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A study of the inclusion complex of amphotericin-B with γ-cyclodextrin

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Summary

Amphotericin-B, a potent antifungal agent, was found to form an inclusion complex with γ-cyclodextrin both in aqueous solution and in the solid state. Complex formation in the solution state was studied using phase solubility and spectral shift methods. The increase in the solubility of amphotericin-B with added γ-cyclodextrin was observed to show a positive deviation from linearity (type A_p phase diagram) indicating the presence of more than one complex in the system. In the γ-cyclodextrin concentration range 0–0.5 M, two complexes, 1 : 1 and 1 : 2, with stability constants of 462 and 42 M^{-1}, respectively, were identified. Using the spectral method, the formation constant for the 1 : 1 inclusion complex was determined to be 422 M^{-1} in 25% v/v methanol. A solid complex of amphotericin-B with γ-cyclodextrin was prepared by using the techniques of lyophilization, kneading and coprecipitation. The solid complex prepared showed improved dissolution as well as enhanced amphotericin-B stability in solution over free amphotericin-B.

Introduction

Amphotericin-B is a polyene antifungal antibiotic, useful in the treatment of many mycotic infections when administered by the intravenous route (Drutz et al., 1968). Despite its toxicity, amphotericin-B remains the single most reliable drug in the treatment of most life-threatening fungal infections.

The solubility of amphotericin-B in water is rather poor (2–4 μg/ml), posing problems in the development of a suitable oral liquid formulation. A commercial formulation, fungizone [1], is a fine colloidal dispersion of amphotericin-B in a micellar solution of sodium deoxycholate. Cyclodextrins have been extensively used in the literature to increase the solubility, dissolution rate and chemical stability of various classes of drugs (Otagiri et al., 1983; Corrigan, 1982). An important characteristic of cyclodextrins is their ability to include guest molecules of suitable size and shape in their relatively hydrophobic cavity leading to an increase in the solubility of the guest molecule (Saenger, 1980; Uekema, 1979).

The present study was undertaken to evaluate if the aqueous solubility of amphotericin-B can be

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[1] E.R. Squibb & Sons, Princeton, NJ 08540, U.S.A.

enhanced by forming an inclusion complex with cyclodextrin. In addition, experiments were also carried out to evaluate different methods for preparing a solid complex of amphotericin-B with cyclodextrin for potential use in the development of a suitable oral formulation.

Materials and Methods

Materials

Amphotericin-B (parenteral grade) was from E.R. Squibb & Sons. α- and β-cyclodextrins (Sigma) were used as received. γ-Cyclodextrin (γ-CyD) was obtained from Japan (U.R. Industries) and used as received. All other materials and solvents were of analytical reagent grade. Double-distilled water was used throughout the study.

Apparatus

Absorption spectra were recorded using a Perkin-Elmer lambda-5 spectrophotometer and 1 cm cuvettes. Measurements of pH were done using the Altex ϕ 71 pH meter. Lyophilization studies were carried out using a laboratory model Usifroid Lyophilizer. Differential scanning calorimetry (DSC) was done using a Perkin-Elmer DSC-2 instrument. The sample size was 10 mg and the scan rate was 10°C/min.

Phase solubility studies

Solubility measurements were carried out according to the method of Higuchi and Lach (1954). Excess amounts of amphotericin-B (8–10 mg) were added to aqueous solutions containing various concentrations of α, β- and γ-cyclodextrins and shaken in a water bath at 25 ± 0.5°C. After equilibrium was attained (approx. 6 days) an aliquot was filtered through a 0.45 μm filter and analyzed for amphotericin-B content by HPLC.

Spectroscopic studies

Complex formation between amphotericin-B and γ-CyD was also studied using the spectral shift method (Connors and Mollica, 1966) in 25 : 75 methanol/water system. The concentration of amphotericin-B in these studies was 7.9×10^{-6} M while the γ-CyD concentration was varied up to 6×10^{-3} M. The change in absorbance of the substrate (amphotericin-B) by the addition of various concentrations of the ligand (γ-CyD) was measured at 415 nm and the data analyzed to evaluate the stoichiometry and stability constant of the complex.

Preparation of solid complex

(a) Lyophilization. A solid complex of amphotericin-B with γ-CyD was prepared by lyophilizing a solution of the complex. γ-Cyd (4.6 g) was dissolved in water (95 ml approx.) and the pH of the solution at 5°C raised to 12.0 by dropwise addition of 2 N sodium hydroxide. Amphotericin-B (100 mg) was added and dissolved under vigorous stirring. The pH of the solution was adjusted rapidly to 7.6 ± 0.1 by dropwise addition of 1 N phosphoric acid and the volume made up to 100 ml by the addition of water. Ten ml aliquots of the solution were transferred into 20 ml vials and lyophilized.

(b) Kneading. To a slurry of γ-CyD, prepared by adding 9.6 g of γ-CyD to 10 ml of water, was added 300 mg of amphotericin-B dissolved in 5 ml of water (at pH 12). This mixture was then transferred to a mortar and kneaded for 15–20 min. At the end of this period, 120 ml of cold isopropyl alcohol (5°C) was added with stirring and the slurry filtered. The solid cake obtained was washed repeatedly with further amounts of cold isopropyl alcohol and dried at 60°C under vacuum.

(c) Coprecipitation. A slurry of γ-CyD was first prepared by adding 9.6 g of γ-CyD to 25 ml of water. To this was added 300 mg of amphotericin-B dissolved in 5 ml of water (at pH 12). This mixture was placed in a water bath at 35–37°C and stirred until a clear solution was obtained and then added dropwise into 200 ml of cold isopropyl alcohol (5°C) with vigorous stirring. The precipitate obtained was collected and washed repeatedly with cold isopropyl alcohol containing 0.05% v/v phosphoric acid and dried at 60°C under vacuum.

Solubility studies

The maximum solubility of amphotericin-B in the complex prepared by the above three methods was determined by shaking excess amounts of the complex in 10 ml of pH 7.4 phosphate buffer. The

solution was filtered through a 0.45 μm filter and the amphotericin-B content of the filtrate determined by HPLC.

Stability studies

The solution stability of amphotericin-B in the complex was evaluated at pH 1.2 and 12. Samples of the lyophilized complex were reconstituted in 0.1 N hydrochloric acid (pH 1.2) or 0.01 N sodium hydroxide (pH 12) at a concentration of 1 mg amphotericin-B/ml. Samples were withdrawn at different time intervals and analyzed for amphotericin-B content by HPLC.

Dissolution studies

The dissolution of the solid complex prepared by all the three methods was evaluated at pH 1.2 and 7.4. Dissolution studies for the samples prepared by kneading and coprecipitation were carried out using the "Dispersed Amount" method. In this method, the equivalent amount of 15 mg amphotericin-B as a 60 mesh powder was weighed and put into a dissolution cell. The dissolution medium (25 ml of pH 1.2 or 7.4 buffer) was maintained at 25°C and stirred at a constant rate. At appropriate time intervals 1 ml samples were withdrawn, filtered and analyzed by HPLC.

Results and Discussion

Solubility studies

The phase solubility diagrams for amphotericin-B with α-, β- and γ-cyclodextrins are shown in Fig. 1. In the case of γ-CyD, the increase in solubility of amphotericin-B with added γ-CyD showed a positive deviation from linearity and the solubility curve can be generally classified as A_p (Higuchi and Connors, 1965). On the other hand, with α- and β-cyclodextrins no appreciable increase in the solubility of amphotericin-B was observed. The A_p type phase diagram observed with γ-CyD indicates formation of higher order complexes with added γ-CyD. As the ligand concentration increases, the contribution of the higher order complexes also increases.

An attempt to calculate the individual stability constants from the A_p phase diagram was made

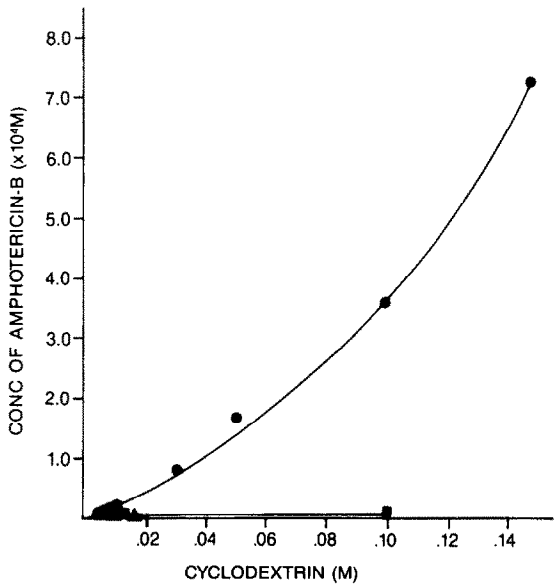

Fig. 1. Solubility of amphotericin-B as a function of α-, β- and γ-cyclodextrin concentration at 25°C. ■, α-cyclodextrin; ▲, β-cyclodextrin; ●, γ-cyclodextrin.

by assuming that only two complexes were formed, SL and SL_2, with the stability constants given by:

$$K_{1:1} = \frac{[SL]}{[S][L]} \tag{1}$$

$$K_{1:2} = \frac{[SL_2]}{[SL][L]} \tag{2}$$

The individual stability constants $K_{1:1}$ and $K_{1:2}$ were calculated using the following equation (Higuchi and Kristiansen, 1970):

$$\frac{S_T - S_0}{L_T - (S_T - S_0)} = K_{1:1} \cdot S_0 + K_{1:1} \cdot K_{1:2}[L_T - (S_T - S_0)] \tag{3}$$

where S_T and L_T are the total molar concentrations of substrate and ligand, respectively, and S_0 the equilibrium solubility in the absence of L. Initial estimates of the stability constants were obtained by plotting the left hand side of the Eqn. 3 versus $[L_T - (S_T - S_0)]$. The final values of the stability constants were obtained by a process of

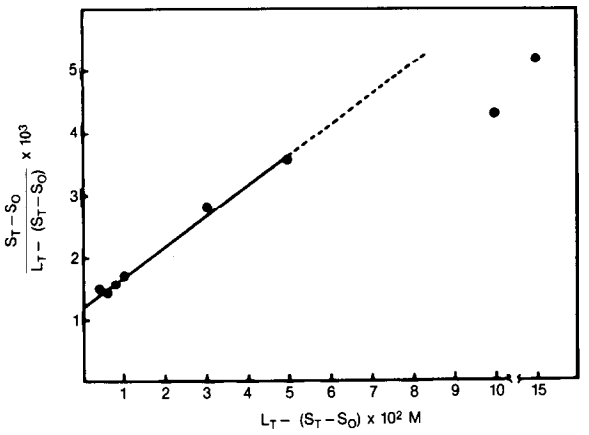

Fig. 2. Plot of Eqn. 3 for determination of $K_{1:1}$ and $K_{1:2}$ for the amphotericin-B/γ-CyD system.

iteration as described by Higuchi and Kristiansen (1970).

As shown in Fig. 2, a plot of Eqn. 3 was observed to be linear only in the γ-CyD concentration range 0–0.05 M. The lack of linearity at γ-CyD concentrations beyond 0.05 M may be due to the formation of other higher order complexes not assumed in the model. An estimate of the $K_{1:1}$ and $K_{1:2}$ values in the γ-CyD concentration range 0–0.05 M was made and observed to be 462 and 42 M^{-1}, respectively. As the solubility data were only fitted over a narrow concentration range these values at best can only be taken as reasonable estimates. The rather moderate value of 462 M^{-1} obtained for the 1 : 1 stability constant indicates that the complex formed is not a strong one.

Spectral studies

The complexation of amphotericin-B with γ-CyD was also studied in 25 : 75 methanol/water mixture by the spectral shift method. The presence of 25% methanol was found to be essential to help keep amphotericin-B in solution and yield a reproducible absorption spectrum. In 25 : 75 methanol/water mixture amphotericin-B shows four absorption maximae at 408.2, 384.5, 365 and 345.7 nm. By addition of γ-CyD to a solution of amphotericin-B, the absorption spectrum showed a red shift. An isosbestic point was observed at 345 nm which was lost at cyclodextrin concentrations greater than 2.0×10^{-3} M, indicating the presence of at least two complexes.

The spectral data in the cyclodextrin concentration range up to 2.0×10^{-3} M were analyzed using the Benesi-Hilderband double reciprocal plot to determine the apparent $K_{1:1}$ stability constant (Eqn. 4):

$$\frac{1}{\Delta A} = \frac{1}{K_{1:1} \cdot S_T \cdot \Delta E_{1:1} \cdot L_T} + \frac{1}{S_T \cdot \Delta E_{1:1}} \qquad (4)$$

where ΔA is the observed change in absorbance at 415 nm, S_T is the total concentration of amphotericin-B, $\Delta E_{1:1}$ the difference in the molar absorptivity between the uncomplexed and complexed drug and L the concentration of the free γ-CyD. At sufficiently high concentrations of γ-CyD, L can be approximated to the total concentration L_T, of γ-CyD. As shown in Fig. 3, a plot of $1/\Delta A$ against $1/L_T$ is linear indicating the presence of 1 : 1 complex. The $K_{1:1}$ stability constant was calculated from the plot to be 422 M^{-1}.

The spectral data were not fitted beyond a γ-CyD concentration of 2.0×10^{-3} M due to the lack of initial estimates for the stability constant of any higher order complex in 25% methanol. This lack of initial estimates would have necessitated the stability constants to be allowed to vary freely in any iterative process. However, as pointed out

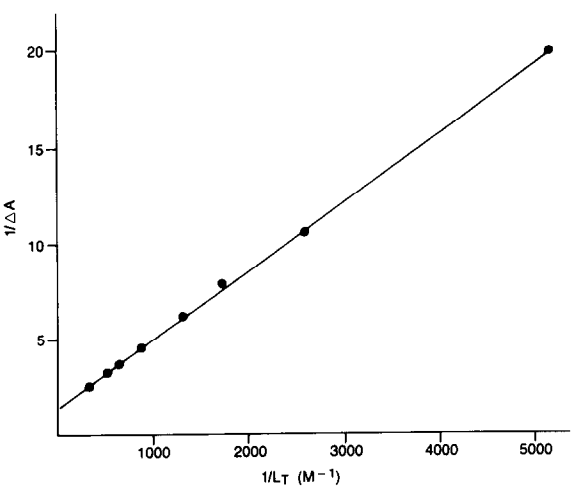

Fig. 3. Benesi-Hilderband plot for the amphotericin-B/γ-cyclodextrin system. ΔA is the change in absorbance at 415 nm and L_T the total ligand concentration.

by Connors and Rosanke (1980), any curve-fitting technique used for extracting the stability constants should be constrained by the condition that the final values must account for the data obtained by at least two independent techniques (e.g. solubility and spectral, etc.). Hence, no attempt was made to analyze the spectral data beyond a γ-CyD concentration of 2.0×10^{-3} M. The $K_{1:1}$ value of 422 M^{-1} obtained by the spectral method appears to be in satisfactory agreement with the value of 462 M^{-1} obtained by the solubility method. The slightly lower value of the 1 : 1 stability constant obtained using the spectral method may reflect some weakening of the complexation forces in 25% methanol.

Preparation of solid complex

The primary objective was to develop a process which could be used for the large scale preparation of the solid complex. Further, based on dose requirement it was desired that the solid complex on reconstitution in water should yield a solution of 1–2 mg/ml amphotericin-B concentration. The A type phase solubility diagram is obtained for systems in which the complex formed is soluble and does not precipitate regardless of the amount of ligand added. Hence, techniques such as kneading, co-precipitation and lyophilization were tried for preparing the solid complex.

Preliminary trials carried out using the kneading technique indicated the process to be unsuitable for large scale preparation of the solid complex due to the poor reproducibility of the technique. The maximum amphotericin-B solubility obtained (on reconstitution at pH 7.4) using this method was observed to strongly depend on such parameters as kneading intensity and time, making the process rather difficult for commercial use. Various weight ratios of amphotericin-B to γ-CyD were tried using the co-precipitation method to achieve the desired amphotericin-B solubility. A weight ratio of 1 : 32 was observed to be optimum. As evident from the data in Table 1, the process of co-precipitation yields approximately a 3-fold higher amphotericin-B solubility than the kneading method. However, considerable batch to batch variation in the maximum amphotericin-B solubility was observed with this technique. It appears

TABLE 1

SOLUBILITY OF AMPHOTERICIN-B IN THREE LOTS OF THE SOLID COMPLEX PREPARED BY KNEADING AND CO-PRECIPITATION IN pH 7.4 BUFFER (20 mM PHOSPHATE) AT R.T.

Method	Solubility (mg/ml)
Kneading	0.61
	0.68
	0.60
Co-precipitation	1.98
	1.83
	2.14

evident that the technique of coprecipitation yields a solid complex, the composition of which is rather difficult to reproduce from one batch to another. Also, a loss in amphotericin-B potency of approximately 10–12% was observed during processing resulting probably from the exposure of amphotericin-B to a high alkaline pH at 35–37°C. Due to the above reasons, the process of co-precipitation was determined to be unsuitable for large scale preparation of the solid complex.

It was observed during the lyophilization studies that a minimum amphotericin-B to γ-CyD weight ratio of 1 : 46 was required to prepare a solution of the desired 1 mg/ml amphotericin-B concentration. Such large amounts of the ligand γ-CyD were evidently required to achieve the target amphotericin-B solubility of 1 mg/ml at pH 7.6. At lower ratios, solutions of the complex were seen to turn cloudy after 15-30 min of storage. The chemical stability of amphotericin-B in the solution prior to lyophilization was monitored under dark storage at R.T. A loss in potency of approximately 1.0% was observed at the end of 24 h storage. Lyophilization of the 1 : 46 complex solution yielded a satisfactory cake which could be rapidly reconstituted in water or 0.1 N HCl to yield a 1 mg/ml amphotericin-B solution. Solutions of the reconstituted lyophile at concentrations greater than 1 mg/ml were observed to turn cloudy after a given length of storage at R.T. In Table 2 are shown data indicating the maximum concentrations of amphotericin-B that can be obtained using the lyophile and the time period over

TABLE 2

EFFECT OF FINAL AMPHOTERICIN-B CONCENTRATION IN RECONSTITUTED SOLUTIONS OF THE LYOPHILE (10 mg AMPHOTERICIN-B/VIAL) ON SOLUTION CLARITY

Reconstitution volume (ml)	Amphotericin-B conc. (mg/ml)	Time period over which solution remains clear
3	3.33	10–15 min
4	2.50	60 min
5	2.00	7 h
10	1.00	> 21 days

which these solutions remained clear. Reconstituted solutions of the lyophile did not show any significant loss in potency under dark storage over a 72 h period at 5°C. The moisture content of the lyophilized cake was determined by Karl Fischer titration to be about 1.1%. The chemical stability of amphotericin-B in the lyophilized cake was monitored over a 15 week period both at R.T. and 40°C. No significant loss in amphotericin-B potency was observed at both the storage temperatures over the 15 week period. Thus, it appears from these studies that the process of lyophilization alone offers a potential method for the large scale preparation of the solid complex.

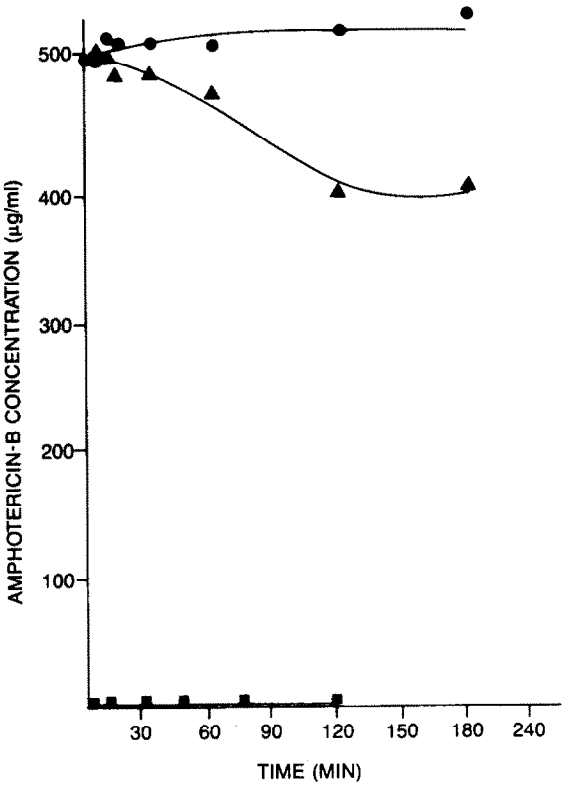

Fig. 4. Dissolution profiles of amphotericin-B and its γ-cyclodextrin complex (co-precipitation method) at 25°C. ■, amphotericin-B alone at pH 7.4; ●, γ-cyclodextrin complex at pH 7.4; ▲, γ-cyclodextrin complex at pH 1.2.

Dissolution studies and stability

Evidence that the lyophilized complex is a true inclusion complex and not a simple physical mixture was substantiated based on the differential scanning calorimetry data. As shown in Fig. 4, the thermograms of amphotericin-B and its physical mixture (the composition being 1 : 46 by weight) show an endothermic peak at about 158°C presumably reflecting a chemical change involving the chromophore in the amphotericin-B molecule. This endothermic peak is not observed in the assumed inclusion complex. The lyophilized complex dissolved rapidly in both pH 1.2 and 7.4 buffer to yield a clear solution of amphotericin-B concentration 1 mg/ml. The dissolution profiles of the complex (prepared by co-precipitation) and amphotericin-B alone are shown in Fig. 5. As seen, the complex exhibits a faster drug dissolution rate than amphotericin-B alone at both the pHs of study. At pH 1.2, the concentration of amphotericin-B after dissolution reached a peak in about 3–4 min and then decreased gradually due to acid catalysis. A loss in amphotericin-B concentration of about 10% was observed after exposure to pH 1.2 for 90 min. The dramatic difference in the dissolution profiles of the complex and free amphotericin-B as illustrated in Fig. 5, highlights the solubility improvement brought about by complexation. The improved dissolution rate may be due to the increase in solubility as well as a decrease in the crystallinity of amphotericin-B brought about by complexation.

Figs. 5 and 6 show the degradation profiles of the lyophilized complex and amphotericin-B alone at pH 1.2 and 12, respectively. The degradation of amphotericin-B was significantly suppressed at both pHs by complexation with γ-CyD. At pH 1.2 after 120 min, the degradation of amphotericin-B

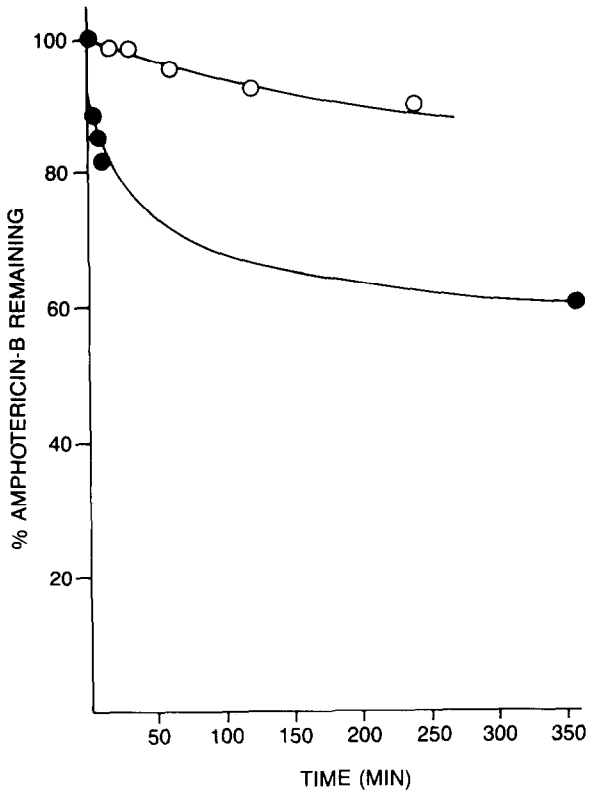

Fig. 5. Degradation profiles of amphotericin-B and lyophilized γ-cyclodextrin complex in pH 1.2 buffer at 25°C. ●, amphotericin B alone; ○, γ-cyclodextrin complex.

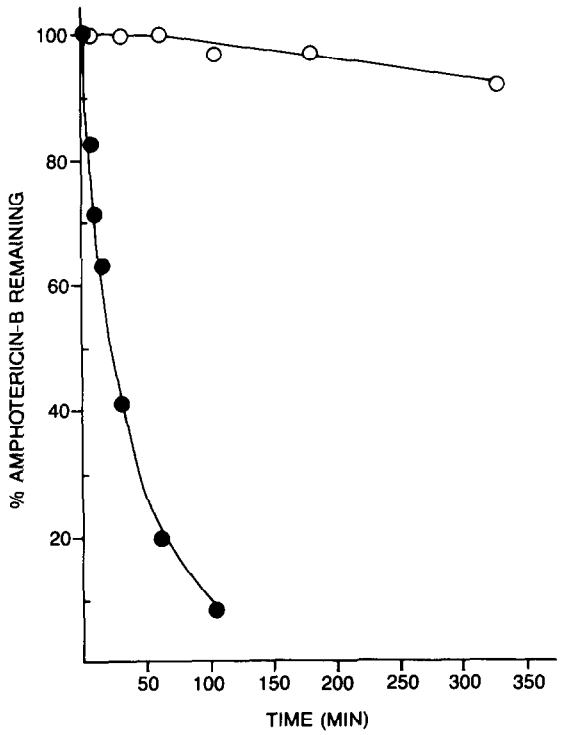

Fig. 6. Degradation profiles of amphotericin B and lyophilized γ-cyclodextrin complex in pH 12 buffer at 25°C. ●, amphotericin B alone; ○, γ-cyclodextrin complex.

in the complex was only 7.5% as compared to 36% for free amphotericin-B during the same time period. At pH 12, the enhanced chemical stability of amphotericin-B in the cyclodextrin complex was rather dramatic. At the end of 3 h at this pH, the chemical loss of amphotericin-B in the complex was only 3.0% as compared to 92% for free amphotericin-B during the same period. Based on the most probable site in the amphotericin-B molecule susceptible to attack by hydroxyl ions at pH 12, a possible mechanism for the formation of 1 : 1 inclusion complex is proposed in Fig. 8. Further support for this inclusion mechanism arises from the fact that the relatively hydrophobic cyclo-

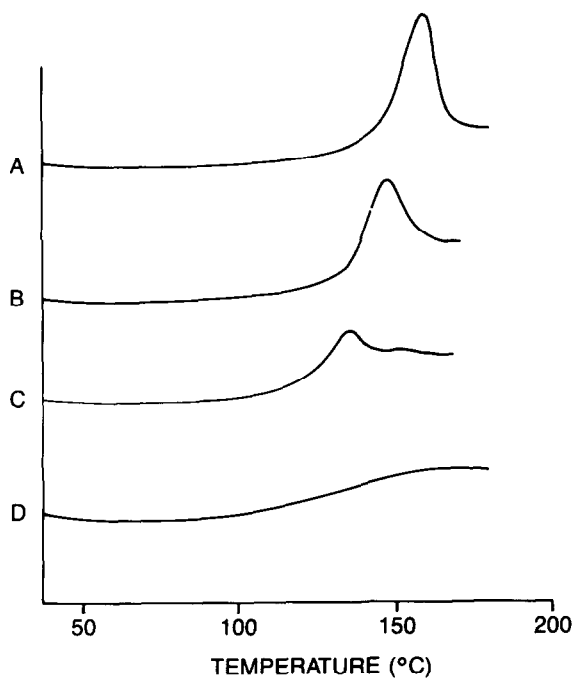

Fig. 7. Differential scanning calorimetry of amphotericin-B (A), γ-cyclodextrin (B), a physical mixture of amphotericin-B and γ-cyclodextrin (C) and the inclusion complex of amphotericin-B with α-cyclodextrin.

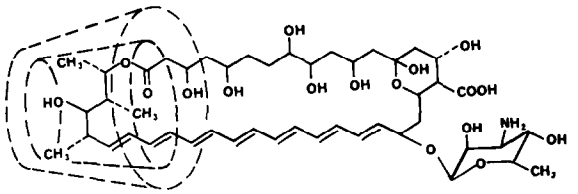

Fig. 8. Proposed mechanism for the formation of amphotericin-B γ-cyclodextrin 1 : 1 inclusion complex.

dextrin cavity should favor inclusion of the hydrophobic part of the amphotericin-B molecule as proposed in this model. However, the proposed model is only tentative and will have to be verified by other techniques.

Thus, the present study indicates that the aqueous solubility of amphotericin-B can be significantly increased by forming an inclusion complex with γ-CyD. This enhancement in solubility brought about by complexation may be of potential use in developing a suitable oral liquid amphotericin-B formulation.

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