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Optimization of Encapsulation Efficiency of Piperine in Soya-Lecithin Multilamellar Vesicles

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Abstract : The objective of the study was to predict, optimize and generate surface contours for encapsulation efficiency of piperine in soya lecithin multilamellar vesicles (MLVs) using artificial neural network (ANN) and factorial design – multiple regression analysis (FD-MRA). Statistica Neural Network was used for ANN while the FD-MRA was performed using the computer program SAS. Nine model formulations were prepared. The formulation variables, the drug and the volume of hydration were taken as independent variables, and the percentage drug entrapment (PDE) was taken as a dependent variable. Experimental data was generated. ANN generated predicted values for the experimental data after several iterations. The best performed network was considered in the predictions. In case of FD-MRA, the prediction numbers were determined using the programming language SAS. ANN showed more error compared with FD-MRA.
Keywords : Optimization, response, surface, piperine, liposomes, error, neural, ANN, MLV and PBS.

Introduction

Optimizing techniques provide depth of understanding and the ability to explore and defend ranges for formulation and processing factors (1). With a rationale approach to the selection of the several excipients and the manufacturing steps for a given product, one qualitatively chooses a formulation. At this point, optimization can become a useful tool to quantitate a formulation that has been qualitatively determined. Optimization is not a screening technique; but is routinely used to improve the trial-and-error methods in industries. There are many methods that can be and have been used for optimization, both classic and otherwise and these are well documented. Several techniques that are practiced in the optimization include evolutionary operation (EVOP), the simplex method, the Lagrangian method, search methods, canonical analysis, and polynomial response surfaces. Of these, several papers routinely published involve polynomial response surfaces. Response surface method used in this study, FD-MRA is a polynomial response surface method.

The best informative method of analysis of results of a factorial experiment depends on the nature of the factors which is a fundamental principle of FD-MRA. The technique is referred to as a response surface methodology (1). If all the factors represent quantitative variables like time, temperature, amount of nitrogen, it is natural to think of the yield or response '**y**' as a function of the levels of these variables. This may be written as:

$y_u = Ø (x_{1u} + x_{2u}, ……., x_{ku}) + e_u$

where u = 1, 2, ……N represents the number of observations in the factorial experiment and x_{iu} represents the level of ith factor in the uth observation. The function Ø is called the response surface. The residual e_u measures the experimental error of the uth observation. Knowledge of the function Ø gives a complete summary of the results of the experiment and also enables one to predict the response for values of the x_{iu} that were not tested in the experiment. When the mathematical form of Ø is not known, this function can sometimes be approximated satisfactorily, within the experimental region, by a polynomial in the variables x_{iu}. Experimental designs and methods of analysis that have been developed for polynomials of the first and second degree are needed to be used in this context (2). Polynomial response surfaces have the great advantage that they are easy to fit. On the other hand, polynomials are untrustworthy when extrapolated. A polynomial surface should be regarded only as an approximation to Ø within the region covered by the experiment. Any prediction made from the polynomial about the response outside the region should be verified by experiments before putting reliance on it. In addition, there are several disadvantages associated with response surface methodology. To overcome the limitations of FD-MRA, ANN was incorporated (3). ANN is a massively parallel-distributed processor made up of simple processing units that has a natural propensity for storing experimental knowledge and making it available for use. It resembles the brain in the way in which knowledge is acquired by the network from its environment through a learning process, and interneuron connection strengths, known as synaptic weights, which are used to store the acquired knowledge. ANN could be applied to quantify a nonlinear relationship between causal factors and pharmaceutical responses by means of iterative training of data obtained from a designed experiment.

Piperine has anti-inflammatory, anti-oxidant and antifibrotic properties due to which it is hepatoprotective and therefore useful in the treatment of cirrhosis and epilepsy (4). Our research group has already demonstrated the hepatoprotective activity of piperine with polycaprolactone-piperine biodegradable microspheres (5). However, our aim is to develop better means for further development of formulations containing piperine. Liposomes offer more advantages for piperine compared to other sustained release dosage forms. Our intention here is to prepare soya lecithin liposomes since soya-lecithin liposomes can be administered by subcutaneous, intraperitoneal, intramuscular and also by intravenous routes. This study aims to prepare liposomes useful for the testing new chemical entity for hepatoprotective activity using animal models. Subsequently this data will be used for the generation of liposomes intended for injection into human beings. The testing of hepatoprotective activity of piperine in mice is easy. Since the formulation is intended for a short term study, the preparation of the liposomes could be tailored as per the need. There is less loss of drug compared to that found during the preparation of microspheres. As our aim is to get a clue for tailoring the formulation techniques for piperine liposomes, the development of a response surface methodology using FD-MRA followed by a neural network was the need of the hour. Thus, the aim here is not only to improve the formulation of piperine liposomes, but also to give leads to researchers working on these lines for scale-up or production of liposomes of piperine for sustained release delivery.

Material and Methods

Piperine was extracted using black pepper (Piper nigrum L.) obtained from a local source. Chloroform and cholesterol from Qualigens Fine Chemicals and SD Fine-Chem Limited, respectively, were used. Heidolph Rotoflash Evaporator (Laborota 4000) was used in the preparation of liposomes. SL 164 Elico Double Beam UV-Vis Spectrophotometer was used to analyze the samples. A Remi R8C Laboratory Centrifuge was used in the study. Statistica Neural Networks version 4.0, Excel, and SAS used in the study were obtained from Statsoft (Tulsa, Oklahoma, USA), Microsoft (Redmond, WA, USA) and SAS Institute (Cary, NC, USA), respectively. Lecithin was a gift sample obtained as Epikuron 145 V from Degusa.

Preparation of Liposomes and Determination of Percentage Encapsulation

In the present study, drug quantity in gms and the volume of hydration in mL were selected as independent variables, whereas percentage drug entrapped (PDE) within the liposomes was selected as a dependent variable. The factors were selected in a 3^2 factorial design methodology. Totally nine different MLVs of various compositions were prepared in the study (Table 1) by thin film hydration. Briefly, a specified amount of lipid with or without the drug was dissolved in chloroform in a round-bottom flask. A thin layer of lipid was allowed to form on the walls of the flask by evaporating chloroform, under reduced pressure. In the preparation of liposome batches, the conditions of the instrument during the film formation were kept constant. To form MLVs, this layer was hydrated with different volumes of PBS. This was performed on the rotavapor. The

temperature of water-bath and the speed of vortex were 75ºC and 90 rpm, respectively. Hydration time was kept as 1 hour for all the liposome batches. To determine the percentage encapsulation, an aliquot of liposome suspension was taken in a centrifuge tube and was centrifuged for 1 hr at 2500 rpm. The supernatant was collected and diluted with required amounts of methanol. To dissolve the pellet,required amount of methanol was added. The absorbance of the samples was determined using a UV-spectrophotometer at a λ_{max} of 343 nm. The amount of piperine in the supernatant and the pellet were determined. PDE was determined using the formula:

PDE = Amount encapsulated in the liposomes X 100

Amount of the lipid taken

Table 1. Compositions Used in the Study to Prepare Liposomes

Composition/ Formulation	PV1	PV2	PV3	PV4	PV5	PV6	PV7	PV8	PV9
Piperine	10	10	10	20	20	20	30	30	30
Cholesterol	10	10	10	10	10	10	10	10	10
Soya Lecithin	100	100	100	100	100	100	100	100	100
Volume of Hydration (PBS)	10	15	20	10	15	20	10	15	20

FD-MRA, ANN and Contour Plots

FD-MRA was performed using SAS program (Table 2). The output gives several statistics which could be useful in interpreting the model. It further gives predicted numbers for the experimental numbers. Two-dimensional contour plots were established with predicted numbers using Excel. The ANN was performed with PDE as output and drug amounts and volume of hydration as inputs. The methodology is the same as previously described by Subramanian et al., (2004) (3). A multilayer feed-forward back-propogation network, which was created by generalizing the Levelberg-Marquardt's learning rule to multiple layer networks and nonlinear differential transfer functions, was used to predict PDE of the liposomal formulations. Two-dimensional contour plots were established using the predicted numbers from the ANN and SAS program using Microsoft Excel. Further, ANN gives the picture of the model it used to generate the numbers along with several statistical parameters.

Table 2. SAS Program for the Assessment of the Relationship Between Dependent and Independent Variables

Data Regression;

Input ID Drugamount Hydrationvolume Encapsulation;

```
DATALINES;
1 10 10 6.4
2 10 15 7.2
3 10 20 7.7
4 20 10 6.4
5 20 15 5.08
6 20 20 5.4
7 30 10 5.82
8 30 15 7.23
9 30 20 10.46;
PROC REG DATA=REGRESSN;
      TITLE 'ENCAPSULATION EFFICIENCY OF PIPERINE IN MLVS'
      MODEL ENCAPSULATION = DRUGAMOUNT  HYDRATIONVOLUME / P R;
RUN;
QUIT;
```

Results

Preparation of MLVs and Determination of Encapsulation Efficiency

MLVs were prepared using a thin film hydration technique. A fine suspension of liposomes was obtained at the end of preparation; the liposomes looked spherical with many layers under the microscope. The encapsulation efficiency is mentioned in Table 2.

FDMRA and Contour Plots

FDMRA was determined using the SAS. The output begins with an analysis of variance table, which looks much as it would be from a standard ANOVA. The sum of squares for model (4.74) tells us how much of the variation in encapsulation is attributable to drug amount and volume of hydration. The mean square for the model (2.37) is the sum of squares (4.74) divided by degrees of freedom for the model. This mean square is then divided by the mean square error (2.584) to produce F statistic for regression (0.92). The p-value for this is reported as 0.4495. In this study, three parameters are estimated: (1) the intercept, or constant, term (2) coefficient for 'Dosage', and (3) the coefficient for 'Hydration Volume'. Each parameter estimate was based on one degree of freedom. For each parameter estimate, a standard error was estimated along with a t-statistic and a p-value for the t-statistic. This part of the printout tells us that it was really both of the independent variables which are not stronger in assessing the dependent variable. Although not closer to being nullified in affecting the dependent variable (p= 0.6130 for drug amount and p= 0.2598 for volume of hydration), the relationship do not mention a clear and strong dependence. The model generated predicted numbers for the experimental numbers (Table 4). The difference between the two and a t-score were determined. Although the relationship is not either clear or stronger, the model is definitely working. This is because the Cooks D value given indicates that all the points are doing fine. Cooks D is a distance measure that helps us determine how strongly a particular data point affects the overall regression. Large absolute values of D (2 or more) indicate possible problems with the model or the data points that require more careful scrutiny. However, it is not the case here. Contour plots were plotted using Excel (Fig 1)

Table 3. Analysis of Variance (ANOVA) of the Model of the Multiple Linear Regression Generated Out of SAS

A. Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	4.73667	2.36833	0.92	0.4495
Error	6	15.50556	2.58426		
Corrected Total	8	20.24222			

Root MSE	1.60756	R-Square	0.2340
Dependent Mean	6.84444	Adj R-Sq	-0.0213
CoeffVar	23.48		

B. Parameter Estimates

Variable	DF	Parameter Estimate	Standard Error	t Value	Pr > |t|
Intercept	1	3.69444	2.42618	1.52	0.1787
DRUGAMOUNT	1	0.03500	0.06563	0.53	0.6130
VOLUMEOFHYDRATION	1	0.16333	0.13126	1.24	0.2598

Table 4. T-Test and Cooks D Value for the Experimental and Predicted Value Generated Out of SAS Output

S.No.	Experimental	Predicted	Error	Cooks D Value
1	6.4000	5.6778	1.0717	0.097
2	7.2000	6.4944	0.8473	0.034
3	7.7000	7.3111	1.0717	0.028
4	6.4000	6.0278	0.8473	0.010
5	5.1000	6.8444	0.5359	0.055
6	5.4000	7.6611	0.8473	0.351
7	5.8000	6.3778	1.0717	0.062
8	7.2000	7.1944	0.8473	0.000
9	10.4000	8.0111	1.0717	1.060

t-value calculated =0.9999; $t_{0.05}$ = 1.860
Since the calculated value of t is less than the table value, the hypothesis is accepted.
The two sets of numbers are the same. The error = 0.72

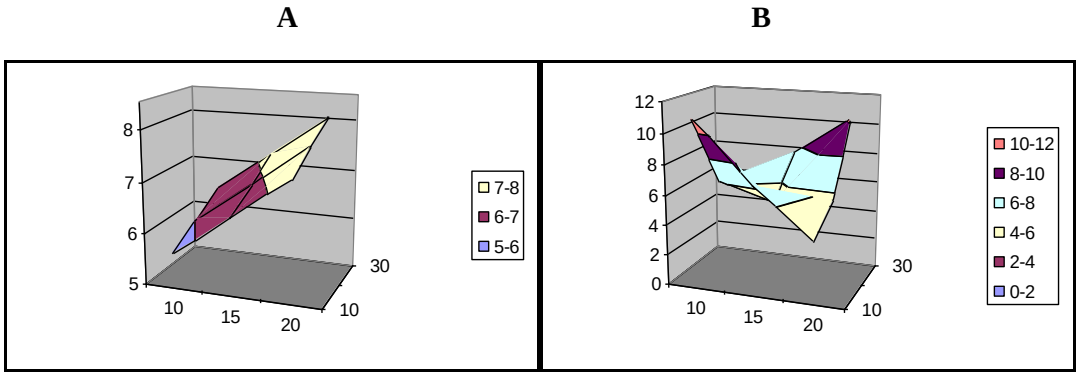

Fig 1. Contour Plots for the PDE Generated Out of Predicted Values Using FD-MRA Technique (A) and ANN (B)

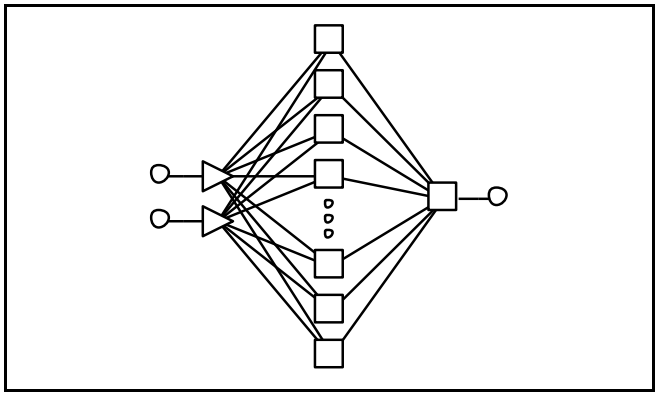

Fig 2. The Best Network Generated Out of ANN Training

ANN and Contour Plots

A multilayer feed-forward back-propagation network using the Levelberg-Marquardts learning rule was used to predict PDE of the liposomal formulations. The network was generated with several permutations and combinations as per one previous study (3). The statistics associated with the ANN are presented in Table 5. The NE observed with optimal ANN structure was 0.000790. For this 63034 networks were tested, 10 were retained in the system. The best network found had excellent performance (regression ratio 0.002136, correlation 1.000000, error 0.000790). The network is shown in Fig 2.

Table 5. T-Test for the Experimental and Predicted Value Generated Out of ANN

S.No.	Experimental	Predicted	Error
1	6.4000	11.1	4.68
2	7.2000	7.2	0.026
3	7.7000	4.03	-3.66
4	6.4000	6.17	-0.228
5	5.1000	5.1	0.0
6	5.4000	5.6	0.18
7	5.8000	5.8	0.0
8	7.2000	7.6	0.4
9	10.4000	10.12	-0.28

t-value calculated =0.8796; $t_{0.05}$ = 1.860
Since the calculated value of t is less than the table value, the hypothesis is accepted.
The two sets of numbers are the same. The error = 2.189

Discussion

Previously, the trend was to perform all the operations involved in response surface methodology using calculator or softwares like Excel. However, we have employed one of the popular programming languages called SAS (6). The tedious job of calculation was conveniently avoided. In the predictions with ANN, the best network after training was considered. Statistics associated with this training are recorded. The ANN showed more error compared with FD-MRA. Compared to the published results with other water soluble liposomes and the liposomes prepared using other techniques, the error obtained with piperine liposomes prepared using thin film hydration technique was very high with either of the optimization techniques employed here. Response surface was plotted and the results generated out of predicted values from SAS and ANN.

For the encapsulation problem presented in this paper, the response surface based optimization scheme could be only used for empirical evaluation of the direction of formulation development. It is neither effective nor efficient in the consideration of the numbers for further development, since the error was very high. This may be because of the method of preparation we have employed. It is a thin film hydration method in which the drug and the polymer are layered and subsequently the layer is hydrated to obtain the liposomes. During this process, the things that could happen include, the formation of vesicles, the partition of the drug between the vesicles and the outer aqueous layer, encapsulation in the vesicles and finally drug solubilization in the aqueous environment. All these factors may affect the outcome. Apart from the manufacturing factors that are kept constant in all the batches, these parameters might also play an important role. Additionally, the lack of fit in this case could be explained by various disadvantages offered by response surface methodology (7). One such disadvantage is the large number of solutions required to compute a fit in many dimensions. For this reason, it is clear that the response surface methodology can become quite inefficient as the number of design variables increase significantly. In this regard, our present experimental work might serve several other design variables which we have not considered in the calculations. These have to be either weeded out or kept constant during the process. Thus, either the time of hydration which influences the stability of the compound has to be increased or the technique of hydration has to be altered accordingly. Similar is the case with other variables. Another significant disadvantage of the response surface method is its inability to mimic highly nonlinear functions. Literature shows that liposomes and other formulations performed on these lines, the response surface based optimization scheme was found to be overall appealing (3). However, in the case of piperine and soya-lecithin liposomes, the same is not true. Either FD-MRA or ANN were not able to predict the numbers, appropriately. Enhanced response surface schemes and other optimization schemes must be explored to utilize fully the methods mentioned in the response surface literature.

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