Formulation Optimization of Paclitaxel Carried by PEGylated Emulsions Based on Artificial Neural Network

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Received March 7, 2004; accepted May 16, 2004

Purpose. To develop paclitaxel carried by injectable PEGylated emulsions, an artificial neural network (ANN) was used to optimize the formulation—which has a small particle size, high entrapment efficiency, and good stability—and to investigate the role of each ingredient in the emulsion.

Methods. Paclitaxel emulsions were prepared by a modified ethanol injection method. A computer optimization technique based on a spherical experimental design for three-level, three factors [soybean oil (X1), PEG-DSPE (X2) and polysorbate 80 (X3)] were used to optimize the formulation. The entrapment efficiency of paclitaxel (Y1) was quantified by HPLC; the particle size of the emulsions (Y2) was measured by dynamic laser light scattering and the stability of paclitaxel emulsions was monitored by the changes in drug concentration (Y3) and particle size (Y4) after storage at 4°C.

Results. The entrapment efficiency, particle size and stability of paclitaxel emulsions were influenced by PEG-DSPE, polysorbate 80, and soybean oil. Paclitaxel emulsions of small size (262 nm), high entrapment efficiency (96.7%), and good stability were obtained by the optimization.

Conclusions. A novel formulation for paclitaxel emulsions was optimized with ANN and prepared. The contribution indices of each component suggested that PEG-DSPE mainly contributes to the entrapment efficiency and particle size of paclitaxel emulsions, while polysorbate 80 contributes to stability.

KEY WORDS: artificial neural network; emulsions; optimization; paclitaxel; PEGylated.

INTRODUCTION

Paclitaxel is widely used as an effective anticancer agent for ovarian, colon, and breast cancer. The commercially available product, Taxol (paclitaxel), is currently a formulation of vehicle containing approximately a 1:1 vol/vol mixture of polyoxyethylated castor oil (Cremophor EL) and ethanol due to its extremely poor solubility in water (0.6 mM) and other pharmaceutical agents. Cremophor EL has been associated with hypersensitivity reactions, nephrotoxicity and neurotoxicity (1). Therefore, premedication with corticosteroids and antihistamine as well as long-term infusion of a 5~20-fold dilution of the product is required to reduce the side effects. However, there are a serious problems associated with dilution of the formulation such as compatibility and stability. The stability of diluted paclitaxel was estimated at 12~24 h since its use was recommended within 12 h of dilution in aqueous medium. Thus there is need for a new formulation of paclitaxel that is efficacious and less toxic than the commercial product.

Recently, many alternative formulations have been developed, such as emulsions (2–7), microspheres (8,9), liposomes (10,11), mixed micelles (12,13), cyclodextrins (14,15), and conjugates (16,17). Several compounds are often used in formulations of paclitaxel, such as polysorbate 80, PEG400, poloxamer, PEGylated lipid, and so forth. A common characteristic of the molecular structure of these compounds is the presence of a polyethyleneglycol group of different lengths and shapes. In fact, a polyethyleneglycol group also exists in the molecular structure of Cremophor EL.

The emulsion (o/w) used for the delivery of paclitaxel has attracted much attention (1). Triacetin provides a high level of solubility, 75 mg/ml, and has been used together with lecithin, pluronic F-68, polysorbate 80 and glycerol to produce a paclitaxel-containing emulsion (2). Wheelar *et al.* (3) manufactured a blend of emulsion and liposome with corn oil, EPC, cholesterol, PEG-lipid (polyethylene glycol derivative, mean molecular weight of PEG 2000) and paclitaxel. Lunberg (4) prepared a paclitaxel emulsion made of triolein, dipalmytoylphosphatidylcholine and polysorbate 80, with polyethylene glycol coated on the emulsion surface. Kan *et al.* (5) developed a paclitaxel emulsion with an oil blend of triacylglycerol, EPC and polysorbate 80 in a glycerol solution, while Simamora *et al.* (6) used polysorbate 80 and sorbitan monolaurate, and Constantinides *et al.* (7) used α-tocopherol, α-tocopherylpolyethyleneglycol-1000 succinate (TPGS), Poloxamer 407 and PEG 400. The optimization of these formulations was based on experience, not computer modeling. Also, the role of each ingredient in the formulation was not elucidated.

Recently we have reported injectable PEGylated emulsions composed of vitamin E, cholesterol and PEG-DSPE (polyethylene glycol derivative of distearoylphosphatidylethanolamine, mean molecular weight of PEG 2000) and an anticancer drug prepared by a modified ethanol injection method (18,19). Such PEGylated emulsions also might encapsulate paclitaxel. We tried to develop a Cremophor-free oil-in-water emulsion of paclitaxel using soybean oil as the internal phase, and polysorbate 80, PEG-DSPE and cholesterol as emulsifiers or co-emulsifiers.

Response surface techniques incorporating an artificial neural network (ANN) and the second-order polynomial regression equation (2PE) were used to achieve an optimal emulsion of paclitaxel with a small particle size, high entrapment efficiency and good stability. The application of ANN in the field of pharmaceutical development has gained interest in recent years (20–22). ANN is a learning system based on a computational technique that can simulate the neurologic processing ability of the human brain (23). Using a computor-program, ALCORA, it can also perform a classic opti-

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ABBREVIATIONS: EPC, egg phosphatidylcholine; PEG-DSPE, polyethylene glycol derivative of distearoylphosphatidylethanolamine, mean molecular weight of PEG 2000; PEG 400, polyethylene glycol 400; ANN, artificial neural network; 2PE, the second-order polynomial regression equation.

0724-8741/04/0900-1692/0 © 2004 Springer Science+Business Media, Inc.

mization technique based on 2PE. The basic concepts of ALCORA and simultaneous optimization of several responses based on ANN have been described fully (24–27).

The aim of the study was to achieve, by applying an optimization, an optimal PEGylated emulsion containing paclitaxel which has a small size, high entrapment efficiency for paclitaxel and good stability, and to investigate the role of each ingredient in the emulsion using the response surface technique.

MATERIALS AND METHODS

Materials

Paclitaxel was kindly supplied by Bristol Pharmaceuticals K.K. (Tokyo, Japan). PEG-DSPE was purchased from NOF Co. Ltd (Tokyo, Japan). Soybean oil and glucose were obtained from Wako Pure Chemical Industries, Ltd (Tokyo, Japan). Cholesterol and polysorbate 80 were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). PEG 400 was purchased from Kishida Chemical Co. Ltd. (Osaka, Japan). Chemicals for high-pressure liquid chromatography (HPLC) were of HPLC grade and all other chemicals were of analytical grade.

Preparation of Paclitaxel Emulsions

Paclitaxel emulsions were prepared by a modified ethanol injection method (18,19,28). Paclitaxel, soybean oil, PEG-DSPE, cholesterol, PEG 400 and polysorbate 80 were dissolved in 40 ml ethanol, then the ethanol was removed with a rotary evaporator till 1–2 ml was left. Next, a constant volume of 5% glucose solution was added to the ethanol solution. The emulsions formed instantly after further evaporation of the residual ethanol. The concentration of paclitaxel was adjusted to 0.6 mg/ml in the final emulsions containing the 5% glucose solution as external phase with drops of Milli Q water. Then the emulsions were filtrated through 0.45-μm Ekikrodisc filters (Gelman Japan, Tokyo, Japan) to homogenize the droplets of emulsion and stored at 4°C for further detection.

HPLC

The HPLC system was composed of an LC-10AS pump (Shimadzu Co., Ltd., Kyoto, Japan), a SIL-10A auto injector (Shimadzu Co., Ltd.), an SPD-10A UV detector (Shimadzu Co., Ltd.), and a C_{18} 4.6 × 150 mm reverse phase column (Shiseido, Tokyo, Japan; Capcell-park, 3 μm particle size). The mobile phase consisted of acetonitrile-0.1% phosphoric acid (wt/vol) in Milli Q water (55:45, vol/vol), at a flow rate of 1.0 ml/min. Chromatography was performed at ambient temperature (20 ± 2°C). The concentration of paclitaxel in each sample was determined with a constructed calibration curve. The internal standard was n-hexyl p-hydroxyl benzoic acid (Tokyo Kasei Kogyo Co., Tokyo, Japan). For UV detection, the wavelength was set to 227 nm (29).

Particle Size and Entrapment Efficiency

The particle size of paclitaxel emulsions was determined using a laser light scattering instrument (ELS800, Otsuka Electronics, Osaka, Japan) by the dynamic light scattering method.

The entrapment efficiency of paclitaxel emulsions was taken as the percentage of paclitaxel carried by the emulsions and was determined by two methods; Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden) column chromatography and filtration. Emulsions were separated from free paclitaxel in the Sephadex G-100 column using a mobile phase of 5% glucose solution. The amounts of paclitaxel in the free fraction and the emulsion fraction were determined by HPLC as described in the "HPLC" section. With the other method, the emulsions were filtrated through 0.45-μm Ekikrodisc and the concentration of paclitaxel in the filtrate was determined by HPLC. It was found that the amount of paclitaxel determined by the two methods was the same in the preliminary experiment. So the simpler method of filtration was selected in this study.

The entrapment efficiency was calculated according to the following equation:

$$\text{Entrapment efficiency (\%)} = (A_e/A_t) \times 100$$

where A_e is the amount of paclitaxel detected in the emulsion form and A_t is the total amount of paclitaxel added.

Stability

After their preparation, paclitaxel emulsions were stored at 4°C in the dark for 10 days. The stability was assessed by monitoring the changes in the particle size and paclitaxel content of emulsions during the storage period, which were calculated using the following equations:

$$\text{Change of particle size (\%)} = (S_{10}/S_0) \times 100$$

where S_{10} was the size on the tenth day and S_0 was the size on the day of preparation;

$$\text{Change of drug concentration (\%)} = (C_{10}/C_0) \times 100$$

where C_{10} was the concentration in the emulsion form on the tenth day and C_0 was the concentration in the emulsion form on the day of preparation. The paclitaxel concentration was determined by the HPLC method.

Experimental Design and Data Analysis

The amounts of soybean oil (X1), PEG-DSPE (X2), and polysorbate 80 (X3) were selected as causal factors in this study. The values listed in Table I in coded form were transformed to physical units as summarized in Table II. A spherical experimental design for three factors was used to prepare the model formulations (Nos. 1–15). The response of the experimental design was adapted to particle size, entrapment efficiency and stability of paclitaxel emulsions on the tenth day.

The software ANN and ALCORA were used to analyze the results of the experiment (25,27). Levels of causal factors were expressed as concentrations (mg/ml).

RESULTS AND DISCUSSION

In this study, a Cremophor-free oil-in-water emulsion of paclitaxel was developed using all excipients, which are less toxic and present in a number of marketed parenteral products. In a preliminary experiment, the particle size and amount of paclitaxel in the emulsion were measured after storage at 4°C for 1 month using oleic acid, vitamin E and soybean oil as an internal phase and polysorbate 80, PEG-

Table I. Spherical (Nos. 1–15) Experimental Design for Three Factors

Formulation no.	X_1	X_2	X_3
1	–1	–1	–1
2	–1	–1	1
3	–1	1	–1
4	–1	1	1
5	1	–1	–1
6	1	–1	1
7	1	1	–1
8	1	1	1
9	–1.73	0	0
10	1.73	0	0
11	0	–1.73	0
12	0	1.73	0
13	0	0	–1.73
14	0	0	1.73
15	0	0	0

DSPE and cholesterol as emulsifiers or co-emulsifiers with a paclitaxel concentration of 0.3 to 1.8 mg/ml. From the results, soybean oil as the oil phase and 0.3 mg/ml of paclitaxel were more stable than the others (data not shown). Therefore, soybean oil was used as the internal phase. Polysorbate 80 with a polyethylene glycol group chain, a surfactant in common use, was used to conduce a paclitaxel formulation. PEG-DSPE was supposed not only to prolong circulation time *in vivo* but also to form an injectable paclitaxel emulsion. Cholesterol was used to stabilize the surface of droplets together with PEG-DSPE (18). Therefore, cholesterol was used in the same amount (weight) as PEG-DSPE. In the formulations of emulsions of paclitaxel, constant amount of paclitaxel, PEG 400 and glucose (constant volume of 5% glucose solution) was added and the final volume of emulsion was adjusted same by drops of Milli Q water.

Spherical Experimental Design

The data of spherical design in the model formulation are listed in Table III, including particle size, entrapment efficiency and stability of paclitaxel emulsions on the tenth day. The data were called responses and marked as Y1 to Y4, respectively. A significant difference in the value of responses can be observed. The entrapment efficiency (Y1) was 79.2%~101.5%, particle size after preparation (Y2) was 256.6~348.5 nm, change of concentration (Y3) was 19.0~99.3% and change of particle size (Y4) was 79.6~106.6%.

ANN was applied to the prediction of responses (Y1–Y4) as a function of causal factors. 2PE was used for comparing the prediction ability.

Three causal factors corresponding to different levels of soybean oil (X1), PEG-DSPE (X2), and polysorbate 80 (X3)

Table II. Levels of Causal Factors* in Physical Form

Factor	Factor level in coded form				
	–1.73	–1	0	1	1.73
Soybean oil (X_1)	4.8	7	10	13	15.2
PEG-DSPE (X_2)	1.3	2	3	4	4.7
Polysorbate 80 (X_3)	15.2	24	36	48	56.8

* Levels of causal factors were expressed as concentrations (mg/ml).

Table III. Results of Experimental Design

	Y1* (%)	Y2† (nm)	Y3‡ (%)	Y4§ (%)
1	90.8 ± 0.5	276.2 ± 3.4	61.9 ± 1.6	99.7 ± 2.1
2	101.5 ± 0.2	277.6 ± 2.3	95.4 ± 2.6	90.4 ± 1.7
3	95.4 ± 1.9	295.3 ± 1.0	35.2 ± 1.3	83.4 ± 1.2
4	86.2 ± 0.9	283.1 ± 2.1	94.2 ± 1.8	94.9 ± 1.1
5	82.1 ± 1.4	328.8 ± 0.5	55.6 ± 2.6	83.9 ± 1.1
6	92.7 ± 1.0	325.0 ± 3.1	99.3 ± 2.9	91.6 ± 1.0
7	90.0 ± 1.0	289.1 ± 3.4	41.7 ± 1.3	79.6 ± 1.3
8	93.4 ± 0.7	335.0 ± 3.1	97.5 ± 2.1	90.2 ± 1.3
9	92.5 ± 1.5	256.6 ± 2.7	88.2 ± 0.1	106.6 ± 1.5
10	85.3 ± 1.6	314.8 ± 2.8	92.2 ± 2.5	93.8 ± 0.7
11	93.7 ± 0.1	348.5 ± 4.1	90.4 ± 1.6	96.4 ± 2.0
12	87.4 ± 2.6	275.0 ± 1.4	74.7 ± 3.6	104.4 ± 0.9
13	90.1 ± 0.4	268.7 ± 4.0	19.0 ± 0.3	93.1 ± 1.4
14	86.2 ± 0.7	291.1 ± 2.5	94.5 ± 2.0	103.4 ± 1.7
15	79.2 ± 0.8	260.5 ± 2.5	58.6 ± 1.2	105.5 ± 1.3

* Entrapment efficiency after preparation.
† Particle size after preparation.
‡ Change of concentration (10 day/0 day).
§ Change of particle size (10 day/0 day).

were used as each nod of the input layer. Reponses were predicted individually with the different sets of ANN. A set of causal factors and responses [45 data pairs; triplicate measurements for 15 formulations (Table I)] was used as tutorial data for ANN. To optimize the structure of ANN, the simulated annealing technique (30) was applied, employing AIC (Akaike's information criterion) as a standard (27). Results are shown in Table IV, suggesting that 4 or 5 nods in the hidden layers were optimal for the prediction of responses, Y1 and Y3, or Y2, and Y4. This means 16 or 20 unknown parameters (3 input nods, 4 or 5 hidden nods, and 1 output nod; i.e., $3\times4 + 4\times1 = 16$ or $3\times5 + 5\times1 = 20$) were required to fit the weights of ANN for the prediction of responses. On the other hand, 2PE requires an estimation of 10 unknown parameters at most as regression coefficients of the polynomial equation [i.e., 3 parameters for the independent term (X1, X2, and X3), 3 for the square term (X1X1, X2X2, and X3X3), 3 for the interaction term (X1X2, X1X3, and X2X3), and 1 for the constant]. To enable an impartial comparison of the predictive ability of ANN and 2PE, we used the coefficient of determination, which was doubly adjusted with degrees of freedom (R**2). As a result, predicted values of

Table IV. Optimal Structures of ANN for Prediction of Responses

Response	Y1	Y2	Y3	Y4
Optimal ANN*	3/4/1	3/5/1	3/4/1	3/5/1
R†	0.984	0.977	0.999	0.991
R**2‡	0.933	0.881	0.996	0.953
AIC§	–80.8	–128	–180	–84.2

* Optimal structure of ANN; input nods/hidden nods/output nods.
† Multiple correlation coefficient between predicted and experimental response values.
‡ Coefficient of determination doubly adjusted with degrees of freedom.
§ Akaike's information criterion. Smaller values of AIC mean a better approximation. The structure of ANN, which gives the smallest value of AIC, was chosen as the optimum.

Table V. Comparisons of Prediction with Results of Experiments

	Y1 (%)	Y2 (nm)	Y3 (%)	Y4 (%)
ANNp*	101	262	96.2	99.7
ANNr†	96.7	262	97.6	98.1
2PEp‡	95.1	267	95.3	97.8
2PEr§	92.6	358	100	88.1

* Predicted by ANN.

† Data of experiment with the optimal formulation predicted by ANN; X1 = 5.50, X2 = 2.18, X3 = 36.0 (mg/ml).

‡ Predicted by 2PE.

§ Data of experiment with the optimal formulation predicted by 2PE, X1 = 5.45, X2 = 2.18, X3 = 42.8 (mg/ml).

responses based on ANN coincided well with the experimental values (Table V). However, approximations of responses based on 2PE were somewhat poorer (R = 0.895 and R**2 = 0.702 for Y1; R = 0.900 and R**2 = 0.714 for Y2; R = 0.977 and R**2 = 0.973 for Y3; R = 0.783 and R**2 = 0.335 for Y4).

Entrapment Efficiency

The contribution indices (27) of the factors in the formulation were calculated by ANN and are summarized in Table VI. The larger the value is the more important the factor. The entrapment efficiency of 0.6 mg/ml paclitaxel after preparation (Y1) was affected in the order PEG-DSPE (X2) > polysorbate 80 (X3) > soybean oil (X1). The soybean oil was relatively less important to entrapment efficiency. The influence of the main factors of PEG-DSPE and polysorbate 80 on the entrapment efficiency is shown in Fig. 1A. It was evident that the entrapment efficiency changed with the amount and ratio of PEG-DSPE and polysorbate 80. The solubility of paclitaxel in soybean oil is not high. Therefore, this finding suggested that most of the paclitaxel might be situated in the surface layer of emulsion droplets due to interaction of the drug with the PEG chain of PEG-DSPE and polysorbate 80 (2–7).

Table VI. Contribution Indices (%) of Factors on the Response in ANN Approximation

	Y1	Y2	Y3	Y4
X1	22.2	32.6	25.3	32.1
X2	40.4	44.2	28.4	32.6
X3	37.5	23.2	46.3	36.2

Particle Size

The particle size after preparation (Y2) was influenced in the order PEG-DSPE (X2) > soybean oil (X1) > polysorbate 80 (X3) (Table VI). The response of particle size to PEG-DSPE and soybean oil is depicted in Fig. 1B. A medium amount (about 2.4 mg/ml PEG-DSPE and 8.96 mg/ml soybean oil) of PEG-DSPE produced small particles (about 269 nm) in the preparation without any homogenization and extrusion of membranes. This finding corresponded well with the result that PEG-DSPE incorporated into emulsions formed small (less than 150 nm) particles (18,19).

Stability

The change in concentration was affected mainly by polysorbate 80 (X3) rather than PEG-DSPE (X2) and soybean oil (X1). Polysorbate 80 (X3) was also important to the change in particle size (Y4) (Table VI). Figures 2A and 2B show the relationship between the two main factors and respective responses in terms of the experimental design. A large amount (>41 mg/ml) of polysorbate 80 and large (>4.4

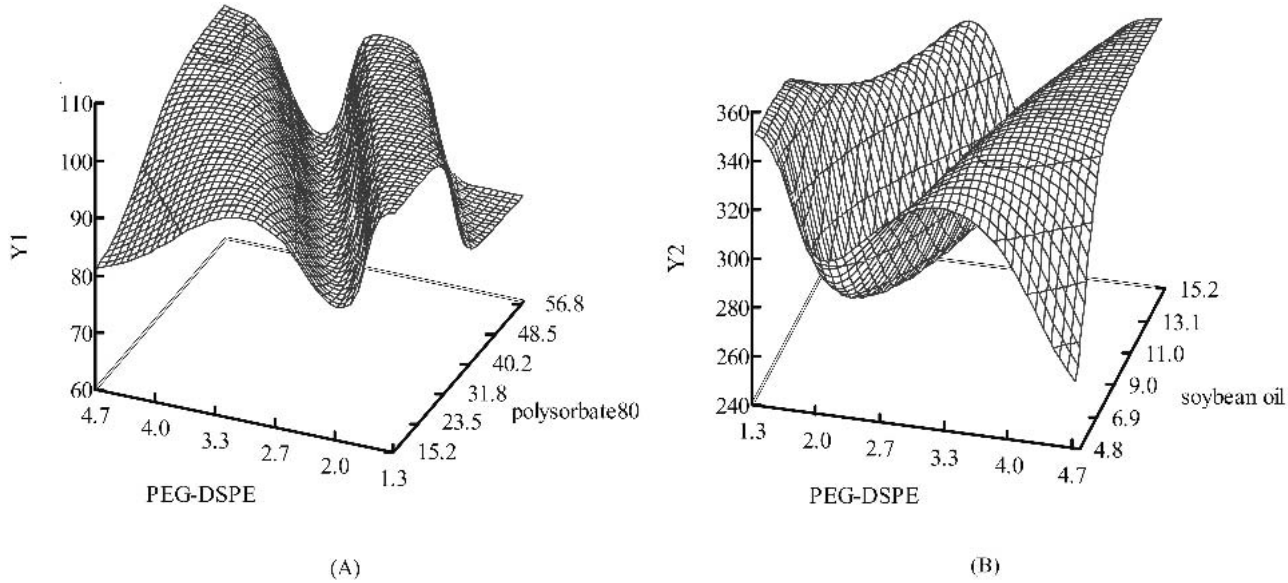

Fig. 1. The effect of PEG-DSPE and polysorbate 80 (mg/ml) on the entrapment efficiency of paclitaxel emulsions (Y1) (A), and the effect of PEG-DSPE and soybean oil (mg/ml) on the particle size of paclitaxel emulsions (Y2) (B).

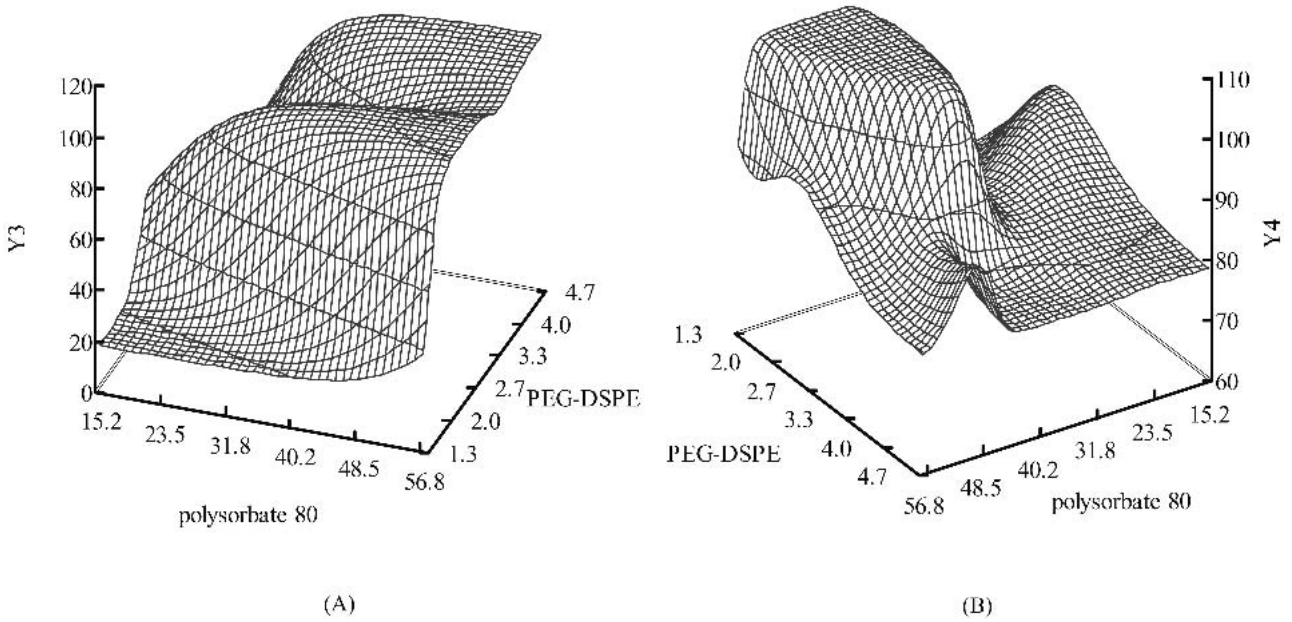

Fig. 2. The effect of polysorbate 80 and PEG-DSPE (mg/ml) on the change in the concentration of paclitaxel emulsions (Y3) (A), and particle size of paclitaxel emulsions (Y4) (B).

mg/ml) or medial (2.1~2.9 mg/ml) amount of PEG-DSPE brought about a more stable concentration of paclitaxel emulsions (Y3) (Fig. 2A), but a large amount (>39 mg/ml) of polysorbate 80 and small amount (<3.3 mg/ml) of PEG-DSPE led to a more stable particle size (Y4) (Fig. 2B). The results indicate that particles modified with a PEG chain proved more stable than those without modification (3).

In some formulations crystals could be observed after ten days and were proved to be paclitaxel by HPLC. And the products of hydrolysis of paclitaxel almost could not be detected by HPLC. Thus it was concluded that the decrease on concentration of paclitaxel was caused mainly by the release of paclitaxel from emulsions and the formation of crystals of paclitaxel in external phase.

Prediction of Optimal Formulation

The software ANN and ALCORA was used to predict the optimal formulation. Two optimal formulations of paclitaxel emulsions were prepared according to the results of predictions by ANN and ALCORA, respectively. The optimal formulations containing 0.6 mg/ml paclitaxel, 30 mg/ml PEG 400, and 50 mg/ml glucose were as follows: by ANN, 5.50 mg/ml soybean oil (X1), 2.18 mg/ml PEG-DSPE (X2), 36.0 mg/ml polysorbate 80 (X3); by 2PE, 5.45 mg/ml soybean oil (X1), 2.18 mg/ml PEG-DSPE (X2), 42.8 mg/ml polysorbate 80 (X3). Then the four parameters of entrapment efficiency after preparation (Y1), particle size after preparation (Y2), change of concentration on the tenth day (Y3) and change of particle size on the tenth day (Y4) were determined. The results are shown in Table V along with the predicted responses. Comparing the predictions and results of experiments, ANN was found to be more suitable for the formulation of paclitaxel emulsions.

Although a number of important formulation parameters remain to be optimized for clinical application, there is indication, at least in paclitaxel PEGylated emulsions, that polysorbate 80 is essential. This information is new with regard to paclitaxel emulsions. PEGylated microemulsions are expected to show long–circulating emulsions *in vivo*. Further research is needed to confirm the efficacy of paclitaxel emulsions *in vivo*.

CONCLUSIONS

We describe here a novel PEGylated emulsion formulation containing paclitaxel that has been optimized by spherical experimental design and ANN for small size (262 nm), high entrapment efficiency (96.7%), and stability. Paclitaxel emulsions were prepared using a very simple procedure and commercialized components for replacement of the toxic Cremophor EL. The contribution indices of each component suggested that PEG-DSPE mainly contributes to the entrapment efficiency and particle size, while polysorbate 80 contributes to the stability of paclitaxel emulsions.

ACKNOWLEDGMENTS

This project was supported in part by a grant from The Promotion and Mutual Aid Corporation for Private Schools of Japan and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. This work was also supported by Hoshi University. There has been a sister-relationship between Hoshi University and the School of Pharmaceutical Sciences, Peking University.

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