*International Journal of Pharmaceutics*, 46 (1988) 223–229
Elsevier



IJP 01568

# A study of the chemical stability of amphotericin B in *N*,*N*-dimethylacetamide

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(Received 20 August 1987)
(Modified version received 8 March 1988)
(Accepted 10 March 1988)

*Key words*: Amphotericin B; Kinetics of degradation; *N*,*N*-Dimethylacetamide; Sodium deoxycholate; Oxidation

## Summary

Studies have demonstrated the potential for using *N*,*N*-dimethylacetamide (DMA) as a solvent for solubilizing amphotericin B. The addition of sodium deoxycholate (NaDOC) to the DMA further enhanced the solubility of amphotericin B. To complement this solubility study the chemical stability of amphotericin B in DMA was investigated. The degradation of amphotericin B in DMA occurs by both an aerobic and an anaerobic pathway with the major route of decomposition at 30 °C involving oxygen. For example, under aerobic conditions at 30 °C, the pseudo-first-order rate constant for the degradation of amphotericin B in 1% (w/v) NaDOC/DMA is $1.23 \times 10^{-2}$ $h^{-1}$ while under anaerobic conditions (carried out in an anhydrous, oxygen-free, nitrogen-filled glove box) the pseudo-first-order rate constant for the degradation of amphotericin B in 1% NaDOC/DMA at 30 °C is $3.50 \times 10^{-3}$ $h^{-1}$. Both of these pathways displayed Arrhenius temperature-dependent kinetics with activation energies of 17.5 and 23.5 kcal/mol for the aerobic reaction and the anaerobic reaction, respectively. In the concentration range studied (0.14 – 1.4% w/w), the percentage of water in the DMA did not alter the rate of anaerobic degradation of amphotericin B in 1% NaDOC/DMA. The influence of light, amphotericin A content, NaDOC level and heavy metal impurities on the rates of degradation of amphotericin B in 1% NaDOC/DMA (under aerobic and anaerobic contitions) was also determined and was found to be negligible.

## Introduction

Amphotericin B (Fig. 1) is the single most important antibiotic for the treatment of systemic mycoses (Abernathy, 1973). However, the compound exhibits extreme instability in aqueous media (Hamilton-Miller, 1973; Andrews et al., 1977, 1979; Beggs, 1978; Lamy-Freund, et al., 1985) and high toxicity (Utz, 1968; Lumpen, 1969; Miller and Bates, 1969; Hamilton-Miller, 1973). It is believed that the major route of degradation (and inactivation) is autoxidation (Andrews et al., 1977, 1979; Lamy-Freund et al., 1985). However, hydrolysis of amphotericin B should also be occurring in solution and evidence exists in the

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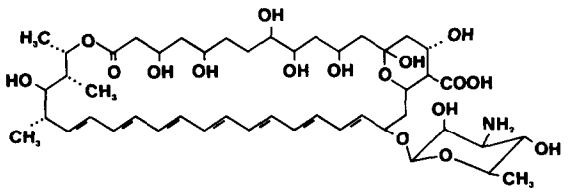

Fig. 1. Structure of amphotericin B.

literature (Hamilton-Miller, 1973) in support of this hypothesis.

Recently, we investigated improving the solubility of amphotericin B through the use of mixed non-aqueous solvent systems (Rajagopalan et al., 1988). These studies demonstrated the potential for using *N,N*-dimethylacetamide (DMA) containing 1% sodium deoxycholate (NaDOC) as a solvent system for solubilizing amphotericin B. In the present study, we have examined the chemical stability of amphotericin B in 1% NaDOC/DMA in order to complement the solubility work.

## Materials and Methods

*Materials*

Amphotericin B was obtained from Dumex A/S, Copenhagen. HPLC analysis of the sample indicated its purity to be 92% (as received) with an amphotericin A content of 1.4%. This sample was used without any further purification, except in studies involving the effect of water content on the rate of thermal degradation. In these studies, amphotericin B was dried by azeotropic vacuum distillation from a slurry of drug substance in 100% ethanol. Sodium deoxycholate (NaDOC) was obtained from Aldrich Chemical Co., Milwaukee, WI (purity 98% as the monohydrate) and used as received except in studies involving the effect of water content on the rate of degradation of amphotericin B where NaDOC was dried by storage at 1 mm Hg, 100 °C for 15 h. Anhydrous *N,N*-dimethylacetamide (DMA) (99$^+$% purity, $< 0.005$%, water, packaged under nitrogen) was purchased from Aldrich Chemical Co. and used without any further purification. 1,10-Phenanthroline was purchased from Aldrich Chemical Co. and used without any further purification (purity 99$^+$%). Acetonitrile, methanol and dimethylsulfoxide (DMSO) were HPLC grade from J.T. Baker Chemical Co. and were used upon filtration and degassing. Water was obtained from a Milli-Q system (Millipore) and was filtered and degassed before use. The filters employed in all cases were Nylon-66 (0.45 $\mu$m pore size) and were obtained from Rainin Instrument Co. The sodium acetate trihydrate and disodium edetate were obtained from JT Baker Chemical Co. and Aldrich Chemical Co., respectively, and were used as received. Glacial acetic acid was purchased from Mallinkrodt Chemical Co. and used without further purification.

*Chromatographic conditions*

HPLC analyses were performed on a Hewlett Packard HP 1090 equipped with a diode array detector for studies involving the effect of water content on the rate of degradation of amphotericin B. All other HPLC assays were performed using a Shimadzu ternary gradient instrument with a single wavelength UV/VIS detector set at 407 nm.

Separations were performed on a Beckman Ultrasphere ODS column (4.6 mm $\times$ 7.5 cm) with 3 $\mu$m particle packing. The mobile phase consisted of 50 mM sodium acetate and 3 mM disodium EDTA adjusted to pH 6 by dropwise addition of acetic acid (component A) and a 60 : 40 (v/v) mixture of methanol and acetonitrile (component B) in a 37 : 63 (v/v) ratio. A flow rate of 1 ml/min was maintained.

An external standard was prepared by accurately weighing approximately 25 mg of amphotericin B into a 25 ml volumetric flask. The drug substance was then dissolved in and diluted to volume with DMSO. A 1 ml aliquot of this standard stock solution was transferred to a 50 ml volumetric flask and diluted to volume with mobile phase, yielding a working standard with a final amphotericin B concentration of approximately 0.02 mg/ml. The injection volume for the external standard was 50 $\mu$l.

Samples were prepared for HPLC analysis by dilution of the amphotericin B in 1% NaDOC/DMA solutions with mobile phase to achieve a final concentration of approximately 0.02 mg/ml

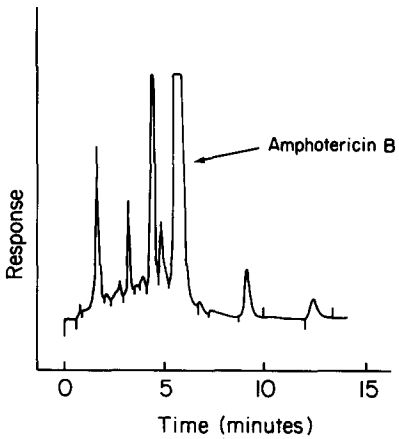


Fig. 2. Representative HPLC chromatogram showing the separation of amphotericin B from its degradation products. Chromatographic conditions are given in Materials and Methods.

in amphotericin B. Both the working standard and the reaction sample solutions were stable (no detected loss of amphotericin B) for 24 h at room temperature. Sample concentrations were determined by comparison of sample response (peak area) to the response of the external standard (peak area). All samples were injected in duplicate.

Using the chromatographic conditions described above, amphotericin B elution times ranged between 5.6 and 6.4 min. with a $k'$ value of 6.2–7.2 (depending on the specific column used in the analysis). The number of theoretical plates per meter for the separation was typically greater than 10,000. The analytical method was shown to be specific for amphotericin B in the presence of known impurities and potential degradation products. Solution degradation products eluted earlier than amphotericin B and were baseline resolved from the parent peak (see Fig. 2).

*Kinetic procedure*

Stock solutions (2 ml total volume) of amphotericin B in 1% (w/v) NaDOC/DMA (unless noted otherwise) were prepared. The initial concentration of amphotericin B was approximately 16.66 mg/ml ($1.80 \times 10^{-2}$ M), unless stated otherwise. Glass screw-cap test tubes (volume of the test tube = 10 ml) were filled with the stock solution and sealed tightly. The test tubes were then placed in a preheated Tecam Dri-block, Model DB-3 (accuracy within 0.5 °C). The vials were shaken frequently in order to replenish any reacted oxygen so that pseudo-first-order reaction conditions could be maintained. At various time periods, the test tubes were removed and aliquots were taken and transferred into 50 ml volumetric flasks and assayed by HPLC as described above. Each aliquot for a single run was taken from a single test tube. The reactions were monitored to at least 50% depletion of the initial amphotericin B concentration with each kinetic experiment being carried out in duplicate.

The anaerobic runs were performed in the same manner as above with the samples being prepared in an oxygen-free, nitrogen-filled glove box using 1% NaDOC/DMA solutions that were deaerated with high purity-grade nitrogen. The reactions were also carried out in the glove box.

Aerobic reactions run under dark conditions were performed in a dark room equipped with a red light that was used only when taking aliquots. All solutions were prepared in this room prior to initiation of the kinetic run. Otherwise, the conditions were identical to those listed above.

For studies of the effect of water content on the rate of degradation of amphotericin B in DMA, anhydrous samples were prepared using components that were dried as described in Materials and Methods. A 40 mg/ml solution of amphotericin B in 3% (w/v) NaDOC/DMA was found to contain 0.14% (w/w) $H_2O$. A similarly prepared sample was spiked with water to yield a solution that was 1.4% (w/w) in $H_2O$. The moisture content in each sample was determined employing a Mitsubishi Moisture Meter (Model CA-05). Samples were maintained at 30 °C.

*Analysis of kinetic data*

For each experiment a standard curve relating integrated peak area to concentration of amphotericin B in the injected solution was prepared. Thus, the concentration of amphotericin B remaining was calculated directly from the HPLC analysis. These data were then plotted and analyzed using "RS/Explore", a linear regression program (BBN Research Systems, Cambridge,

MA). The coefficients of determination ($r^2$) in all cases were 0.94 or greater.

## Results

*Effect of oxygen on the rate of degradation*

A 1% NaDOC/DMA solution of amphotericin B (initial concentration = 16.66 mg/ml) was prepared under aerobic conditions, placed at 30 °C and monitored by HPLC. The degradation of amphotericin B with time is plotted in Fig. 3A. The calculated rate constant for this reaction was $1.23 \times 10^{-2}$ $h^{-1}$ giving a $t_{90}$ of 8–9 h for the decomposition of amphotericin B in 1% NaDOC/DMA. When the reaction was performed under anaerobic conditions the rate constant for the degradation of amphotericin B was $3.50 \times 10^{-3}$ $h^{-1}$ (see Fig. 3B). This value yields a $t_{90}$ of approximately 30 h which corresponds to a 3.5-fold increase when compared to the $t_{90}$ value for the aerobic reaction.

*Effect of temperature on the rate of degradation*

The degradation of amphotericin B in 1% NaDOC/DMA was further studied at 40 °C and 50 °C under both aerobic and anaerobic conditions. The results are shown in Fig. 3A, B respectively, where they are compared to the data that were obtained at 30 °C. The apparent activation energies for the aerobic and anaerobic reactions have been determined from Arrhenius plots (see Fig. 4). The apparent activation energy for the aerobic reaction is 17.5 kcal/mol while, for the anaerobic reaction, it is 23.5 kcal/mol.

*Effect of water content on the rate of degradation*

The effect of water content on the rate of degradation of amphotericin B was studied by comparison of the drug stability in DMA solutions that contained either 0.14% (w/w) $H_2O$ or 1.4% (w/w) $H_2O$. Samples were stored at 5 °C and 30 °C for 72 h and analyzed at initial, 24 h, 48 h, and 72 h time points. No loss of amphotericin B was detected in the 5 °C samples over the time course of the experiment. Both the anhydrous and the water-spiked samples degraded approximately 30% after 72 h when stored at 30 °C. A comparison of the % recoveries of amphotericin B at each time point for the 30 °C samples is given in Fig. 5. These data indicate that there was no significant difference in the rate of degradation of amphotericin B in the anhydrous and water spiked samples.

*Effect of light on the rate of degradation*

To determine the role of light in the rate of degradation of amphotericin B in 1% NaDOC/DMA, the reaction was performed in a dark room at 30 °C under aerobic reaction conditions and compared to a control run in the presence of normal room light. No difference in the rate of degradation was observed.

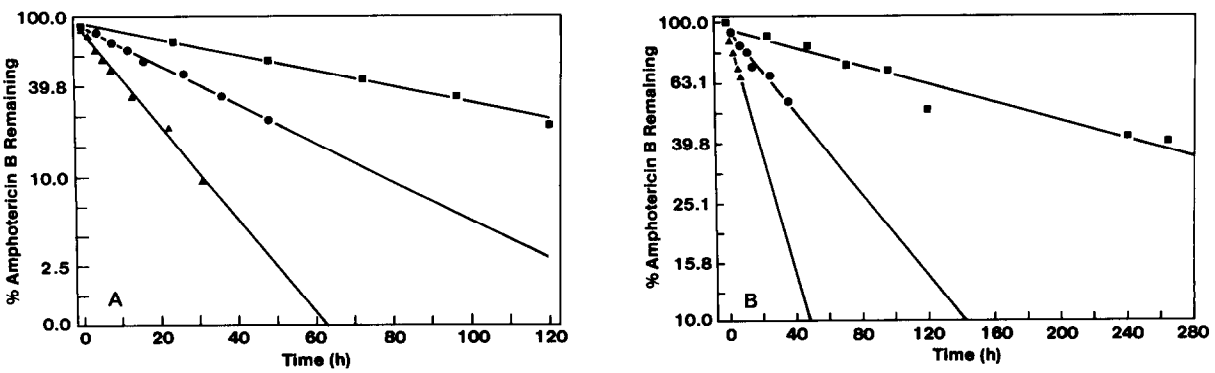


Fig. 3. A: degradation of amphotericin B (initial concentration = 16.66 mg/ml) in 1% NaDOC/DMA under aerobic reaction conditions at 30 °C (square), 40 °C (circle) and 50 °C (triangle). B: degradation of amphotericin B (initial concentration = 16.66 mg/ml) in 1% NaDOC/DMA under anaerobic reaction conditions at 30 °C (square), 40 °C (circle) and 50 °C (triangle).

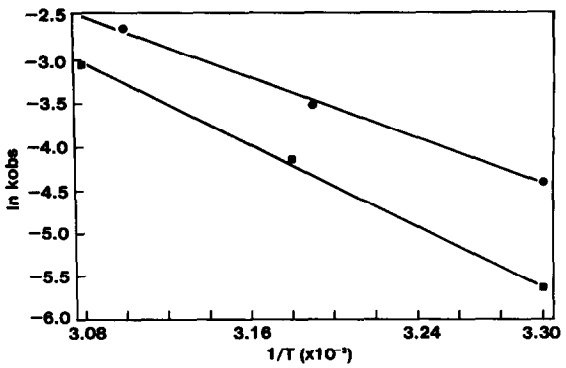


Fig. 4. Arrhenius plots of the degradation of amphotericin B (initial concentration = 16.66 mg/ml) in 1% NaDOC/DMA under aerobic (circle) and anaerobic (square) reaction conditions.

*Effect of amphotericin A content on the rate of degradation*

Purification of amphotericin B is rather difficult and samples usually contain varying amounts of structurally similar polyenes such as amphotericin A. The lot of amphotericin B used for these studies contained 1.4% amphotericin A. In order to determine whether or not the presence of amphotericin A played a role in the degradation of amphotericin B in 1% NaDOC/DMA, the kinetics were carried out at 30 °C (both aerobically and anaerobically) using a second batch of amphotericin B that had a higher amphotericin A content of 4.5%. The amount of amphotericin A present in the starting material did not affect the rate of degradation of amphotericin B under either set of reaction conditions suggesting that, at least at these levels, amphotericin A does not play an appreciable role in the decomposition of amphotericin B in 1% NaDOC/DMA.

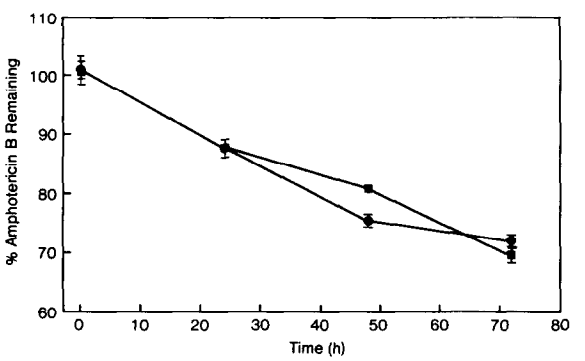


Fig. 5. Degradation of amphotericin B (initial concentration = 40.00 mg/ml) in 3% NaDOC/DMA at 30 °C in the presence of 0.14% w/w (square) and 1.4% w/w (circle) water. Each time point is the average of triplicate runs.

*Effect of sodium deoxycholate on the rate of degradation*

The degradation of amphotericin B in DMA (at an initial concentration of 5 mg/ml) was investigated in the absence and presence of 1% NaDOC (w/v) under both aerobic and anaerobic reaction conditions at 30 °C. The addition of NaDOC has no effect on the rate of decomposition in either case.

*Effect of heavy metal impurities on the rate of degradation*

It is believed that the mechanism of biological inactivation of amphotericin B in aqueous solution is autoxidation via free radical formation (Andrews, et al., 1977; Lamy-Freund, et al., 1985). If this route is important, then the presence of heavy metals, such as iron, would likely accelerate the inactivation process. This same autoxidative pathway could also play a role in the chemical instability of amphotericin B evidenced here. Therefore, the 3 components employed in this study (amphotericin B, NaDOC and DMA) were subjected to elemental analysis. Only the amphotericin B sample deviated significantly from the expected analytical result. Inductively coupled plasma (ICP) discharge analysis of amphotericin B showed that two heavy metals were present in appreciable quantities — iron at 55 ppm and calcium at 740 ppm. Of the two, iron is the only one known to play a catalytic role in autoxidations.

In order to determine whether or not lowering the level of iron impurity that was present in amphotericin B would decrease the rapid rate of degradation of amphotericin B, 1,10-phenanthroline was added to a 1% NaDOC/DMA solution of amphotericin B. Iron complexes of 1,10-phenanthroline are known to have significantly higher oxidation potentials than the uncomplexed iron species (Koenig, 1968) and, therefore, the complexing agent would be expected to alter the

degradation profile of amphotericin B if iron played a major catalytic role. Some of the phenanthroline/iron complex precipitated from solution and was removed via filtration. The degradation of amphotericin B was monitored at 30 °C under aerobic reaction conditions and compared to a control that did not include the prior addition of 1,10-phenanthroline. No difference in the rate of degradation of the filtered solution vs the control was observed, suggesting that iron, present in the sample, did not contribute significantly to the breakdown of amphotericin B.

## Discussion

Inspection of the structure of amphotericin B (Fig. 1) suggests that the most likely pathways of chemical decomposition when in solution are oxidation along the heptaene portion of the molecule, hydrolysis of the lactone fuctionality and/or hydrolysis of the amino sugar. In fact, ample literature precedence exists in support of these routes of degradation. Andrews et al. (1977, 1979) have shown that the biological activity of amphotericin B could be prolonged in vitro when suitable antioxidants were added to the fungus-containing culture medium for up to 30 h at 37 °C while Hamilton-Miller (1973) has studied the effect of pH on the stability of amphotericin B at 37 °C in aqueous, buffered media and has generated a pH rate profile (utilizing biological activity data) that is suggestive of a hydrolytic mechanism. The relative role of these two processes in the rate of degradation of amphotericin B will be dependent on a number of factors such as the polarity and pH of the solution medium in question, the amount of oxygen present, the quantity and activity of any water that is present, the influence of light and the presence of any impurities capable of catalyzing oxidation (such as heavy metals) or hydrolysis (e.g. a base). Therefore, we have investigated the chemical degradation of amphotericin B in DMA with these factors in mind and have quantified the aerobic and anaerobic pathways in order to evaluate their relative roles in the observed degradation of amphotericin B when it is in a medium such as DMA (dielectric constant = 37.8).

Results obtained by monitoring the degradation of amphotericin B in 1% NaDOC/DMA at 30 °C under aerobic and anaerobic conditions demonstrate that the primary decomposition pathway for the drug involved oxidation. As evidenced in Fig. 3A, B, the rate of oxidative degradation of amphotericin B in 1% NaDOC/DMA at 30 °C is 2.5 times faster (determined by: ((rate constant under aerobic conditions) − (rate constant under anaerobic conditions))/(rate constant under anaerobic conditions)) than when the reaction is monitored under conditions where the oxygen has been purged from the system. Since oxidation of amphotericin B is the major degradation pathway in DMA at this temperature the roles of light and heavy metal impurities on the rate of oxidation were investigated. Surprisingly, neither factor had any significant effect on the observed rate of amphotericin B oxidation. The ability of NaDOC and amphotericin A, in the concentration ranges studied, to influence the rate of oxidative degradation of amphotericin B were found, as well, to be negligible.

The apparent activation energy for the degradation of amphotericin B in 1% NaDOC/DMA under aerobic reaction conditions was determined and was found to be 17.5 kcal/mol. This value is similar to the value of 16.4 kcal/mol determined by Hamilton-Miller (1973) for the aerobic degradation of amphotericin B in an aqueous, buffered medium (pH 7). Another interesting comparison between the results reported here and those of Hamilton-Miller (1973) is that the half-life of amphotericin B at 4 °C in 1% DOC/DMA is 37 days while the half-life under similar conditions in an aqueous medium at pH 7 is 34 days (both values were obtained from extrapolation of Arrhenius plots), i.e. the chemical stability of amphotericin B in 1% DOC/DMA is very similar to its chemical stability in water at pH 7 (at 4 °C).

The role of an anaerobic pathway in the degradation of amphotericin B in 1% NaDOC/DMA has been suggested from data obtained under anaerobic conditions. As stated above, this route was approximately 2.5 times slower than the oxidative route at 30 °C. The apparent activation energy for this process was determined and was found to be 23.5 kcal/mol, appreciably higher

than the apparent activation energy for the aerobic reaction and resulting in the anaerobic pathway(s) assuming an increasingly greater role in the overall degradation of amphotericin B in DMA as the reaction temperature is increased.

Since data in the literature suggest that amphotericin B is susceptible to hydrolysis (Hamilton-Miller, 1973), the influence of employing dried materials on the degradation of amphotericin B in DMA was investigated by comparing the rates of decomposition for the reaction where anhydrous components (as defined in Materials and Methods) were used to the reaction where water was added to the solution. No significant differences were observed in the two reactions suggesting that drying the components in the scrupulous manner described herein does not decrease the rate of decomposition of amphotericin B in DMA at 30 °C.

Other components in the medium that could be significant to any anaerobic pathway of degradation were also evaluted. Varying the amounts of NaDOC or amphotericin A present in the solution had no effect on the rate of degradation of amphotericin B in DMA when carried out under anaerobic or aerobic conditions.

In conclusion, at room temperature or below (conditions that would be employed in the storage and usage of any parenteral formulation) the major route of degradation of amphotericin B in DMA is oxidation. Nevertheless, there is an unidentified anaerobic process in DMA that contributes sufficiently to drug degradation under these temperature conditions to limit the use of DMA as a solubilizing agent for amphotericin B formulations. In practice, formulations of amphotericin B would have to be reconstituted with the DMA before use (taking precautions to limit oxygen content) and, if stored at 5 °C or lower, it could be kept for up to 5 days.

## Acknowledgements

The authors wish to express their gratitude to Eufrosynia Dubynin, Lynn Malinoski, David Thornton, Brenda Uhrig and Jeanette Wilson without whose dedicated efforts the work reported in this manuscript would not have been accomplished and to Dr. William Vincek and Dr. Robert Siegler for their helpful comments. We would also like to acknowledge Dr. Lee Webb and his group (Department of Analytical Chemistry, SK&F Labs) for the purity analysis of amphotericin B, Ms. Edith Reich (Department of Analytical Chemistry, SK&F Labs) for the elemental analysis results, Mr. Gary Zuber (Department of Analytical Chemistry, SK&F Labs) for his analytical assistance and Dr. James McArdle (Department of Analytical Chemistry, SK&F Labs) for carrying out the ICP experiments.

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