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Spectral study of cholesteryl linoleate - amphotericin B interaction and behaviour of cholesteryl esters in electric field

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Cholesteryl esters are abundant in biological membranes. Their presence alongside fatty acids, free or bound to phospholipids, modulates the lipid membrane behaviour. This study describes the unfacilitated passive diffusion electric model of some mixtures of cholesteryl linoleate and glycerol in order to explain the nature of relaxation time. Relaxation time responds by equivalent electric capacity of transmembrane process. This time depends on the nature of transported compounds and decreases with their concentration. Also, the purpose of the present work is the study of the interaction between amphotericin B and a cholesteryl ester, namely cholesteryl linoleate.

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1. Introduction

Poly and monounsaturated fatty acids are essential as far as their presence in membrane structured is concerned. Studies have been carried out to demonstrate that the major changes in the membrane structure occur as a result of a weak modification of the non-saturation state, which can elucidated the crucial role of the double link in the membrane functions.

Linoleic acid is the most common polyunsaturated fatty acid in animal tissues and plants; it is an essential fatty acid for animals and is one of the major fatty acids in the coronary arterial wall [1]. Cholesteryl linoleate (Ch-Lin, Fig. 1), the most abundant cholesteryl ester (CE) in lipoproteins, showed a reduction of 70% in LDL, while other CEs showed a lower reduction (50%) [2]. Influence of Ch-Lin – low-density lipoprotein complex was studied into some tumour cells [3,4].

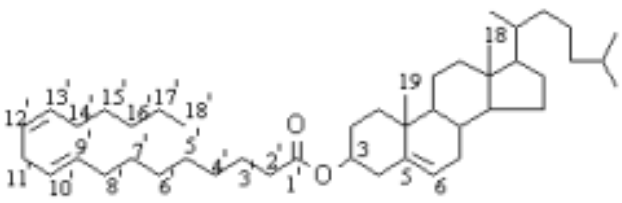


Fig. 1. Chemical structure of Ch-Lin.

Amphotericin B (AmB, Fig. 2) is rod-shaped molecule with the hydrophilic part (consisting of seven hydroxyl groups and one carboxyl group) of the macrolide ring forming an opposing face to the lipophilic polyenic fragment, composed by seven conjugated double bonds.

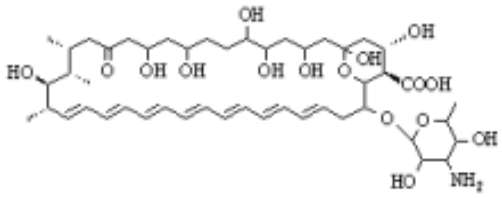


Fig. 2. Chemical structure of AmB

This antibiotic, in principle, acts at the level of fungi cellular membranes [5], causing their enhanced permeability by the formation of pores or channel through which a number of vital elements, like potassium ions and small organic molecules, escape from cells, causing eventually their destruction.

In literature there is a controversy as regards the role of sterols in the phospholipid membrane. In this way, two mechanisms have been postulated. According to the first model, the action of sterols is indirect as they are supposed to modify the packing arrangement of the membrane phospholipids, facilitating in this way the incorporation of antibiotic molecules [6,7]. The other model suggests the existence of some complexes between amphotericin B and sterols (ergosterol, cholesterol, etc.), which are responsible for the channel formation in cellular membranes [8,9].

Recently, the existence of strong interactions between amphotericin B and different sterols was demonstrated by the monolayer technique [10], confirming the indirect mechanism of complex formation between the components. The interactions do exist between polar groups of both compounds as well as between their apolar parts, due to the formation of hydrogen bonds and hydrophobic attraction (van der Waals) forces, respectively. Other investigations showed that the

interactions between AmB and ergosterol are stronger than in the AmB - cholesterol system [11,12].

The properties of membrane channels, the associated state of the AmB and the molecular aspects of interactions between AmB and the surface of a phospholipid membrane have been studied. It was postulated that AmB dimers, rather than monomers, form AmB - membrane channels [13,14]. Two mechanisms have been proposed [15]. First, the so-called "sequential mechanism", in which monomeric AmB molecules enters the cell membrane and then form channels. In this case, the AmB molecules may interact with sterols or other lipids and form complexes prior to channel formation. Since AmB molecules form monolayers at air - water interface, another mechanism of channel formation called the "one step" mechanism assumes that AmB may also form aggregates on the membrane surface. These higher associates formed on the membrane surface may enter the membrane in one step and form the channel.

In spite of fact that the effect of AmB on organization of lipid membranes and the effect of lipid phase on molecular organization of the drug have been established, there are the unclear aspects as regard the surface topography or the mechanism of lipid - AmB interaction in a layer. Therefore, we think that the determination of the relaxation time of Ch-Lin, mixtures of Ch-Lin with cholesterol in glycerol solution and the study of the interaction between AmB and Ch-Lin, by UV-Vis absorption spectroscopy, presented in this work, will contribute at understanding the effect of this drug on membrane lipids.

2. Experimental set-up and procedures, used substances

The time variation of the depolarising electric current intensity was recorded by means of a data-acquisition system, employing an interface of the UT 70B type. The absorption spectra were recorded on a Perkin-Elmer Lambda 25 UV-VIS spectrophotometer using the 1cm optical path length quartz cell, at room temperature.

Linoleic acid and cholesterol were Merck products. Cholesteryl linoleate was synthesized from cholesterol and linoleic acid, in the presence of the *p*-toluensulphonic acid, according to the indications in the literature [16]. Ch-Lin was crystallized several times from amyl alcohol. The purity of cholesteryl linoleate was checked by thin-layer chromatography. The stock solutions of Ch-Lin for spectral analyses were prepared in anhydrous ethanol.

Amphotericin B from *Streptomyces sp.* was Sigma-Aldrich product. The stock solutions of amphotericin B were prepared in ethanol and their concentration was determined using the following molar absorption coefficient value: $\varepsilon_{407nm}=160000 M^{-1}cm^{-1}$ [17,18].

The manner in which the experiment was carried out is the classic one; a potential difference of one volt was applied across the two electrodes immersed in the electrolytic solution. After a certain time was allowed in order to reach the stationary regimen for the functioning of the electrolysor, the electrolysor was commuted in other circuit, were there is no external power supply, but there is instead a high resistance resistor (1.5 MΩ) connected serially between the two electrodes. To obtain the experimental results presented in the last column of the Table 1, the measurements were repeated for all the samples, and after that the relaxation time was determined for each measurement and, finally, its average value was calculated, as well as the average square derivation.

3. Results and discussion

The physicochemical properties of AmB, mainly its amphiphilic and zwitterionic nature, attributed respectively to the apolar and polar sides of the lactone ring and to the presence of ionisable carboxyl and amino groups, as well as its asymmetrical distribution of hydrophobic and hydrophilic groups, make the drug extremely insoluble in water and polar organic solvents.

The self-association of AmB has been followed by measurement of the electronic absorption in the UV-VIS region as a function of drug concentration. In polar organic solvents, like ethanol, AmB exists as a monomer and its UV-Vis spectrum (Fig. 3) shows the sharp bands at 428, 407, 383 and 364nm.

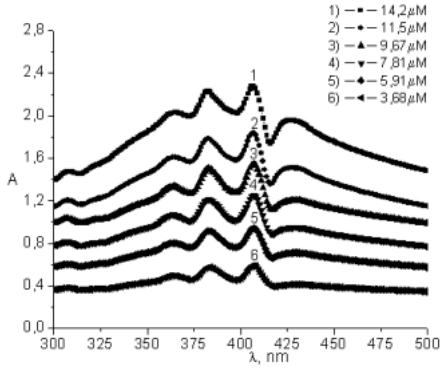


Fig. 3. Absorption spectra of AmB in ethanol, at different drug' concentrations.

The evolution of the spectra with the increase of AmB concentration attests the formation of molecular aggregates and allows the following assignment of the bands: the band at 407nm – monomer, the band at 428nm – dimer and the band at 364nm – higher aggregates, in accordance with other literature data [19-23].

The ratio of absorbance $\frac{A_{364}}{A_{407}}$ has been applied as the measure of degree of AmB self-association. For monomeric AmB, the ratio is about 0.25 while for the aggregated forms increases with concentration and saturates at a level about 2. In addition, we have determined a self-association constant of ~5000 M^{-1} for AmB, by two methods [17].

In order to determine the relaxation time of the electrolytic solutions, an electrolytic cell is used, which is

polarised through the agency of a direct current power source. After the polarisation, the voltage source is removed, and its electrodes are linked up to a metal conductor, by means of a high resistance.

By considering the expressions of the λ and ω_0 parameters [24], the expression of the relaxation time results as:

$$\tau = \frac{2\lambda}{\omega_0^2} = (R + R_0) \cdot C \qquad (1)$$

Let us admit that the electric parameters of the metallic circuit portions remain unaltered. Let us further admit that the geometry of the electrolysor stays the same, as well. In these conditions, for a certain type of electrolytic solution, the relaxation time is dependent only upon the concentration of the electrolyte solution. As the geometry of the system is constant, the variation of the electric resistance of the electrolyte column is determined only by the modification of the electric conductibility of the solution, at the same time as the concentration of the electrolytic solution rises. As is known, the electric conductibility of a monovalent electrolytic solution is given by the relation:

$$\sigma = e \cdot n \cdot (\mu_+ + \mu_-) \qquad (2)$$

where:
e represents the electric charge of an ion, *n* represents the ion concentration in the electrolytic solution, μ_+ and μ_- represent the mobilities of the anions and those of the cations [25].

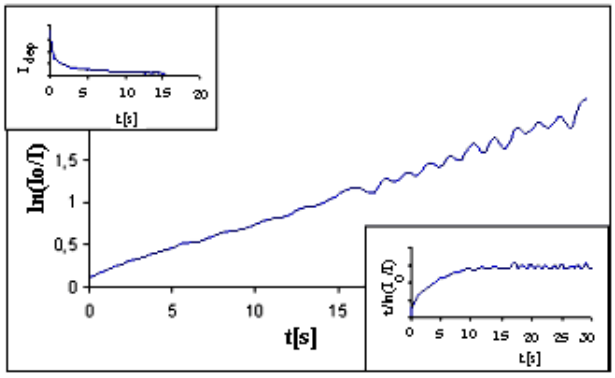
It is found that the electric resistance of the electrolytic solution column must decrease with the increase in its concentration. By experimental determination of the time of relaxation for various concentrations of the electrolytic solution, the magnitude of $\mu_+ + \mu_-$ along the slope of the straight line can be determined:

$$\tau = a \cdot n^{-1} + b \qquad (3)$$

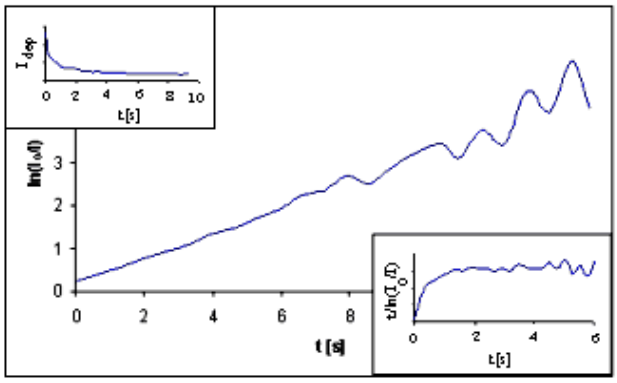
where *a* and *b* are two constants.

To fully understand the way each individual relaxation time was calculated; let us concentrate on one of the experimental graphs (for instance, the graph in Fig. 4).

In the top left corner of the image, the experimental evolution of the intensity of the depolarising electric current is presented in keeping with the time factor. What one can find is that, as naturally expected, the intensity of the electric current keeps on decreasing in time (except for certain relatively small fluctuations).



(a)



(b)

*Fig. 4. Time evolution of the depolarising current for samples **1** (a) and **3** (b).*

In the centre is shown the dependency of the logarithm of ratio between the initial intensity of the electric current and its instantaneous value in keeping with the time factor, after achieving the necessary corrections [25].

In the right bottom corner is shown the dependency of the parameter $\frac{t}{\ln(I_0 / I)}$ upon time, which is necessary in determining the relaxation time in the Honciuc - Paun method [26]. By ignoring the insignificant deviations from linearity within the region of the high time periods, caused by the high measuring errors, corresponding to the very low depolarising currents, we find that the shape of experimental curves is identical to the one deduced by means of the equivalent electric scheme.

The samples analysed, the chemical composition, both qualitative and quantitative, as well as the values of the relaxation time determined experimentally are presented in Table 1.

Table 1. The composition and the relaxation times for the samples investigated.

Sample	Compound (mols)			Relaxation time (s)
	Cholesteryl linoleate	Cholesterol	Glycerol	
1	$4.39 \cdot 10^{-5}$	-	$7.24 \cdot 10^{-3}$	16±3
2	$7.43 \cdot 10^{-5}$	-	$7.24 \cdot 10^{-3}$	13±3
3	$2.71 \cdot 10^{-5}$	$2.25 \cdot 10^{-5}$	$7.24 \cdot 10^{-3}$	2.5±0.5
4	$3.82 \cdot 10^{-5}$	$2.72 \cdot 10^{-5}$	$10.61 \cdot 10^{-3}$	2.3±0.7
5	$3.84 \cdot 10^{-5}$	$2.25 \cdot 10^{-5}$	$7.24 \cdot 10^{-3}$	2.0±0.3

The behaviour in an external field of Ch-Lin was discussed, also this CE with cholesterol. This system is useful for modelling simple biological membranes. Evolution of thermodynamic equilibrium state is a relaxation process. The presence of Ch in mixtures of Ch-Lin in glycerol (samples **3** - **5**) determines a decreasing of the electric relaxation time, determines a major modification of the mechanism of scattering. Mixtures of CE and Ch in glycerol solution at low concentration are characterized by a single relaxation time, not two at the same time.

A family of curves obtained at the titration of AmB solutions with Ch-Lin is presented in Fig. 5. It may be observed that the complex of AmB with Ch-Lin is characterized by the decrease of the major bands at small and medium Ch-Lin to AmB concentration ratios ($\frac{\text{Ch - Lin}}{\text{AmB}}$, noted p).

The estimation of the binding affinity in AmB – Ch-Lin system is based on changes in the UV-VIS absorption spectra of drug occurring upon interaction with cholesteryl ester. Supposing a 1:1 binding ratio and do not account explicitly for either the dimerization of the drug or cooperativity effects on the binding, we have determined the binding constant of AmB to Ch-Lin by Benesi-Hildebrand, Scott, Scatchard and Wolfe methods [18].

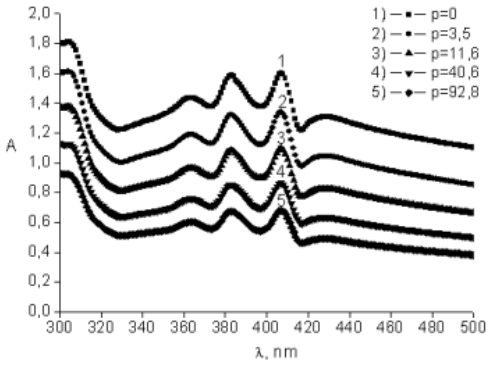


Fig. 5. Absorption spectra of AmB - Ch-Lin system, at different p ratios.

We have obtained the following values for the binding constants: $1.01 \cdot 10^4$ M^{-1}, $0.93 \cdot 10^4$ M^{-1}, $0.68 \cdot 10^4$ M^{-1} and $1.41 \cdot 10^4$ M^{-1}, taking into account the assumptions from Benesi-Hildebrand, Scott, Scatchard and Wolfe theories.

The experimental data were also processed in terms of Scatchard theory [27], on basis of equations corresponding to a single class of binding sites that do not exhibit cooperative behaviour. So, the experimental data for the system of AmB with Ch-Lin were fitted either to the linear Scatchard plot,

$$\frac{r}{C_F} = (n - r) \cdot K \tag{4}$$

or to a non-linear regression:

$$r = \frac{n \cdot K \cdot C_F}{1 + K \cdot C_F} \tag{5}$$

where:
r is the binding ratio, C_F, C_B are the concentrations of free and bound drug, respectively, n is the number of binding sites and K is the binding constant.

On basis of assumption that the absorption is due only to the free form of the drug, the concentrations of free and bound drug are given by:

$$C_B = C^0 \cdot \frac{A - A_0}{A_0} \tag{6}$$

$$C_F = C^0 - C_B \tag{7}$$

A_0 being the absorbance of the free drug,
A - the absorbance of the drug measured at each Ch-Lin concentration,
C^0 - the total drug concentration.

The Scatchard plot presented in Fig. 6, attest the presence of two processes. The solid line with a negative slope is characteristic for non-cooperative binding to one class with n equivalent binding sites. Considering this linear segment, the binding constant $K=4.35 \cdot 10^5$ M^{-1} and the number of sites n=0.01 were obtained.

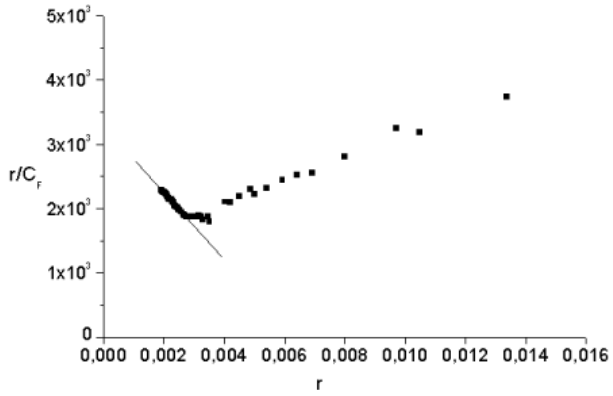


Fig. 6. Scatchard plot in AmB - Ch-Lin system.

We have used the Schwarz procedure [28] consisting in analysing the binding curves. So, the fitter of the binding ratio values (Equation. 5, Fig. 7) yields similar binding parameters in this system.

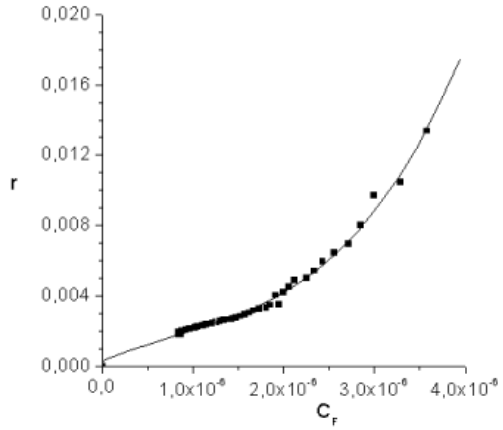


Fig. 7. Non-linear regression fit (equation 5) of the binding data.

In conclusion, the analysis of AmB – Ch-Lin system points out two binding types: the first process analysed by Benesi-Hildebrand, Scott, Scatchard and Wolfe methods, which supposes a 1:1 binding ratio and does not account explicitly for either the dimerization of the drug or cooperativity effects on the binding, and the second process analysed by Scatchard and Schwarz methods, which supposes a linear lattice of equivalent binding sites with nearest neighbour cooperativity.

References

[1] S. I. Pomposiello, M. Alva, D. W. Wilde, O. Carretero, Hypertension **31**, 615 (1998).
[2] R. Luechapudiporn, N. P. Morales, S. Fucharoen, U. Chantharaksri, Clinical Chemical & Laboratory Medicine **44**, 574 (2006).
[3] P. F. Hall, M. Nakamura, Journal of Biological Chemistry **254**, 12547 (1979).
[4] M. Elleder, A. Chlumska, J. Ledvinova, H. Poupetova, Virchows Archiv **436**, 82 (2000).
[5] B. De Kruijff, R. A. Demel, Biochim. Biophys. Acta **339**, 57 (1974).
[6] M. E. Kleimberg, A. Filkenstein, J. Membr. Biol. **80**, 257 (1984).
[7] C. C. Hsuchen, D. S. Feingold, Biochem. Biophys. Res. Commun. **51**, 972 (1973).
[8] J. Barwicz, W. I. Gruszecki, I. Gruda, J. Colloid Interf. Sci. **158**, 71 (1993).
[9] M. Saint-Pierre-Chazalet, C. Thomas, M. Dupeyart, C. M. Gary-Bobo, Biochim. Biophys. Acta **944**, 477 (1988).
[10] R. Seoane, J. Minones, O. Conde, M. Casas, E. Iribarnegaray, Biochim. Biophys. Acta **1375**, 73 (1998).
[11] R. Seoane, J. Minones, O. Conde, M. Casas, E. Iribarnegaray, Langmuir **15**, 3570 (1999).
[12] R. Seoane, J. Minones, O. Conde, E. Iribarnegaray, M. Casas, Langmuir **15**, 5567 (1999).
[13] M. Gagos, R. Koper, W. I. Gruszecki, Biochim. Biophys. Acta. **1511**, 90 (2001).
[14] W. I. Gruszecki, M. Gagos, P. Kernen, FEBS Lett **524**, 92 (2002).
[15] K Sternal, J. Czub, M. Baginski, J. Mol. Model. **10**, 223 (2004).
[16] I. Baciu, C. Topala, C. Draghici, Rev. Chim. (Bucharest) **52**, 110 (2001).
[17] L. E. Vijan., C. M. Topala, Rev. Chim. (Bucharest) **59**, 297 (2008)
[18] L. E. Vijan, C. M. Topala, Rev. Chim. (Bucharest) **59**, 756 (2008).
[19] J. Bolard, M. Cheron, Journal of Photochemistry and Photobiology B: Biology **72**, 101 (2003).
[20] W. Huang, Z. Zhang, X. Han, J. Tang, J. Wang, S. Dong, E. Wang, Biophys J. **83**, 3245 (2002).
[21] J. Bolard, P. Legrand, F. Heitz, B. Cybulska, Biochemistry **30**, 5707 (1991).
[22] G. Fujii, J.E. Chang, T. Coley, B. Steere, Biochemistry 36, 4959 (1997).
[23] C. Chapados, J. Barwicz, I. Gruda, Biophys. Chem. 51, 71 (1994).
[24] B. Oprescu, C. Topala, Rom. Journ. Phys. **50**, 1179 (2005).
[25] B. Oprescu, C. Topala, Rev. Chim. (Bucharest) **55**, 341 (2004).
[26] M. Honciuc, V. Puiu-Paun, Rev. Chim. (Bucharest) **54**, 117 (2003).
[27] G. Scatchard, Ann. N. Y. Acad. Sci. **51**, 660 (1949).
[28] G. Schwarz, Biophys. Chem. **6**, 65 (1976).

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