# Bootstrap Resampling Technique to Evaluate the Reliability of the Optimal Liposome Formulation: Skin Permeability and Stability Response Variables

Sureewan Duangjit,[a,c] Praneet Opanasopit,[a] Theerasak Rojanarata,[a] Jun Takayama,[b] Kozo Takayama,[c] and Tanasait Ngawhirunpat*[,a]

[a] *Faculty of Pharmacy, Silpakorn University; Sanamchandra Palace Campus, Nakhon Pathom 73000, Thailand:* [b] *Research Fellow of Japan Society for the Promotion of Science; 5–3–1 Kojimachi, Chiyoda-ku, Tokyo 102–0083, Japan: and* [c] *Department of Pharmaceutics, Hoshi University; 2–4–41 Ebara, Shinagawa-ku, Tokyo 142–8501, Japan.*
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**A nonlinear response surface method incorporating multivariate spline interpolation (RSM-S) is a useful technique for the optimization of pharmaceutical formulations, although the direct reliability of the optimal formulation must be evaluated. In this study, we demonstrated the feasibility of using the bootstrap (BS) resampling technique to evaluate the direct reliability of the optimal liposome formulation predicted by RSM-S. The formulation characteristics ($X_n$), including vesicle size ($X_1$), size distribution ($X_2$), zeta potential ($X_3$), elasticity ($X_4$), drug content ($X_5$), entrapment efficiency ($X_6$), release rate ($X_7$), and the penetration enhancer (PE) factors as formulation factors ($Z_n$), with the type of PE ($Z_1$) and content of PE ($Z_2$) were used as causal factors of the response surface analysis. The intended responses were high skin permeability (flux, $Y_1$) and high stability formulation (drug remaining, $Y_2$). Based on the dataset obtained, the simultaneous optimal solutions were estimated using RSM-S. Leave-one-out-cross-validation showed satisfying reliability of the optimal solution. Concurrently, similar BS optimal solutions were estimated from the BS dataset that was generated from the original dataset through BS resampling at frequencies of 250, 500, 750, and 1000. The analysis and simulation indicated that $X_4$, $X_5$, and $Z_2$ were the prime factors affecting $Y_1$ and $Y_2$. These findings suggest that this approach could also be useful for evaluating the reliability of an optimal liposome formulation predicted by RSM-S and would be beneficial for the pharmaceutical development of liposomes for transdermal drug delivery.**

**Key words** bootstrap; response surface; simultaneous optimal solution; transdermal drug delivery

Optimization techniques using computer-based rationales to research and develop pharmaceutical formulations have recently become attractive and interesting. A non-linear response surface method incorporating multivariate spline interpolation (RSM-S) is a powerful method for pharmaceutical optimization.[1] RSM-S has shown that the complex relationships between causal factors and response variables could be simply comprehended and that the simultaneous optimal solutions obtained would be stable and reproducible.[2] Several intensive studies successfully developed novel pharmaceutical formulations using RSM-S (*e.g.*, water-in-oil-water multiple emulsion of insulin for intestinal delivery,[3] sustained release of diltiazem tablets for oral delivery[4] and ultra-deformable liposome of meloxicam for transdermal delivery[5]). RSM-S was determined to be a promising technique for formulation optimization.[3–7] Simultaneously, it is considerable to evaluate the accuracy and reliability of each optimal formulation estimated by RSM-S. The leave-one-out-cross-validation (LOOCV) method was also employed. The LOOCV method can evaluate the generalization error of a given response surface.[8] Moreover, the reliability of optimal formulation estimated by certain response surface can be directly evaluated using bootstrap (BS) resampling methods. The BS method is a simulation technique based on the empirical distribution of the experimental data that introduced by Efron.[9] BS resampling is generally used to estimate confidence intervals and the bias and variance of an estimator. The basic idea of BS resampling is randomly sampling from original dataset (experimental data). A BS samples ($X^*=X_1^*,X_2^*,\cdots,X_n^*$) is randomly sampled that replacement from the original data ($X=X_1,X_2,\cdots,X_n$) by reproducing the BS resampling procedure.

When designing and developing liposome for transdermal drug delivery, the safety, stability and efficacy of formulation must be simultaneously optimized. Generally, the liposome formulation is composed of various formulation characteristics and several formulation factors. The formulation characteristics and formulation factors are the major parameters directly affecting the skin permeability of a liposome formulation.[10] The development of liposomes has previously been based primarily on trial and error to obtain an appropriate formulation for satisfying multiple characteristics of the formulation. Designing and testing on a case-by-case basis (or by trial and error techniques) was considered a wasteful method for designing each liposome formulation. The acceptable liposome formulation for one characteristic was often not satisfactory for other characteristics. Thus, these restrictions incurred difficulties in the design and development of liposome formulations. The optimal liposome formulation is generally influenced by a mixture of acceptable formulation characteristics and formulation factors. Therefore, an understanding of the actual relationships between causal factors (*e.g.*, formulation characteristics and formulation factors) and pharmaceutical responses (*e.g.*, skin permeability and stability of formulation) is required to develop satisfying liposome for transdermal drug delivery.

In this study, the original dataset used was obtained from the experiment. The formulation characteristics ($X_n$) and formulation factors ($Z_n$) of 30 model liposome formulations

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* To whom correspondence should be addressed. e-mail: tanasait@su.ac.th

were selected as causal factors of response variables ($Y_n$). Using multivariate statistical techniques, the significant causal factors were chosen as effective causal factors for certain response analyses. When developing an optimal liposome formulation for transdermal drug delivery, having a proper mixture of high skin permeability and good stability formulation should be considered. For this objective, RSM-S was applied in our study. The LOOCV and BS resampling methods were also used to evaluate the reliability of the simultaneous optimal solutions predicted by RSM-S.

## MATERIALS AND METHODS

**Materials** Phosphatidylcholine (PC) was obtained from LIPOID GmbH (Ludwigshafen, Germany). Sodium hexadecyl sulfate (SHS) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Hexadecylpyridinium chloride (HPC), dodecylpyridinium chloride (DPC), and butylpyridinium chloride (BPC) were purchased from MP Biomedicals (Santa Ana, CA, U.S.A.). Meloxicam (MX) was supplied by Sigma-Aldrich Production GmbH (Buchs, Switzerland). Cholesterol (Chol) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals used were of reagent grade.

**Preparation of Model Liposome Formulation** Thirty model formulations were prepared according to the optimal liposome formulation obtained in our previous study.[11] As shown in Table 1, model formulations that were composed of a controlled amount of PC, Chol, MX, and various types and content of penetration enhancers (PE) (*e.g.*, SHS, HPC, DPC, BPC) were prepared using a sonication method. A previous study reported that alkyl pyridinium surfactants exhibit the ability for saturation and solubilization of the bilayer,[12] which could enhance the transdermal delivery of anti-inflammatory drugs.[13] Briefly, all ingredients were dissolved and mixed in methanol–chloroform (1 : 2, v/v), and the solvent was evaporated under a nitrogen gas stream. The lipid thin film was dried in a desiccator for 6 h to remove the remaining solvent. The dried lipid film was hydrated with an acetate buffer solution (pH 5.5). Model vesicle formulations were subsequently sonicated for 30 min using a sonicator bath (5510J-DTH; Branson Ultrasonics, Danbury, CT, U.S.A.). All model liposome formulations were freshly prepared or preserved in airtight containers at 4°C prior to further studies.

**Determination of Vesicle Size, Size Distribution and Zeta Potential of Liposomes** The vesicle size, size distribution and zeta potential of model formulation were measured by photon correlation spectroscopy (Zetasizer Nano series, Malvern Instrument, U.K.). Twenty microliters of liposomes was diluted with 1480 $\mu$L of deionized water. All determinations were performed at room temperature, at least three independent samples were collected and the vesicle size, size distribution and zeta potential were measured in triplicate.

Table 1. Formulation of Model Liposome Formulation for Simultaneous Optimization

| Formula | mM |
|---|---|
| Phosphatidylcholine[a)] | 10.00 |
| Cholesterol[b)] | 1.05 |
| Meloxicam | 2.20 |
| Penetration enhancers[c–f)] | 0.00–2.90 |

*a*) Bilayer forming liposome. *b*) Membrane stabilizer. *c*) Sodium hexadecyl sulfate. *d*) Hexadecylpyridinium chloride. *e*) Dodecylpyridinium chloride. *f*) Butylpyridinium chloride.

**Determination of Elasticity of Liposomes** The elasticity value of the model liposomes formulation was directly proportional to $J_{Flux} \times (r_v/r_p)^2$, which was obtained from the previous study.[14,15]

$$\text{Elasticity value} = J_{Flux} \times \left( \frac{r_v}{r_p} \right)^2 \quad (1)$$

where $J_{Flux}$ is the rate of penetration through a permeable barrier ($mg \cdot s^{-1} \cdot cm^{-2}$), $r_v$ is the average liposome size after extrusion (nm) and $r_p$ is the pore size of the membrane (nm). To measure $J_{Flux}$, the liposomes were extruded through a polycarbonate membrane (Nuclepore, GE Healthcare Life Sciences, Buckinghamshire, U.K.) with a pore diameter of 50 nm ($r_p$) at a pressure of 0.5 MPa. Five minutes after extrusion, the extrudate was weighed ($J_{Flux}$), and the average liposome diameter ($r_v$) was measured by photon correlation spectroscopy.

**Determination of Drug Content in Liposome Formulations and Entrapment Efficiency** The MX content in the liposome formulations and entrapment efficiency were determined by HPLC. The liposome vesicles were broken down with Triton® X-100 (0.1% w/v) at a 1 : 1 v/v ratio and appropriately diluted with phosphate buffer solution (PBS, pH 7.4). The liposome/Triton® X-100 was centrifuged at 10000×***g*** at 4°C for 10 min. The supernatant was filtered with a 0.45 $\mu$m nylon syringe filter. The entrapment efficiency of the MX loaded in the liposome formulations was calculated according to the following Eq. 2:

$$\% \text{ Entrapment efficiency} = \left( \frac{C_L}{C_i} \right) \times 100 \quad (2)$$

where $C_L$ is the concentration of MX loaded in the liposome formulations, as described in the above methods, and $C_i$ is the initial concentration of MX added to the liposome formulations.

**Evaluation of the Release Profile of Liposomes** The release profile of MX from the MX loaded liposome formulation was determined using a dialysis bag with a molecular weight cut off; MWCO 6000–8000. Fifty milliliters of PBS at a control temperature of 32±1°C was used as the receiving medium and was constantly stirred at 150 rpm. This condition was chosen to obtain sink condition in the receiving medium. Five hundred microliters of MX loaded liposome formulation was filled in a dialysis bag and immersed in the receiving medium. The samples were withdrawn and filtered at intervals of 5, 10, 15, 20, 30, 45, and 60 min and 2, 4, 6, 8, 10, and 12 h. The concentration of MX was determined by HPLC.

**Determination of the Response Variables** The skin permeability (flux, $Y_1$) and stability of the formulation (drug remaining, $Y_2$) were selected as the response variables to be evaluated in the resulting liposome formulation.

a) Skin Permeability of Liposomes: The excised skins of hairless mice (Laboskin®, HOS: HR-1 Male, 7 weeks, Sankyo Labo Service Corporation, Inc., Tokyo, Japan) were used as skin models for the *in vitro* skin permeation study. This animal study was performed at Hoshi University and complied with the regulations of the committee on Ethics in the Care and Use of Laboratory Animals. Side-by-side diffusion cells

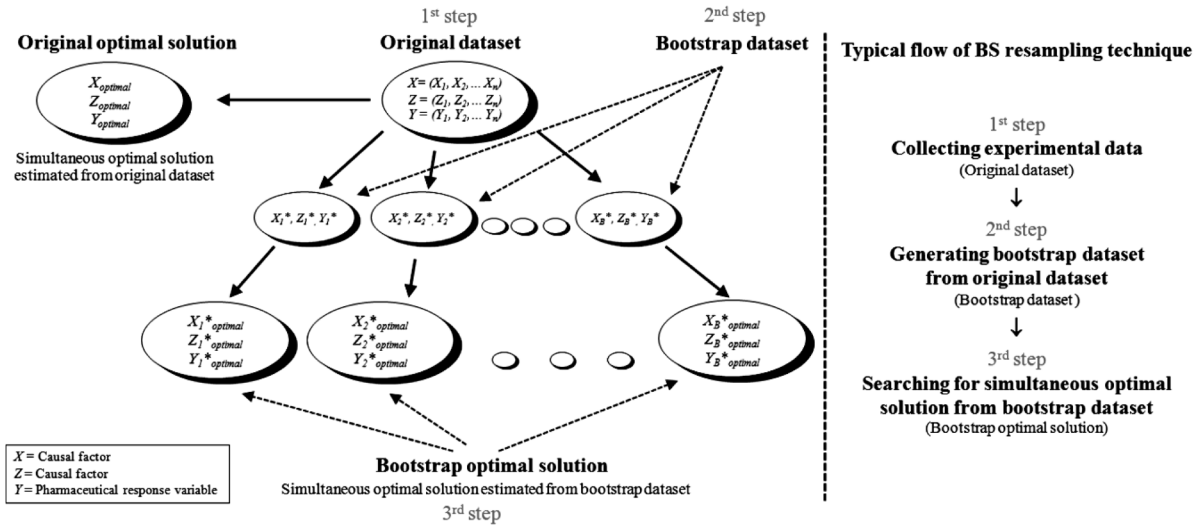


Fig. 1. The Process of Bootstrap Resampling Technique to Evaluate the Reliability of Simultaneous Optimal Solutions

with an available diffusion area of 0.95 $cm^2$ were employed. The receiving chambers were filled with 3 mL of PBS (pH 7.4 at 32°C), and the donor chambers were filled with 3 mL of the MX loaded liposome formulation. At the appropriate times, the receiving medium was withdrawn, and the same volume of fresh buffer solution was replaced in the receiving chambers. The concentration of MX in the aliquot was analyzed using HPLC. The cumulative amount of MX per area was plotted against time, and the flux value ($Y_1$) was determined as the slope of linear portion of the plot.

b) Stability of Liposomes: The MX loaded liposome formulations were kept in glass bottles with plastic plugs and stored at 25±1°C for 30 d. The drug remaining in the MX loaded liposome formulations ($Y_2$) was determined by HPLC. The concentration of MX in the liposome formulation after preparation at day 0 was normalized to 100%.

**HPLC Analysis** The HPLC system consisted of a SIL-20 A autosampler, an LC-20AT liquid chromatography and an SPD-20AUV detector (Shimadzu Corporation, Kyoto, Japan). The analytical column was YMC-Pack ODS-A (150 mm×4.6 mm i.d., S-5, YMC Co., Ltd., Kyoto, Japan). The mobile phase was composed of methanol–acetate buffer solution (pH 4.6) (50 : 50, v/v). The flow rate was set at 0.8 mL/min, and the wavelength used was UV-detected at 272 nm. All samples were freshly prepared or stored at 4°C until analysis. The calibration curve for MX was in the range of 1–100 $\mu$g/mL, with a correlation coefficient of 0.9997. The percent recovery ranged from 99.9–100.3%, and the relative standard deviations for both the intra- and inter-day measurements were less than 2%.

**Simultaneous Optimization and Reliability Evaluation of the Optimal Solution Using DataNESIA and BS Resampling** The formulation characteristics ($X_n$) (*e.g.*, vesicle size ($X_1$), size distribution ($X_2$), zeta potential ($X_3$), elasticity ($X_4$), drug content ($X_5$), entrapment efficiency ($X_6$), release rate ($X_7$)) and penetration enhancers (PE) used in the model formulations as formulation factors ($Z_n$) (*e.g.*, type of PE ($Z_1$) and content of PE ($Z_2$)) were used as causal factors of the response variables. The model formulation of sufficient skin permeability ($Y_1$) and good stability formulation ($Y_2$) was defined as the optimal liposome formulation. High skin permeability and high stability formulation were considered ideal for seeking simultaneous optimal solution. The significant causal factors were selected as effective causal factors for dataNESIA analysis using the multiple regression analysis (MRA) incorporating the stepwise way of factor selection. The software JMP (Version 8, SAS Institute Inc., Cary, NC, U.S.A.) was employed for MRA. The simultaneous optimal solution was estimated using the dataNESIA software (Version 3.2, Azbil Corp., Fujisawa, Japan), which was based on a RSM-S, using the original dataset obtained from 30 model formulations. As shown in Fig. 1, the simultaneous optimal solution from the original dataset was called "the original optimal solution." The accuracy and reliability of the original optimal solution were also determined by LOOCV. The statistical significance of accuracy and reliability was tested based on Pearson's $R$ test. Finally, the BS resampling method was employed to estimate confidence ranges of the original optimal solution. An enormous number of BS samples was generated from the original dataset through BS resampling at a frequency of 250, 500, 750, and 1000. The simultaneous optimal solutions for all BS samples were also estimated using RSM-S. The simultaneous optimal solutions from the BS samples dataset are hereafter called "the BS optimal solution." The details of the reliability assessment of the original optimal solution and the BS optimal solutions have been fully described in previous studies.[1,16)]

## RESULTS AND DISCUSSION

**Prediction of Response Variables and Simultaneous Optimization** In this study, the formulation characteristics (*e.g.*, vesicle size ($X_1$), size distribution ($X_2$), zeta potential ($X_3$), elasticity ($X_4$), drug content ($X_5$), entrapment efficiency ($X_6$), release rate ($X_7$)) and formulation factors (*e.g.*, type of PE ($Z_1$) and content of PE ($Z_2$)) were used as the causal factors of the response variables. The selection of significant causal factors as the original dataset for the dataNESIA analysis was key to generating an accurate optimal solution because the evaluation of the precise optimal solution depended significantly on the integrity and the correctness of the original dataset. The result indicated that the elasticity ($X_4$), drug content ($X_5$), and content

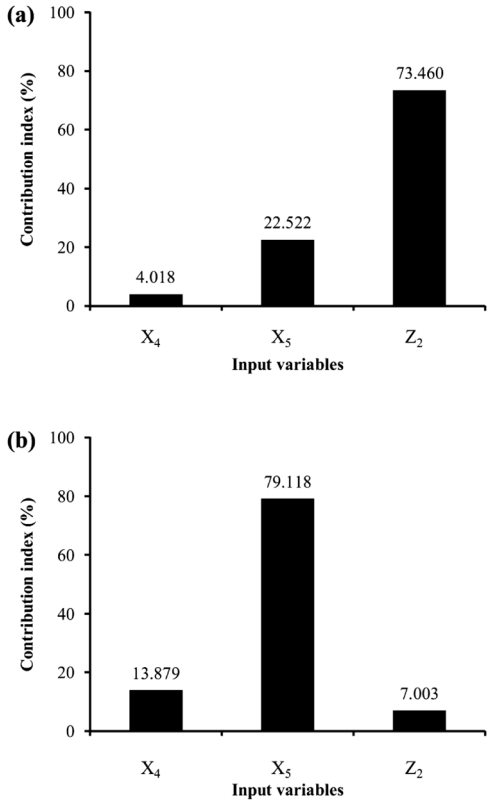


Fig. 2. The Contribution Index of Effective Causal Factor for Predicting Response Variables

(a) Skin permeability ($Y_1$) and (b) stability of formulation ($Y_2$).

of PE ($Z_2$) were selected as effective causal factors for RSM-S by MRA, incorporating a stepwise way of factor selection. The correlation coefficients for the skin permeability ($Y_1$) and the stability of formulation ($Y_2$) were sufficiently high (0.7601 and 0.9700, respectively), suggesting that $X_5$ and $Z_2$ and $X_4$, $X_5$ and $Z_2$ were important to $Y_1$ and $Y_2$, respectively. The contribution index of the effective causal factor for predicting $Y_1$ and $Y_2$ is shown in Fig. 2.

The liposome formulation was optimized based on the original dataset using RSM-S. $X_4$=74.5 ($mg \cdot s^{-1} \cdot cm^{-2}$), $X_5$=514 ($\mu$g/mL), and $Z_2$=0.0689 (%mol) were estimated as optimal formulation characteristics and formulation factors variables. The following were predicted to be the optimal response variables: $Y_1$=0.269 ($\mu g/cm^2/h$) and $Y_2$=375 ($\mu$g/mL). The results indicated that the original optimal solution, which was considered ideal, had a relatively high elasticity ($X_4$), high drug content ($X_5$), and high content of PE ($Z_2$). The formulation characteristics and formulation factors could directly affect the effectiveness of liposome formulation for improving skin permeability, as reported in a previous study.[17] The stability of liposome formulation could be modified by altering aspects of the composition of the liposome, such as the presence of cholesterol.[18] Thus, these effective causal factors might be factors affecting both the efficacy and stability of liposome formulation. The approximate actual relationship between causal factors (formulation characteristics and formulation factors) on response variables (skin permeability and stability of formulation) is shown in Fig. 3.

Figures 3a, b and c show the response surfaces of the skin permeability estimated by RSM-S. Each response surface exhibited relationships among three effective causal factors ($X_4, X_5, Z_2$) and response variables ($Y_1, Y_2$) by fixing one effective causal factor at an optimal constant value and then generating the response surface of two remain causal factors to one response variable. The results indicated that as the elasticity ($X_4$) was held constant (74.5 $mg \cdot s^{-1} \cdot cm^{-2}$), the increase in the drug content ($X_5$) and the content of PE ($Z_2$) to high values (over 350 $\mu$g/mL and 0.06%mol, respectively) resulted in higher skin permeability, as shown in Figs. 3a and c, respectively. When the drug content ($X_5$) was constant, as shown in Fig. 3b, the content of PE ($Z_2$) was demonstrated to be a major factor inducing higher skin permeability. Zucker *et al.* noted that the capability to entrap sufficient drug content in the formulation was necessary in pharmaceutical liposome formulation to achieve therapeutic efficacy.[19] These results indicated that the skin permeability of the liposome formulation in our study was influenced by the drug content ($X_5$) and the content of PE ($Z_2$); thus, these responses were confirmed by the contribution index shown in Fig. 2a.

Figures 3d, e and f show the response surfaces of the stability of formulation predicted by RSM-S. The results revealed that as the content of PE ($Z_2$) was kept steady, the increase in elasticity ($X_4$) and drug content ($X_5$) to high values (over 60 $mg \cdot s^{-1} \cdot cm^{-2}$ and 350 $\mu$g/mL, respectively) tended to increase both the drug content remaining in the formulation and the stability of the formulation, as shown in Fig. 3d. Good stability of formulation was exhibited when the elasticity ($X_4$) and the content of PE ($Y_2$) was higher than 60 $mg \cdot s^{-1} \cdot cm^{-2}$ and 0.06%mol, respectively, as shown in Fig. 3e. Elsayed *et al.* revealed that a single-chain surfactant (as PE) with a high radius of curvature could destabilize or increase the deformability of the vesicle.[20] The present results suggested that our liposome formulation still displayed good stability; The present results suggested that our liposome formulation had high elasticity characteristics but still displayed good stability because all of the model liposome formulations used in this study contained an optimal amount of cholesterol as a membrane stabilizer.[18,21] Liposome formulations with high elasticity values could improve the *in vitro* and *in vivo* skin permeability of various drugs.[22–24] Moreover, the level of high drug content remaining in the formulation after storage at 25°C for 30 d was obtained (Fig. 3f) as the content of PE ($Y_2$) decreased and the drug content ($X_5$) increased. These results could be summarized as follows: the stability of liposome formulation in our study was affected by the elasticity ($X_4$), the drug content ($X_5$) and the content of PE ($Z_2$). These responses were also confirmed by the contribution index shown in Fig. 2b.

The approximate relationships obtained from our study were consistent with the results of a previous study: the formulation characteristics and formulation factors directly affected the skin permeability effectiveness of the liposome formulation. Furthermore, the present findings could provide beneficial basic knowledge and help determine the essential causal factor information for the further development of liposome in transdermal drug delivery. An effective liposome formulation should contain both acceptable skin permeability and good stability in one liposome formulation. The elasticity ($X_4$), drug content ($X_5$), and content of PE ($Z_2$) were significant factors that should be considered in liposome optimization. Therefore, the chosen liposome composition should extremely affect these significant factors, and this technique can be ap-

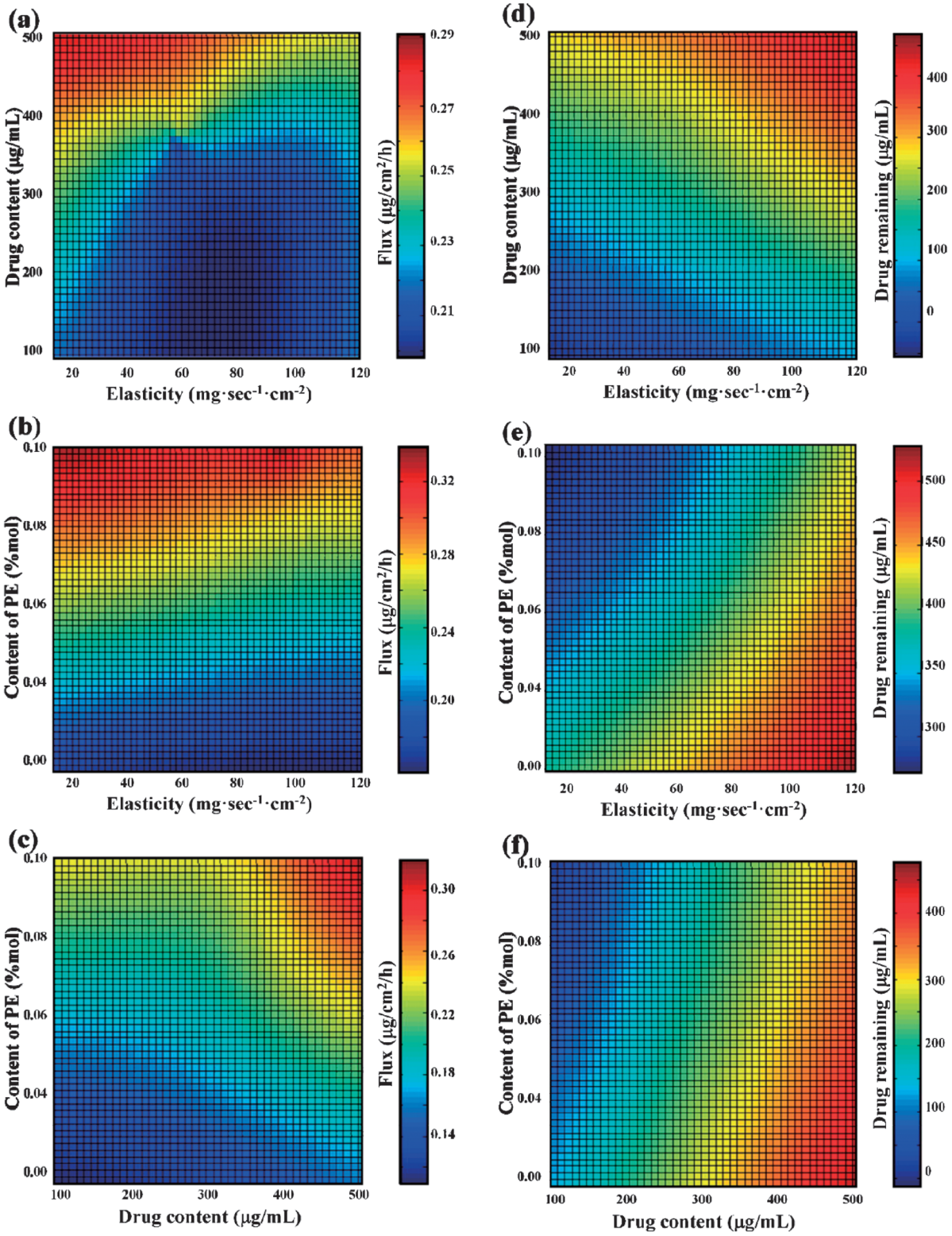


Fig. 3. The Response Surface of Skin Permeability (Flux, $Y_1$) (Left) and Stability of Formulation (Drug Remaining, $Y_2$) (Right) as Function of $X_4$ and $X_5$ (a, d), $X_4$ and $X_9$ (b, e) and $X_5$ and $X_9$ (c, f) at a Constant of $Z_2$ (0.0689%mol), $X_5$ (514 $\mu$g/mL) and $X_4$ (74.5 mg·s$^{-1}$·cm$^{-2}$), Respectively

plied for selecting the liposome composition. To date, it has been difficult to interpret all of the influences on the confounded relationships between formulation characteristics (as latent variables) and formulation factors,[5] although several recent pharmaceutical studies have been successful in formulation optimization. However, our study was successful in achieving this purpose by using both formulation characteristics and formulation factors as causal factors for RSM-S analysis, to understand the relationships of formulation characteristics (as latent variables), formulation factors and pharmaceutical response variables.

The accuracy and reliability of the response surface of original optimal solution were determined by LOOCV, as shown in Fig. 4. The correlation coefficients of the estimated and

experimental values for the skin permeability ($Y_1$) and the stability of formulation ($Y_2$) were extremely high ($R_{LOOCV}$=0.9653 and 0.9984, respectively). These results suggested that RSM-S successfully predicted the relationship between the causal factors (formulation characteristic and formulation factors) and pharmaceutical response variables.[6,7] These results indicated that an original optimal solution with acceptable characteristics (*e.g.*, high skin permeability and good stability formulation) could be estimated with RSM-S.

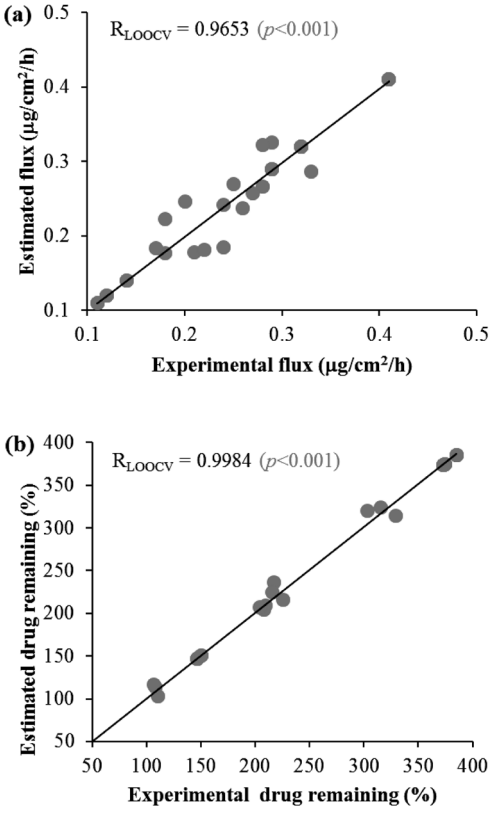


Fig. 4. The Leave-One-Out-Cross-Validation (LOOCV) Estimated Accuracy and Reliability of the Response Surface Variables

(a) Skin permeability (flux) and (b) stability of formulation (drug remaining at 25°C for 30 d) predicted by $X_4$, $X_5$, and $Z_2$.

**Evaluation of the Reliability of the Optimal Solution Using BS Resampling** In evaluating the reliability of the optimal solution, the LOOCV method efficiently provided a versatile assessment of the response surfaces.[25] The correlation coefficients are values that indicate the stability of the response surface. Thus, the reliability of the original optimal solution cannot be quantitatively evaluated using these values.[13] Therefore, BS resampling was needed to evaluate the reliability of the optimal solution[8,26,27] estimated by RSM-S. The BS datasets were generated from the original datasets through BS resampling at a frequency of 250, 500, 750, and 1000. The BS optimal solution and predicted responses are shown in Table 2. The BS optimal solutions and their standard deviation were stable, regardless of altering the frequency of resampling, indicating that a resampling frequency of more than 250 was adequate to determine the stability of the optimal solutions. Consistent with a previous study,[16] a small frequency size of more than 50 resamplings was also sufficient to evaluate the stability of the optimal pharmaceutical formulation.

The confidence intervals of the original optimal solution are shown in Table 3. The ranges of confidence intervals of most of the factors ($X_4$, $X_5$, and $Z_2$) were quite narrow for practical studies of liposome formulations. While further study is required to confirm the potential of the optimal solution predicted by RSM-S compared with the optimal formulation found in the experiment, a previous study[25] suggested that the characteristic values predicted by RSM-S were quite similar to the experimental values. Therefore, these results support the hypothesis that the RSM-S method can be employed to estimate simultaneous optimal solutions. The reliability of the optimal solution improved with an increase in the size of the experimental original dataset, although the precision of

Table 2. Bootstrap Optimal Solutions and Bootstrap Standard Deviations by Different Frequencies of Bootstrap Resampling

| BS resampling frequency | Optimized formulations | | | Predicted responses | |
|---|---|---|---|---|---|
| | $X_4$[c] (mg·s$^{-1}$·cm$^{-2}$) | $X_5$[d] (μg/mL) | $Z_2$[e] (%mol) | $Y_1$[f] (μg/cm²/h) | $Y_2$[g] (μg/mL) |
| $N$=0[a] | 74.5 | 514 | 0.0689 | 0.2692 | 375 |
| $N$=250[b] | 74.4 (0.194) | 514 (0.202) | 0.0690 (0.0001) | 0.2690 (0.0000) | 375 (0.266) |
| $N$=500[b] | 74.3 (0.189) | 514 (0.219) | 0.0689 (0.0001) | 0.2690 (0.0001) | 375 (0.210) |
| $N$=750[b] | 74.3 (0.185) | 514 (0.217) | 0.0689 (0.0001) | 0.2690 (0.0001) | 375 (0.215) |
| $N$=100[b] | 74.4 (0.187) | 514 (0.200) | 0.0689 (0.0001) | 0.2690 (0.0001) | 375 (0.213) |

*a*) Original optimal solution. *b*) BS optimal solution. *c*) Elasticity. *d*) Drug content. *e*) Content of penetration enhancer. *f*) Skin permeation flux. *g*) Drug remaining at 25°C, 30 d. ( ) bootstrap standard deviation.

Table 3. Confidence Interval of Simultaneous Optimal Solution Estimated by RSM-S

| Causal factor | Original optimal solution | 250[a] | | 500[b] | | 750[c] | | 1000[d] | |
|---|---|---|---|---|---|---|---|---|---|
| | | Lower | Upper | Lower | Upper | Lower | Upper | Lower | Upper |
| $X_4$ (mg·s$^{-1}$·cm$^{-2}$) | 74.5 | 74.2 | 75.0 | 73.8 | 74.8 | 73.9 | 74.9 | 73.8 | 74.9 |
| $X_5$ (μg/mL) | 514 | 513 | 514 | 513 | 515 | 513 | 515 | 513 | 514 |
| $Z_2$ (%mol) | 0.0689 | 0.0688 | 0.0691 | 0.0687 | 0.0691 | 0.0688 | 0.0692 | 0.0688 | 0.0691 |
| $Y_1$ (μg/cm²/h) | 0.269 | 0.2689 | 0.2690 | 0.2688 | 0.2692 | 0.2689 | 0.2692 | 0.2689 | 0.2691 |
| $Y_2$ (μg/mL) | 375 | 374 | 376 | 374 | 375 | 374 | 376 | 374 | 375 |

*a*) BS resampling frequency at 250. *b*) BS resampling frequency at 500. *c*) BS resampling frequency at 750. *d*) BS resampling frequency at 1000.

the optimal solutions was ensured, even with a small size of original dataset.

## CONCLUSION

The multivariate statistical technique based on the BS resampling method was useful to evaluate the accuracy and reliability of the optimal solution determined by RSM-S. In our study, the elasticity ($X_4$), drug content ($X_5$), and content of PE ($Z_2$) were primary causal factors that should be intensively considered in the development of a liposome formulation for transdermal drug delivery because these were the most important factors that significantly correlated with efficient and effective simultaneous optimal liposome formulation.

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