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APPLICATION OF DIFFERENTIAL SPECTRA IN THE ULTRAVIOLET-VISIBLE REGION TO STUDY THE FORMATION OF AMPHOTERICIN B-STEROL COMPLEXES

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Summary

The extent of complex formation between the polyene antibiotic, amphotericin B, and cholesterol or ergosterol was investigated and a method for a quantitative measurement of the complex formation was developed.

The effect of experimental conditions on the magnitude of the amphotericin B-sterol interaction and on the selectivity of this interaction showed that there was only a narrow range of solvent composition in which the differential selectivity of amphotericin B towards these two sterols could be observed.

Introduction

Amphotericin B is a potent antifungal antibiotic that damages fungal cell membranes by binding to sterols [1]. The relatively low toxicity of this antibiotic to the host, compared to other polyenes, allows it to be given parenterally to treat patients with invasive fungal infections. Since both host and fungal cells contain sterols, it has been assumed that the relative specificity of amphotericin B for fungi is based on a more avid binding to ergosterol, the principal fungal sterol, than to cholesterol, the sterol found in animal cell membranes. In contrast, filipin, a polyene too toxic for clinical use, was shown to bind more avidly to cholesterol than to ergosterol. The notion that polyene antibiotic toxicity is related to the type of sterol incorporated into membrane is supported by several different kinds of studies utilizing fungi [2,3], erythrocytes

Abbreviation: Me_2SO, dimethyl sulfoxide.

[4], mycoplasma [5] and phospholipid bilayer membranes [6].

One might expect that if both sterols complex with the polyene antibiotics in a comparable fashion, the extent of filipin spectral change induced by complexing with sterol should be greater for cholesterol and amphotericin B spectral change should be greater for ergosterol. There are reports that cholesterol induced greater changes in the spectral characteristics of filipin than did ergosterol [7,8]. However, for amphotericin B, there are observations which could be interpreted as indicating a lack of selectivity. For example, amphotericin B undergoes a relatively constant enhancement in fluorescence polarization in the presence of various lecithin/sterol vesicles [9], and lecithin/cholesterol vesicles produce nearly equal enhancement in fluorescence excitation and emission spectra [10].

In preliminary experiments, we found that under some experimental conditions the spectral changes of amphotericin B in the ultraviolet-visible region, due to the formation of antibiotic-sterol complexes, were more pronounced in the presence of ergosterol than with cholesterol. These experiments were performed in the presence of a non-ionic detergent, Triton X-100, which is known to interfere with polyene antibiotic-sterol complex formation [7]. The formation of complexes was monitored by the ratios of the absorbance at 363 and 408 nm (peak 3/peak 1 absorbance ratio), according to the method of Norman et al. [11].

We were not persuaded that the peak 3/peak 1 absorbance ratio could be considered as a real measure of antibiotic-sterol interaction and compared in a quantitative way for different sterols. Therefore, we have adopted the method of differential spectra to investigate amphotericin B-cholesterol and amphotericin B-ergosterol complex formation. This method eliminates the concentration- and solvent composition-dependent changes in the spectrum and records only the changes caused by the interaction between sterols and the antibiotic. It therefore makes possible a quantitative approach to selectivity in amphotericin B-sterol complex formation.

Materials and Methods

Amphotericin B (A grade) was purchased from Calbiochem, La Jolla, CA, and cholesterol (certified grade) was supplied by Fischer Scientific Company, Montréal, Québec. Ergosterol (95%) was purchased from Sigma, St. Louis, MO, and was twice recrystallized from ethanol. Triton X-100 was obtained from Packard Instruments Company, Downers Grove, IL.

Amphotericin B stock solutions were prepared by dissolving the powder (4.60 ± 0.08 mg) in 1.0 ml of Me_2SO and increasing the volume to 100.00 ml with water. The molecular weight of amphotericin B is 924. Triton X-100 stock solution was prepared by dissolving the detergent in water. Stock solutions of sterols were prepared by dissolving the compound in alcohol. All solutions were prepared in a 20° C thermostatically controlled bath and were stored at 4° C for not more than 2 days before use for the amphotericin B solution and not more than 1 week for the sterol solutions. The water/alcohol solutions of amphotericin B and sterols were prepared at 20° C by adding to a calculated amount of amphotericin B stock solution, alcohol, water and, in the last place,

the appropriate sterol stock solution. Spectra were recorded within 15—45 min after preparation of the samples.

Absorbance at two selected peaks (408 and 363 nm) was measured on a Coleman Junior Spectrophotometer. The concentration of amphotericin B used in this experiment was $8 \cdot 10^{-6}$ M and the final concentration of Me_2SO was 0.2% and of ethanol, 2.5%.

The differential spectra were recorded with a Cary 17D double-beam spectrophotometer. In the sample beam, two cuvettes were installed in series; one with an antibiotic/sterol solution and the other with the solvent used in the experiment. In the reference beam, one cuvette contained the antibiotic solution and the other the appropriate sterol solution. Since all concentrations were identical, the recorded differential spectrum represented the direct effect of sterol-antibiotic interaction. The concentration of amphotericin B used in this experiment was $9 \pm 0.4 \cdot 10^{-6}$ M and the antibiotic : sterol ratio was 1 : 1, a ratio considered as optimal by others [12,13]. The final concentration of Me_2SO was 0.2%.

To monitor the amphotericin B-sterol interaction we have used the negative peak of the differential spectra at 408 nm which measured the disappearance of free amphotericin B. We have devised a method allowing us to calculate the contribution of the formed amphotericin B-sterol complexes to the value of this peak.

Results

Fig. 1 shows the effect of sterols on the amphotericin B peak 3/peak 1 absorbance ratio at different Triton X-100 concentrations. The most pronounced difference between the effects of cholesterol and ergosterol occurs when 0.2% of the detergent is added (Fig. 1c). Little or no selectivity can be observed at lower detergent concentrations (Fig. 1a and b) and there is almost no interaction between the antibiotic and both sterols at a higher detergent concentration (Fig. 1d). Figs. 2—4 present typical differential spectra of amphotericin B with sterols in 10% methanol (Fig. 2), 10% methanol +0.2% Triton X-100 (Fig. 3) and 25% propanol (Fig. 4). One can see that in 10% methanol solution, the spectral changes are comparable for both sterols. Addition of 0.2% Triton X-100, the most effective concentration of the detergent determined by our preliminary study (Fig. 1), gives a spectrum which indicates a greater effect of both sterols, the effect of ergosterol is much more pronounced than with cholesterol (Fig. 3). In 25% propanol solution, there is no effect of cholesterol, whereas ergosterol strongly decreases the free amphotericin B concentration.

The previous experiments demonstrate that the antibiotic-sterol interaction is strongly dependent on experimental conditions. The effect of experimental conditions, such as the kind of alcohol and the alcohol : water ratio, was further examined. Table I shows the amphotericin B-sterol interaction as a function of solvent composition. This interaction was monitored by differential absorption at 408 nm, the wavelength of maximum absorption of free amphotericin B. The negative peak at 408 nm in the differential spectra reflects the disappearance of free amphotericin B but its magnitude may be diminished by

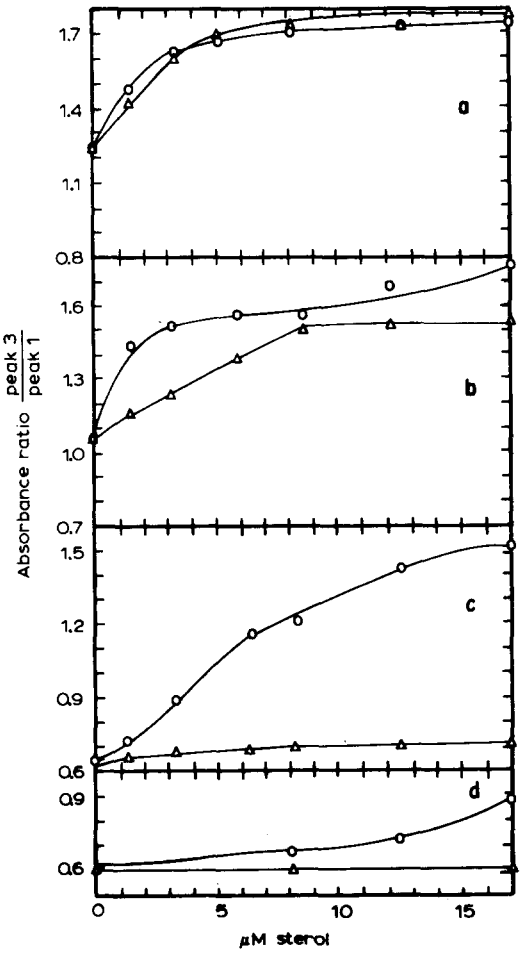

Fig. 1. Effect of sterols on the amphotericin B absorbance ratio (peak 3/peak 1) at different Triton X-100 concentrations: a, 0.025%; b, 0.05%; c, 0.2%; and d, 0.5%. ○, with ergosterol; △, with cholesterol.

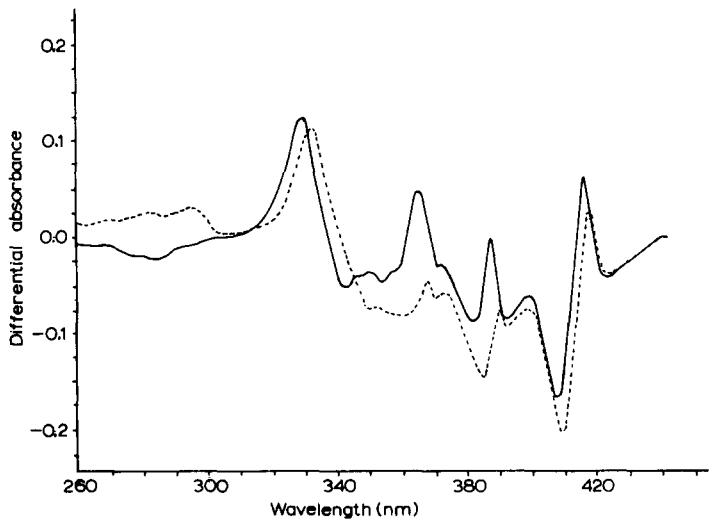

Fig. 2. Differential absorption spectra of $9 \cdot 10^{-6}$ M amphotericin B with sterols (1 : 1) in 10% methanol. - - - - - -, with ergosterol; ———, with cholesterol.

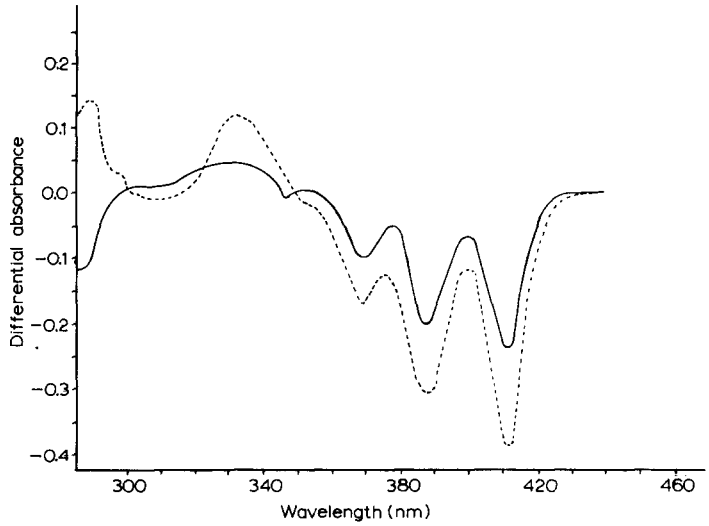

Fig. 3. Differential absorption spectra of $9 \cdot 10^{-6}$ M amphotericin B with sterols (1 : 1) in 10% methanol in the presence of 0.2% of Triton X-100. - - - - - -, with ergosterol; ———, with cholesterol.

contribution of the formed complex. To evaluate this effect we have measured the spectra of 10^{-5} M amphotericin B solution in 17% propanol, varying the amphotericin B : sterol molar ratio. Fig. 5 shows the resulting plot of absorption at 408 nm as a function of the molar ratios. Extrapolation to infinite sterol concentration yields an estimate of the absorption of a 10^{-5} M solution of amphotericin B-sterol complex. The calculated extinction coefficients of the complexes were $1.4 \cdot 10^4$ l · mol^{-1} · cm^{-1} for ergosterol and $1.9 \cdot 10^4$ l · mol^{-1} · cm^{-1} for cholesterol. The extinction coefficient at 408 nm of free amphotericin B solution was determined to be $1.4 \cdot 10^5$ l · mol^{-1} · cm^{-1} (Fig. 6).

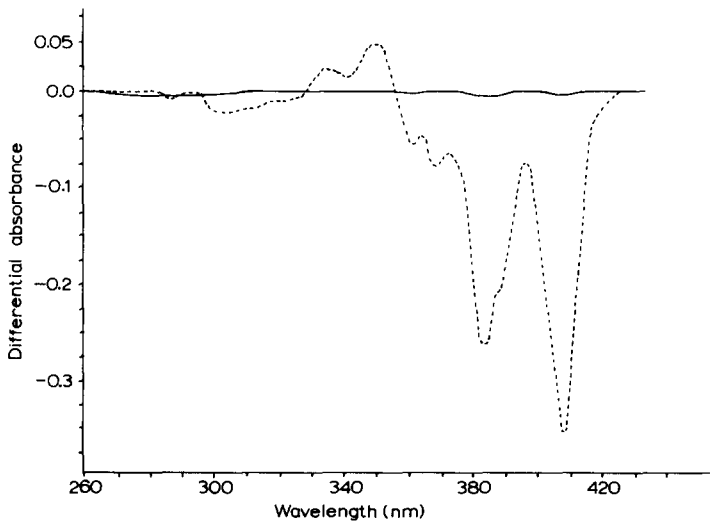

Fig. 4. Differential absorption spectra of $9 \cdot 10^{-6}$ M amphotericin B with sterols (1 : 1) in 25% propanol. - - - - - -, with ergosterol; ———, with cholesterol.

TABLE I

EFFECT OF CHOLESTEROL AND ERGOSTEROL ON THE AMPHOTERICIN B ABSORPTION SPECTRUM

%V, the volume concentration of the alcohol. A_{erg}, the magnitude of the negative peak at 408 nm for amphotericin B/ergosterol solution. A_{chol}, the magnitude of the negative peak at 408 nm for amphotericin B/cholesterol solution. $[Cx]_{erg}$, concentration of amphotericin B/ergosterol complex. $[Cx]_{chol}$, concentration of amphotericin B/cholesterol complex. $S = [Cx]_{erg} - [Cx]_{chol}$.

Solvent	%V	A_{erg}	A_{chol}	$[Cx]_{erg}$ (mol/l) ($\times 10^6$)	$[Cx]_{chol}$ (mol/l) ($\times 10^6$)	S (mol/l) ($\times 10^6$)
Methanol	10	−0.198	−0.160	1.57	1.32	0.25
	15	−0.188	−0.155	1.49	1.28	0.21
	20	−0.265	−0.232	2.10	1.92	0.18
	25	−0.299	−0.221	2.37	1.83	0.54
	30	−0.880	−0.678	6.98	5.60	1.38
	35	−0.980	−0.970	7.78	8.02	−0.24
	40	−0.880	−0.840	6.98	6.94	0.04
	50	−0.220	−0.150	1.74	1.24	0.50
	60	−0.010	0.00	0.08	0	0.08
	70	0.00	0.00	0.00	0.00	0.00
Methanol + 0.2% Triton X-100	10	−0.390	−0.235	3.09	1.94	1.15
Propanol	10	−0.210	−0.210	1.67	1.73	−0.06
	15	−0.665	−0.529	5.28	4.37	0.91
	17	−1.065	−0.887	8.45	7.33	1.12
	20	−0.928	−0.720	7.36	5.95	1.41
	25	−0.360	0.00	2.85	0.00	2.85
	30	0.00	0.00	0.00	0.00	0.00

Since the concentration of the formed complex is equal to the concentration of missing free amphotericin B, we can describe the differential spectra by the following equations:

$$A_{erg} = -\epsilon_A \cdot [Cx]_{erg} + \epsilon_{AE}[Cx]_{erg} = -1.26 \cdot 10^5 [Cx]_{erg}$$

and

$$A_{chol} = -\epsilon_A \cdot [Cx]_{chol} + \epsilon_{AC}[Cx]_{chol} = -1.21 \cdot 10^5 [Cx]_{chol}$$

A_{erg} and A_{chol} are values of negative peaks at 408 nm for the solutions of amphotericin B with ergosterol and cholesterol, respectively; ϵ_A, ϵ_{AE} and ϵ_{AC} are the extinction coefficients of free amphotericin B, amphotericin B-ergosterol complex and amphotericin B-cholesterol complex, respectively; and $[Cx]_{erg}$ and $[Cx]_{chol}$ are the concentrations of the respective complexes. The selectivity of amphotericin B interactions with the two sterols may be defined as:

$$S = [Cx]_{erg} - [Cx]_{chol}$$

As one can see from Table I at low alcohol concentrations, the effect of any sterol on the spectrum is small; at high alcohol : water ratios there is no effect at all. The region of discernible amphotericin B-sterol complex formation is 0—60% for methanol and 0—30% for propanol. The region in which one can observe a preference in the interaction of amphotericin B with ergosterol to that with cholesterol is 10—60% for methanol and 15—25% for propanol. The mag-

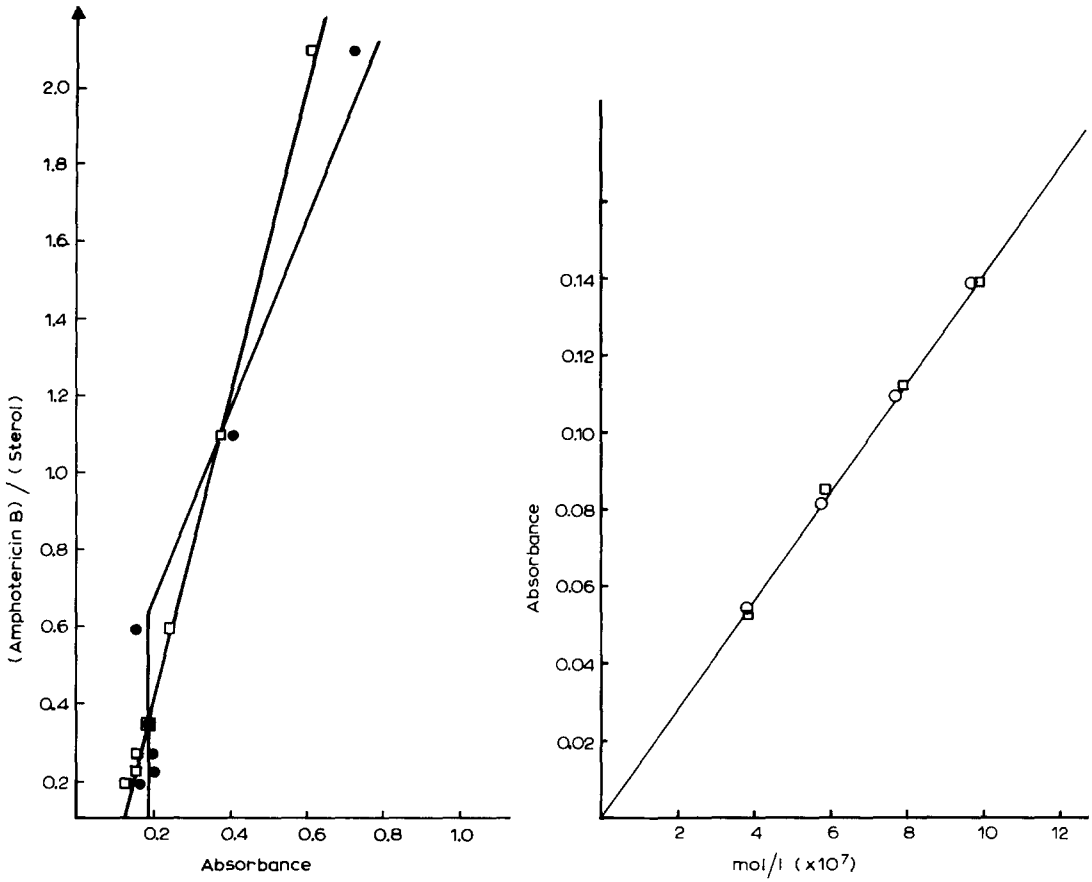

Fig. 5. Effect of the amphotericin B : sterol molar ratio on the absorbance at 408 nm. □, with ergosterol; ●, with cholesterol.

Fig. 6. Effect of the amphotericin B concentration in propanol containing 0.4% of Me_2SO on the absorbance at 408 nm. ○, experiment 1; □, experiment 2.

nitude of the selectivity is greater in propanol than in methanol and the most pronounced selectivity is observed in 25% propanol solution.

The interpretation of our results has been based on the assumption that ergosterol and cholesterol are equally available to form complexes with amphotericin B, or, at least that ergosterol is not more available than cholesterol. This assumption was based on the known higher solubility of cholesterol than of ergosterol in alcohols and in water [14]. To support our argument we have recorded a differential spectrum of another polyene antibiotic, filipin. Our results (Fig. 7) demonstrate that filipin binds more to cholesterol than to ergosterol, which is in agreement with previous findings (Ref. 15 and references contained therein). Hence, the differences in spectra we have observed cannot be attributed to the differential availability of sterols in solution but reflect the selectivity of the interaction of the polyenes with the sterols.

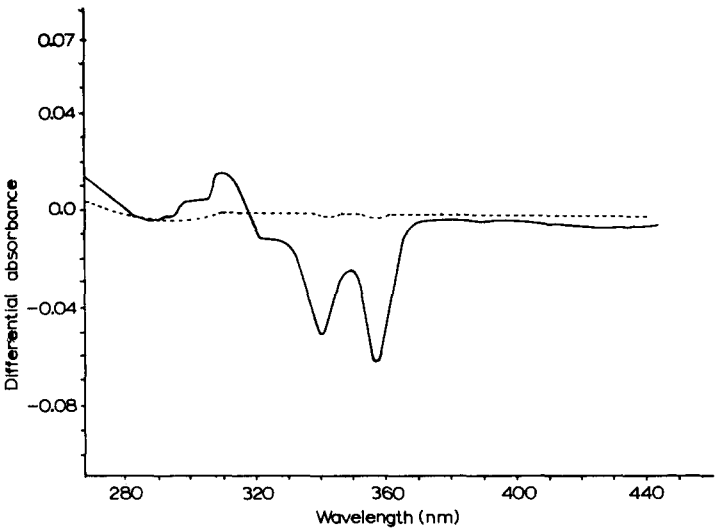

Fig. 7. Differential absorption spectra of $9 \cdot 10^{-6}$ M filipin with sterols (1 : 1) in 50% methanol. - - - - -, with ergosterol; ———, with cholesterol.

Discussion

There are several reports that demonstrate that the effects of amphotericin B on biological membranes depend on the type of incorporated sterol and are much more pronounced on membranes containing ergosterol than on those with cholesterol [3,16,17]. The fact that this phenomenon had not been observed in spectral investigations in solution was quite puzzling. The only plausible explanation was that the experimental conditions used in the spectral studies were very different from the biological conditions. In other words, it is possible that inside a biological membrane, amphotericin B forms complexes with sterols previously bound to other membrane components and the antibiotic has to compete for sterol. Our present study involved the development of conditions which stressed the amphotericin B-sterol complexes in solution so that differential avidity could be determined by a spectroscopic investigation.

At low alcohol : water ratios, the amphotericin B-sterol complex formation is low (Table I). The antibiotic exists under these conditions mainly as aggregates and sterols have to compete with amphotericin B-amphotericin B aggregation forces. At high alcohol : water ratios, there is no complex formation because of the interference of alcohol molecules which interrupt the amphotericin B-amphotericin B aggregates as well as the amphotericin B-sterol complexes. There is only a narrow range of solvent composition when enough of the free antibiotic molecules are available to react with sterols and the concentration of alcohol is not sufficiently high to break the amphotericin B-sterol complexes. This region of solvent composition is larger in methanol than in propanol. The role of Triton X-100 is similar to that of alcohols, but the concentration necessary to break aggregates and complexes is much lower. This explanation is also in agreement with the hypothesis that the sterol-polyene antibiotic complexes are hydrophobic (Ref. 12 and references contained ther-

ein) because propanol is more effective at breaking complexes than methanol and Triton X-100 is more effective than both alcohols.

The region of solvent composition in which we observed the interaction between amphotericin B and sterols is limited to the conditions under which the antibiotic exists, at least to a certain degree, as a monomer and the concentration of complex-breaking agents is not too high. The region in which we observe an important selectivity is more narrow than the region of complex formation. In aqueous propanol solutions and in the presence of Triton X-100, we observe the most pronounced selectivity under conditions which maximally stress the complexes. The same phenomenon is observed in methanol/water solutions, but in this mixture there is a second region of pronounced selectivity in 30% methanol for which we have no explanation. Although the selectivity of polyene antibiotic-sterol interactions is well established, no quantitative method for its determination is known. The method presented in this work for amphotericin B may be also adapted to the study of other polyene antibiotic interactions with sterols and may help in better understanding of their biological activity.

Acknowledgements

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