International Journal of Pharmaceutics, 44 (1988) 117–123
Elsevier

IJP 01490

A stability study of amphotericin B in aqueous media using factorial design

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(Received 8 June 1987)
(Modified version received 30 October 1987)
(Accepted 30 November 1987)

Key words: Amphotericin B; Ion-pair HPLC; Stability study; Factorial design

Summary

A reversed phase ion-pair high-performance liquid chromatography assay has been developed for the determination of amphotericin B (AmB) in aqueous media. This assay has been applied to a factorial stability study of AmB. The effects of temperature, pH, ionic strength, surfactant concentration, oxygen and light on AmB have been investigated using a $2^5 \times 3$ factorial design. Results indicate that oxygen, light and temperature have significant effects on the stability of AmB solutions. Interactions between temperature and ionic strength, ionic strength and surfactant, pH and temperature and surfactant have also been identified. It is concluded that AmB is most stable in the absence of light, in oxygen-free environment and at low temperatures.

Introduction

Amphotericin B is an antibiotic used in the treatment of systemic mycoses (Medoff et al., 1983). It has been reported that polyene antifungal agents are unstable, especially in the presence of light and in acidic or in alkaline solutions (Walksman et al., 1965). However, no comprehensive stability studies on AmB have been reported in the literature. Furthermore, in most reported stability studies bioassay or colorimetric analysis were employed for the determination of AmB (Gallelli, 1967; Shadomy et al., 1973; Tripple et al., 1975). These assays are neither sensitive nor specific for AmB.

In the present investigation, a reversed phase ion-pair HPLC assay for AmB is reported. This assay has been applied to a stability study of AmB in aqueous media. A factorial approach has been employed in the experimental design, and in the data analysis of the stability study (Ahlneck and Waltersson, 1986; Bolton, 1983; Waltersson 1986).

In conventional drug stability studies, a single variable is investigated each time, with remaining variables kept constant. This approach is extremely time-consuming, and interactions of factors are not considered. The advantage of factorial experimental design over that of the conventional approach is its ability to identify those factors that affect the stability of the drug with minimal experimental work. Moreover, interactions among the factors are also assessed.

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Theory

If the degradation process is assumed to follow first order kinetics in all situations, the concentration of AmB at a given time (τ) can be described by the following equation

$$C = C_0\, e^{-k\tau} \text{ or } (\ln C_0 - \ln C) = k\tau \qquad (1)$$

where C is the concentration of AmB remaining, C_0 is the concentration of AmB at time zero and k is the first-order decomposition rate constant. While the decomposition rate constant at different storage conditions is usually chosen for analysis (Ahlneck and Waltersson, 1986; Bolton, 1983; Waltersson, 1986), its determination is time-consuming. In this study, the drug concentration remaining at a fixed time is suggested as the dependent variable. By transforming k as a function of temperature and the activation energy using the Arrhenius equation, the dependent variable ln (ln $C_0 - \ln C$) can be written as

$$\ln(\ln C_0 - \ln C) = \ln A - \frac{E}{RT} + \ln \tau \qquad (2)$$

where A is the Arrhenius factor, E the activation energy, T the temperature and R is the gas constant. By fixing the storage time (τ) and the initial concentration (C_0), any factor affecting A or E will result in change of ln (ln $C_0 - \ln C$). The log–log transformation of the concentration response under different storage conditions can then be analysed using analysis of variance (ANOVA). Results obtained using this method will indicate those factors and interactions of factors which have significant effect on the stability of the drug. However, before making any interpretation, the validity of using the ln (ln $C_0 - \ln C$) expression must be verified by analysing the residual plots of the data (Box, et al., 1978).

Materials and Methods

Apparatus and materials

The chromatographic system for the quantitation of AmB consisted of a Waters Associate M45 or a LKB 2100 HPLC pump, a Waters M440 detector with a mercury light source and a Rheodyne 7125 injector with 20 μl sample loop. The 100×4.6 mm i.d. chromatographic column was slurry-packed with 5 μm MOS-Hypersil (C8) (Shandon). The glass-door oven used for the stability study was a Thelco Model 19. The UV-spectrum of the chromatographic peak was recorded with a Shimadzu UV240 detector equipped with an 8 μl flow cell using the stop flow scan technique.

AmB was kindly donated by Squibb Pharmaceutical (Auckland, NZ). Orthophosphoric acid, sodium chloride, sodium hydrogen phosphate, sodium lauryl sulphate (SLS) were obtained from BDH. Polysorbate-20 was supplied by Sigma Chemicals. Acetonitrile (HPLC grade) was purchased from J.T. Baker. Glassware was silanised with Aquasil from Price Chemical. Water was double glass-distilled and MilliQ-filtered.

Chromatographic conditions

The mobile phase was a mixture of acetonitrile in water (50% v/v) containing 100 mM disodium hydrogen phosphate, 10 mM SLS and adjusted to pH2 with orthophosphoric acid. A flow rate of 2 ml/min and detection at 405 nm were employed. Peak height was used for quantitation.

Factorial design

The factor levels chosen for this investigation are summarized in Table 1. A glass-door oven was employed for those treatments requiring high light intensity and at high temperature. High light level was achieved by positioning a 25 W (1200 lumen) fluorescent bulb at 15 cm from the samples. All vials stored in darkness were covered with tin foil. The high ionic strength media were prepared with 5 mM disodium hydrogen phosphate, the buffer salt, and 485 mM sodium chloride, the non-buffer salt. High levels of surfactant concentration was obtained using 0.4 mM polysorbate-20 and the low surfactant levels had no added surfactant. High oxygen levels were obtained by purging the solutions for 1.5 min with 95% oxygen and low oxygen levels were ensured by purging the sample with dry nitrogen for 1.5 min. The media were adjusted to the desired pH with 0.1 M sodium hydroxide or 0.1 M hydrochloric acid.

TABLE 1

Treatment plan for the factorial design

Factor	Level		
	Low	Medium	High
A. temperature	$20 \pm 4°C$ (t)		$70 \pm 2°C$ (T)
B. light	darkness (l)		25 W fluorescent bulb at 15 cm (L)
C. ionic strength	< 0.001 (i)		0.5 M added salt (I)
D. surfactant concentration	0 (s)		0.4 mM Tween 20 (S)
E. oxygen	N_2 purged		O_2 purged
F. pH	4	7.4	10

Each combination of these factor-levels, as listed in Table 1, was tested, resulting in $2^5 \times 3$ storage conditions. The 96 treatments were then randomized for testing.

Aqueous solutions of AmB, with an initial concentration of 10 μg/ml, were stored in silanized containers under appropriate conditions for 6 h. The pH of all treatments was measured at the end of the experiment and was found to vary not more than 0.1 pH unit. The remaining drug content was then assayed immediately by the ion-pair HPLC. Each measurement was made in triplicate and if an individual measurement differed from the others by more than 5%, further replicate measurements were performed. The data were then analysed using the SAS computer package (SAS, 1982). The coefficient of variation of the experiment based on 4 determinations on one treatment where AmB was stored at t, L, i, S, N_2 purged and pH 7.4 was about 11%.

Results and Discussion

Chromatography

AmB is a Zwitter ion with pK_a values of 5.5 and 10 for its carboxyl and amino functional groups respectively (Smith and Rawlins, 1973). Chromatographic retention of AmB has been obtained using mobile phase of mixtures of aqueous buffer and organic modifiers, with or without the inclusion of the zwitter pairing ion, ethylenediaminetetraacetic acid (Leclercq et al., 1985; Mayhew et al., 1983; Margosis and Aszalos, 1984; Nilsson-Ehle et al., 1987). These systems were found to either give poor reproducibility, due to the retention of AmB being very sensitive to pH changes of the mobile phase (Knox and Jurand, 1979), or poor peak shape. However, this compound should be retained on the C8 support in presence of anionic hydrophobic pairing ions at low pH (Hung

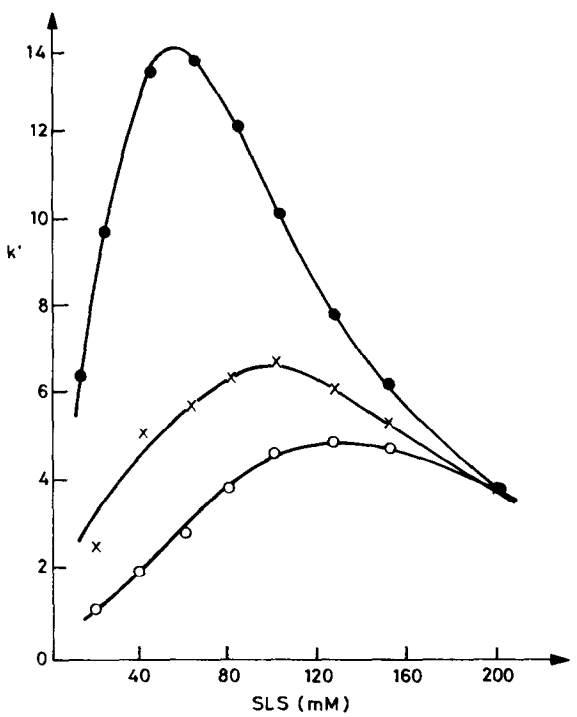

Fig. 1. Effect of pairing ion SLS concentration on k' of AmB. Chromatographic conditions: 100 mm × 4.6 mm MOS-Hypersil column; mobile phase of acetonitrile aqueous buffer, 10 mM Na_2HPO_4 at pH 2; flow rate 2 ml/min. (●), Acetonitrile aqueous buffer, 45% v/v; (×), acetonitrile aqueous buffer, 50% v/v; (○), acetonitrile aqueous buffer, 55% v/v.

and Taylor, 1981). C8 stationary support was chosen because it produced sharper peaks than that of the C18. SLS was used as the pairing ion because of its absorption characteristic on the hydrophobic stationary phase (Hung and Taylor, 1981).

In this study, the pH and ionic strength of the mobile phase were empirically optimized as previously described (Hung and Perrier, 1985). The variation in the capacity factor (k') of AmB as a function of mobile phase pairing ion concentration, with different acetonitrile content, is shown in Fig. 1. The chromatographic behaviour of the ionized AmB in the above chromatographic conditions can be explained by the ion-exchange desolvation mechanism previously proposed (Hung and Taylor, 1980, 1981). A mobile phase containing 50% v/v acetonitrile in buffer and 100 mM sodium lauryl sulphate was used for subsequent stability study as it provided acceptable and reproducible retention of AmB. A typical chromatogram obtained using this mobile phase is shown in Fig. 2. Standard curve of peak height (20 μl injected) vs concentration (0.1 to 20 μg/ml) was found to be linear with coefficient of determination value > 0.98. The within-day coefficient of variation based on 6 determinations of concentrations of 0.1, 1 and 10 μg/ml was less than 5%. Taking a signal to noise ratio of 3 as the criterion, the detection limit of this assay for AmB is about 0.1 μg/ml. However, by simply increasing the volume of injection to 200 μl, a 10-fold decrease in the detection limit of this assay can be achieved without loss of column efficiency (Taylor et al., 1987).

Factorial stability study

The stress factors employed in this study were based on the potential sources of degradation present in commercial AmB infusions. Sodium desoxycholate, a solubilizing agent included in commercial AmB infusions, is insoluble in aqueous media at low pH. Hence, polysorbate-20, instead of sodium desoxycholate, was used as the surfactant in this investigation. In addition, polysorbate-20 has similar HLB value as sodium desoxycholate (Martin et al., 1983) and therefore should exhibit equivalent solubilizing activity. Three pH levels were employed to identify any

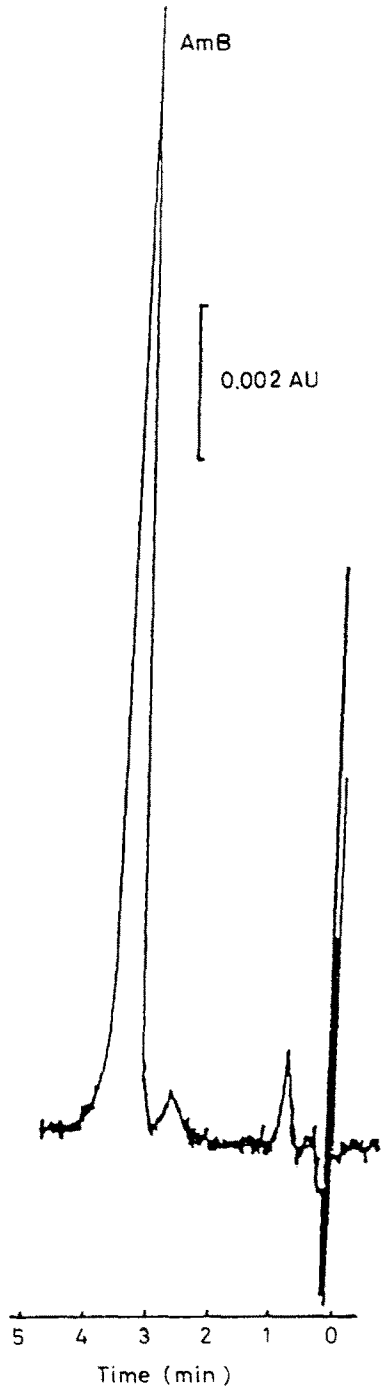

Fig. 2. Representative chromatogram of amphotericin B (10 μg/ml) in aqueous media. Chromatographic conditions: see text.

non-linear relationship in the rate–pH profile of AmB decomposition (Martin et al., 1983). Preliminary investigations indicated that AmB could be detected even after storage under the most unfavourable conditions for 6 h. Hence a stress period of 6 hours was used for each experiment.

However, upon conducting the stability study, amphotericin B could not be detected after the 6-h period in 8 storage conditions. Because of the log–log transformation of data, a response of zero would result in an erroneous estimate. Hence, all drug concentrations were expressed in units of ng/ml with a C_0 of 10,000 ng/ml. Those treatments with no detectable drug content were assigned to 1 ng/ml, a negligible but finite value. The purity of the AmB peak for all treatments was confirmed by comparing its UV spectrum with that of the authentic compound. In all studies, only AmB was detected at 405 nm in the reacted mixtures.

The analysis of residual plots of the data indicate that the ln (ln C_0 − ln C) expression of the dependent variable is appropriate in this study, i.e., first-order decomposition of AmB can be assumed under all storage conditions. The concentration of AmB remaining under different storage conditions varied between 0 to 91%. However, the fit of data using zero-order transformation is not adequate. Higher-order transformation has not been attempted in view of their complexity. Since the experiments were not repeated, no estimate of the experimental error was available. The 6-way interaction was therefore used as the error term in the preliminary analysis (Davies, 1954). Three main factors and 6 interactions were found to be significant at 5% level. All 4- and 5-way interactions were found to be insignificant. Hence in the second analysis, these high-order interactions sum of squares were pooled into the error sum of squares to increase the sensitivity of the analysis. The same main effects and interactions remained significant. These were (P values in brackets) light (0.0001), temperature (0.0001) and oxygen (0.0355); the two-way interactions of pH–temper-

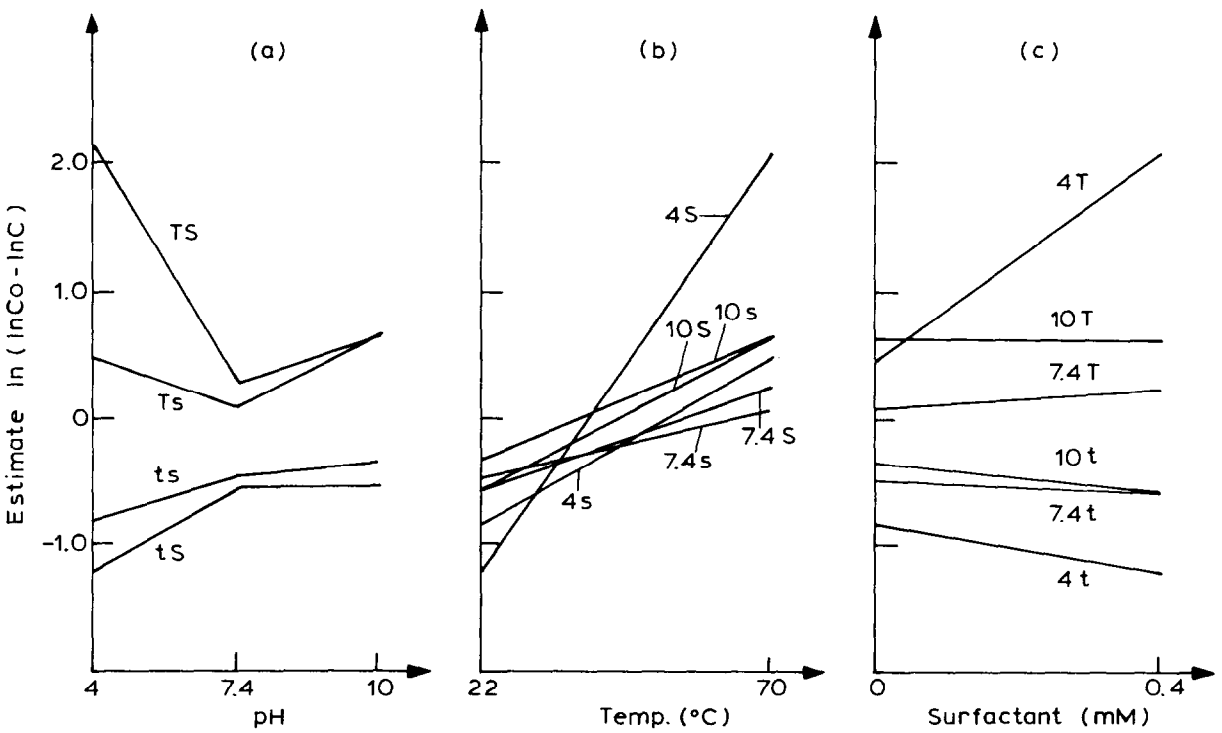

Fig. 3. Estimate plot of the three-way interaction between pH, temperature and surfactant concentration.

ature (0.00017), pH–surfactant (0.0259), ionic strength–surfactant (0.0055), temperature–ionic strength (0.0471) and temperature–surfactant (0.0006); and the 3-way interaction of pH–temperature–surfactant (0.0018). Plots of estimates of the dependent variable, ln (ln C_0 − ln C), for each significant interaction are shown in Fig. 3–5. Upper case and lower case letters refer to high and low levels of factors, respectively (see Table 1).

Fig. 3a shows that at room temperature, with high surfactant concentration, an acidic pH of 4.0 provides optimum stability of AmB. However, an aqueous medium of pH 7.4 is preferred if the solution temperature can not be controlled. At this pH the stability of AmB is least sensitive to the variation of temperature. Fig. 3b shows that room temperature is preferred regardless of the pH and surfactant concentration. Fig. 3c indicates that AmB is most stable at room temperature in the presence of high surfactant concentration, and acidic pH. In cases where the storage temperature is high, low surfactant concentration is preferred. The data presented in Fig. 3b, c also indicate that the presence of surfactant at low pH and high temperature will have a catalytic effect on the decomposition of AmB.

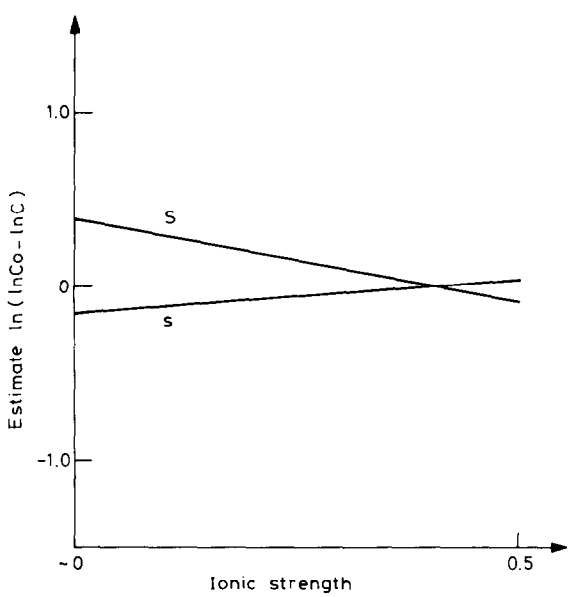

Fig. 4. Estimate plot of the two-way interaction between surfactant concentration and ionic strength.

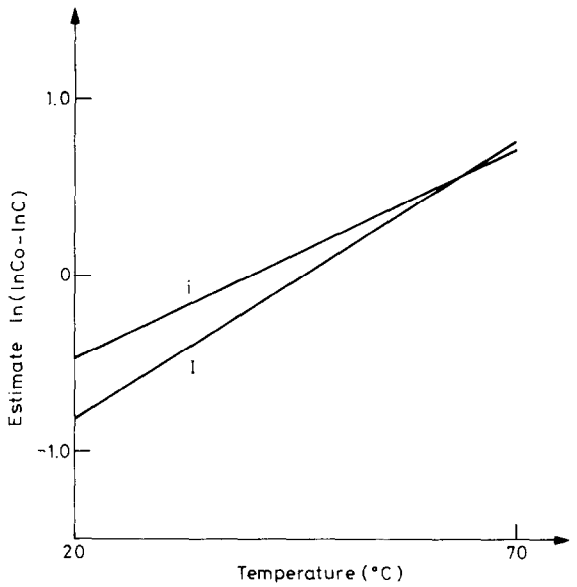

Fig. 5. Estimate plot of the two-way interaction between temperature and ionic strength.

Fig. 4 demonstrates that a combination of either high surfactant concentration with high ionic strength, or low surfactant concentration with low ionic strength would provide better storage conditions for AmB. Fig. 5 shows that at room temperature, greater stability is achieved with high ionic strength of the aqueous media.

Of the other significant main factors, low oxygen levels and darkness are required for optimum stability, regardless of the level of any other factor. The log–log estimates for the high and low level of oxygen were 0.123 and −0.109 respectively (i.e. 32.3 and 40.8% remaining). Similarly the log–log estimates for strong light and in darkness were 0.286 and −0.273, respectively (i.e. 26.4 and 46.7% remaining).

Conclusion

A simple and sensitive ion-pair HPLC for AmB is presented. The factorial stability study of AmB indicated that AmB is most stable in darkness, in the absence of oxygen and at low temperature. Interactions between temperature and ionic strength, ionic strength and surfactant, pH–tem-

perature–surfactant have also been detected. This investigation demonstrates the advantages of factorial experiments in testing the stability of drugs. Important factors and their interactions can easily be detected without prior information of the degradation pathways of the compound. In addition this study will be of assistance in designing comprehensive experiments on the physical–chemical evaluations of the compound.

Acknowledgements

This investigation was in part supported by the UGC and Lottery Board of New Zealand. A.J.S. gratefully acknowledges receipt of a New Zealand Pharmacy Education and Research Foundation summer research grant.

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