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Separation of overlapping spectra from evolving systems using factor analysis. 2. Amphotericin B in aqueous propanol and in aqueous lauroyl sucrose

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Abstract

A method based on factor analysis is used to separate the spectra of individual species from the experimental absorption spectra obtained from a fixed amount of amphotericin B dissolved in aqueous solutions. In aqueous propanol solutions and in aqueous lauroyl sucrose solutions, five species are identified: two monomers and three aggregates. Although the spectra of the different species in the two media are similar, the intensity signatures of the two systems are quite different and serve to characterize them.

Key words: Factor analysis; Eigenspectra; Real spectra; Constraints; Absorption spectroscopy; Amphotericin B; Lauroyl sucrose; Aggregation; Monomer; Oligomer

1. Introduction

Amphotericin B (AmB, Fig. 1) is an important antibiotic used to treat systemic mycoses which are particularly frequent and very often fatal in immunocompromised patients. The usefulness of this drug is limited by its toxicity. There are evidences that the toxicity of AmB is related to its aggregation state [1]. It is therefore very important to have an efficient method for studying the species present in the solutions used for injections and to determine their distribution.

AmB is a polyene and its electronic spectra are very sensitive to the environmental conditions of the molecules. Drastic changes occur when the concentration of the amphiphilic substance in the aqueous solvent is modified [2,3]. Many mono- and oligo-meric species are almost always present in aqueous solutions of AmB. The spectra obtained from these solutions are intrinsically overlapped. It is therefore difficult to identify the spectra of the individual species and consequently it is not easy to determine which species is responsible for the toxicity of the drug [3–8]. The same difficulties hinder the studies of inter-

Abbreviations: MF, multiplication factors (which are relayed to concentrations); AmB, amphotericin B; LS, lauroyl sucrose; cmc, critical micelle concentration.

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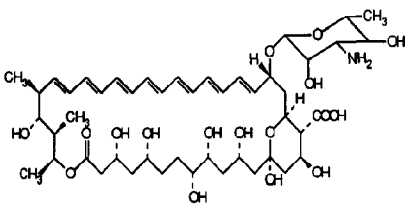

Fig. 1. Molecular model of amphotericin B (AmB).

actions of AmB with membranes or serum components. Such studies are essential to the determination of the mechanism of the selective toxicity of the drug.

Factor analysis is a computer method that permits the association of attributes in a large data bank containing information on the system under study. In spectroscopy, factor analysis can be used to determine: (1) the number of absorbing or emitting species in a series of spectra of an evolving system; (2) the concentration of each species in this system; (3) the spectrum of each species [9].

The spectra and concentrations of the species retrieved by factor analysis are usually in abstract form which are not readily useful for analytical purposes. In part 1 [10] we gave the theory of how to obtain real spectra and real multiplication factors (MF, related to the concentrations; see below) by applying some constraints to the abstract factors. These constraints are non-negative intensities, non-negative concentration, and maximum entropy criteria. The precision of the bands in the retrieved spectra is the same as that of the original spectra. The precision of the relative MF is better than 1%.

The multiplication factors (MF) that we used in part 1 and that we use in this paper are the values that are multiplied with the retrieved spectra to obtain the original spectra. The term MF is better suited than the term concentration which is often used in factor analysis but in absorption spectroscopy the two terms are directly proportional. To transform MF into concentrations one identifies in the original experimental spectra a spectrum that contains only one species and uses this spectrum to determine the normalisation factor that will transform the MF into absolute concentrations. The same normalisation factor is used to transform the retrieved spectra, whose intensity is dependent on MF, into absolute spectra.

In this article we use the method of factor analysis to obtain the real spectra and real MF of the species present in aqueous solutions of AmB. The solvents in these solutions are made with different amount of propanol or lauroyl sucrose in water.

2. Method

The mathematical formulation to factor analysis can be found in the book of Malinowski and Howery [9] and in other works [10–14]. We gave in part 1 the specific equations and the details of the procedure that we use here.

Briefly, the different steps in factor analysis that we use to retrieve the real spectrum of the individual species from a series of experimental spectra are the following. The n spectra are first transformed into a data matrix, **[D]**, the starting point in the factor analysis procedure.

Step 1 transforms **[D]** by matrix multiplication into the covariance matrix, **[Z]**.

Step 2 diagonalizes and decomposes **[Z]** into the abstract factors **[R]** and **[C]** which are related to the spectra and MF, respectively. The subroutine EIGEN given by Shurvell et al. is used to obtain the eigenvalues [15].

Step 3 reduces **[R]** and **[C]** into the abstract factors reduced **[R′]** and **[C′]** by using the minimum number of eigenfactors that will reproduce **[D]**. These matrices are transformed into spectra and MF curves to evaluate the result of the operation.

Step 4 reproduces the data matrix called now **[D′]** by combining **[R′]** and **[C′]**.

Step 5 subtracts **[D′]** from **[D]** to give **[DIFF]**, which is converted into spectra to evaluate the effectiveness of step 3. If the elements of **[DIFF]** are not near zero, then we return to step 3 and add some other eigenfactors to obtain a new set of **[R′]** and **[C′]**.

Step 6 applies the constraints to the abstract factors [R'] and [C'] to obtain the real factors unoptimized [R''] and [C'']. The constraints are: non-negative MF and non negative spectral intensities. The BOTM algorithm developed by Powel [16] is used to apply these constraints.

Step 7 further optimizes the real spectra by scanning for the most likelihood spectra while maintaining the constraints imposed in step 6.

Step 8 combines the real factors optimized [R'''] and [C'''] to generate the data matrix [D'] which are compared with the original data [D].

3. Experimental

3.1. Sampling

AmB was purchased from Sigma Co. (St. Louis, MO); *n*-propanol (spectronic grade) was purchased from Aldrich Chem. Co. (Milwauke, WI); lauroyl sucrose (LS) was obtained from Mitsubishi-Kassi Food Co. (Tokyo, Japan, catalogue number SM-1200).

AmB dissolved in dimethyl sulfoxyde (DMSO) was diluted with water to obtain a 50 μM stock solution. The final concentration of DMSO in the stock solution was 1% (V/V).

A first series of 30 solutions of 6.5 μM AmB in different mixtures of propanol in water was prepared by adding alcohol and water to the AmB stock solution. The concentration of propanol varied from 0 to 80% (V/V).

A second series of 30 solutions of 6.5 μM AmB in different mixtures of LS in water was prepared by adding the required amount of a LS stock solution to the stock solution of AmB and then diluting with water to the proper concentration. The concentration of lauroyl sucrose in the samples varied from 0.0 to 4.0 mM.

3.2. Spectroscopy

The absorption spectra were recorded using a Milton-Roy Spectronic 3000 array spectrophotometer with the following instrumental parameters: 240–540 nm range; 2 nm spectral slitwidth; 0.37 nm detector slitwidth; 540 ms exposure time.

Three spectra were taken for each sample mentioned in section 3.1. Since the solutions tend to vary with time, the spectra were recorded within 10 min after the samples were prepared.

3.3. Computer treatment of the data

The data points ($I(\lambda)$ versus λ (in nm)) stored on floppy diskettes were transferred to a central computer (IBM RS6000) where the computations were carried out. First, the average of the three spectra for each solution is made. Then the 30 spectra obtained are transformed into the data matrix, [D] (600×30), which are successively transformed into abstract factors and into real factors by the method described in section 2. The abstract and real factors are transformed into spectra and MF which are traced on a plotter (IBM 6187-2) to give the figures presented here.

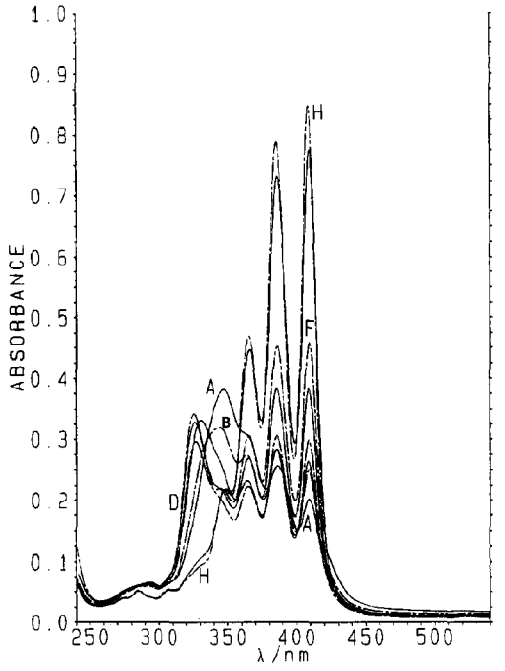

Fig. 2. Spectra of 6.5 μM AmB in propanol–water solutions: (A) 0%; (B) 3.0%; (C) 4.0%; (D) 5.5%; (E) 8,0%; (F) 10.0%; (G) 15.0%; (H) 80% propanol (V/V).

4. Results and discussion

4.1. AmB in aqueous propanol

Fig. 2 shows 8 typical spectra of AmB (6.5 μM) in aqueous propanol where the concentration of alcohol varied from 0.0 to 80%. As we can see on this figure, the spectra of a fixed amount of AmB vary considerably with the concentration of propanol. After obtaining the covariance matrix, [Z], made the diagonalization and performed the decomposition, the matrix of the abstract factors, [R] and [C], are obtained.

4.1.1. Identification in the experimental spectra of three non-redundant spectra

From Fig. 2 we estimate that at least three species are present in the system. The reduction process then starts by using the first three eigenfactors. This gives the abstract factors reduced,

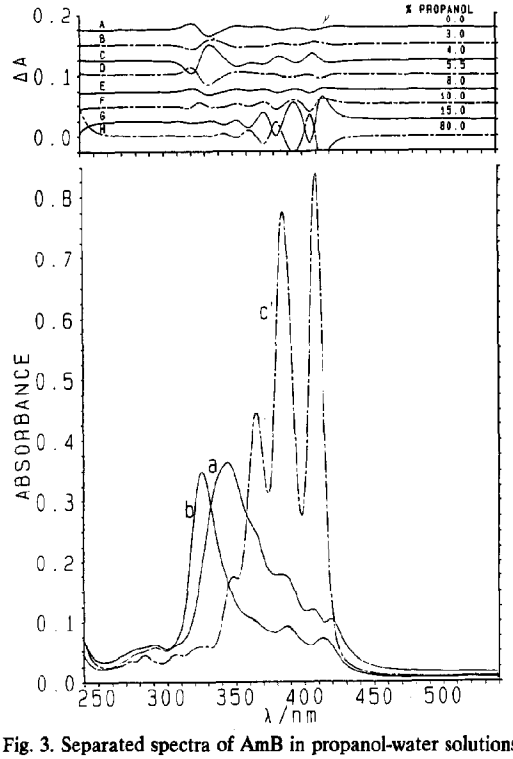

Fig. 3. Separated spectra of AmB in propanol-water solutions for the case of three species. Top: difference between the calculated and experimental spectra.

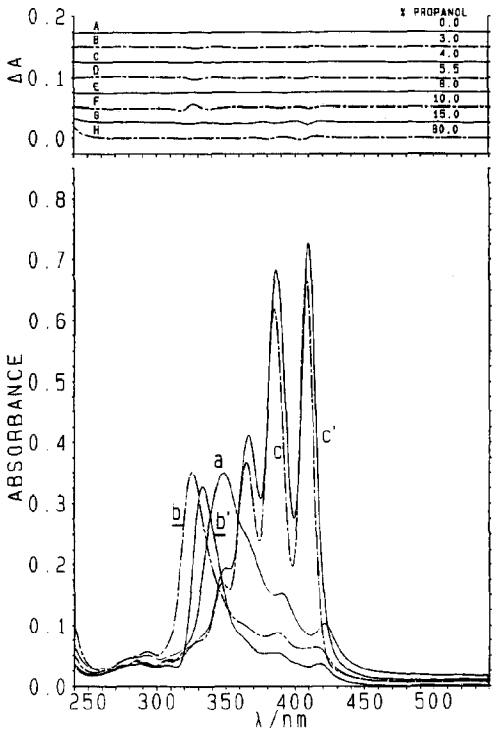

Fig. 4. Separated spectra of AmB in propanol–water solutions for the case of five species. Top: difference between calculated and experimental spectra of Fig. 2.

[R'] and [C']. After imposing the constraints on [R'] and [C'], the real spectra are obtained. In Fig. 3 (bottom), we give these spectra that we identify as that of two aggregates (trace a, λ_{max} = 344 nm and trace b, λ_{max} = 326 nm) and one monomer (trace c', λ_{max} = 409 nm) [17]. After multiplying the MF with the retrieved spectra and subtracting the results from the original spectra, the residuals (Fig. 3 top) show two distinct series of anomalies: one situated near 330 nm and one situated near 400 nm. This indicates that at least two more species are present in this system.

4.1.2. Reduction trial with five non-redundant spectra

Our next trial is to go back to the reduction step (step 3) by using the first five eigenfactors. With these eigenfactors, the data matrix are reproduced and subtracted from the original data

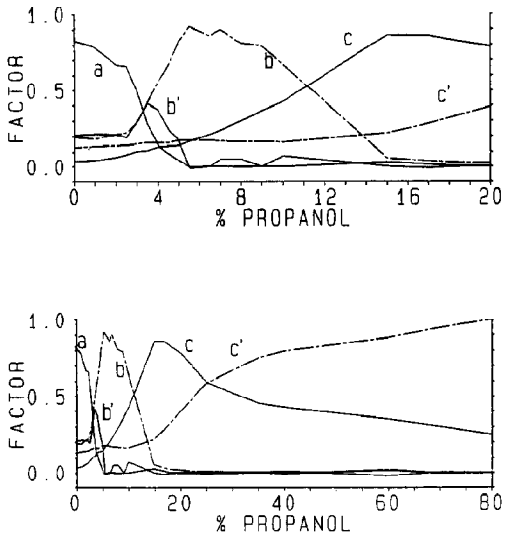

Fig. 5. Multiplication factors (MF) for the five identified species of AmB in propanol–water solutions. Bottom, concentration range from 0 to 80% propanol; top: range from 0 to 20%.

to give the difference matrix. This matrix is used to generate the spectra at the top of Fig. 4. The residuals in these spectra are almost at the zero absorbance level which indicates that the trial is valid. From this we conclude that five species define this system.

After imposing all the constraints indicated in section 2, the five real spectra and MF are retrieved. These are shown in Figs. 4 and 5, respectively. On the basis of the λ_{max} of the strongest band, we identify the spectra as that of two monomers (Fig. 4c and 4c') and three aggregates (Figs. 4a, 4b, and 4b'). The position of the bands in these spectra is given in Table 1. The MF (Fig. 5) reveals the evolution of the species with increasing propanol concentration. In pure water, aggregate a whose λ_{max} of the strongest band is situated at 348 nm is the principal species. In this solution, a small amount of aggregates b' and b and monomers c and c' are also present. As the proportion of propanol is progressively increased in the solution, aggregate a is transformed into aggregates b' ($\lambda_{max} = 333$ nm) and b ($\lambda_{max} = 326$ nm). At about 5% propanol, aggregate a is completely transformed. Species b' is not very abundant and disappears rapidly as the concentration of the alcohol increases. Species b peaks near 5.5% propanol and is decomposed firstly into monomer c ($\lambda_{max} = 408$ nm) and secondly into monomer c' ($\lambda_{max} = 410$ nm). At about 20% propanol, only monomeric AmB is present. Table 2 gives the MF of all the species as each species becomes the most abundant. Table 2 gives also the relative MF which are obtained by dividing MF by the sum of the MF. The relative MF gives an approximate molar fraction of the AmB species at the concentration of alcohol indicated.

The spectra of species b and b' are similar but slightly displaced one from the other (Fig. 4). The MF curve (Fig. 5) of species b' lies underneath that of species b. These results enable us to consider that these two species are similar with the difference that they are solvated slightly differently and that aggregate b' is much less abundant than aggregate b.

The shift between spectrum of aggregate a and that of aggregate b' is bigger than that observed between the two b species. Species a predominates in solution in water and in aqueous solutions with low propanol (< 3%) contents whereas the b species predominate at higher alcohol concentration. These results allow us to consider that aggregate a is very different than the b aggregates.

The two monomers c and c' have similar spectra (Fig. 4) with a shift of less than 2 nm between

Table 1
Position of the bands (in nm) of the absorption spectra of 6.5 μM AmB in aqueous propanol [a]

Fig. 4a aggregate	Fig. 4b' aggregate	Fig. 4b aggregate	Fig. 4c' monomer	Fig. 4c monomer
420.8	417.8	413.0	408.3	409.5
389.3	384.5	387.0	384.8	386.0
367.8	363.5	363.8	365.0	366.5
348.0	344.3	340.0	347.8	349.0
337.3	333.3	325.5	329.5	330.3

[a] The underline indicate λ_{max} of the most intense band in the spectrum.

Table 2
Distribution of the species of 6.5 μM AmB in aqueous propanol (Fig. 5) [a]

% Propanol	Aggregate a λ_{max} = 348 nm		Aggregate b' λ_{max} = 333 nm		Aggregate b λ_{max} = 325 nm		Monomer c λ_{max} = 408 nm		Monomer c' λ_{max} = 410 nm		Σ MF
	MF	rel.	MF	rel.	MF	rel.	MF	rel.	MF	rel.	
0.0	0.83	0.59	0.21	0.15	0.20	0.14	0.04	0.03	0.13	0.09	1.40
3.5	0.28	0.20	0.47	0.30	0.42	0.30	0.11	0.08	0.16	0.12	1.39
5.4	0.00	0.00	0.00	0.00	0.92	0.73	0.17	0.13	0.18	0.14	1.27
15.0	0.03	0.03	0.00	0.00	0.06	0.05	0.86	0.74	0.22	0.19	1.17
80.0	0.00	0.00	0.00	0.00	0.00	0.00	0.25	0.20	1.00	0.80	1.25

[a] MF: multiplication factor; rel: relative MF.

the two but their distribution curves are very different (Fig. 5). Monomer c predominates at low alcohol content peaking at 15% propanol. As the concentration of alcohol is increased, the amount of monomer c decreases to the profit of monomer c'. Higher than 25% propanol, monomer c' is the predominant species. To explain these results one has to consider that the environment of these two species are different. Since species c is present at low alcohol concentrations where the OH groups prevail, this monomer must be solvated by the OH groups of water and propanol. On the contrary, species c' is present at high alcohol concentrations where the aliphatic chains are abundant, therefore this monomer

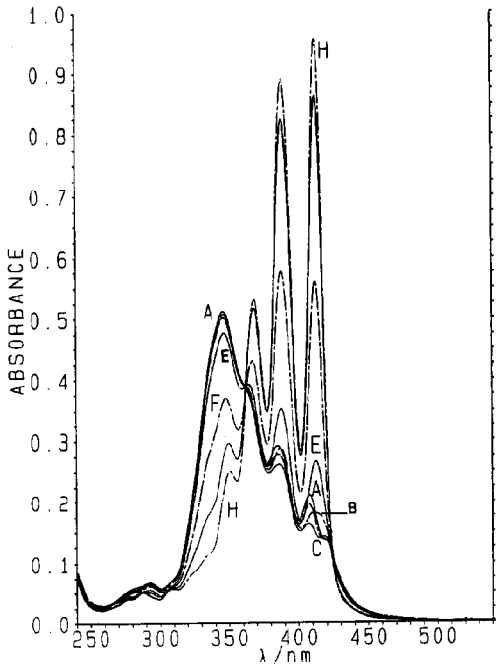

Fig. 6. Spectra of 6.5 μM AmB in aqueous lauroyl sucrose: (A) 0.0; (B) 5 μM; (C) 100 μM; (D) 340 μM; (E) 450 μM; (F) 800 μM; (G) 1.4 mM; (H) 4.0 mM lauroyl sucrose.

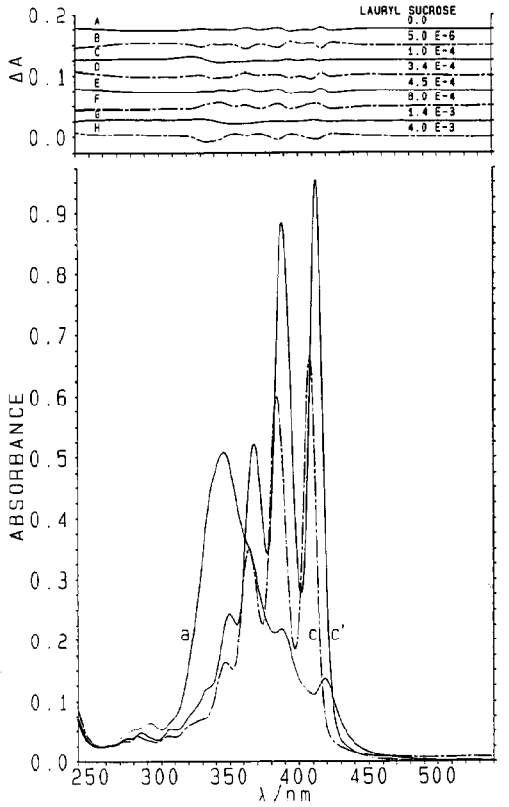

Fig. 7. Three separated spectra of AmB in aqueous lauroyl sucrose. Top: difference between calculated and experimental spectra of Fig. 6.

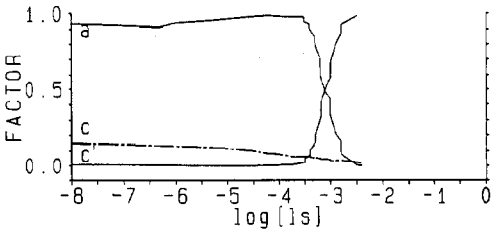

Fig. 8. Multiplication factors (MF) for the three identified species of AmB in aqueous lauroyl sucrose.

must be solvated by the aliphatic chains of propanol. The presence of species c′ at 0% propanol concentration can be explained by the small amount of DMSO in the solution.

4.2. AmB in aqueous lauroyl sucrose

Fig. 6 shows 8 typical spectra of AmB (6.5 μM) in aqueous lauroyl sucrose where the concentration of the surfactant varied from 0.0 to 4.0 mM. As for AmB in propanol, the spectra are transformed into the data matrix and the abstract factors, [**R**] and [**C**], are obtained.

4.2.1. Reduction trial with three non-redundant spectra

The data reproduced are compared with the original data to give the difference spectra shown in Fig. 7 at the top. The residuals of the difference spectra are small. The real spectra of the three species of AmB retrieved by factor analysis are given in the bottom of Fig. 7 and the MF in Fig. 8. From the position of the λ_{max} we see that

Table 3
Position of the bands (in nm) of the absorption spectra of 6.5 μM AmB in aqueous lauroyl sucrose [a]

Fig. 7a aggregate	Fig. 7c monomer	Fig. 7c′ monomer
419.0	408.0	412.8
387.8	384.3	388.5
366.5	365.0	368.5
346.3	347.5	350.5
335.3	329.0	332.8

[a] The underline indicate λ_{max} of the most intense band in the spectrum.

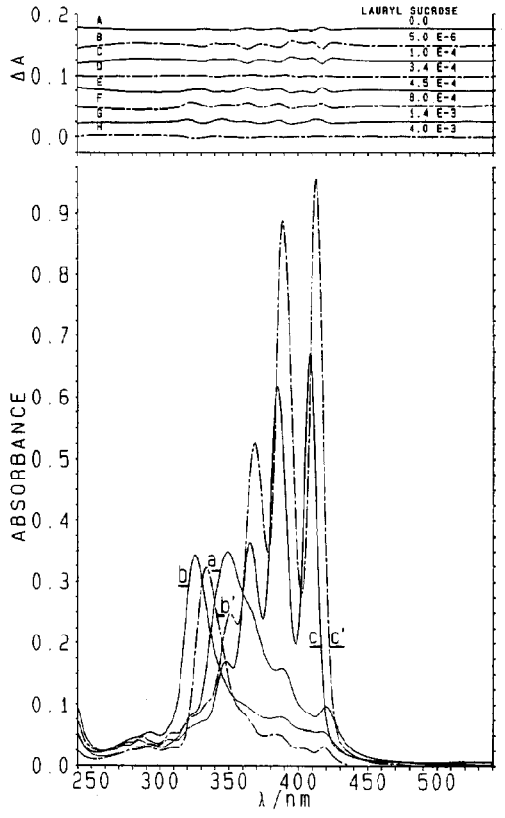

Fig. 9. Five separated spectra of AmB in aqueous lauroyl sucrose. Top: difference between the calculated and experimental spectra of Fig. 6.

species a is an aggregate and species c and c′ are monomers. The position of the bands is given in Table 3.

The spectrum of aggregate a retrieved from the solution of AmB in aqueous LS (Fig. 7a) is very similar to that of aggregate a obtained from the solution of AmB in aqueous propanol (Fig. 4A). To compare them effectively we subtracted one spectrum from the other after normalization. The result showed a band situated at 328 nm. This indicates that beside aggregate a some other aggregate exist in aqueous LS. A reduction trial using 4 species did not decrease appreciably the 328 nm band. We therefore made a reduction trial with five species using as target the spectra b and b′ of AmB in aqueous propanol. This trial did reduce appreciably the 328 nm band.

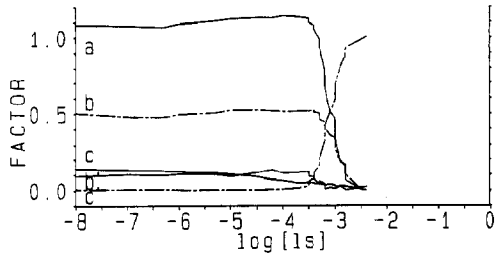

Fig. 10. Multiplication factors (MF) for the five identified species of AmB in aqueous lauroyl sucrose.

Table 4
Position of the bands (in nm) of the absorption spectra of 6.5 μM AmB in aqueous lauroyl sucrose [a]

Fig. 9a aggregate	Fig. 9b′ aggregate	Fig. 9b aggregate	Fig. 9c monomer	Fig. 9c′ monomer
420.0	418.8	418.8	408.3	411.8
390.5	388.5	390.0	384.8	388.5
367.0	363.8	363.0	365.0	368.5
348.5	344.5	344.5	346.8	350.8
320.8	331.5	323.5	328.5	332.8

[a] The underline indicate λ_{max} of the most intense band in the spectrum.

4.2.2. *Reduction trial with five non-redundant spectra*

The spectra and the traces of the MF resulting from this trial are given in Figs. 9 and 10, respectively. The residuals from the difference between the calculated and the original spectra (Fig. 9, top) have slightly improved compared to the trial with three species (Fig. 7, top). But since this trial did reduce appreciably the 328 nm band we consider that, in this system, at least five species are present. From the λ_{max} of the strongest band in the spectra, we identify two species as monomers and three as aggregates (Table 4). Table 5 gives the MF of all the species as each species becomes the most abundant.

The MF curves (Fig. 10) show that in pure water, aggregate a (λ_{max} = 349 nm) is the predominant species, aggregate b (λ_{max} = 324 nm) is half as abundant as aggregate a, and the other species are not very abundant (Table 5). The concentration of the latters is almost constant until the concentration of LS is 320 μM (log (−3.5)) where aggregates a, b, and b′ and monomer c are transformed to give the monomer c′ (λ_{max} = 412 nm). Near the cmc of lauroyl sucrose (600 μM, log(−3.2)) the concentrations of aggregate a and monomer c′ are the same.

In pure water, the concentration of the species of Amb in aqueous LS sucrose (Fig. 10) is different than that obtained for AmB in aqueous propanol (Fig. 5). The difference is only superficial. Indeed consider that, as we mentioned in section 4.1.2, that aggregates b and b′ are almost the same species, the sum of the relative MF of these two aggregates in aqueous LS is 0.33 (Table 5) whereas in aqueous propanol it is 0.29 (Table 2). The difference is within experimental error.

For the two systems, the difference that we observe for the relative amount of aggregates b

Table 5
Distribution of the species of 6.5 μM AmB in aqueous lauroy sucrose (Fig. 10) [a]

Conc. log[ls]	Aggregate a λ_{max} = 349 nm		Aggregate b′ λ_{max} = 332 nm		Aggregate b λ_{max} = 324 nm		Monomer c λ_{max} = 408 nm		Monomer c′ λ_{max} = 412 nm		Σ MF
	MF	rel.	MF	rel.	MF	rel.	MF	rel.	MF	rel.	
8.0	1.082	0.59	0.088	0.05	0.506	0.28	0.139	0.08	0.006	0.00	1.82
5.0	1.127	0.61	0.088	0.05	0.524	0.28	0.108	0.06	0.006	0.00	1.85
4.2	1.135	0.60	0.135	0.07	0.521	0.28	0.084	0.04	0.006	0.00	1.88
4.0	1.144	0.62	0.116	0.06	0.518	0.28	0.066	0.04	0.007	0.00	1.85
2.4	0.000	0.00	0.029	0.03	0.000	0.00	0.000	0.00	1.000	0.97	1.03

[a] MF: multiplication factor; rel: relative MF.

and b′ in pure water indicates the variation in the two samples which were prepared from two stock solutions at two different occasions.

5. Conclusion

The factor analysis of the spectroscopic results obtained for AmB in aqueous propanol (Figs. 4 and 5) shows clearly the presence of five spectroscopically different species, three aggregates and two monomers. In pure water, one type of aggregate is the predominant species. As the percentage of alcohol is increased, this aggregate is transformed into two other aggregates which are in turn transformed into two monomers.

It may be concluded, from the above results obtained from the spectra of AmB in propanol/water solutions, that the aggregates b and b′ are favoured by the presence of propanol. It may be hypothesised that propanol replaces the solvating water near the polar heads of AmB molecules thus changing their conformation inside the aggregate. Given the important spectral shifts between the two types of aggregates (a and b′) it must be concluded that the structures and dimensions of these two types of aggregates are very different.

A quite different situation is observed when LS is added to the aqueous solution of AmB (Figs. 9 and 10). Increasing concentrations of LS do not change the a:b ratio, the aggregate a being always the predominating species. The amount of both aggregates start to decrease at LS concentration of about 316 μM giving place to the monomeric AmB solvated by LS (species c′).

We have shown previously that AmB in LS solutions is less toxic to mice that the commercial form, Fungizone (1). The spectra of concentrated solutions of Fungizone show that the aggregate whose λ_{max} is at 328 nm is the predominating species (2). This λ_{max} is the position of the average of the λ_{max} of the species b and b′ that we have identified in this work. Therefore, it may be thought that these aggregates of AmB are the ones which are most toxic to animals. Other studies are necessary in order to confirm this hypothesis.

Although the spectra of the different species of AmB in aqueous propanol and in aqueous LS are quite similar, the abundance of the species of the two systems are very different and the MF curves serve to characterize them. The method described here is an efficient method for the identification of the spectra of the different species present in the AmB solutions and the determination of their abundance. This method is well suited to evaluate the different preparations used in the *in vivo* experiments.

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