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THERMAL BEHAVIOUR AND STABILITY OF AMPHOTERICIN B

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ABSTRACT

Thermoanalytical methods (DTA, TG, DTG) and other techniques (UV–VIS spectroscopy, thin-layer chromatography, biological assay) have been employed to examine the thermal behaviour of the polyene antibiotic amphotericin B. Heated to 410 K, in either argon or self-generating atmospheres, amphotericin B does not undergo chemical changes and does not lose biological activity. However, the compound decomposes completely on heating up to 720 K and this process is accompanied by a partial volatilization of light products. The thermal decomposition pattern is markedly affected by the nature of the gaseous phase.

INTRODUCTION

Amphotericin B (AmB) has a distinctive place among over 200 polyene macrolide antibiotics reported [1], since it is the most effective drug currently available for the treatment of a wide variety of deep-seated fungal infections in humans [2,3]. The antibiotic is commonly used with immunosuppressed patients, such as AIDS victims and transplant patients [4], who are especially susceptible to the mycotic infections. The compound also exhibits several other activities, among which its antiviral and antitumour activities are of some importance [5,6].

The complete structure of AmB (Fig. 1) has been elucidated by chemical methods [7] and X-ray single crystal analysis [8]. The compound is a macrocyclic (38-atom ring) lactone containing seven conjugated double bonds (all *trans*) and glycoside-linked amino-sugar, mycosamine. The polyfunctional character of the molecule determines the unique biological and physico-chemical properties of AmB, including its stability. Numerous studies have revealed that AmB exhibits a rather poor stability when exposed to heat [9–12], light [9,10,12–14], γ-radiation [15] and extremes of pH [9,16],

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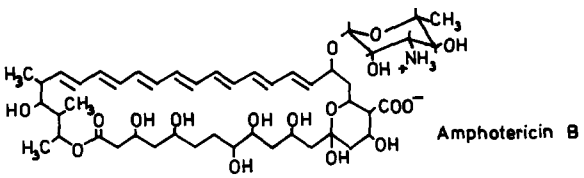

Fig. 1. Structure of AmB.

and in the presence of oxygen (air) [10,12,17,18]. Knowledge of the thermal properties and stability of AmB is of great importance, since these determine the clinical utility of the compound. The present work is devoted to these problems.

EXPERIMENTAL

AmB was provided by the Rhône–Poulenc Company (France). The thermal analyses were carried out using a Q-1500 derivatograph (Monicon), with α-Al_2O_3 as reference. For the dynamic measurements, the sample was placed in a standard platinum crucible (see ref. 19, Appendix 1, No. 2). For the quasi isobaric–isothermal experiments, a special platinum labyrinth crucible [20] was used. Other operating conditions are indicated in the captions of Figs. 2–5.

UV–VIS spectra were recorded on a Beckman model 3600 spectrophotometer.

Thin-layer chromatography (TLC) analyses were carried out with Kieselgel 60 plates (Merck) using two developing systems: (A) chloroform + methanol + water (10 : 6 : 1, v/v), and (B) ethyl acetate + acetic acid + water (4 : 1 : 1, v/v).

Antifungal activity was measured in terms of the concentration of the compound causing 50% inhibition of cell growth (IC_{50}) of two model organisms, *Saccharomyces cerevisiae* (AT CC 9763) and *Candida albicans* (1440), as has been previously described [21].

RESULTS AND DISCUSSION

Thermal analyses of AmB under various conditions are shown in Figs. 2–5. The thermoanalytical curves demonstrate the multi-step decomposition pattern. The behaviour of AmB depends markedly on the nature of the atmosphere over the heated sample. Figure 2 presents the results of thermoanalytical measurements in a dynamic argon atmsophere. The TG curve shows a gradual loss of mass of the sample with rising temperature. The remainder in the crucible at 720 K accounts for ca. 16% of the mass of the

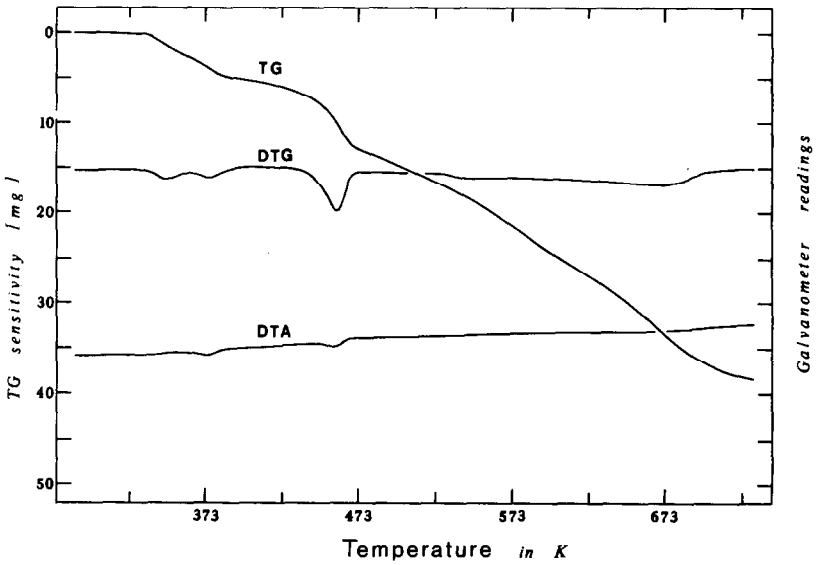

Fig. 2. Thermal analysis of AmB in a dynamic atmosphere of argon. Conditions: mass of sample, 45 mg; sensitivities of TG, DTG and DTA measurements, 50 mg, 500 μV and 500 μV, respectively; heating rate, 2.5 K min^{-1}.

original sample. Two characteristic peaks can be distinguished in the DTG curve, at 375 and 455 K. The corresponding energetic effects are seen as weak endothermic peaks in the DTA curve.

Figure 3 shows thermoanalytical curves for AmB recorded in a static atmosphere of air. The TG curve is very similar to that characterizing decomposition of the compound in argon (Fig. 2). However, on heating up to 720 K, the sample volatilizes almost completely. The DTG and DTA curves exhibit four characteristic peaks. The lowest temperature peak in the DTG curve, at 365 K, is accompanied by a weak endothermic effect shown in the DTA curve. However, the sharp mass loss indicated by the peak in the DTG curve at 430 K is accompanied by an exothermic effect. It is worth mentioning that on analysis of AmB in an argon atmosphere, the peak occurring in the DTG curve in the same temperature region is also accompanied by an exothermic effect (Fig. 2). At higher temperatures, two broad peaks are seen in both the DTG and DTA curves. The accompanying chemical processes are exothermic.

The results of thermal analyses performed in a dynamic atmosphere of oxygen (Fig. 4) are completely different from those obtained in argon or air atmospheres. Loss of a few percent of the mass is observed on heating the sample up to 420 K. At 425 K a decomposition process begins which is accompanied by a very strong exothermic effect and fast volatilization of the sample. At 470 K the thermal processes seem to be essentially completed, although the mass loss does not correspond to 100%.

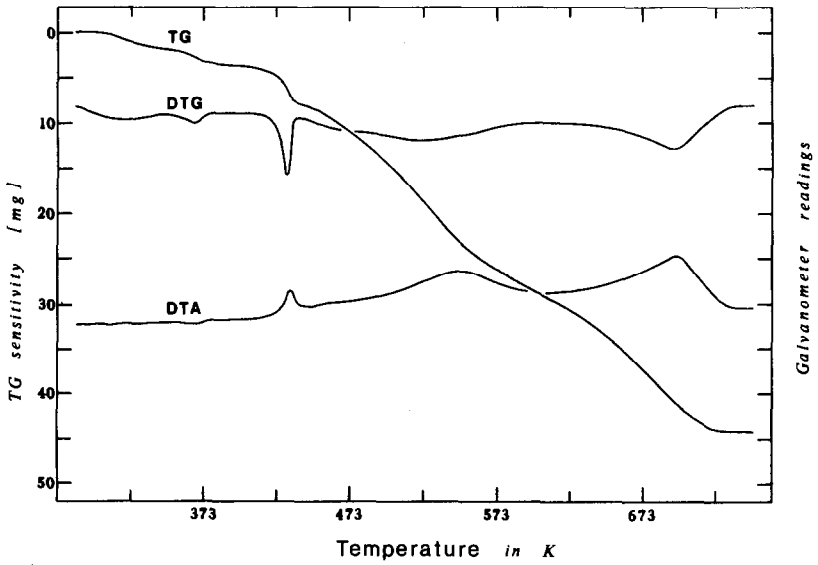

Fig. 3. Thermal analysis of AmB in a static atmosphere of air. Conditions: mass of sample, 45 mg; sensitivities of TG, DTG and DTA measurements, 50 mg, 500 μV and 500 μV, respectively; heating rate, 2.5 K min^{-1}.

In an attempt to improve the accuracy of our thermoanalytical measurements, we performed analyses of AmB in quasi isobaric–isothermal conditions in a crucible which ensured a self-generating atmosphere over the

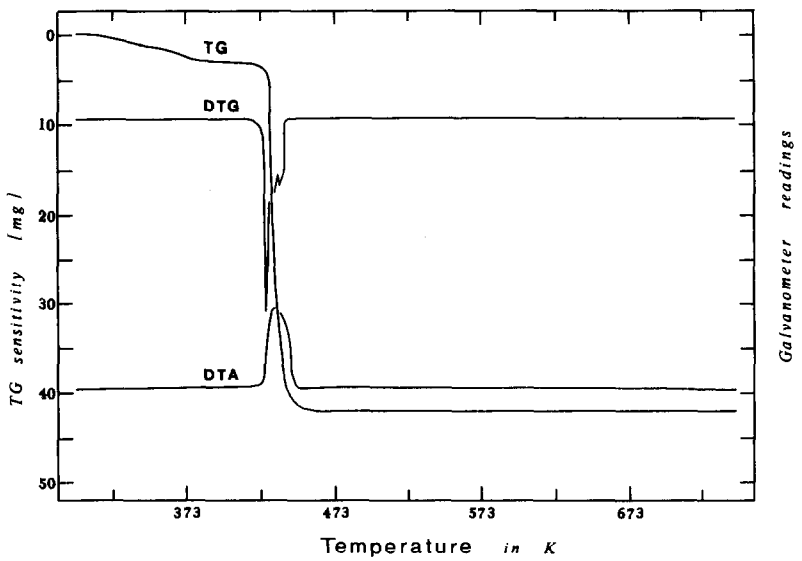

Fig. 4. Thermal analysis of AmB in a dynamic atmosphere of oxygen. Conditions: mass of sample, 50 mg; sensitivities of TG, DTG and DTA measurements, 50 mg, 2500 μV and 2500 μV, respectively; heating rate, 2.5 K min^{-1}.

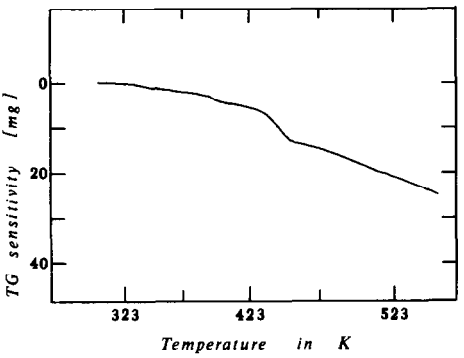

Fig. 5. Thermal analysis of AmB in Q mode. Conditions: mass of sample, 40 mg; sensitivities of TG and DTG measurements, 50 mg and 250 μV, respectively; heating rate, 3 K min^{-1}; rate of decomposition, 0.6 mg min^{-1}.

sample (Fig. 5). The TG curve was recorded only in the range from ambient temperature to 540 K, owing to time restrictions. (The Q-analysis for the temperature range indicated in Figs. 2–4 would require several hours.) The TG curve presented in Fig. 5 does not differ markedly from those obtained for decomposition of AmB in static air and dynamic argon atmospheres (Figs. 2 and 3) over the same temperature range. Thus, better resolution was not achieved, though this analysis did confirm the behaviour of AmB at moderate temperatures discussed earlier.

From the thermoanalytical data presented in Figs. 2–5 and the discussion above it can be seen that AmB undergoes complex chemical changes upon heating. These measurements also reveal that oxygen present in the gaseous phase above the sample participates in chemical processes initiated by changes in temperature. The influence of oxygen is pronounced at temperatures above 420 K, but the shapes of the thermoanalytical curves are essentially independent of the composition of the gaseous phase in the temperature range from room temperature to 420 K. Within these limits the effects recorded are weak and diffuse, and this suggests rather minor changes in the heated sample.

In order to determine the extent of degradation of the compound, we heated samples of AmB in a derivatograph to various chosen temperatures under various conditions (for details see captions of Figs. 6 and 7) and subjected the remainders of the samples to UV–VIS and TLC analyses. A complementary assay of the biological activity of the heated samples was also performed.

Owing to the presence of seven conjugated double bonds, the molecule of AmB exhibits very characteristic absorption in the region 300–425 nm [10,22]. The long-wavelength band of the monomeric form is characterized by the presence of three sharp intense maxima at 363, 382 and 407 nm [22]. Therefore, UV–VIS spectroscopy can be used to evaluate the extent of

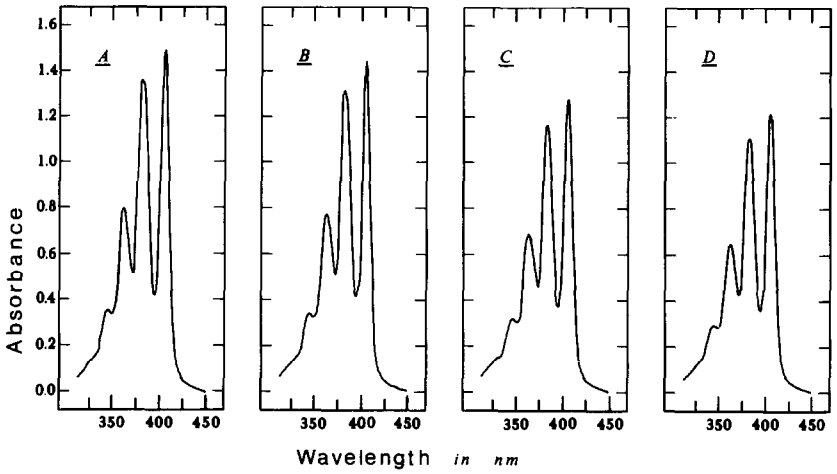

Fig. 6. Long wavelength absorption spectra of: (A) AmB; (B) AmB heated in an argon atmosphere to 408 K; (C) AmB heated in an oxygen atmosphere to 408 K; (D) AmB heated in an argon atmosphere to 428 K. Conditions: $c = 10\ \mu g\ cm^{-3}$ in MeOH; $l = 1$ cm.

chemical changes upon heating. As can be seen from Fig. 6, heating of AmB under any of the conditions chosen does not bring about changes in the shape of the long wavelength band system. This means that the structure of the chromophore is preserved, which implies that isomerization inside the chromophore does not take place. Heating of AmB in an argon atmosphere up to 408 K causes only a very minor decrease in the absorption, which indicates that the compound remains practically unchanged under these conditions. The decrease in the optical density is more pronounced when AmB is heated in an oxygen atmosphere up to 408 K or in an argon atmosphere up to 428 K (Fig. 6 (C) and (D), respectively). In these two cases, then, partial decomposition of the compound may occur.

Further examination of the extent of degradation of AmB upon heating was carried out by TLC. As can be seen from Fig. 7, all the heated samples give essentially one spot at the same R_F as untreated AmB, although chromatograms of samples heated in an oxygen atmosphere up to 408 K and in an argon atmosphere up to 428 K indicate traces of unidentified substances remaining at the starting point. These observations suggest that the major part of AmB remains unchanged upon heating under the conditions chosen in the present work.

Biological assays are immensely important, since these should be most sensitive to any changes in the structure of the molecule. The procedures applied in the present work revealed that the biological activity of AmB heated under any of the chosen conditions (for details see the legends accompanying Figs. 6 and 7) remains the same as that of an untreated sample, within the uncertainty limits of the biological tests.

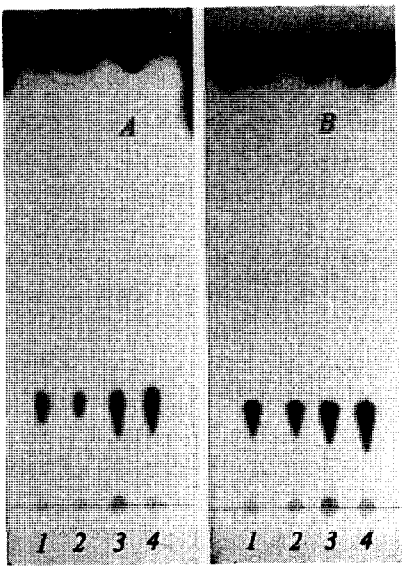

Fig. 7. TLC analyses of: (1) AmB; (4) AmB heated in an argon atmosphere to 408 K; (3) AmB heated in an oxygen atmosphere to 408 K; (2) AmB heated in an argon atmosphere to 428 K. A and B denote developing systems.

All the observations above clearly demonstrate that the molecule of AmB remains practically unchanged upon heating to temperatures somewhat exceeding 400 K, and that the thermal behaviour of AmB is not affected by the character of the environment. It was therefore interesting to determine how far the compound can be heated without degradation. The characteristic thermal effect in a dynamic argon atmosphere occurs at 455 K, and a similar effect is observed in a static atmosphere of air at 430 K. Therefore, we heated a sample of the compound, in Q-conditions, to a temperature somewhat above these, i.e. to 470 K. The remainder of the sample was subjected to UV–VIS and TLC analyses. Both methods revealed considerable decomposition of AmB. The temperature of 410 K seems, therefore, to be the upper limit to which the compound can be heated without observable degradation.

On the basis of these experimental data and information from the literature [9,11,18] the following picture of the behaviour of AmB under rising temperature can be outlined. The compound is thermally stable up to 410 K. The slight mass loss on heating up to this temperature is presumably due to the release of physically bound light molecules, e.g. water, atmospheric components, etc. Further increase in temperature initiates chemical changes which are dependent on the atmosphere in which the heating takes place. In the inert gas, the compound undergoes pyrolysis to form light products which are instantaneously released to the gaseous phase. The DTA curve recorded in an argon atmosphere (Fig. 2) does not exhibit any

exothermic effect, which means that the molecule must be provided with energy to undergo decomposition. The pyrolysis is therefore accompanied by the formation of energy-rich products. Taking the above into account, the occurrence of self-oxidation (auto-oxidation) processes, which can be expected to be exothermic, is not very likely. Owing to the complex structure of the AmB molecule, it is rather difficult to predict the possible pathways of its degradation. As has been demonstrated by others, the pyrolysis pathways and thus the pyrolysis products are specific to particular antibiotics, and this forms the basis for differentiation between various such derivatives [10,23].

A completely different pattern of chemical changes is expected if AmB is heated in an atmosphere of oxygen. The strong exothermic effect occurring simultaneously with volatilization of the sample clearly demonstrates that oxygen participates in the degradation of the antibiotic. Under the experimental conditions used, oxygen causes oxidation of the parent AmB molecules. Since oxidation is usually exothermic, a certain amount of heat is released during the process. This brings about an increase in the temperature of the sample and consequently accelerates the degradation and volatilization. The process is thus self-accelerating until oxidation is completed. Owing to the complex structure of the AmB molecule its degradation and oxidation can begin at various sites. Furthermore, secondary processes must be complex because of the wide variety of possible reaction pathways and local changes in composition of the gaseous phase participating in the degradation of the compound. Besides volatile compounds, carbonization products can also occur, forming a residue at the bottom of the crucible. A residue of this kind actually occurs in both inert and active atmospheres (see Figs. 2 and 4). The thermoanalytical curves characterizing the thermal behaviour of AmB in a static atmosphere of air show features of the corresponding curves recorded in argon and oxygen atmospheres. Owing to the lack of a sufficient amount of oxygen in the reaction zone, the decomposition process is not self-accelerating. However, the influence of oxygen on the degradation of AmB is seen across the whole range of temperature at which the process was examined (see exothermic effects in Fig. 3).

Three important conclusions can be drawn from the above discussion. First, pure AmB remains unchanged upon linear heating from ambient temperature to 410 K. Secondly, self-oxidation of the AmB molecule is not very likely. Thirdly, oxidation of AmB by molecular oxygen practically initiates burning of the compound at temperatures as low as 420–430 K.

The oxidation of AmB has been studied extensively in the past [11,18] since it is thought that this process plays an important role in ageing of the antibiotic. Lamy-Freund et al. [18] have recently proved that free radicals are formed during interaction of AmB with oxygen. These radicals are centred at carbon atoms localized in the moiety of the original molecule. The same authors have also pointed out that consumption of oxygen is directly

connected with loss of the drug's activity. These data are in full agreement with the results of our studies, although we think that the term "auto-oxidation" for the process, used by the authors of ref. 18, is somewhat confusing, since it would seem to suggest that oxygen atoms originally present in the AmB molecule are involved in the process.

The oxidation process is highly exothermic and thus should proceed spontaneously. In other words, from the thermodynamical point of view, AmB should immediately react with oxygen present in the system. Since the compound can be isolated and is fairly stable at ambient temperatures, there must exist some kinetic barrier against the reaction with oxygen. However, this barrier cannot be very high, because spontaneous oxidation is initiated at relatively low temperatures (420–430 K). Moreover, a low energy level for the products of reaction AmB with oxygen may be achieved by a tunnel effect. This discussion reveals that the rate of oxidation of AmB at ambient temperatures must be a measurable quantity. Therefore, the presence of oxygen is the main factor causing the antibiotic inactivation. In our opinion, samples under inert gas or in evacuated ampoules could be stored unchanged for a very long time, even at relatively high temperatures. It is generally recommended that the drug be stored at low temperatures. This precaution most probably protects against ageing by inhibiting oxidation processes.

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