DRUG DEVELOPMENT AND INDUSTRIAL PHARMACY, 14(12), 1657-1671 (1988)

INVESTIGATION OF THE STABILITY OF DOXORUBICIN HYDROCHLORIDE USING FACTORIAL DESIGN

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Abstract

Using drug concentration remaining at a given time as the criterion, a 2^4 x 3 factorial design has been employed to investigate the effects of temperature, light, media (aqueous or organic/aqueous), ionic strength and pH on the stability of doxorubicin hydrochloride. Following the application of first order kinetics, and assuming an additive model, the statistical significance of the factors and their interactions have been determined using analysis of variance (ANOVA) on the dependent variable $\ln(\ln C_0 - \ln C)$. The results indicate that temperature, pH and media are the major factors responsible for the stability of drug. The two-way interaction between temperature and pH, and the three-way interaction between temperature, light and ionic strength are also significant. It is found that doxorubicin is more stable in non-aqueous media at low temperature and low pH values. A combination of darkness and low ionic strength is also conducive to its stability.

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Introduction

Doxorubicin hydrochloride (Adriamycin hydrochloride®) is an anthracycline antibiotic widely used in the treatment of acute leukemias, lymphomas and a variety of solid tumours (1-3). In order to reduce the incidence of cardiotoxicity associated with this cytotoxic agent, efforts have been made towards the synthesis and evaluation of different kinds of controlled and targeted drug delivery systems (4-8). To collect a meaningful information regarding the in vitro release profile of doxorubicin from these delivery devices, it is essential that its stability is maintained throughout the course of the study. In addition, the information on the factors conducive to its stability is essential for situations where it is administered as an infusion for long periods (9-11).

In common with other anthracyclines, doxorubicin is sensitive to light (12,13). It has been shown that in dark, 10 μg/ml aqueous solution of doxorubicin can be stored for at least one week, without affecting its stability (13). This study also demonstrated the dependence of photo-degradation of doxorubicin on the nature of the solvent. The degradation of drug in ethanol was found to be less prominent than that observed in saline, water or Ringer-Kreb's bicarbonate media. However because of the study design, no information was provided regarding the interaction between light and the nature of the solvent (13).

Whereas some studies have demonstrated absolute stability of doxorubicin at temperatures ≤ 4°C (14,15), other reports have indicated considerable degradation of this drug at 37°C (14,16,17). It has been recommended by the official compendia that doxorubicin is stable in saline for up to 24 hr at room temperature (18), and that this drug is unstable at both acidic as well as alkaline pH (19). This information appears to conflict with other studies where low pH has been shown to preserve the stability of doxorubicin (14-16).

Routine stability studies involve use of one factor at a time, while keeping other factors constant (13,14,17). The results obtained from such studies therefore do not provide any information about the potential interactions between factors, and their significance, if any. Conversely, simultaneous variation of all the included factors during the course of a study, permits the collection of information regarding the effects of individual factors as well as about the interactions among them. These objectives can be fulfilled by using factorial experimental design (20,21). In addition, this statistical technique also reduces the experimental work involved by an appreciable extent (20-22).

Theoretical

The conventional accelerated stability studies are based on computing degradation rate constant of a drug under different test conditions. However such studies are often tedious. Using amphotericin B as the model drug, Hung et al. (23)

have recently suggested the use of amount remaining at a given time as the dependent variable, in the determination of its stability. Hence this parameter has been employed in the present study to investigate the effect of five different factors on the stability of doxorubicin.

Doxorubicin is known to exhibit first-order degradation kinetics under different conditions (13,14,17). Hence the concentration of this drug at any time can be estimated using the relationship

$$(\ln C_0 - \ln C) = k.\tau \quad (1)$$

where C is the concentration of drug at time τ, C_0 is the initial concentration of drug (i.e., at $\tau = o$), and k is the apparent first-order degradation rate constant. It has been suggested that if τ and C_0 are kept constant, the effect of a given factor can be monitored by estimating the amount remaining at that time (23). The log-log transformation, ln ($\ln C_0$ - ln C), can then be analysed using ANOVA. However, this method requires establishment of the validity of using $\ln(\ln C_0 - \ln C)$ as the dependent variable, which can be performed by examining residual plots of the data (21).

a) Temperature - The effect of temperature on the activation energy (E_a), and hence the degradation rate constant (k) of a drug, may be expressed using the Arrhenius Equation (24)

$$k = A.e^{-E_a/RT} \quad (2)$$

where A is the Arrhenius factor, R is the gas constant and T is the absolute temperature. Here k from Eq (1) can be transformed as a function of T and Ea, in such a manner that the dependent variable $\ln(\ln C_0 - \ln C)$ may be expressed as

$$\ln(\ln C_0 - \ln C) = \ln A - Ea/RT + \ln \tau \quad (3)$$

b) Solvent - Assuming a bi-molecular reaction of the type

$$A + B \rightleftharpoons (A.....B)^* \longrightarrow \text{Products}$$

where A and B are the reactants and (A.....B)* is the activated complex, the degradation rate constant k for the reaction can be expressed as (24)

$$k = k_0.e^{V^*(\Delta\delta_A + \Delta\delta_B - \Delta\delta^*)/RT} \quad (4)$$

where k_0 is the rate constant in an infinitely dilute solution; $V = V_A + V_B - V^*$, representing overall molar volume of the reactants and activated complex; $\Delta\delta$ represents the difference in the solubility parameter of the solvent and the reactants or activated complex. Eq. (4) can be transformed into the first-order rate expression and finally into the form of the dependent variable $\ln(\ln C_0 - \ln C)$ as

$$\ln(\ln C_0 - \ln C) = \ln k_0 + V^*(\Delta\delta A + \Delta\delta B - \Delta\delta^*)/RT + \ln \tau \quad (5)$$

c) Ionic strength - Again, assuming a bi-molecular reaction of the form

$$A^{ZA} + B^{ZB} \rightleftharpoons (A.....B)^{*(ZA+ZB)} \longrightarrow \text{Products}$$

where ZA, ZB and *(ZA + ZB) are the charges for the reactants and the activated complex, the degradation rate constant may be expressed as (24)

$$k = k_0.e^{1.02*ZAZB\sqrt{\mu}} \quad (6)$$

where k_0 is as defined above, and μ is the ionic strength of the solution. This Equation may be transformed into the first-order rate expression and then into the form of $\ln(\ln C_0 - \ln C)$ as

$$\ln(\ln C_0 - \ln C) = \ln k_0 + 1.02* ZAZB\sqrt{\mu} + \ln\tau \quad (7)$$

d) pH: In a buffered solution, drug degradation may not accompany noticeable change in the concentration of acid or base, so that the reaction may be considered to be catalysed by hydrogen or hydroxyl ions (24). For specific acid-base catalysis type reaction, the degradation rate constant k may be estimated using the relationship (24)

$$k = k_2.k_w.e^{pH} \quad (8)$$

where k_2 is the rate constant for hydrogen ion catalysed reaction, and k_w is the rate constant for water catalysed reaction. Transformation of this relationship into the first-order rate expression gives

$$\ln C = \ln C_0 - k_2.k_w.e^{pH}.\tau \quad (9)$$

and the log transformation of Eq. (9) gives

$$\ln(\ln C_0 - \ln C) = \ln k_2.k_w + pH + \ln\tau \quad (10)$$

e) Light - Like heat, light may promote the activation of molecules for a reaction to occur. Despite the fact that initial photochemical reaction may be followed by thermal reactions, in most instances, the rate of activation of photochemical reactions is independent of temperature (24).

Materials and Methods

Apparatus

The chromatographic system used for the analysis of doxorubicin hydrochloride consisted of a Waters Associate 6000A pump, a Rheodyne 7125 injector fitted with a 100 µl sample loop and a Schoeffel FS970 fluorescence detector. The chromatograms were recorded on a Linesis L650 recorder. The

chromatographic column was a stainless steel, 100 x 4.6 mm i.d., packed with ODS-Hypersil (Shandon Southern Products, UK).

A glass door oven, model 19 Thelco, was used for the experiments requiring low or high levels of temperature. A 25 W (1200 lumen) fluorescent bulb, positioned about 15 cm from the oven, was used for the samples requiring light. The vials were covered with tin foil when they were stored in dark. All pH measurements were performed using a Solstat EPM 1500 pH meter (Solstat Industries Ltd, Christchurch, NZ) equipped with a microelectrode.

Materials

Doxorubicin hydrochloride and daunomycin hydrochloride (internal standard) were kindly donated by Farmitalia Carlo Erba (Milan, Italy). HPLC grade acetonitrile and methanol were obtained from J.T. Baker. Analytical grade disodium hydrogen phosphate, orthophosphoric acid, potassium dihydrogen phosphate, sodium chloride and sodium hydroxide were supplied by BDH Chemical Co. (Poole UK). Sodium lauryl sulphate (99% pure) was from Sigma Chemical Co. (St. Louis, MO). All glassware was silanised using Aquasil® (Pierce Chemical Co., Rockford, IL) and water was double glass distilled and MilliQ® filtered.

Chromatography

Doxorubicin hydrochloride was quantitated using reversed-phase ion-pair HPLC (25). The mobile phase consisted of 50% v/v acetonitrile-aqueous buffer containing 80 mM sodium lauryl sulphate and 30 mM potassium dihydrogen phosphate. The final pH of the solvent was adjusted to 2.0 with orthophosphoric acid and a flow rate of 2 ml/min was maintained. The excitation and emission wavelengths were fixed at 470 and 580 nm, respectively. The injection volumes varied between 20 to 100 µl. For aqueous samples the detection limit of doxorubicin, based on this method, was ~ 2 ng/ml, with the coefficient of variation ≤ 3%.

Stability study

Table 1 shows the various factors and their levels investigated in the factorial stability study of doxorubicin hydrochloride. The selection of these factors and their levels was based on previous information (12-17, 26). Use of a 2^4 x 3 factorial approach resulted in a total of 48 treatments, which were randomised and investigated according to the protocol listed in Table 2. The initial concentration of doxorubicin hydrochloride (C_o) for all the treatments was fixed at 2.2 µg/ml. This drug concentration has been used in the previous stability studies (17) as well as for the continuous intravenous infusion in cancer patients (11). A treatment time of 8

Table 1

Various factors and their levels investigated in the factorial stability study of doxorubicin hydrochloride

Factors	levels		
	low	medium	high
Temperature	20 ± 2°C (t)		70 ± 2°C (T)
Media	aqueous buffer		organic/aqueous buffer
Light	dark (l)		light (L)
Ionic strength	μ = 0.02 (i)		μ = 0.20 (I)
pH	2	7	11

hr was considered. In preliminary experiments, this combination of initial drug concentration and treatment duration was found to permit analysis of undecomposed drug even under unfavourable conditions.

To prepare an aqueous buffer of low ionic strength (μ = 0.02), 5 mmol disodium hydrogen phosphate and 5 mmol sodium chloride were added to one liter of pure water. The sodium chloride level was increased to 185 mmol for the high ionic strength (μ = 0.2) aqueous buffer. For preparing organic/aqueous buffers, aqueous buffer with ionic strengths of 0.04 and 0.4 were mixed with equal volume of methanol. This was assumed to resemble the ionic strength levels of 0.02 and 0.2, used in the pure aqueous buffers. The media were adjusted to a required pH using 0.1 M orthophosphoric acid or 0.1M sodium hydroxide.

Typically, to a 8.75 ml of a given treatment medium, with appropriate ionic strength and pH, 0.25 ml of stock doxorubicin hydrochloride solution (7.92 mg/100 ml) was added to give a final volume of 9 ml. The tube was briefly vortexed and the resulting solution divided equally into three silanised test tubes. All the three tubes were then subjected to a stress condition as specified for that treatment in Table II. After 8 hr of storage, each tube was covered with a tin foil, 150 μl of daunomycin hydrochloride (1 mg/100ml) was spiked and the samples either analysed immediately or stored at -70°C until analysed within 48 hr. The amount of doxorubicin remaining for a given treatment was calculated based on the average of three determinations. If the variation of these determinations exceeded 5%, further replicated experiments for that treatment were performed. Reproducibility of the experiment, based on four replicates on a treatment, where

the drug was stored at pH 2, under light, in organic/ aqueous buffer of low ionic strength, and at 70°C, demonstrated less than 10% variation.

Analysis of data

The concentration of doxorubicin remaining at the end of the experiments, was determined from a plot of peak height ratio (PHR) of doxorubicin to internal standard versus doxorubicin concentration. The standard curve resulted in the Equation

$$PHR = 0.0035^{*} \text{ doxorubicin concentration (ng/ml)} - 0.0088$$

which was linear (concentration range 0.01 to 2.0 μg/ml) with coefficient of determination greater than 0.99. When PHR was zero, the above equation resulted in a drug concentration of 2.5 ng/ml, which is close to the detection limit of the assay. Use of this figure as amount remaining permitted ready log-log transformation, which is not possible when drug concentration is recorded zero (23).

The model relating the response variable $\ln(\ln C_0 - \ln C)$ and the independent variables temperature, media, light, ionic strength and pH, and all their interactions, was assumed to be additive and analysed using ANOVA (20,21). All statistical analyses were performed using SAS computer package (27).

Results and Discussion

Routine accelerated stability studies involve maintenance of drug samples at various stress conditions, e.g., temperature or pH, and fitting Arrhenius Equation to the derived drug degradation rate constants (24). This requires collection of at least three data points to obtain a meaningful estimate of the degradation rate constant. Conversely, use of the amount remaining at a given time as a dependent variable requires collection of only one data point per treatment (23). This therefore leads to a considerable decrease in the total time required to conduct a stability study. In addition, use of factorial designs make such studies ideal for the determination of the effects of several factors and their interactions on the stability of a given drug (20,21).

In view of the fact that factorial experiments assume additivity of effects of various factors and their interactions, if the decomposition follows first-order kinetics, it is necessary to apply log-log transformation to a given concentration data before subjecting them to ANOVA (23). This transformation linearises the effects of various factors which affect the stability of the drug. A typical additive model used for the ANOVA, e.g., one relating doxorubicin hydrochloride concentration with the potential factors affecting its stability, takes the form (21).

Table 2

Randomised protocol for investigating 48 treatments during factorial stability study of doxorubicin hydrochloride, and the percentage of drug remaining after each treatment.

Treatment	Temperature (°C)	Media	Light	Ionic strength (μ)	pH	Percentage drug remaining[a]
1	70	organic/aqueous buffer	dark	0.02	2	29.69
2	20	organic/aqueous buffer	dark	0.20	2	95.89
3	70	aqueous buffer	light	0.20	2	9.59
4	20	aqueous buffer	dark	0.20	7	36.91
5	70	aqueous buffer	light	0.20	11	0.0[b]
6	70	organic/aqueous buffer	light	0.20	7	13.40
7	20	organic/aqueous buffer	dark	0.02	11	31.81
8	20	organic buffer	light	0.20	7	90.00
9	70	aqueous buffer	light	0.02	2	8.30
10	20	organic/aqueous buffer	dark	0.02	7	100.08
11	70	organic/aqueous buffer	light	0.02	7	42.81
12	20	organic/aqueous buffer	dark	0.20	7	85.91
13	70	aqueous buffer	dark	0.20	7	11.01
14	20	aqueous buffer	dark	0.02	2	91.05
15	70	aqueous buffer	light	0.02	7	18.69
16	70	organic/aqueous buffer	dark	0.02	11	4.23
17	20	organic/aqueous buffer	light	0.20	2	95.39
18	70	organic/aqueous buffer	dark	0.02	7	20.34
19	20	organic/aqueous buffer	light	0.02	2	94.32
20	20	organic/aqueous buffer	dark	0.20	11	12.54
21	70	organic/aqueous buffer	light	0.20	11	0.0[b]
22	70	organic/aqueous buffer	light	0.02	11	0.0[b]
23	70	aqueous buffer	dark	0.02	2	11.38
24	70	organic/aqueous buffer	light	0.20	2	36.68
25	20	aqueous buffer	light	0.02	11	6.01
26	20	aqueous buffer	light	0.02	2	77.88
27	20	organic/aqueous buffer	light	0.02	7	88.55
28	20	organic/aqueous buffer	dark	0.02	2	100.02
29	70	aqueous buffer	light	0.20	7	0.0[b]
30	20	aqueous buffer	dark	0.02	11	5.58

Table 2 Continued.....

Treatment	Temperature (°C)	Media	Light	Ionic strength (μ)	pH	Percentage drug remaining[a]
31	20	aqueous buffer	dark	0.20	11	6.30
32	20	aqueous buffer	light	0.02	7	73.86
33	20	aqueous buffer	light	0.20	2	91.54
34	70	aqueous buffer	dark	0.02	11	0.0[b]
35	20	aqueous buffer	light	0.20	11	5.48
36	70	organic/aqueous buffer	light	0.02	2	58.25
37	70	organic/aqueous buffer	dark	0.20	7	34.39
38	70	aqueous buffer	dark	0.02	7	20.42
39	20	aqueous buffer	dark	0.20	2	81.78
40	20	aqueous buffer	light	0.20	7	83.52
41	70	organic/aqueous buffer	dark	0.20	11	0.0[b]
42	20	organic/aqueous buffer	light	0.20	11	7.74
43	70	organic/aqueous buffer	dark	0.20	2	41.55
44	70	aqueous buffer	dark	0.20	11	0.0[b]
45	70	aqueous buffer	dark	0.20	2	15.60
46	70	aqueous buffer	light	0.02	11	0.0[b]
47	20	aqueous buffer	dark	0.20	7	95.89
48	20	organic/aqueous buffer	light	0.20	11	55.01

[a] Data obtained after storage of drug samples for 8 hr under the specified conditions.

[b] These figures are recorded as zero mainly because the PHR of these samples was zero. However the use of standard curve equation (see Materials and Methods) generated 2.5 ng/ml for zero PHR, which corresponds to 0.11% drug remaining.

$$\ln(\ln C_0 - \ln C) = \mu + \text{temperature} + \text{pH} + \ldots\ldots + \text{pH*temperature} + \ldots\ldots$$

where μ is a constant.

The pH of all the 48 samples was monitored at the end of the treatment period and was found to vary not more than ± 0.15 pH units. Fig. 1 shows the chromatogram of a fresh doxorubicin sample, and a chromatogram obtained from the sample of doxorubicin, following its 8 hr of storage at 70°C in organic/aqueous buffer with ionic strength 0.02 and at pH 7. At least three unidentified peaks, x, y

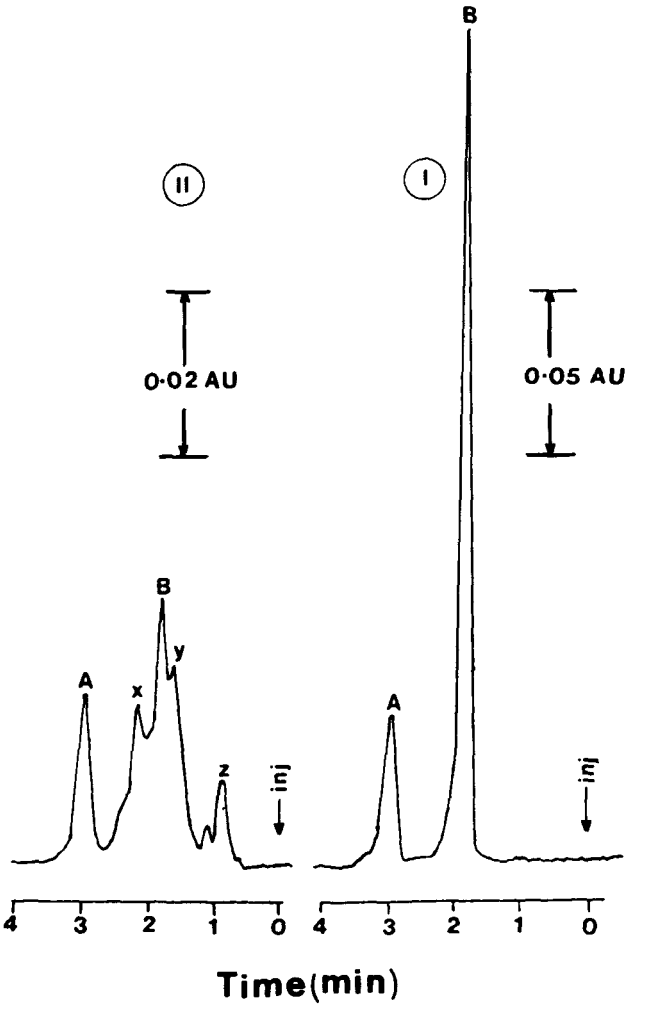

Figure 1

Representative chromatograms of doxorubicin hydrochloride: (I) in organic/aqueous buffer with high ionic strength ($\mu = 0.20$) at pH 7, before subjecting to 70°C; and (II) after 8 hr of storage in organic/aqueous buffer of low ionic strength ($\mu = 0.02$) at pH 7 and 70°C in dark. Peak identification: (A) daunomycin hydrochloride, (B) doxorubicin hydrochloride, (x, y and z) unidentified heat-decomposed products of doxorubicin hydrochloride.

and z, were detected in the samples where the drug was appreciably decomposed (see Fig. 1).

Table 2 lists the percentage of drug remaining undecomposed after subjecting the drug samples to various stress conditions. The drug decomposition varied between 0 to 100%. Two treatments involving the use of low levels of temperature and ionic strength, along with organic/aqueous buffer, in dark, preserved 100% drug after 8 hr of storage (see treatments 10 and 28 in Table 2). Under these two conditions, the use of media with pH of 2 and 7 demonstrated no effect on the stability of drug. However it is interesting to note that under the favourable conditions of temperature, medium and ionic strength, but in presence of light, increase in pH from 2 to 7 increases the drug degradation from 6 to 12% (treatments 19 and 27, Table 2). Hence when the samples of doxorubicin hydrochloride are intended to be exposed to light, it is essential that a medium with low pH be used. These observations are in close agreement with those reported previoulsy (14-16).

Whereas the use of organic/aqueous buffer of low pH and high ionic strength at 20°C and in dark, leads to only 4% degradation of the drug (treatment 2, Table 2), use of aqueous buffer instead increases the decomposition of drug to ~ 19% (treatment 39, Table 2). In general, preservation of ≥ 90% parent drug for up to 8 hr invariably warrants use of low temperature along with low or medium levels of pH (treatments 2,8,10,14,17, 19,28,33 and 47, Table 2). The use of high pH with low temperature however leads to 50 to 95% degradation of the drug. High levels of temperature and pH are by far the the most important factor affecting the stability of doxorubicin. This is apparent from the observation that seven out of eight treatments involving high levels of these factors resulted in total decomposition of the drug. One treatment at these levels preserved approximately 5%of the parent drug after 8 hr of storage (treatment 16, Table 2). This may have been due to comparatively favourable conditions offered to this treatment by one or more of the three factors: organic/aqueous buffer, low ionic strength (μ = 0.02) and/or darkness.

Since the experiments were not repeated, the five-way interaction was used as the error term in the preliminary analysis of data. It was found that all the four-way interactions were not significant. Hence, another analysis was carried out after pooling all the four and five-way interactions into the error term. This increased the degree of freedom for the error term from 2 to 11 and hence increased the sensitivity of the statistical analysis of the data (28). Three main factors were found to be significant at the 5% level. These were (p values in brackets): temperature (0.0000), pH (0.0000) and media (0.0002). The two and three-way interactions which were found to be significant at a similar level were: light-ionic strength

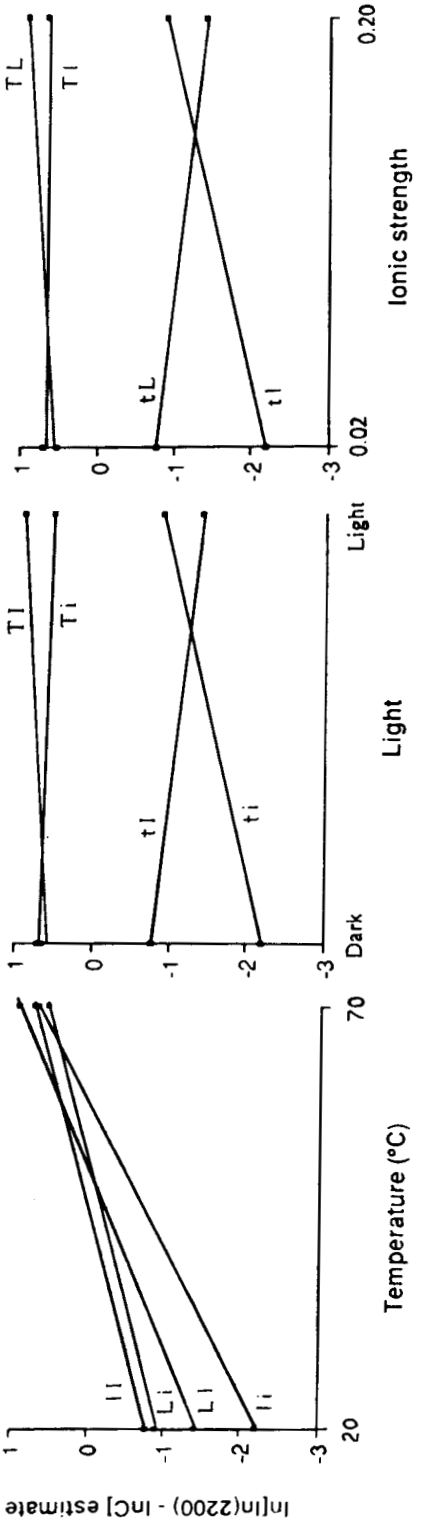

Figure 2

Estimate plots for the three-way interaction between temperature, light and ionic strength. Symbol representation: (l) dark; (L) light; (i) $\mu = 0.02$; (I) $\mu = 0.20$; (t) 20 °C; and (T) 70°C.

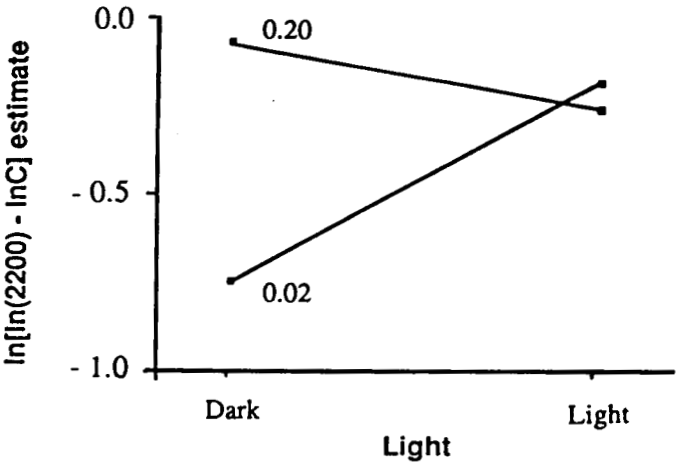

Figure 3

Estimate plots for the two-way interaction between light and ionic strength (μ = 0.02 and 0.20).

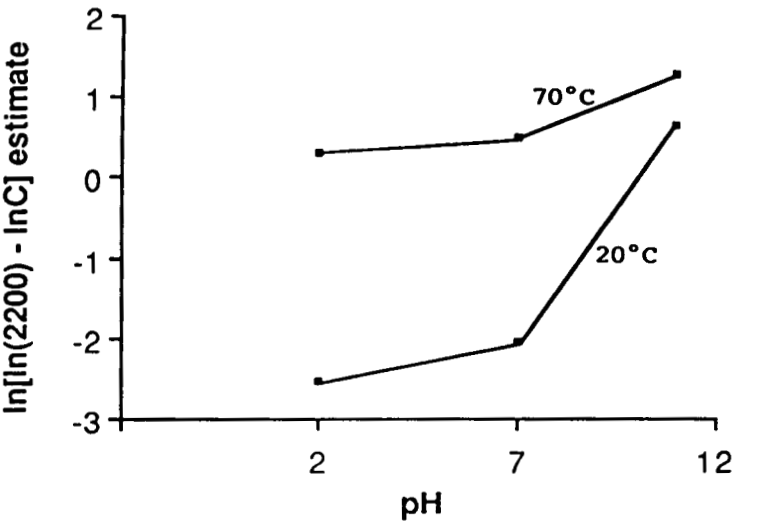

Figure 4

Estimate plots for the two-way interaction between pH and temperature (20 and 70°C).

(0.0216), temperature-pH (0.0001) and temperature-light-ionic strength (0.0016). The analysis of residual plots indicated that the additive model on the response variable $\ln(\ln C_0 - \ln C)$ was appropriate for these data.

The estimates of the dependent variable $\ln(\ln C_0 - \ln C)$ for these significant interactions are depicted in Figs. 2 through 4. The use of upper and lower cases for the different alphabets represent high and low levels of a factor, respectively (see Table 1). Fig. 2 shows that a combination of low temperature, low ionic strength and darkness provides optimum stability of doxorubicin hydrochloride. It is obvious that irrespective of the combinations of light and ionic strength, storage of the drug samples at high temperature results in an unacceptably high degradation of drug. These observations are also reinforced in Fig. 3. Fig. 4 indicates that low pH is favourable for the stability of doxorubicin. At both temperatures (20 and 70°C) the rates of degradation of drug are relatively slow when the pH of the media varies between 2 and 7. However, between pH values of 7 and 11, the drug degrades at an appreciably higher rate. This is particularly true for the samples stored at 20°C. However there is no ready explanation for this observation.

In conclusion, this study indicates that doxorubicin hydrochloride should best be stored in dark in a media with low levels of dielectric constant, ionic strength, pH and temperature.

Acknowledgments

This work is supported by the Medical Research Council grants 84/93 and 86/96. The gifts of doxorubicin hydrochloride (adriamycin hydrochloride) and daunomycin hydrochloride by the Farmitalia Carlo Erba, Milan (Italy) is gratefully acknowledged.

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