ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
17 (1998) 133–140

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

A computer-based expert system designs and analyzes a $2^{(k-p)}$ fractional factorial design for the formulation optimization of novel multicomponent liposomes

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Received 14 April 1997; received in revised form 18 June 1997; accepted 11 July 1997

Abstract

A computer-based technique based on a $2^{(k-p)}$ fractional factorial design was applied for the optimization of recently described multicomponent protective liposomal formulations. These formulations contain sodium ascorbate (vitamin C) as a model drug sensitive to photochemical oxidation, as well as oil red O and/or oxybenzone as oil soluble light absorbers, incorporated into the lipid bilayers and sulisobenzone as a water soluble light absorber incorporated into the aqueous phase of liposomes. The three light absorbers (present or absent) incorporated in multilamellar liposomes and the drug in free or in complexed with α-cyclodextrin form comprised the four factors of the system. The stabilization ratio and the percentage entrapment in the liposomes of the vitamin were the two response variables of the system to be optimized. The entrapment values were calculated for all the materials either spectrophotometrically or by using second order derivative spectrophotometry. The response variables were predicted by multiple regression equations comprising combinations of the four formulation factors. Both the higher entrapment and the higher protection for the drug should characterize the optimum formulation.

Keywords: Experimental design; Fractional factorial design; Optimization; Sodium ascorbate; Vesicular systems; Analytical techniques

1. Introduction

Drugs sensitive to photochemical oxidation are known to degrade on exposure to light and oxygen and lose their activity. Such drugs when used topically for medical or cosmetic reasons must be formulated in such a way so as to achieve maximum stability. Known stabilizing systems from the literature include the use of certain antioxidants and light absorbers in the same preparation with the drug or the use of cyclodextrins as complexing system, providing moderate stability against the examined external factors (light and oxygen). We have recently proposed [1–3] a novel multicomponent stabilizing system based on liposomes, which provides high protection to sensitive

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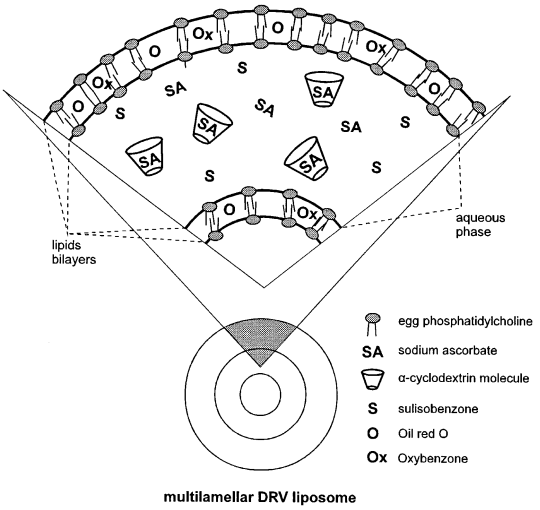
0731-7085/98/$19.00
PII S0731-7085(97)00173-8

drugs. This system is based generally, on the known ability of liposomes to accommodate both hydrophobic and hydrophilic substances into their lipid membranes and their aqueous phases respectively. In brief, multilamellar liposomes consisted of phosphatidylcholine and cholesterol entrap the water-soluble sensitive drug, as such or in the form of a cyclodextrin complex, in the aqueous phase and one or more light absorbers either in the aqueous phase or in the lipid bilayers, depending on their characteristics.

In the present study, sodium ascorbate (vitamin C) was chosen as a model drug sensitive to photochemical oxidation with a rapid decomposition on exposure to light and air ($t_{50\%} = 0.9$ h) [4]. In order to increase the stability of the vitamin, sodium ascorbate was entrapped as such or in the form of an α-cyclodextrin complex in dehydration-rehydration multicomponent liposomes containing one or more of the light absorbers oil red O, oxybenzone and sulisobenzone (Scheme 1). A liposomal formulation can be characterized as being efficient when it contains the vitamin in high entrapment value with the higher stabilization ratio (the ratio k_0/k_L, where k_0 and k_L are the degradation rate constants of the vitamin in free form and after being entrapped in liposomes respectively).

From the four factors mentioned above (the presence/absence of the α-cyclodextrin cavity, oil red O, oxybenzone and sulisobenzone), each one reporting different behavior on the two responses of interest (stabilization ratio and %entrapment of the vitamin), it is not obvious how the optimum formulation can be achieved. In the present study, the experimental design [5] can be used in order to derive valid and robust statistical significance tests for the examined factors with a minimum number of experiments. It is sufficient to consider the factors affecting the responses at two levels; for instance the concentration of each light absorber may either be set zero or to a constant molar ratio with the vitamin, the vitamin may either be in free or complexed form (Table 1). The most intuitive approach to study these factors and how they affect the examined responses, would be to vary the factors of interest in a 2^k full factorial design



Scheme 1.

(k factors at two level), that is, to try all possible combinations. This would work fine, except that the number of necessary liposomal preparations will increase geometrically. For example, the four factors examined in the present study requires $2^4 = 16$ preparations. Because each liposomal preparation is time-consuming and requires costly materials, the use of a $2^{(k-p)}$ fractional factorial design [6] will reduce considerably the number of preparations (from 16 preparations to eight, in the present case of four factors at two levels each).

Table 1
Low and high settings (levels) for the four examined factors

Factor name		Factor Setting	
		Low	High
1. Free-complex	Q*	Free	Complex
2. OilredO	C*	Out	In
3. Oxybenzone	C	Out	In
4. Sulisobenzone	C	Out	In

* The letters Q and C denote a qualitative factor (cannot be varied continuously) and a continuous factor (can be varied continuously) respectively.

2. Experimental section

2.1. Materials and instrumentation

Sodium ascorbate (SA) and α-cyclodextrin (αCD) were obtained from Aldrich Chemical (Poole, Dorset, UK). Oil red O, oxybenzone, sulisobenzone and cholesterol were from Sigma Chemical (Poole, Dorset, UK). Phosphatidylcholine (PC) was from Lipids Products (Nuthill, Surrey). All other reagents were of analytical grade. Double distilled water was used throughout. Photooxidation studies of SA were carried out using a Blak-Ray longwave (365 nm) UV lamp with 6 W rating and 460 μW cm^{-2} dm^{-1} intensity (model UVGL-58, UVP, San Gabriel, USA). Measurement of SA degradation kinetics in various preparations and assay of the components entrapped into liposomes were carried out in a Compuspec UV/visible spectrophotometer (Wallac) connected to a personal computer which can also analyze the spectra to their derivatives.

2.2. Preparation of SA:αCD complex and multilamellar liposomes

The inclusion complex of SA with αCD was prepared according to the freeze-drying method [7]. Multilamellar liposomes were prepared according to the dehydration-rehydration method with some modifications: Briefly, small unilamellar vesicles (SUV) prepared from equimolar PC and cholesterol were mixed with SA (free or complexed) dissolved in deionized water, diluted to 10 ml with water and freeze-dried overnight. The dry powder was subjected to controlled rehydration and then centrifuged at $27\,300 \times g$ for 20 min to separate the entrapped and non-entrapped SA. The liposomal pellet containing multilamellar dehydration-rehydration vesicles (DRV) was washed three times by centrifugation in 0.1 M sodium phosphate buffer containing 0.9% NaCl, pH 7.4 (PBS) and resuspended in 4 ml PBS before use. DRV liposomes incorporating the vitamin and the lipid soluble light absorbers in their lipid bilayers were prepared as above with the absorbers and the lipids dissolved in chloroform, prior to the generation of the SUV precursor vesicles. When the water-soluble light absorber sulisobenzone was also entrapped into DRV liposomes, this was dissolved together with free or complexed SA in the aqueous solution to be subsequently mixed with SUV.

2.3. Estimation of liposome-entrapped materials

Entrapment values for SA and light absorbers were estimated by measuring the concentrations of materials in both the obtained DRV liposomal pellets and the separated pooled supernatants by derivative UV spectroscopy [8]. In the present work, the use of the second-order derivative (D2) of the spectra was found to provide both good resolution and high signal-to-noise (S/N) ratios.

2.4. Photooxidation studies

The photooxidation of SA into the different DRV formulations exposed to UV light was calculated spectrophotometrically ($\lambda_{max} = 265$ nm). The assay briefly is as follows: The liposomal suspension of SA (3 ml) was transferred into an open quartz cuvette and was placed in front of the UV lamp. The liposomal suspension was stirred continuously in order to be homogenous during the study and in order for the whole suspension to be equally irradiated. At time intervals, 100 μlt of the liposomal suspension were dialyzed with 200 μlt isopropanol and the resultant clear solution was diluted to 3 ml with water and was measured at 265 nm.

2.5. $2^{(k-p)}$ fractional factorial design at 2 levels

In the present study, the above-mentioned four factors are examined in two levels and how they affect two different responses (stabilization ratio and %entrapment value) (Table 1). This specific design is described as a $2^{(4-1)}$ design of resolution IV (four) [9]. This means that we study $k = 4$ factors overall (the first number in parentheses); however, $p = 1$ of those factors (the second number in parentheses) was generated from the interactions of a full $2^{((4-1)=3)}$ factorial design. As a result, the design does not give full resolution; that is, there are certain interaction effects that

are confounded with (identical to) other effects. In this design of resolution IV can be concluded that no main effects of the examined factors are confounded with any other interaction of order less than $R = 4 - 1 = 3$. In this design then, main effects are not confounded with two-way interactions, but only with three-way interactions. Also, no two-way interactions are confounded with any other interaction of order less than $R = 4 - 2 = 2$. Thus, the two-way interactions in this design are confounded with each other. In the present study, the calculation of the variability of measurements (pure error), through whole of partial replications, is omitted in order to simplify the study [10].

We used a statistical software package [11] with experimental design capabilities to perform the calculations and to illustrate all the interactive graphics. The eight formulations listed in Table 1 were evaluated in random order to nullify the effect of extraneous or nuisance variables. After the two responses (Table 2) had been collected, the system was ready for analysis.

Table 2
Eight liposomal formulations in randomized order and the estimated responses

Case	(Factor 1) Free-complex	(Factor 2) OilredO	(Factor 3) Oxybenzone	(Factor 4) Sulisobenzone	(Response 1) Stabilization ratio	(Response 2) %Entrapment
1	Complex	In	In	In	136	7
2	Complex	In	Out	Out	105	16
3	Complex	Out	In	Out	55	18
4	Complex	Out	Out	In	18	8
5	Free	In	In	Out	90	61
6	Free	In	Out	In	69	32
7	Free	Out	In	In	25	31
8	Free	Out	Out	Out	5	65

3. Results and discussion

3.1. Calculation of entrapped materials

The interest for the entrapment values is concentrated not only on SA but also on the light absorbers since their entrapment values affect the stability and probably the entrapment value of SA. In the present study, the pellets were dissolved with isopropanol and the resulting solutions were calculated by derivative UV spectrophotometry for SA and the light absorbers. Also, the pooled supernatants were measured for the unentrapped materials by disruption of possible small unilamellar vesicles (SUV) and solubilization of the unentrapped light absorbers with isopropanol. The three combined supernatants were, also, measured by derivative spectrophotometry. The entrapment values for each compound were calculated according to the formula:

$$\%\text{entrapment} = \frac{A_P}{A_P + A_S} 100$$

Table 3
Estimated factors main effects and the coefficients for the predictive mathematical models

Factor	(Response 1) Stabilization ratio		(Response 2) %entrapped SA	
	Effect	Coefficient	Effect	Coefficient
Mean/Intercept	62.875	62.875	29.750	29.750
1. Free-Complex	31.250	15.625	−35.000	−17.500
2. Oil red O	74.250	37.125	−1.500	−0.750
3. Oxybenzone	27.250	13.625	−1.000	−0.500
4. Sulisobenzone	−1.750	−0.875	−20.500	−10.250

where A_P is the absorbance of the materials in the pellets and A_S is the absorbance of non-entrapped materials in the pooled supernatants, after a dilution correction to achieve identical dilutions for both A_P and A_S. Specifically, in the liposomal preparation no 1 in Table 2, where all the compounds are present, the entrapment values for all the compounds were calculated indirectly according to the formula:

$$\%\text{entrapment} = \frac{A_0 - A}{A_0} 100$$

where A_0 is the absorbance of the initial concentration of materials and A denotes the absorbance of non-entrapped materials in the organic and aqueous phases (obtained on extraction of the combined supernatants with chloroform) after a dilution correction to achieve identical dilutions for both A_0 and A (the procedure is described in details elsewhere) [12].

3.2. Determination of the four factors on the two responses

3.2.1. Check of main effects and anova results

Table 2 presents the eight runs (liposomal preparations) in random order and the calculated two different responses. After the calculations, the system is ready for analysis beginning with the calculation of the main effects of the factors (the design is of resolution IV; hence the two-way interactions are confounded each other and they cannot be estimated from this design). In Table 3, the first numeric column for each response contains the effect estimates that can be interpreted as deviations of the mean of the negative settings from the mean of the positive settings for the respective factor (15). For example, if the vitamin is entrapped in complexed form instead of the free form, then we can expect an improvement of the stabilization ratio by 31.25 and a decrease of the entrapment value by 35 (Table 3; negative values for the effects denote a decrease of the response value). Furthermore, the presence of oil red O increases the stabilization ratio by 74.25 and does not change significantly the entrapment value of the vitamin (Table 3). The second numeric column for each response in Table 3 contains the effect coefficients. These are the coefficients that could be used for the prediction of each response for new factor settings, via the linear equation:

$$y_{\text{pred.}} = b_0 + b_1x_1 + \ldots b_4x_4$$

where $y_{\text{pred.}}$ stands for the predicted response (stabilization ratio or %entrapment), x_1 through x_4 stand for the settings (1 through 4), b_1 through b_4 are the respective coefficients and b_0 stands for the intercept or mean. For this design the main effect estimates does not show the standard errors, because this is a saturated design [13], where all degrees of freedom (i.e. information) is used to estimate the factors main effects and no independent assessment of the error variance is available.

After the estimation of the factors main effects, the determination of the significant factors affecting the dependent variables of interest (responses) is following by performing ANOVA for each response separately (Table 4 and Table 5). In these Tables the sum of squares (SS) are the information that was used up to estimate the factor effects, the F-ratios (F) are the ratio of the

Table 4
ANOVA for the stabilization ratio

Factor	SS	df	MS	*F*	*p*
1. Free-Complex	1953.13	1	1953.13	20.6044	0.020018
2. OilredO	11 026.13	1	11 026.13	116.3196	0.001705
3. Oxybenzone	1485.13	1	1485.13	15.6673	0.028788
4. Sulisobenzone	6.13	1	6.13	0.0646	0.815772
Error	284.37	3	94.79		
Total SS	14 754.88	7			

R-sqr = 0.98073.

respective mean-square-effect and the mean-square-error. Furthermore, because the factors in this study have two levels, each ANOVA main effect has 1 degree of freedom (df). Finally, from the p values it appears when the main effect of each factor is statistically significant ($p < 0.05$) or marginally significant ($p < 0.10$).

Therefore, the ANOVA data for the first response (Table 4) support the conclusion that, indeed, factors 1, 2 and 3 significantly affect the stabilization ratio of the vitamin. As we can see the same three factors show the largest parameter estimates (Table 3); thus the settings of these three factors were most important for the resultant stabilization ratio. This means that the vitamin expresses the highest stability when in the complexed form (SA:αCD) is entrapped in the aqueous phase of liposomes containing oil red O and oxybenzone in their bilayers. Similarly, from the ANOVA in Table 5 it appears that the factors 1 and 4 are the only important for the %entrapment values of the vitamin, meaning that the liposomal formulation composed from 1:1 egg PC and cholesterol provide highest entrapment values when the vitamin is in the free form and the hydrophilic sulisobenzone is absent. From the above observations, the formulator can easily conclude that the presence of the two hydrophobic light absorbers in a liposomal formulation containing the vitamin in free form provide both the better stability and the higher entrapment value. Also, the presence of the hydrophilic sulisobenzone adds little to the overall stability decreasing at the same time the entrapment value considerably. Finally, if the main scope is the highest stability, then the vitamin can be used in complexed form 'sacrificing' the highest entrapment value.

3.2.2. Diagnostic plots of residuals and pareto charts of effects

From the ANOVA tables we have concluded to particular 'models' that include a particular number of effects for each of the two responses (see above). We should also examine the distribution of the residual values [10], which are the differences between the predicted values (as predicted by the current models) and the observed values. Fig. 1 presents the normal probability plot of residuals for each response separately, by assessing how closely the set of observed values follow a theoretical distribution. Since all values fall onto a straight line it can be concluded that they follow the normal distribution.

Another useful plot for identifying the factors that are important is the Pareto chart of effects (Fig. 2). This graph will show the ANOVA effect estimates plotted against the horizontal axis. This plot will also include a vertical line to indicate the $p = 0.05$ threshold for statistical significance (an effect that exceeds the vertical line may be considered significant).

3.2.3. Normal probability plot of effects

Another useful, albeit more technical summary graph, is the normal probability plot of effects [10] which is constructed as follows (Fig. 3): First, the effect estimates are rank ordered. From these ranks, z values (i.e. standard values of the normal

Table 5
ANOVA for the percentage entrapment value

Factor	SS	df	MS	*F*	*p*
1. Free-Complex	2450.000	1	2450.000	29.81744	0.012069
2. OilredO	4.500	1	4.500	0.05477	0.830028
3. Oxybenzone	2.000	1	2.000	0.02434	0.885928
4. Sulisobenzone	840.500	1	840.500	10.22921	0.049396
Error	246.500	3	82.167		
Total SS	3543.500	8			

R-sqr = 0.93044.

distribution) can be computed based on the assumption that the estimates come from a normal distribution with a common mean. These z values are plotted on the left Y-axis in the plot, and the corresponding normal probabilities are shown on the right Y-axis in the plot. If the actual estimates (plotted on the X-axis) are normally distributed, then all values should fall onto a straight line in the plot. This plot is very useful for separating random noise from 'real' effects. The estimates for effects that are actually zero in the population will assume a normal distribution around a common mean of zero; effects that truly exist will be shown as outliers. In Fig. 3a the point for the oil red O and in Fig. 3b the point for the free-complex main effects appear different from the other effects.

In the present study, after completing the proposed design, it is becoming evident, from the

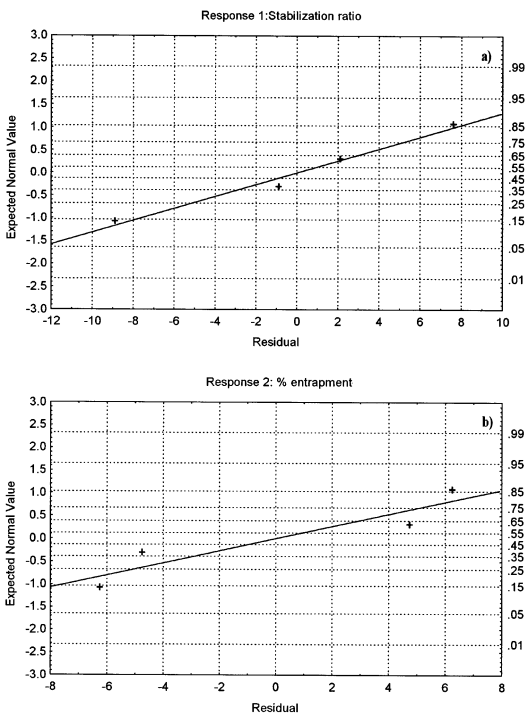


Fig. 1. Normal probability plots of residual values for the stabilization ratio (a) and the percentage entrapment (b).

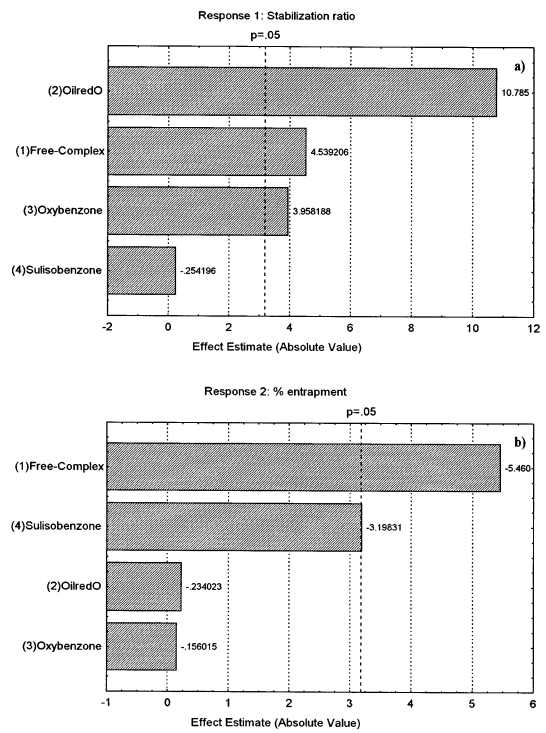


Fig. 2. Pareto charts for the factors main effect on stabilization ratio (a) and on percentage entrapment (b).

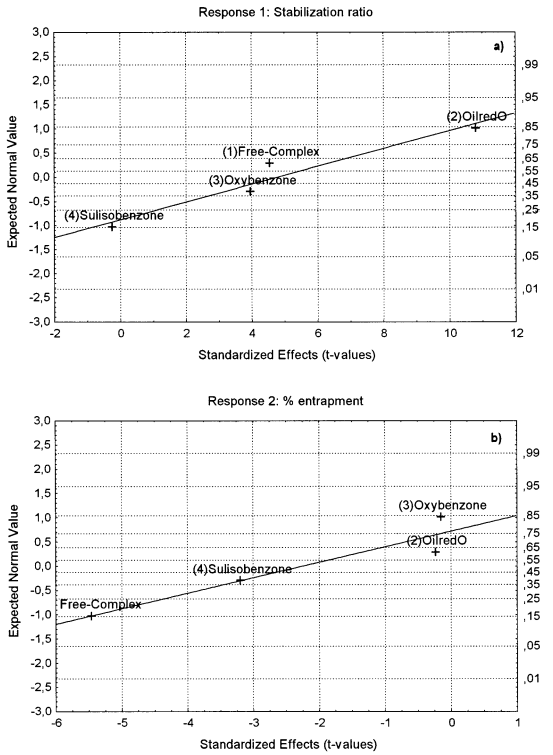


Fig. 3. Normal probability plot of factors main effects on the stabilization ratio (a) and on percentage entrapment (b).

ANOVA tables and the graphical representations, that if the major purpose of a liposomal preparation is to increase the stability of the entrapped material, then the formulator has to use the complexed form of this material together with oil red O (Table 2, case 2). The addition of sulisobenzone in this preparation (Table 2, case 1) almost halved the entrapment value without any further, significant, increase in the stabilization ratio and thus, it can be avoided. On the other hand, if the major purpose of the preparation is to maximize the entrapment value then the formulator has to entrap the material in its free form with oil red O and oxybenzone (Table 2, case 5) and to omit sulisobenzone. In the same manner, the other cases in Table 2 could be evaluated, with the same simplicity and could be used in specific tailored circumstances. All the above conclusions were drawn on the basis of the results extracted from only eight preparations needed for the fractional factorial instead of sixteen would needed for the full factorial design.

In conclusion, such multicomponent liposomal formulations may include more factors during their preparation (i.e. the lipid:cholesterol molar ratio, the presence of a second lipid and its molar ratio to the first lipid, different combinations of light-absorbers, other cyclodextrins for the complexation of the drug, different preparation method for the liposomes to name some of them) making the interpretation of the system extremely complicated. In order for all the factors to be used at their optimal level and the best responses to be achieved, a lot of experiments must be performed, including all the possible combinations between the different factors. The use of a fractional factorial design, as described in the present study, can decrease the number of experiments, give useful conclusions for the main effects and interactions between the examined factors, and clarify complicated interactions through graphical representations.

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