the entrapment efficiency of highly loaded formulations and conventionally loaded ones. However, no significant difference was observed between drug association and entrapment efficiency data of preloaded and conventionally loaded formulations. Pronounced enhancement of drug loading with highly loaded formulations may be attributed to the fact that more drug could be entrapped because of the presence of excess amount of drug during nanosphere preparation. However, preloaded nanospheres are limited to the amount of drug included in the preformed complexes. Considering that a certain amount of drug may be lost from the complex during loading process, it is expected that loading will be lower for preloaded nanospheres.

Bifonazole has a very high association constant to mother β-CD whereas clotrimazole has a significantly low association constant. Consequently, it can be observed in Table I that bifonazole entrapment was significantly higher ($p < 0.05$) in the case of preloaded and highly loaded nanospheres. There was no statistical difference between bifonazole and clotrimazole entrapment to conventionally loaded nanospheres. This is rather expected because the affinity of drug to CD may play an active role for loading when nanospheres are prepared from preformed complexes. During the preparation of conventionally loaded nanospheres, very little

**Table I.** Loading Characteristics of Preloaded, Conventionally Loaded, and Highly Loaded Amphiphililc β-Cyclodextrin Nanoparticles with Bifonazole and Clotrimazole (n = 6)

| | Bifonazole | | | Clotrimazole | | |
|---|---|---|---|---|---|---|
| Formulation | Entrapped drug quantity (μg) ± SD | Entrapment efficiency | Associated drug (%) | Entrapped drug quantity (μg) ± SD | Entrapment efficiency | Associated drug (%) |
| Preloaded | | | | | | |
| 1:1 β-CDC6 | 35 ± 5 | 32 | 32 | 30 ± 2 | 25 | 25 |
| 1:1 6 N CAPROβCD | 49 ± 5 | 32 | 34 | 36 ± 6 | 23 | 22 |
| Conventionally loaded | | | | | | |
| β-CDC6 | 68 ± 3 | 55 | 34 | 74 ± 4 | 54 | 37 |
| 6 N CAPROβCD | 81 ± 2 | 47 | 41 | 83 ± 3 | 44 | 42 |
| Highly loaded | | | | | | |
| 1:1 β-CDC6 | 112 ± 11 | 102 | 36 | 99 ± 1 | 83 | 31 |
| 1:1 6 N CAPRO β CD | 173 ± 8 | 117 | 50 | 124 ± 6 | 78 | 35 |

time is left to form an eventual complex between the loaded drug and the nanosphere constituent; amphiphilic β-CD. These data also suggest that when conventional loading techniques are used, drug is adsorbed to the large surface of the nanospheres.

Preparation of nanospheres directly from preformed complexes (preloading) or reloading this preloaded nanosphere with excess amount of drug during preparation (highly loading) resulted in considerable increase in loading properties. In techniques based on the use of preformed complexes, drug is already included either in the cavity or entangled within the long acyl chains. A second possibility for drug entrapment is the interaction and possible entrapment of drug in the acyl chains. High loading is found to increase the molar drug entrapped per molar CD by 2-fold compared with conventional loading.

### *In Vitro* Release Characteristics of BF and CL from Amphiphilic β-CD Nanospheres

In vitro release data were evaluated according to the same parameters as those affecting drug loading. Release profiles of model drugs bifonazole and clotrimazole were assessed in water:PEG400 (60:40). It can be observed that amphiphilic β-CD modification site has a significant effect on drug release. Amphiphilic β-CD modified on the primary face, 6-N-CAPRO-β-CD, displayed slower release profiles than β-CDC6 modified on the secondary face. This difference is observed only for preloaded and highly loaded nanospheres. Both of these techniques include preformed inclusion complexes; however, the slower release profile of 6-N-CAPRO-β-CD suggests that this molecule holds the drug longer in its cavity.

Loading technique has a high influence on release profile with preloading resulting in the slowest release profiles. High-loaded nanospheres are believed to liberate the excess drug adsorbed on the surface followed by the slower liberation of drug included in the cavity. Conventionally loaded nanospheres liberated model drugs immediately within the first 15 min because of the burst effect. However, preloaded and highly loaded nanospheres prolonged the release up to 1 h and reduced the burst effect. It has been previously reported that conventionally loaded amphiphilic γ-CD nanospheres entrap drug molecules by adsorption on particle surfaces (16,17). However, for nanospheres consisting of preformed inclusion complexes, drug entrapped in the CD cavity is also released by time dependent mechanisms such as dissociation upon dilution and competitive displacement of drug from the cavity by release medium constituents (18). Highly loaded nanospheres that are believed to entrap the drug both in the CD cavity and adsorbed on the particle surface, release a considerable amount of the drugs immediately but nevertheless continue the release process for an additional hour. Differences in release values of nanospheres in different time points are statistically significant ($p < 0.05$).

Affinity of mother β-CD to model drug plays a major role on drug release from amphiphilic β-CD nanospheres (19). It can be observed clearly in Figs. 4 and 5 that bifonazole which has a very high association constant ($K_{1:1}$ 11,000 $M^{-1}$) with β-CD is released significantly slower than clotrimazole which has the lowest association constant ($K_{1:1}$ 500 $M^{-1}$) within imidazole derived antifungals ($p < 0.05$).

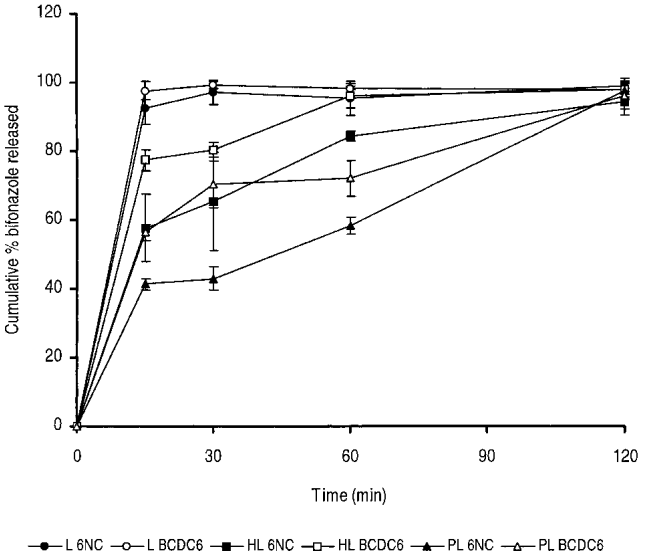


**Fig. 4.** *In vitro* release profile of bifonazole from amphiphilic β-CD nanospheres in water:PEG 400 (60:40; n = 6, SD). L6NC, conventionally loaded 6-N-CAPRO-β-CD nanospheres; L B-CDC6, conventionally loaded β-CDC6 nanospheres; HL 6NC, highly loaded 6-N-CAPRO-β-CD nanospheres; HL B-CDC6, highly loaded β-CDC6 nanospheres; PL 6NC, preloaded 6-N-CAPRO-β-CD nanospheres; PL B-CDC6, preloaded B-CDC6 nanospheres.

### Hemolytic Evaluation of Amphiphilic β-CD Nanospheres

Figure 6 displays hemolysis caused by amphiphilic β-CDs compared with the mother β-CD in whole blood and red blood cell suspension. Hemolysis induced by amphiphilic β-CDs were evaluated because β-CD has been questioned for its hemolytic properties arising from their ability to disrupt cell membrane components of red blood cells, namely phospholipids and cholesterol. In this study, β-CD, and nanospheres of 6-N-CAPRO-β-CD and β-CDC6 were evaluated for hemolysis in RBCs and total blood.

As expected, amphiphilic β-CDs were both less hemolytic ($p < 0.05$) than the natural β-CD because of their hydrophobic substituents in all concentrations covered by this study. Besides hydrophobicity, self-assembly of amphiphilic β-CDs in the form of nanospheres was also believed to reduce interaction and contact of the CDs directly with the red blood cells. Hemolytic effect was much more pronounced on red blood cell suspension ($p < 0.05$), which is also expected because it is believed that the presence of other blood components and proteins protect the disruption of erythrocyte cell membrane (20). When amphiphilic β-CDs are compared regarding hemolysis, it was found that β-CDC6 caused significantly lower hemolysis in red blood cells and total blood ($p < 0.05$) with less than 10% hemolysis up to a concentration of 4 mM. In the same concentration, 6-N-CAPRO-β-CD caused 15% hemolysis whereas β-CD displayed almost 20%.

The concentration range covered by the hemolysis experiment was very wide and regarding that amphiphilic β-CD concentration in nanospheres does not exceed 0.6 mM. It may be pointed out that both amphiphilic β-CDs do not cause hemolysis more than 20% at this molar concentration. it can be concluded that amphiphilic β-CDs are non-hemolytic in injectable nanosphere form and the grafting of lipophilic moi-

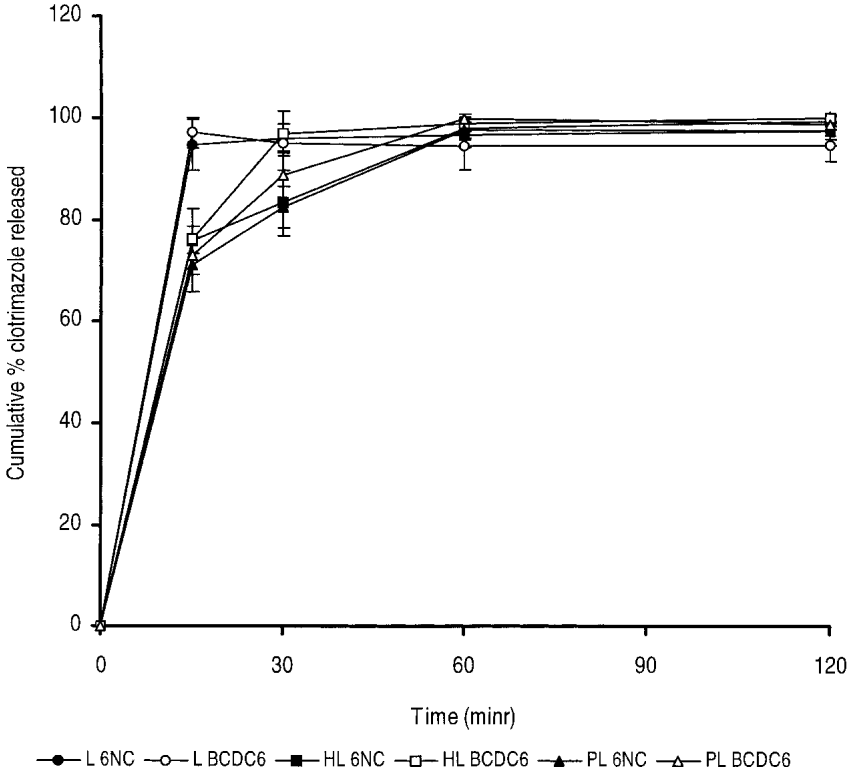


**Fig. 5.** *In vitro* release profile of clotrimazole from amphiphilic β-CD nanospheres in Water:PEG 400 (60:40; n = 6, SD). For abbreviations, see legend to Fig. 4.

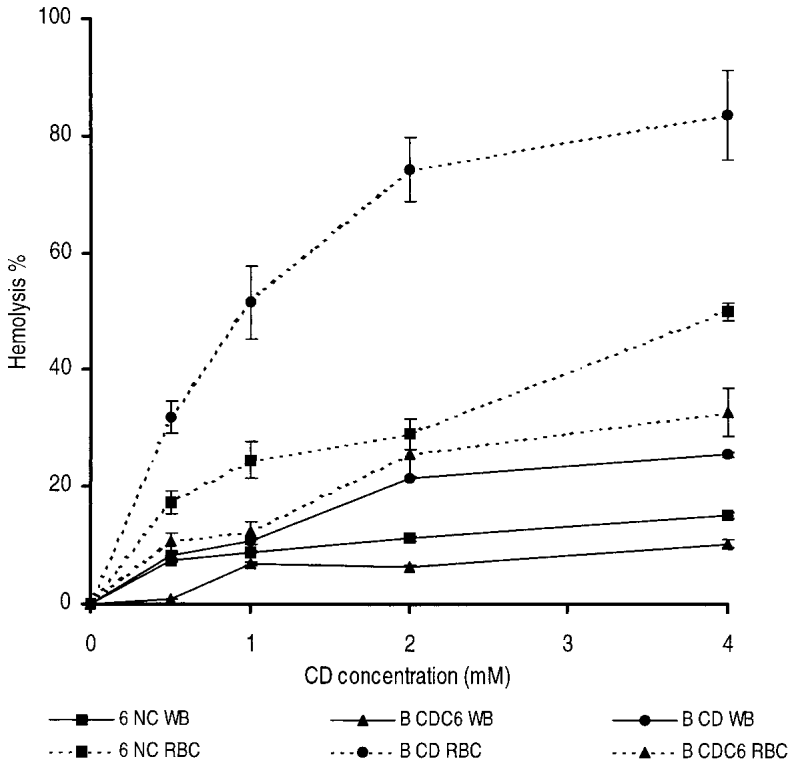


**Fig. 6.** Hemolytic evaluation of mother β-CD and amphiphilic β-CD derivatives in WB and RBCs (n = 3, SD). 6NC WB, whole blood-treated 6-N-CAPRO-β-CD nanospheres; 6 NC RBC, erythrocyte suspension treated with 6-N-CAPRO-β-CD nanospheres; β-CDC6 WB, whole blood treated with β-CDC6 nanospheres; β-CDC6 RBC; erythrocyte suspension treated with β-CDC6, β-CD WB, whole blood treated with β-CD solution; β-CD RBC, erythrocyte suspension treated with β-CD solution.

eties to the CD molecule reduces the hemolytic activity of this excipient.

### Antimycotic Activity of Amphiphilic β-CD Nanospheres

Antimicrobial activity of the samples were assessed by the determination of their MIC values on *C. Albicans* ATCC 90028. MICs against *Candida albicans* are listed in Table II for ethanolic solutions of model drugs and amphiphilic β-CDs, aqueous dispersions of drug-loaded nanospheres and aqueous solutions of model drug:β-CD complexes. Insoluble antifungals drugs were administered in ethanolic solution form and the ineffectiveness of ethanol on *C. albicans* culture was verified. It is seen that clotrimazole has a lower MIC value than bifonazole probably because of its much higher solubility than bifonazole, which is in accordance with previous data (21). Interestingly, both amphiphilic β-CDs displayed a certain weak antimycotic activity, as seen in Table II. When drug-loaded amphiphilic β-CD nanospheres were incubated with *C. Albicans*, synergistic effect was very pronounced since MIC values of BF was lowered by 2-fold whereas MIC values of CL was lowered 10-fold. This phenomenon was previously encountered when clotrimazole or other imidazole derived antifungals such miconazole or econazole was complexed to β-, hydroxypropyl-β-, or γ-CD. Lowering of the MIC values may be attributed to the supersaturation of growth medium with antifungals, which are released from nanospheres in soluble form (16,22). Thus, clotrimazole which is already more soluble than bifonazole yielded a MIC value 10 times smaller than in ethanolic solution form. Bifonazole has a very limited water-solubility and reduced MIC value by 2-fold when associated to nanospheres. β-CD:model drug complexes were administered at the same molar ratio as nanospheres and MIC values are seen in Table II. It is observed that more drug is solubilized by encapsulation into nanospheres than complexation to β-CD.

## CONCLUSIONS

In this study, amphiphilic β-CDs were demonstrated to form 1:1 inclusion complexes with antifungal model drugs, bifonazole and clotrimazole. Nanospheres were prepared directly from these inclusion complexes without any surface active agents. Significantly high entrapment values were achieved in comparison to conventionally loaded CD nanospheres by using novel techniques based on the preparation of nanospheres from preformed inclusion complexes. This phenomenon may present an advantage in the preparation of parenteral formulation of poorly soluble drugs as in the case of bifonazole; thus, facilitating the formulation of such drugs for the parenteral route. In vitro release characteristics of amphiphilic β-CD nanospheres loaded with different techniques were somewhat similar with immediate release in a period of 1 to 2 h. Amphiphilic β-CD nanospheres are promising delivery systems for drugs with poor water-solubility facilitating the therapeutic use of these drugs because they prove to have very low hemolytic effect and enhanced therapeutic efficacy.

**Table II.** MIC Values Amphiphilic β-CDs, Antimycotic Model Drugs, and Drug-Loaded Amphiphilic β-CD Nanospheres against *Candida albicans* (n = 3, SD)

| System component | MIC value (μg/mL ± SD) |
|---|---|
| Bifonazole ethanolic solution | 0.48 ± 0.13 |
| Clotrimazole ethanolic solution | 0.12 ± 0.07 |
| 6-N-CAPRO-β-CD ethanolic solution | 12.5 ± 3.6 |
| β-CDC6 ethanolic solution | 25 ± 7.2 |
| Bifonazole:6-N-CAPROβ-CD nanosphere | 0.19 ± 0.11 |
| Bifonazole:β-CDC6 nanosphere | 0.19 ± 0.05 |
| Clotrimazole:6-N-CAPRO-β-CD nanosphere | 0.012 ± 0.006 |
| Clotrimazole:β-CDC6 nanosphere | 0.012 ± 0.006 |
| β-CD:Bifonazole complex aqueous solution | 125 ± 36 |
| β-CD:Clotrimazole complex aqueous solution | 7.8 ± 4.5 |

## REFERENCES


1. D. Duchene, D. Wouessidjewe, and G. Ponchel. Cyclodextrins and carrier systems. *J. Control. Release* **62**:263–268 (1999).
2. D. Woussidjewe, M. Skiba, F. Leroy-Lechat, E. Lemos-Senna, F. Puisieux, and D. Duchene. A new concept in drug delivery based on skirt-shaped cyclodextrin aggregates, present state and future prospects. *STP Pharma Sci.* **6**:21–28 (1996).
3. P. C. Tchoreloff, M. M. Boissonnade, A. W. Coleman, and A. Baszkin. Amphiphilic monolayers of insoluble cyclodextrins at the water/air interface. Surface pressure and surface potential studies. *Langmuir* **11**:191–196 (1995).
4. C. Ringard-Lefebvre, A. Bochot, E. Memişoğlu, D. Charon, D. Duchene, and A. Baszkin. Interfacial behavior of the oil/water system in the presence of spread amphiphilic cyclodextrins. *Coll. Surf. B. Biointerfaces* **25**:109–117 (2002).
5. E. Memişoğlu, A. Bochot, D. Charon, A. A. Hıncal, and D. Duchene. Characterization of nanocapsules composed of amphiphilic beta-cyclodextrins modified on the primary face. *Eur. J. Pharm. Sci.* **11**(Suppl):S29 (2001).
6. E. Memişoğlu, A. Bochot, M. Şen, D. Charon, D. Duchene, and A. A. Hıncal. Amphiphilic β-cyclodextrins modified on the primary face: Synthesis, characterization and evaluation of their potential as novel excipients in the preparation of nanocapsules. *J. Pharm. Sci.* **91**:1214–1224 (2002).
7. D. O. Thompson. Cyclodextrins-enabling excipients. Their present and future use in pharmaceutics (review). *CRC Crit. Rev. Ther. Drug Carrier Syst.* **14**:1–104 (1997).
8. H. Van Doorne, E. H. Bosch, and C. F. Lerk. Formation of antimicrobial activity of complexes of β-cyclodextrin and antimycotic imidazole derivatives. *Pharm. Weekblad (Sci.)* **10**:80–85 (1988)
9. A. R. Hedges. Industrial applications of cyclodextrins. *Chem. Rev.* **98**:2035–2044 (1998).
10. H. Fessi, J.P. Devissaguet, C. Thies. Process for the preparation of dispersible colloidal systems of a substance in the form of nanospheres. US Patent, 5,118,528 (1988)
11. National Committee for Clinical Laboratory Standards. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically,* 4th Ed, Approved Standard. M7 A4, 17(2), NCCLS, Villanova, PA (1997).
12. J. L. Ford and P. Timmins. *Pharmaceutical Thermal Analysis,* Ellis Horwood Limited. Chichester, 1989
13. H. Takahashi, Y. Irinatsu, S. Kozuka, and W. Tagaki. Host-guest complexation of a lipophilic heptakis(6-dodecylamino-6-deoxy)-β-cyclodextrin with nitrophenols in chloroform. *Mem. Fac. Eng. Osaka City Univ.* **26**:93–99 (1985).
14. H. Nakahara, H. Tanaka, K. Fukuda, M. Matsumoto, and W. Tagaki. Selective inclusion of naphthalene derivatives by monolayers of amphiphilic cyclodextrins at the air-water interface. *Thin Solid Films* **284-285**:687–690 (1996).
15. N. Morin, G. Crini, C. Cosentino, J. Milet, and J. Vebrel. JC, Rouland. Formation of two particular structures between β-cyclodextrin and bifonazole: β-cyclodextrin-bifonazole and $(\beta\text{-cyclodextrin})_i$ _bifonazole (where $2<i<3$). *J. Chem. Soc. Perkin-Trans.* **2**:2647–2651 (1999).

16. E. Lemos-Senna, D. Wouesidjewe, S. Lesieur, F. Puisieux, G. Couarrazze, and D. Duchene. Evaluation of the hydrophobic drug loading characteristics in nanoprecipitated amphiphilic cyclodextrin nanospheres. *Pharm. Dev. Technol.* **3**:1–10 (1998).
17. E. Lemos-Senna, D. Wouessidjewe, S. Lesieur, and D. Duchene. Preparation of amphiphilic cyclodextrin nanospheres using the emulsion solvent evaporation method, influence of the surfactant on preparation and hydrophobic drug loading. *Int. J. Pharm.* **170**: 119–128 (1998).
18. F. Hirayama, M. Yamanaka, T. Horikawa, and K. Uekama. Characterization of peracetylated β-cyclodextrins with different chain lengths as a novel sustained release carrier for water-soluble drugs. *Chem. Pharm. Bull.* **43**:130–136 (1995).
19. F. Hirayama and K. Uekama. Cyclodextrin-based controlled drug release system. *Adv. Drug Del. Rev.* **36**:125–144 (1995).
20. J. Szejtli, T. Cserhati, and M. Szögyi. Interactions between cyclodextrins and cell membrane phospholipids. *Carbohyd. Polym.* **6**: 35–49 (1986).
21. H. Yamaguchi, T. Hiratani, and M. Plempel. In vitro studies of a new imidazole antimycotic, bifonazole in comparison with clotrimazole and miconazole. *Arzneim. Forsch./Drug Res.* **33**:546–551 (1983)
22. M. Pedersen, S. Bjerregaard, J. Jacobsen, and A. Mehlsen Sorensen. A genuine clotrimazole-γ-cyclodextrin inclusion complex. Isolation, antimycotic activity, toxicity and unusual dissolution rate. *Int. J. Pharm.* **176**:121–131 (1998).