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A Plackett–Burnam screening design directs the efficient formulation of multicomponent DRV liposomes

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Abstract

A computer-based technique was applied for the optimization of recently described multicomponent protective liposomal formulations. These formulations contain riboflavin in either free form or complexed with γ-cyclodextrin as a model drug, sensitive to photochemical degradation, as well as various light absorbers and antioxidants incorporated into the lipid bilayer and/or the aqueous phase of liposomes. During the liposomal preparation, a series of 11 factors were isolated as important to affect their effectiveness as stabilization systems. These factors were related, first, to the composition of liposomes and, second, to variations during the preparation procedure. The Plackett–Burnam design described in this study was applied for the isolation of the significant factors in order to concentrate more on them. The stabilization ratio of the vitamin was the response variable of the system to be optimized. In order to assure the presence of the examined components in liposomes, the entrapment values were calculated for all the materials, either spectrophotometrically or using second-order derivative spectrophotometry. The optimum formulation should be characterized from the higher protection of the drug.

Keywords: Screening designs; Plackett–Burman; Liposomal preparations; Stability

1. Introduction

Drugs sensitive to ultraviolet radiation are known to degrade on exposure to light losing their activity. Such drugs, when they are going to be used topically for medical or cosmetic reasons, must be prepared 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 a complexing system that also provides 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 drugs. This system is based generally on the known ability of liposomes to accommodate both hydrophobic and hydrophilic

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substances into their lipid membranes and their aqueous phases, respectively. In brief, multilamellar liposomes consisting 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, riboflavin was chosen as a model photosensitive drug with a rapid decomposition on exposure to light ($t_{50\%} = 0.5$ h) [4]. In order to increase the stability of the vitamin, it was entrapped as such or in the form of a γ-cyclodextrin complex in multilamelar liposomes containing one or more of the light absorbers oil red O, oxybenzone, dioxybenzone, sulisobenzone and the antioxidant β-carotene. The multilamelar liposomes were prepared either by the dehydration–rehydration technique or by the disruption of lipid film method containing cholesterol in low or high concentration, DSPC as an alternative lipid, and sonicated through a bath or probe sonication for a low or higher period of time. All these variations comprise the 11 factors that directly affect the physical stability of liposomes as well as the chemical stability of the entrapped vitamin. A liposomal formulation can be characterized as being efficient when achieving the highest 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 in liposomal formulations, respectively). From the aforementioned facts, it is becoming evident that the design of efficient liposomal preparations is a multivariate procedure in which many factors could affect the desired liposomal properties. The formulator is that it is impossible to guess the effects of these factors on the final results. Furthermore, it is impossible to isolate the significant from the insignificant factors, a fact that could diminish dramatically the number of the experiments, which are in certain cases highly costly and time consuming. In the literature there is no other effort to screen so many involved factors during liposomal preparations, using a Plackett–Burnam design (PBD). As will be concluded later in this study, the application of the described screening design will extract valuable information for the efficient design of liposomes with the lowest number of experiments.

From the aforementioned 11 factors (Table 1), each one reporting different behavior on the response of interest (stabilization ratio 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 response 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; and the vitamin may either be in free or complexed form (Table 1). An intuitive first approach to study these factors and how they affect the examined response would be a PBD. To perform a full factorial design for the examination of 11 factors, at two levels for each one, it would be necessary to prepare $2^{11} = 2048$ liposomal formulations. Because each liposomal preparation is time consuming and requires costly materials, the use of a PBD [6] can reduce considerably the number of preparations, from 2048 preparations

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

Factor name	Factor setting	
	Low	High
1) Free-complex, Q[a]	Free[b]	Complex[b] (0.2 mmol)
2) Oil red O, C[a]	Out	In (0.2 mmol)
3) Oxybenzone, C	Out	In (0.2 mmol)
4) Deoxybenzone, C	Out	In (0.2 mmol)
5) Sulisobenzone, C	Out	In (0.2 mmol)
6) β-Carotene, C	Out	In (0.2 mmol)
7) DRV or MLV, Q	DRV	MLV
8) Cholesterol, C	0.5 mmol	1 mmol
9) DSPC, C	Out	In (0.5 mmol)
10) Sonication time, Q	Low	High
11) Sonication type, Q	Probe	Bath

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

[b] In all the liposomal preparations, egg PC and cholesterol were kept at 1 mmol and R (free or complexed) at 0.1 mmol.

to 12 in the present case of 11 factors at two levels each. Of course, the amount of information is not the same performing 12 instead of 2048 experiments but, on the other hand, the time and cost to perform 2048 experiments is not comparable with that of performing 12 experiments. More specifically, with the PBD one will have an estimate of the factors main effects only and no other information concerning higher order interactions.

2. Experimental

2.1. Materials and instrumentation

Riboflavin (R) and γ-cyclodextrin (γCD) were obtained from Aldrich Chemical Company (Poole, Dorset, UK). Oil red O, oxybenzone, dioxybenzone, sulisobenzone, β-carotene and cholesterol were from Sigma Chemical Company (Poole, Dorset, UK). Phosphatidylcholine (PC) and DSPC were from Lipid Products (Nuthill, Surrey). All other reagents were of analytical grade. Double-distilled water was used throughout. Photostability studies of R 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 R degradation kinetics in various preparations was performed fluorometrically (excitation, 445 nm; emmission, 520 nm) and assays of the components entrapped into liposomes were carried out in a Compuspec UV/visible spectrophotometer (Wallac) connected to a personal computer that can also analyze the spectra to their derivatives.

The inclusion complex of R with γCD was prepared according to the freeze-drying method [2]. Multilamellar liposomes were prepared according to the dehydration–rehydration method [3]. Entrapment values for R and light absorbers were estimated by measuring the concentrations of the materials in both the obtained DRV liposomal pellets and the separated pooled supernatants fluorometrically for R and by derivative UV spectroscopy for the rest of the components [3]. Derivative UV spectrophotometry is a useful technique for the analysis of multicomponent systems with extensive absorbance overlaps and spectra without a clear maximum. Derivative UV spectrophotomerty is a useful technique for the analysis of multicomponent systems with extensive absorbance overlaps and spectra without a clear maximum. The photostabilization of R into the different DRV formulations exposed to UV light was calculated fluorometrically [3].

2.2. Plackett–Burman screening designs

PBD [6,7] are used to investigate $n-1$ variables in n experiments proposing experimental designs for more than seven factors, and especially for $n \times 4$ experiments, i.e. 8, 12, 16, 20, etc., that are suitable for studying up to 7, 11, 15, 19, etc. factors, respectively. One useful characteristic is that the sample size is a multiple of 4 rather than a power of 2. There are no two-level fractional factorial designs with sample sizes between 16 and 32 runs. However, there are 20-run, 24-run, and 28-run PBDs. In some cases, where $n \times 4 = 2^k$, the PBD is a specific fraction of a full factorial design, and saturated fractional factorial designs can be used as well. However, this is not the case for multiples of 4 that are not equal to the power of 2. The main effects are orthogonal and two-factor interactions that are only partially confounded with main effects. This is different from the resolution three-fractional factorial, where two-factor interactions are indistinguishable from main effects. Let us consider the case of 12 experiments for 11 factors as happens in the present study. The PBDs have the particularity that they are cyclical. Consider, for example, the 11-factor, 12-experiment design. It is obtained from a first line, which describes the first experiment and in this case is [+ − + − − − + + + − +] where the sign + denotes the factor in its high level and the sign − denotes the factor in its low level. To make the second line, we move the minus sign at the far right to the beginning of the next line and slide the rest of the signs one place. Experiments 2–12 are obtained by writing down all cyclical permutations of this line. The last experiment, 12, always contains only minus signs (all the factors at their low levels). The complete design is therefore presented in Table 2. It is also

Table 2
Twelve liposomal formulations and the estimated responses

	Complex	Oil red O	Oxybenzone	Deoxybenzone	Sulisobenzone	Carotene	DRV or MLV	Cholesterol	DSPC	Sonication time	Sonication type	Stabilization ratio
1	Complex	Out	In	Out	Out	Out	MLV	High	In	Low	Bath	50
2	Complex	In	out	In	Out	Out	DRV	High	In	High	Probe	270
3	Free	In	In	Out	In	Out	DRV	Low	In	High	Bath	130
4	Complex	Out	In	In	Out	In	DRV	Low	Out	High	Bath	90
5	Complex	In	Out	In	In	Out	MLV	Low	Out	Low	Bath	160
6	Complex	In	In	Out	In	In	DRV	High	Out	Low	Probe	260
7	Free	In	In	In	Out	In	MLV	Low	In	Low	Probe	150
8	Free	Out	In	In	In	Out	MLV	High	Out	High	Probe	14
9	Free	Out	Out	In	In	In	DRV	High	In	Low	Bath	24
10	Complex	Out	Out	Out	In	In	MLV	Low	In	High	Probe	60
11	Free	In	Out	Out	Out	In	MLV	High	Out	High	Bath	110
12	Free	Out	Out	Out	Out	Out	DRV	Low	Out	Low	Probe	6

possible to verify that each factor is examined at six + and six − levels, and it is also possible to verify that the main factor is not confounded when the effects are determined in the following way:

$$\text{Effect} = 1/6\left[\sum(y \text{ at} + \text{level}) - \sum(y \text{ at} - \text{level})\right]$$

In a previous study [8], this was described as a geometric 2^{k-p} fractional factorial design where the interactions were confounded with the main effects or with each other. In the case of the geometric designs (as the 2^{k-p} fractional factorials), each interaction is totally confounded with one of the main effects. Aliasing in the non-geometric 12 runs (as in the present study), experimental designs are rather different. For example, the effect of the interaction X_1X_2 is partially confounded with all of the main effects except those of X_1 and X_2. Therefore, with these designs, there is a reduced likelihood of drawing a wrong conclusion on the main effects where there is one important interaction. Analysis taking interactions into account is quite complex [9].

Comparing further PBD with the fractional factorial designs (FFD), it should be noted that PDB are used when there are more than seven factors, while FFD could be used in situations with less factors. Using the FFD design of reference [8] with Resolution IV, we would need to perform 64 runs, almost five times more experiments, with the gain of the effects of some two-way interactions, not necessary in the present case of study. If we wanted to find a modeling equation for predicting the performance of liposomes then we had to isolate the significant factors from the PBD and then to transfer these factors on a FFD to examine the modeling procedure. In other words, in multivariate systems (such as the liposomal systems), the PDB should be used before any other FFD. Furthermore, it should be noted that in PBD one should use dummy factors [7,10], something not recommended in FFD, while FFD are more flexible during the fold-over procedure, something not appropriate in PBD. The 12 formulations suggested by PBD, which are presented in Table 2, were evaluated in random order to nullify the effect of extraneous or nuisance variables. After the responses (Table 2) had been collected, the system was ready for analysis.

3. Results and discussion

3.1. Calculation of entrapped materials

The interest for the entrapment values is concentrated not only on R, but also on the light absorbers since their entrapment values affect the stability and probably the entrapment value of R. In the present study, the pellets were first disrupted with isopropanol and the resulting solutions calculated fluorometrically for R and by UV derivative for the light absorbers. Secondly, 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 fluorometrically and by UV derivative. The entrapment values for each compound were calculated according to the procedure that is described in detail elsewhere [3,11].

3.2. Determination of the 11 factors on the two responses

3.2.1. Pareto chart of effects

A useful plot for identifying the factors that are important is the Pareto chart of effects (Fig. 1). This graph will show the factors main effect estimates plotted against the horizontal axis. The factors main effects are rank ordered according to their significance and, if there is an estimate of error variability available (standardized effects), this chart will include a vertical line to indicate the $P = 0.05$ threshold for statistical significance. In the present study, there are no degrees of freedom left to estimate the error variability; therefore, the plot for standardized effect is not produced. A common way to obtain an estimate of error variability is to pool some insignificant effects into error (see later). After completing the 12 runs, the Pareto chart for the stabilization ratio showed that the most significant effect is the presence of the light absorber oil red O and the

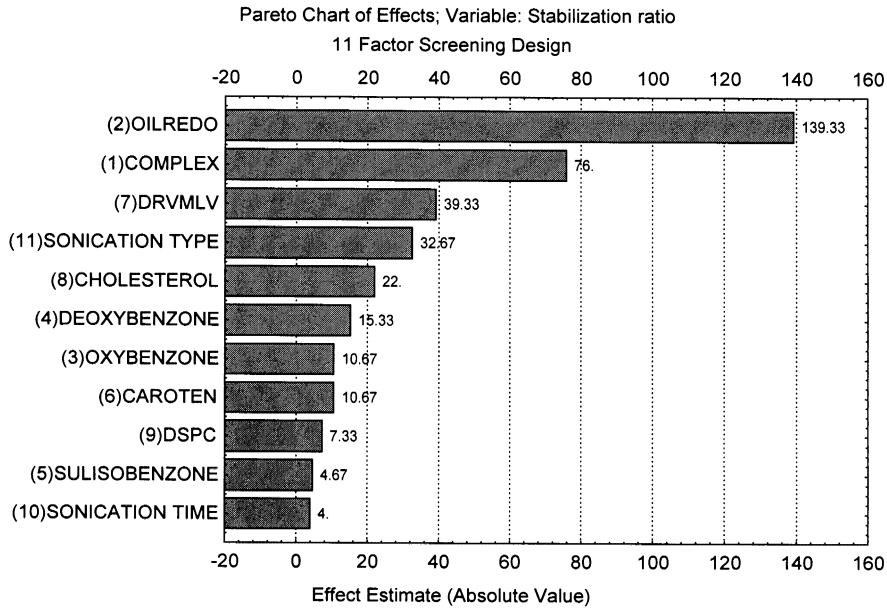

Fig. 1. Pareto charts for the factors main effect on stabilization ratio and on percentage entrapment.

second most important factor is the complex with the γ-cyclodextrin form of the vitamin. The third most important factor is the preparation method and indicates that the DRV is superior to that of MLV preparation method. The molar ratios of cholesterol as well as the presence of the rest light absorbers do not play an important role in the stabilization of vitamin. The two most insignificant factors, as becoming evident from the Pareto chart, are the duration of sonication and the presence of the light absorber sulisobenzone, and so it is decided these two factors should pool their effects into error.

3.2.2. Regression coefficients and analysis of variance results

After the collection of the 12 runs and the calculated responses (Table 2), the system is ready for analysis beginning with the calculation of the regression coefficients. These are the coefficients that could be used for the prediction of each response for new factor settings, via the multiple linear equation:

$$y_{\text{pred}} = b_0 + b_1x_1 + \ \ldots \ + b_{11}x_{11}$$

where y_{pred} stands for the predicted response (stabilization ratio), x_1–x_{11} stand for the settings (1–11), b_1–b_{11} are the respective coefficients, and b_0 stands for the intercept or mean. For this design, the main effect estimates do not show the standard errors, because this is a saturated design [12,13] where all degrees of freedom (i.e. information) are used to estimate the factors main effects and no independent assessment of the error variance is available. In the present study of 12 experiments, we have to calculate 11 parameters (effects) plus a constant term. There is a unique solution to the problem and the results themselves do not give any evidence of the precision of the results. Of course, the experimental data are not exact so the calculated parameters are not exact either, but are estimates of the true values b_i. If we define the imprecision of each experimental result by a standard deviation σ, then the corresponding standard deviation of the estimate b_i can then be calculated as $\sigma_b = \sigma/\sqrt{12}$.

After the estimation of the factor regression coefficients, the determination of the significant factors affecting the dependent variables of interest (responses) is following by performing analysis of variance (ANOVA) (Table 3). It is obvious

Table 3
ANOVA for the stabilization ratio

Factor	SS[a]	Degrees of freedom	MS[b]	*F*	*P*
(1) Complex	17328.00	1.00	17328.00	–	–
(2) Oil Red O	58241.33	1.00	58241.33	–	–
(3) Oxybenzone	341.33	1.00	341.33	–	–
(4) Deoxybenzone	705.33	1.00	705.33	–	–
(5) Sulisobenzone	65.33	1.00	65.33	–	–
(6) Carotene	341.33	1.00	341.33	–	–
(7) DRV or MLV	4641.33	1.00	4641.33	–	–
(8) Cholesterol	1452.00	1.00	1452.00	–	–
(9) DSPC	161.33	1.00	161.33	–	–
(10) Sonication time	48.00	1.00	48.00	–	–
(11) Sonication type	3201.33	1.00	3201.33	–	–
Error	0	0			
Total SS	86526.667	11			

[a] Sum of squares.
[b] Mean square effect.

from Table 3 that the *F* statistics and *P* values are not available since all the available degrees of freedom were used for the calculation of the factors main effects. A common way to avoid this difficulty is to pool some factors into error and, especially, the two less important factors; namely, the sulisobenzone and the sonication time. Care should be taken that, in this particular case, the *P* values should not be interpreted too literally due to the fact that we hypothesized the insignificant role of the two less important factors. In Table 4, the new ANOVA table presents the sum of squares (SS) as the information that was used up to estimate the factor main effects, the *F*-ratios (*F*) as the ratio of the respective mean-square effect (ms) and the mean-square error. From the *P* values in Table 4, it appears when the main effect of each factor is statistically significant ($P < 0.05$) or marginally significant ($P < 0.10$). The factors with the asterisks seem to be the significant ones, based this time on statistical computations (ANOVA) and not visually as in the case of the Pareto chart of the non standardized values presented in Fig. 1.

Therefore, the transformed ANOVA data (Table 4) support the conclusion that, indeed,

Table 4
ANOVA with two main effects pooled into error[a]

Factor	SS	Degrees of freedom	MS	*F*	*P*
(1) Complex	17328*	1.00	17328.00	305.79	0.0032*
(2) Oil Red O	58241.33*	1.00	58241.33	1027.79	0.0009*
(3) Oxybenzone	341.33	1.00	341.33	6.02	0.1336
(4) Deoxybenzone	705.33	1.00	705.33	12.45	0.0718
(6) Carotene	341.33	1.00	341.33	6.02	0.1336
(7) DRV or MLV	4641.33*	1.00	4641.33	81.91	0.0119*
(8) Cholesterol	1452.00*	1.00	1452.00	25.62	0.0368*
(9) DSPC	161.33	1.00	161.33	2.85	0.2336
(11) Sonication type	3201.33*	1.00	3201.33	56.49	0.0172*
Error	113.33	2.00	56.67		
Total SS	86526.67	11.00			

[a] * Significant factors.

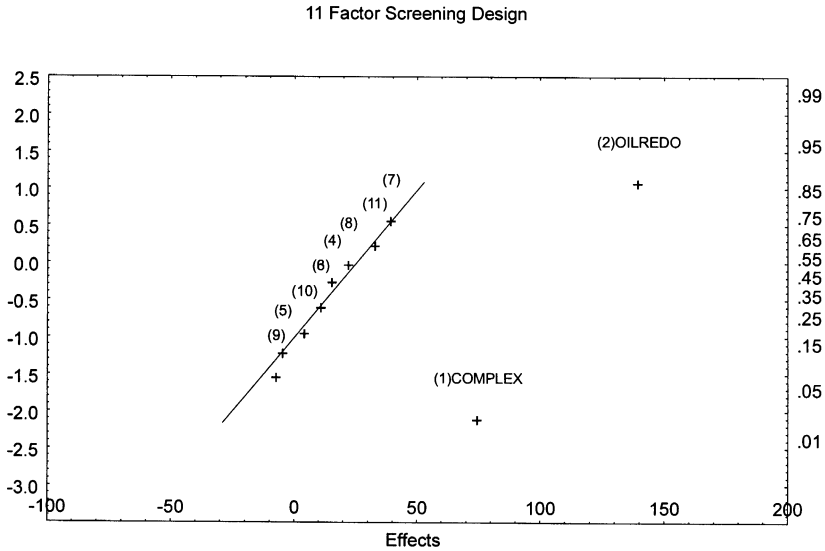

Fig. 2. Normal probability plot of factors main effects on the stabilization ratio. (The right y axis denotes the percentage cumulative frequency, which equals the cumulative frequency divided by $(n+1)$, where cumulative frequency for a measurement denotes the measurements less than or equal to that measurement, and n is the total number of measurements).

factors 1, 2, 7, 8 and 11 significantly affect the stabilization ratio of the vitamin; thus, the settings of these five factors were most important for the resultant stabilization ratio. This means that the vitamin expresses the highest stability when in complexed form (R:γCD) and is entrapped in the aqueous phase of DRV liposomes of PC:cholesterol 1:1 molar ratio, containing oil red O in their bilayers and prepared using probe instead of bath sonication. From the presented observations, the formulator can easily conclude that the presence of at least one hydrophobic light absorber in a liposomal formulation, containing the vitamin in complexed form, provides very good stability. On the other hand, the presence of the hydrophilic sulisobenzone adds little to the overall stability.

3.2.3. Normal probability plot of effects

Another useful, albeit more technical summary graph, is the normal probability plot of effects [7] (Fig. 2), which is constructed as follows. First, the effect estimates are rank ordered. From these ranks, z values (i.e. standard values of the normal 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. 2, the points for the oil red O and the free-complex main effects appear much different from the other effects.

4. Conclusion

In conclusion, such multicomponent vesicular formulations may include more factors during the preparation, making the interpretation of the

system extremely complicated. In order to isolate the most important 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 screening design, as described in the present study, diminished considerably the number of experiments (liposomal preparation), and gave as much as possible information and useful conclusions for the main effects of the examined factors. The PBD identified quickly and efficiently the most significant factors, with the only drawback being the lack of information for any kind of interaction between the factors. The PBD almost halved the number of factors, making the drawing of conclusions easier. In order to examine further the isolated significant factors for their main effects and their interactions, other experimental design techniques should be followed, such as fractional factorial, central composite or full factorial designs, depending on the number of factors and the runs recommended.

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