An Improved Formulation Screening and Optimization Method Applied to the Development of a Self-Microemulsifying Drug Delivery System

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This study focused on the development of an improved formulation screening and optimization method for a self-microemulsifying drug delivery system (SMEDDS). Solubility study and construction of a ternary phase diagram were carried out to determine the primary formulation components. Experimental design combined with a desirability study was employed to obtain the optimal formulation composition. The obtained bufalin SMEDDS formulation was Maisine 35-1 and Miglyol 812N (1 : 1, w/w) of 29.5%, Cremophor EL of 39.5%, and Transcutol P of 30.5%. It showed desired properties with droplet size of 33.9 nm; polydispersity index of 0.126; equilibrium solubility of 12.6 mg/ml, and 73.6% of soluble drug post-digestion. A rapid release of up to 21% occurred in the first 10 min. A bufalin SMEDDS was well absorbed at all intestinal segments. The absorption of bufalin from a SMEDDS was 2.38-fold higher than that of bufalin suspension in terms of relative bioavailability. The studies on solubility and ternary phase diagrams combined with experimental design may offer a valuable and efficient strategy for developing and optimizing a SMEDDS to obtain optimal formulations with desired characteristics.

Key words self-microemulsifying drug delivery system; bufalin; *in vitro* digestion; *in situ* intestinal absorption

A self-microemulsifying drug delivery system (SMEDDS) is an isotropic mixture of oil, surfactant, co-surfactant, and drug. A microemulsion can be rapidly generated upon gentle mixing with aqueous media. SMEDDSs have many advantages: enhanced drug-loading capacity, improved bioavailability; less irritation to the gastrointestinal tract; and a reduced effect on food intake.[1—5] A SMEDDS is therefore a very attractive option when formulating a drug that is poorly soluble in water to be delivered *via* the oral route.

How to design and develop a SMEDDS formulation efficiently is of great interest to formulators. Recently, an experimental design method, central composite design (CCD), has been used to optimize SMEDDS formulation.[6] It was suggested to be a suitable alternative to the traditional "one-factor-at-a-time" experimental approach. Responses included droplet size, polydispersity, equilibrium solubility, and *in situ* intestinal absorption rate. Although it showed good prediction capability, many animals were required because of the application of *in situ* intestinal absorption rate as one response. Evaluation of *in situ* intestinal absorption is also time-consuming. A more convenient and indicative response is therefore needed. The *in vivo* performance of lipid-based drug delivery systems is affected by digestion and interactions between the formulation and the gastrointestinal environment.[7] A correlation between the solubilization behavior of the formulation during *in vitro* lipolysis and oral bioavailability was noted.[8] *In vitro* lipolysis may therefore be an appropriate indicator of performance, and models of *in vitro* lipid digestion have recently been developed to assist in the design of lipid-based formulations.[7,9,10] Based on these findings, solubilization capacity under a digestion condition was established as a valuable response instead of *in situ* intestinal absorption in this study.

Bufalin (Fig. 1) is isolated from the traditional Chinese medicine "Chan'su" (toad venom). The anti-cancer effect of bufalin has prompted much attention. Bufalin has exhibited significant inhibitory activities against human leukemia cells,[11] hepatoma cells[12] and prostate cancer cells.[13] Established mechanisms include: inhibiting the proliferation and angiogenesis of tumor endothelial cells[14]; inducing apoptosis[11]; inducing differentiation[15]; and reversing multidrug resistance.[16] It was also demonstrated that apoptosis was not induced by bufalin in normal mononuclear cells and polymorphonuclear cells.[17] Bufalin is a very promising antitumor agent, but its poor solubility in water and narrow therapeutic index has limited clinical application. In our preformulation study, we found bufalin was well absorbed in rat intestine.[18] SMEDDSs may be a useful approach to the oral delivery of bufalin.

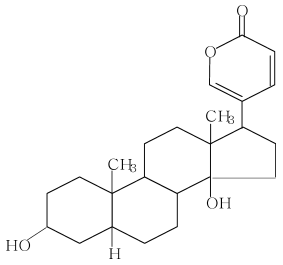

Fig. 1. Chemical Structure of Bufalin

The aim of this study was the development of an improved formulation screening and optimization method for a SMEDDS. The SMEDDS was based on bufalin. It was characterized through morphology observation, analysis of droplet size and polydispersity, and measurement of drug solubility pre- and post-digestion media. *In vitro* release of bufalin from a SMEDDS was studied in comparison with suspension. *In situ* intestinal absorption and *in vivo* bioavailability study were also assessed and discussed.

Experimental

Materials Bufalin (purity 98%) was purchased from Jiangxi Herbfine Hi-Tech Co., Ltd. (Nanchang, China). Cremophor EL® was a gift from BASF (Germany). Miglyol® 812N was from Sasol (Germany). Maisine® 35-1 and Transcutol® P were kindly supplied by Gattefosse (France). Sodium taurodeoxycholate (NaTDC, 99%), 4-bromophenylboronic acid (4-BPB), 3-*sn*-phosphatidylcholine (PC) and porcine pancreatin were purchased from

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Sigma-Aldrich (U.S.A.). All other chemicals used were of analytic grade.

Animals Experiments were approved by the Institutional Animal Ethical Committee of Shanghai University of Traditional Chinese Medicine (Shanghai, China). Experiments complied with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Male Sprague-Dawley rats (180—220 g) were supplied by the Laboratory Animal Center of Shanghai University of Traditional Chinese Medicine.

Solubility Study The solubilities of bufalin in vehicles were determined (Table 1). Excess bufalin was introduced into the vehicle in a screw-capped test tube. The mixture was stirred for 48 h at 25 °C, followed by centrifugation at 2500×***g*** for 20 min. The supernatant was withdrawn and filtered through a membrane filter (pore size, 0.45 μm). Bufalin concentration was determined by high-performance liquid chromatography (HPLC) after dilution with methanol. HPLC analyses were carried out on an Agilent system (HP1100; Agilent, Palo Alto, CA, U.S.A.). Chromatographic conditions were as follows: octadecyl silica (ODS) column (Kromasil C18; 250 mm×4.6 mm; 5 μm; Dikma Technology, Shanghai, China); the mobile phase of methanol/water (70 : 30) was pumped at a flow rate of 1.0 ml/min; column temperature was maintained at 25 °C; and the ultraviolet (UV) detector was set at 300 nm.

Construction of the Ternary Phase Diagram Oil, surfactant, co-surfactant and drug (0.5%, w/w) were mixed at various concentrations. Mixtures were stirred until the drug was entirely dissolved. Phase clarity was visually checked under dilution with distilled water and gentle agitation at 37 °C. Ternary phase diagrams comprising oil, surfactant and co-surfactant were constructed to show the self-microemulsification region.

Preparation of a Bufalin SMEDDS The formulation composition is shown in Table 2. Formulations contained an identical amount of bufalin (0.5%, w/w) except those for equilibrium solubility studies in CCD. A SMEDDS was prepared by dissolving bufalin in a mixture of Maisine 35-1, Miglyol 812N, Cremophor EL and Transcutol P at ambient temperature. Components were gently vortex-mixed and stirred until a transparent preparation was obtained. An optimized formulation was prepared by the same method.

Formulation Optimization A CCD was applied with two variables (oil percentage and Sur/Co-s ratio) and four responses (droplet size, polydispersity, equilibrium solubility, and solubilization capacity under an *in vitro* digestion condition). The design matrix is shown in Table 2. The data were fitted to a classical second-order polynomial model.

$$Y=b_0+b_1X_1+b_2X_2+b_3X_1^2+b_4X_2^2+b_5X_1X_2 \quad (1)$$

where X_1 and X_2 correspond to studied factors, Y denotes the measured response, b_0 is an intercept, and b_1—b_5 are the regression coefficients.

Data were analyzed by nonlinear estimation using Statistica 6.0 software. Analysis of variance (ANOVA) and *F*-test were employed to validate the resulting model.

The desirability function for the response to be minimized is defined as:

$$d=\begin{cases}0 & (Y_i \geq Y_{max}) \\ \dfrac{Y_{max}-Y_i}{Y_{max}-Y_{min}} & (Y_{min}<Y_i<Y_{max}) \\ 1 & (Y_i \leq Y_{min})\end{cases} \quad (2)$$

where Y_{min} and Y_{max} represent the lowest and highest possible value, respectively, and Y_i indicates the experimental value.

For a response to be maximized, the desirability function is defined as:

$$d=\begin{cases}0 & (Y_i \leq Y_{max}) \\ \dfrac{Y_i-Y_{max}}{Y_{max}-Y_{min}} & (Y_{min}<Y_i<Y_{max}) \\ 1 & (Y_i \geq Y_{min})\end{cases} \quad (3)$$

Where, Y_{min}, Y_{max} and Y_i represent the values with the same meaning as those expressed in Eq. 2. The limits were selected according to the observed response values and the practicle requirments for which each response should fit, as similar with previous reports.[19—21] The constraints are $Y_1 : Y_{max}=250$ (largest acceptable droplet size, because above which the dispersion is not considered to be microemulsion) and $Y_{min}=20$ (desirable droplet size); $Y_2 : Y_{max}=0.43$ (largest acceptable polydispersity index) and $Y_{min}=0.09$ (desirable polydispersity index, from the practical viewpoint much lower polydispersity is not necessary since it would have little merit to the quality of SMEDDS); $Y_3 : Y_{max}=17.2$ (desirable equilibrium solubility) and $Y_{min}=8.2$ (lowest acceptable solubility); $Y_4 : Y_{max}=87$ (desirable solubilized drug post-digestion) and $Y_{min}=54$ (lowest acceptable solubilized drug percentage post-digestion and below which the formulation fails to be SMEDDS). Among these objectives, Y_1 and Y_2 were minimized, whereas Y_3 and Y_4 were maximized.

Overall desirability (*D*), is calculated by combining the individual desirabilities using the geometric mean as in the following equation:

$$D=\left(\prod_{i=1}^{n} d_i\right)^{1/n} \quad (4)$$

where d_i is the individual desirability determined from the responses, respectively, n is the total number of responses.

Table 1. Solubility of Bufalin in Various Vehicles at 25 °C (n=3)

Vehicle	Solubility (mg/ml)
Maisine 35-1	2.45±0.33
Miglyol 812N	2.22±0.40
Isopropyl myristate	0.45±0.12
Cremophor EL	11.67±1.14
Transcutol P	23.76±2.31
PEG 400	18.63±1.50
Labrasol	1.24±0.21

Table 2. Central Composite Design: Run Matrix and Obtained Responses

Number	Variables		Responses				*D*
	Oil%	Sur/Co-s	Droplet size (nm)	Polydispersity index (PI)	Solubility (mg/ml)	Solubilized drug post-digestion (%)	
1 (+1, +1)	37.84	1.277	73.7	0.420	9.5	80.8	0.227
2 (+1, −1)	37.84	0.880	249.1	0.377	12.0	76.5	0.115
3 (−1, +1)	27.20	1.277	28.0	0.095	14.5	66.1	0.703
4 (−1, −1)	27.20	0.880	48.9	0.381	17.1	60.5	0.396
5 (+α, 0)	40.00	1.092	119.0	0.372	8.3	86.4	0.180
6 (−α, 0)	25.00	1.092	34.0	0.224	16.6	55.8	0.413
7 (0, +α)	32.50	1.350	21.0	0.120	10.5	80.2	0.655
8 (0, −α)	32.50	0.830	102.5	0.381	13.7	69.2	0.402
9 (0, 0)	32.50	1.092	66.9	0.204	11.9	76.3	0.619
10 (0, 0)	32.50	1.092	61.1	0.182	11.5	74.9	0.611
11 (0, 0)	32.50	1.092	68.0	0.225	12.4	73.8	0.605
12 (0, 0)	32.50	1.092	63.5	0.217	12.4	73.8	0.582
13 (0, 0)	32.50	1.092	65.0	0.198	12.5	74.6	0.636

α=1.414.

The obtained D was analyzed by ANOVA and F-test. A three-dimensional graph of the response against the two factors (X_1, X_2) was plotted, from which the region corresponding to the optimal value for D was obtained.

Determination of Droplet Size and Polydispersity The sample was prepared by diluting with distilled water (1 : 100) before measurement. Samples were analyzed for droplet size and polydispersity by a Nicomp™ 380 ZLS Zeta Potential/Particle sizer (PSS Nicomp, Santa Barbara, CA, U.S.A.). The measurement conditions were: He–Ne laser; angle, 90°; temperature, 23 °C; reflection index, 1.333; wavelength, 635 nm. Each sample was analyzed in triplicate.

***In Vitro* Digestion Experiments** Drug solubilization capacity was investigated by digestion experiments. *In vitro* digestion experiments were done as previously described.[8] Digestion buffer comprised 50 mM TRIS maleate, 150 mM NaCl and 5 mM $CaCl_2 \cdot 2H_2O$ (pH 7.5). One gram of SMEDDS was dispersed in 36 ml of digestion buffer containing 5 mM NaTDC and 1.25 mM PC. Experiments were initiated by introduction of 4 ml of freshly prepared pancreatic extract (containing 40000 tributyrin units of pancreatic lipase activity) and lasted for 60 min with pH maintained at 7.5 by NaOH titration. Aliquots (4.4 ml) were withdrawn, followed by immediate addition of 4-BPB (0.5 M in methanol, 9 μg/ml digestion medium). The mixture was centrifuged at 334000×g for 30 min (Optima LE-80K, Beckman, Palo Alta, CA, U.S.A.). After ultracentrifugation, the mixture was separated into an aqueous phase and a pellet phase. The obtained aqueous sample was diluted with methanol before HPLC. The drug in the pellet was extracted and dissolved using 2 ml of ethyl acetate/methanol (1 : 1). After filtration, the filtrate was diluted with methanol and analyzed by HPLC.

Morphology Morphology was studied using a transmission electron microscope (JEM 1230; JEOL, Tokyo, Japan). The bufalin SMEDDS was diluted with distilled water (1 : 100) and gently mixed. One drop of diluted sample was placed on copper grids. It was then stained with a 1% solution of phosphotungstic acid for 30 s. The specimen was air-dried for 3 h before observation.

***In Vitro* Release Test** Bufalin release from the SMEDDS was determined by reverse dialysis.[22] Briefly, six dialysis bags of identical size containing 2 ml of blank phosphate-buffered saline (PBS, pH 6.8) were immersed in 250 ml of PBS without enzymes for 12 h. The bufalin SMEDDS was introduced into the dissolution medium at 50 rpm at 37 °C. At predetermined time intervals, one dialysis bag was removed and followed by addition of 2 ml of PBS (37 °C) to the curette. Then, 20 μl of sample was carefully withdrawn from the dialysis bag and injected into the HPLC system. A release study of bufalin suspension was also carried out to investigate the effect of the preparations on drug release. Bufalin suspension was prepared by dispersing bufalin in aqueous solution containing 1% (w/v) of glycerin and 0.5% (w/v) of sodium carboxymethyl cellulose. Results represent the mean±S.D. of triplicate experiments.

***In Situ* Intestinal Absorption Study** An *in situ* recirculation perfusion technique was applied, as previously reported.[6] Briefly, rats were fasted overnight and anesthetized by ethylcarbamate (100 mg/100 g; i.p.). They were then placed on a thermostatic surface maintained at 37 °C. The intestinal segment to be perfused was exposed and rinsed with physiological saline. Physiological saline remained in the intestine and was rinsed by air. The intestinal segment was connected to a constant-flow pump by the catheters. The perfusate was prepared by dispersing the bufalin SMEDDS in Krebs–Ringer's solution (100 ml) containing phenol red (20 μg/ml) at 37 °C. Perfusion rate was 5 ml/min for the first 10 min, followed by 1.0 ml/min. Samples (2 ml) were taken at predetermined times, and the same amount (2 ml) of Krebs–Ringer's solution containing phenol red (20 μg/ml) replenished. The sample was filtered through a membrane filter (0.45 μm), followed by detection of the concentration of bufalin (by HPLC) and phenol red (by UV detection) at 558 nm. Absorption rate (K_a) was calculated from the slope of the plot of the log of the remaining drug *versus* time. The percentage uptake was determined by dividing the amount of drug absorbed in 3 h by the total amount of drug at 0 h. The statistical difference between K_a of different intestinal segments was evaluated by analysis of variance. $p<0.05$ was considered significant.

Bioavailability Study The study was approved by the Institutional Animal Care and Use Committee, Shanghai University of T.C.M. Male SD rats (250±20 g) were provided by the Laboratory Animal Center of Shanghai University of T.C.M. Rats were fasted for 12 h before experiment. We investigated the pharmacokinetic characteristics of bufalin SMEDDS and bufalin suspension. The suspension was prepared by mixing bufalin in sodium carboxymethyl cellulose (CMC-Na) solution containing 1.0% glycerin (v/v). After oral gavage of equal amounts of the preparations (bufalin, 2.4 mg/kg), blood samples (0.5—1 ml each) were collected *via* retro-orbital puncture at 30, 45, 60, 90, 120, 150, 240, 360 and 480 min. Plasma samples were obtained by centrifugation of blood at 3000×g for 10 min and were stored at −20°C until analysis. The plasma samples were vortexed with 1.0 ml of ethyl acetate : diethyl ether (4 : 1, v/v) for 3 min and centrifuged at 3000×g for 10 min. The supernatant organic layer was withdrawn and extracted twice. The resultant organic layer was evaporated to dryness under a light stream of nitrogen. The residue was dissolved in 100 μl of methanol : water (70 : 30, v/v) and centrifuged at 11700 g for 10 min at 4 °C. The supernatant (35 μl) was injected into an HPLC system for analysis. The conditions were: mobile phase methanol : water (70 : 30, v/v); flow rate 1.0 ml/min; column temperature 25 °C; wavelength 300 nm. The intra- and inter-day assay precision was 5.7±1.6% and 6.8±2.9%, respectively. The linear regression equation was $A=45.06C+6.6526$ ($r=0.9998$). The linear regression range was 10—1000 ng/ml. The mean extraction recovery was 81.3±0.8%. The mean spiked recovery of bufalin in plasma was 96.4±1.6%. The pharmacokinetic parameters were determined using DAS 2.0 software.

Results and Discussion

Determination of the Formation Conditions of the SMEDDS The formation conditions under which microemulsions can be generated must be ascertained before a SMEDDS, as a means of delivering a poorly water-soluble drug, can be optimized. In general, the main conditions involve selection of the excipient or component in the SMEDDS formulation, and identification of the microemulsification region. It has been suggested that the SMEDDS composition should be simple, safe, compatible, and possesses good solubility to the drug.[23,24] Solubilizing capacity is essential for composing a SMEDDS, particularly for screening the oil phase. Table 1 shows bufalin solubilities in vehicles. The sequence of bufalin solubility in candidate oils was isopropyl myristate<Miglyol 812N≈Maisine 35-1. Miglyol 812N or Maisine 35-1, or their mixture, may be the oil phase in terms of solubilizing capacity. Further screening was done according to their phase behavior. Compared with Miglyol 812N alone or Maisine 35-1 alone, the mixture of Maisine 35-1 and Miglyol 812N (1 : 1, w/w) showed maximal microemulsification (Fig. 2), and was duly selected as the oil phase. Cremophor EL has been successfully used in several SMEDDSs as a surfactant because it has an excellent solubilizing capacity for poorly water-soluble drugs, and high self-microemulsifying efficiency.[1,3,8] Cremophor EL was therefore used as the surfactant, and determined the feasibility (by phase diagram) and characterization of the SMEDDS. Transcutol P provided higher drug solubility than polyethylene glycol-400 (PEG-400). A system composed of Maisine 35-1 and Miglyol 812N (1 : 1), Cremophor EL and PEG-400 produced phase separation when maintained at ambient temperature for >48 h, whereas a system comprising Transcutol P instead of PEG-400 was stable. Thus, Transcutol P was selected as the co-surfactant. According to the phase diagram and assessment of self-microemulsification efficiency, the range of oil percentage and Sur/Co-s ratio at which microemulsion could be efficiently formed was an oil percentage of 25—40 and a Sur/Co-s ratio of 0.83—1.35. Further optimization of the formulation within this range was carried out by the experiments described below.

Formulation Optimization Oil percentage and Sur/Co-s ratio have an important bearing on the formation of fine microemulsions upon introduction of a SMEDDS into aqueous media. They were therefore used as CCD factors for further investigation to obtain a desirable formulation composition. We fixed the drug content capable of meeting the needs of

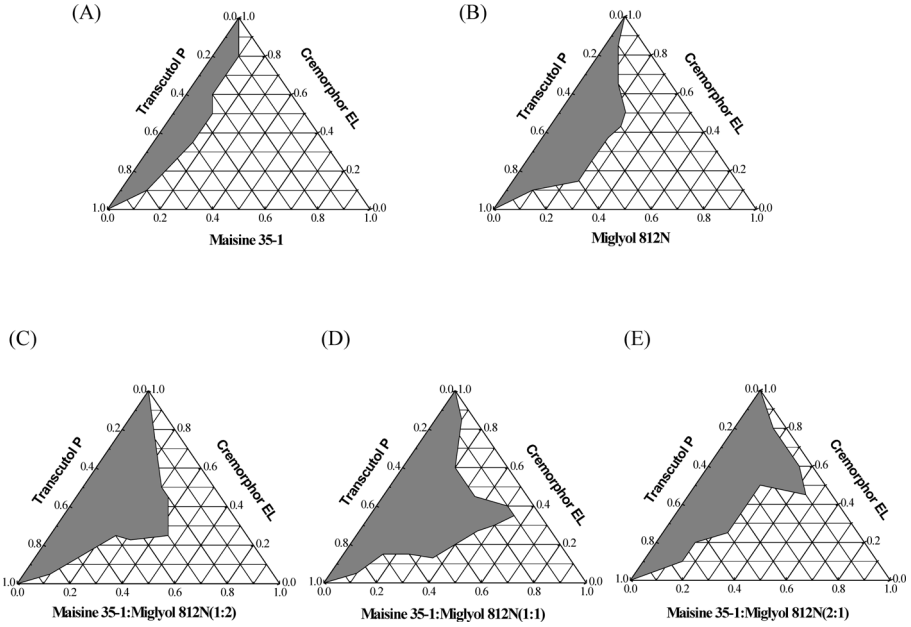

Fig. 2. Pseudo-ternary Phase Diagrams Consisting of Oil, Cremophor EL and Transcutol P

Oil phases were: Maisine 35-1 (A), Miglyol 812N (B), Maisine 35-1 : Miglyol 812N (1 : 2) (C), Maisine 35-1 : Labrafac CC(1 : 1) (D), Maisine 35-1 : Miglyol 812N (2 : 1) (E). The shadow area represents the region of self-microemulsification.

medical use even though drug content has been reported to influence the properties of a SMEDDS.[10] Responses, as indicators of the properties of a SMEDDS, were particle size, polydispersity, equilibrium solubility, and solubilization capacity under an *in vitro* digestion condition. Table 2 lists the results of measured responses; data were computed by Statistica 6.0 software. The four responses were individually fitted to the second-order polynomial model shown in Eq. 1. Each obtained model was validated by ANOVA combined with the *F*-test. The determination coefficient (R^2, agreement between the experimental results and predicted values obtained from the model) and the model *F*-value (Fisher variation ratio, the ratio of mean square for regression to mean square for residual) were listed followed with each fitting model.

In general, small size of droplet, narrow dispersion of droplet, high equilibrium solubility, and high drug solubilization capacity under a digestion condition are desired for a SMEDDS. The desirability function was used for further optimization to combine these responses into a single parameter, and obtain the best compromise condition for all responses. The responses of droplet size (Y_1), polydispersity (Y_2), equilibrium solubility (Y_3) and solubilization capacity under a digestion condition (Y_4) were transformed into the individual desirability d_1, d_2, d_3 and d_4, respectively. Y_1 and Y_2 should be minimized, whereas Y_3 and Y_4 should be maximized. *D* was calculated by Eq. 4 and the results are shown in Table 2. The resulting models were shown as follows:

$$Y_1 = -596.453 + 19.630X_1 + 579.623X_2 + 0.457X_1^2 + 199.586X_2^2 - 37.520(X_1 \cdot X_2) \quad (5)$$

R^2 0.9109 *F*-ratio 36.55 $P<0.0001$

$$Y_2 = 6.07611 - 0.19783X_1 - 4.86581X_2 + 0.00195X_1^2 + 0.90714X_1^2 + 0.07690(X_1 \cdot X_2) \quad (6)$$

R^2 0.9390 *F*-ratio 134.50 $P<0.0001$

$$Y_3 = 58.8467 - 1.4151X_1 - 21.9335X_2 + 0.0137X_1^2 + 7.0258X_2^2 + 0.0103(X_1 \cdot X_2) \quad (7)$$

R^2 0.9645 *F*-ratio 922.50 $P<0.0001$

$$Y_4 = -105.590 + 7.120X_1 + 50.19X_2 - 0.079X_1^2 - 12.324X_2^2 - 0.209(X_1 \cdot X_2) \quad (8)$$

R^2 0.9575 *F*-ratio 2293.83 $P<>0.0001$

$$D = -8.65888 + 0.40570X_1 + 5.18454X_2 - 0.00592X_1^2 - 1.51566X_2^2 - 0.04268(X_1 \cdot X_2) \quad (9)$$

R^2 0.9413 *F*-ratio 141.24 $P<0.0001$

All of the regression models yielded a good fit with high determination coefficient and *F* value. All the determination coefficients R^2 are larger than 0.9, indicating that over 90% of the variation in the response could be explained by the model and the goodness of fit of the model was confirmed. The obtained *F* value is compared with the theoretical value (Fisher test critical value) $F_{\alpha(p-1,N-p)}$ (α, chosen risk, p the number of terms of the model, N the number of the experiments) to test the significance of the regression model. The theoretical value $F_{0.05(6,7)}$ is 3.87. The *F*-ratio was found to be far greater than the theoretical value with very low probability of less than 0.0001 for each regression model, indicating that the regression model is significant with a confidence level of 95%. Additionally, as previously reported, checking the adequacy of the model needs verifying on lack of fit, which can be explained by the residual analysis. A plot of normal probability of the residuals and a plot of the observed *versus* the predicted value are efficient in investigating the systemic departure from the assumptions such as the normality and constant variances of errors. Figure 3A is a plot of normal probability of the residuals for overall desirability (*D*). As shown in Fig. 3A, the residuals generally fall on a straight line, implying they followed the normal distribution. In addition, it was found that none of the individual residual exceeded the residual variance (twice the square root of the residual variance), indicating the experimental values are well in agreement with the predicted values, as similar re-

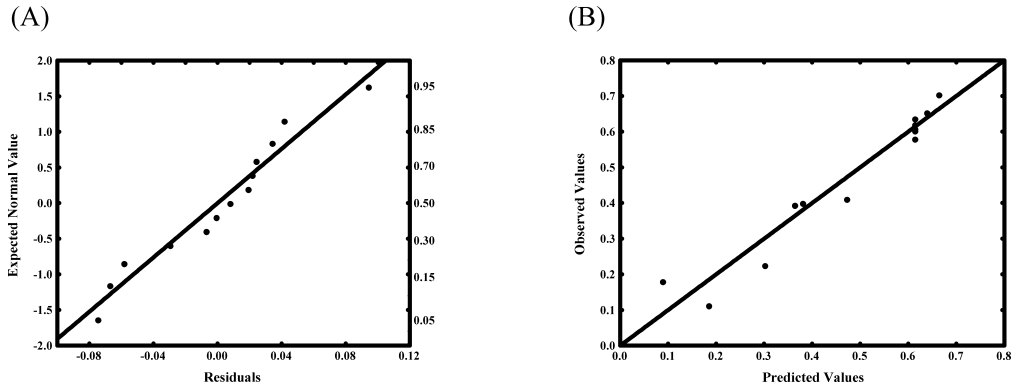

Fig. 3. (A) Normal Probalility, (B) Observed *versus* Predicted Values for Overall Desirability (*D*)

ported by Liu and Chiou.[25] The observed values and the predicted values for *D* are illustrated in Fig. 3B. The points of the experimental values are close to the diagonal line, which indicated a good fit of the model and the deviation between the predicted and observed values was less. Further, the chi-square (χ^2) test was carried out to check the significance of the difference between the predicted responses and the observed values. It was found that the obtained chi-square value (0.1644) for *D* is less than the tabulated value ($\chi^2_{0.05,12}$, 21.03). This means that there was no significant difference between the observed data and the predicted values. In other words, it confirms the deviation between the observed data and the predicted values is acceptable. Therefore, we can conclude that the obtained regression models are reliable and adequate for predictions and for optimization.

Figure 4 shows the response surface for the *D*-holding variables X_1 and X_2. The region where optimization was studied was the pure black domain marked in Fig. 4. A larger *D* value indicates a better overall expression performance, so the maximum value of *D* should be found in this region (X_1: 27—31.5; X_2: 1.16—1.35). Computer script calculation was done by Visual Basic Language with a step width of 0.01. The functional expression used in script calculation was the regression Eq. 9. After 920 runs, the maximum function value was obtained at $X_1=29.6$ and $X_2=1.29$ ($D=0.699$). It is interesting to find that the predicted maximum *D* value of 0.6991 calculated from the regression model at $X_1=29.6$ and $X_2=1.29$ is lower than the experimental *D* result of 0.703 as shown in Table 2 at $X_1=27.2$ and $X_2=1.28$. Actually, the predicted *D* value at $X_1=27.2$ and $X_2=1.28$ according to the same regression model should be 0.663, which is obvious lower than the predicted maximum *D* value of 0.699. Therefore, setting the variables responsible for the maximum response at $X_1=29.6$ and $X_2=1.29$ is true. As concerns the deviation of the experimental value (0.703) with the predicted value (0.663) at the point of $X_1=27.2$ and $X_2=1.28$, it is confirmed that this deviation is acceptable according to the regression model validation analysis mentioned above. Corresponding predicted response values at $X_1=29.6$ and $X_2=1.29$ were 30.7 nm (droplet size), 0.127 (polydispersity index (PI)), 11.9 mg/ml (equilibrium solubility) and 71.7% (soluble drug percentage post-digestion). To confirm the model adequacy for prediction, five batches of formulations under the optimal composition were prepared, and the four responses evaluated for each formulation, respectively. The results of

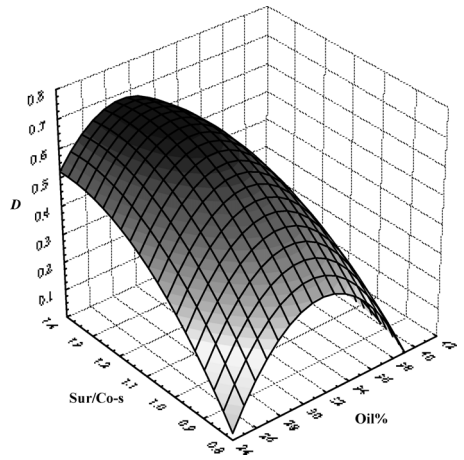

Fig. 4. Response Surface for Overall Desirability (*D*) as a Function of Oil Percentage and Sur/Co-s Ratio

Table 3. Comparison between the Observed Values and Predicted Values for Optimized Bufalin SMEDDS ($n=3$)

Response	Predicted values	Observed values	Bias (%)
Droplet size (nm)	30.7	33.9±2.3	−10.4
Polydispersity	0.127	0.126±0.017	0.787
Equilibrium solubility (mg/ml)	11.9	12.6±0.5	−5.9
Solubilization capacity	71.7	73.6±2.2	−5.4

Bias (%)=(predicted value−observed value)/predicted value ×100%.

the bias (Table 3) indicated good agreement between predicted and observed results. Oil percentage (X_1) and Sur/Co-s ratio (X_2) were optimized to be 29.6 and 1.29, respectively. The corresponding SMEDDS formulation containing bufalin (0.5%, w/w) was: Maisine 35-1 and Miglyol 812N (1 : 1, w/w) of 29.5%, Cremophor EL of 39.5%, and Transcutol P of 30.5%.

As concerns as the methods of combining and comprising the multiple responses, it is noteworthy that the desirability function approach used in the present study is not the only possible approach. A few strategies to multiple responses optimization have been reported in the past two decades, such as desirability function approach, generalized distance approach, loss function approach. Among them, desirability approach is a predominant method, in which the overall desirability (*D*) is defined by combining the d_i either by a geomet-

ric mean (Eq. 4)[26—29] or by a weighted geometric mean[30] ($D=(\prod_{i=1}^{n} d_i^{w_i})^{1/n}$, where w_i is the weighting of the ith response). The latter introduced weighting factors to reflect the relative importance assigned to the response. Weight assignments need individual or group convictive judgments.[31] As to the SMEDDS optimization, by now, which response is more important than another and priority were few reported, which result in difficulty in identifying and assigning weighting factors for each response. With the further development of SMEDDS and possible assigning weighting factors in common agreements, the desirability approach by a weighted geometric mean is suggested to be an alternative procedure to solve this problem.

Morphology The morphology of the optimized formulation was observed (Fig. 5): the droplets were very spherical.

***In Vitro* Release Study** The profile of bufalin release from the SMEDDS formulation and suspension is shown in Fig. 6. A rapid release up to 21% occurred for the SMEDDS in the first 10 min, whereas only 5% bufalin was released from suspension in the same period. The accumulated amount of drug released from the SMEDDS in 120 min was approximately 90%, as opposed to 35% from suspension. The results showed the superior, faster release of the SMEDDS in comparison with suspension. Spontaneous formation of a microemulsion with a small droplet size may have provided the faster rate of drug release. The SMEDDS is expected to improve bioavailability compared with suspension, and further investigations will be carried out.

Study of *in Situ* Intestinal Absorption K_a values for the duodenum, jejunum and ileum were $0.0625\pm0.0116\,h^{-1}$, $0.0758\pm0.0210\,h^{-1}$ and $0.0783\pm0.0164\,h^{-1}$, respectively. There were no significant differences in K_a for the tested intestinal segments ($p>0.05$). This indicates that there were no obvious main sites for the bufalin SMEDDS absorption. Percentage uptake after perfusion for 3 h at the duodenum, jejunum and ileum was 14.6±3.9%, 17.6±4.6%, and 17.9±3.5%, respectively ($p>0.05$). Physical absorption of the drug on the intestinal wall and the perfusion catheter was detected as the reported method,[32] and obvious absorption of the drug was not observed. Based on the results in the present study, it could be concluded that bufalin was well absorbed at all tested segments, and no main site for bufalin SMEDDS absorption in the small intestine was found. Intestinal absorption is a complex process, in which various factors (apart from those mentioned above) may influence absorption parameters (*e.g.*, intestinal disease, metabolism, drug transporters, surgery, choice of anesthetics).[33,34] It is very difficult to evaluate all of the influencing factors on drug absorption just by one individual method. Combining two or more techniques may provide more information regarding drug absorption.

Bioavailability Study The bioavailability of bufalin SMEDDS was studied in comparison with that of bufalin suspension. The plasma profile of bufalin SMEDDS and suspension is shown in Fig. 7, two peaks in the plasma concentration profiles for both preparations, which was possibly caused by enterohepatic circulation. Therefore, the pharmacokinetic parameters of bufalin SMEDDS and bufalin suspension were calculated using a non-compartmental model rather than a compartmental model. The mean pharmacokinetic parameters for bufalin SMEDDS and suspension are as follows: C_{max}, (552.48±102.73) and (237.36±44.61) ng/ml; t_{max}, (48±6.7) and (60±1.0) min; $AUC_{(0—t)}$, (58182.45±13188.20) and (24402.10±5684.05) ng min/ml; $AUC_{(0—\infty)}$, (64686.30±13125.71) and (43509.39±12451.45) ng min/ml. The mean relative bioavailability of bufalin SMEDDS was 2.38-fold higher compared with that of the suspension. SMEDDSs have been reported to improve the bioavailability of many hydrophobic drugs, which may be related to the improved properties, such as enhanced drug solubility in gastrointestinal tract (GIT), reduced food effect, stimulation of lymphatic transport and altered intestinal permeability.[4,5,35] In this study, after optimization of the bufalin SMEDDS formulation, we obtained a formulation that had good properties *in vitro*, which greatly contributed to the drug behavior *in vivo*. The formulation had significantly improved bioavailability compared with that of the suspension. Therefore, peroral administration *via* SMEDDS offers a promising route for administration of bufalin.

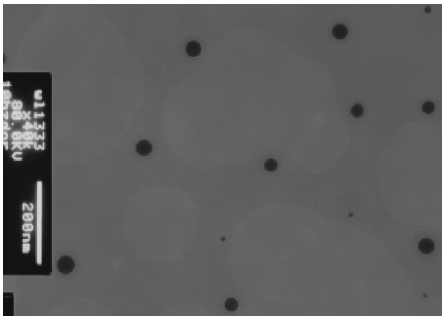

Fig. 5. Transmission Electron Micrograph of a Bufalin Microemulsion (Magnification, ×40000)

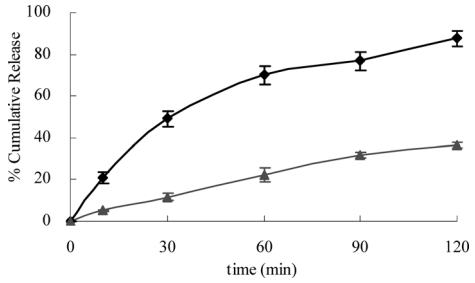

Fig. 6. *In Vitro* Release Profiles of Bufalin from SMEDDS (—◆—) and Suspension (—▲—) ($n=5$)

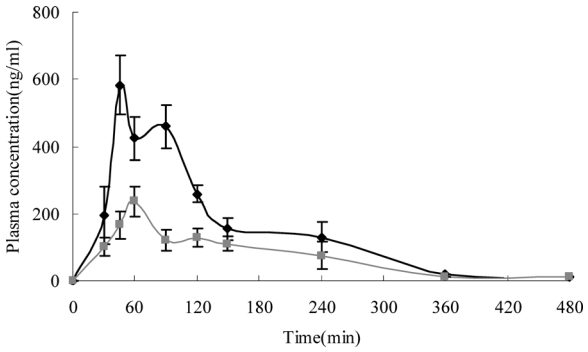

Fig. 7. Plasma Concentration Profile of Bufalin after Oral Administration of SMEDDS (—◆—) and the Suspension (—■—) in Rats ($n=5$)

Conclusion

An improved and efficient experimental approach was developed to design and optimize SMEDDS formulation with two variables (oil percentage and Sur/Co-s ratio) and four responses (droplet size, polydispersity, equilibrium solubility, and solubilization capacity under an *in vitro* digestion condition). The obtained bufalin SMEDDS showed small droplet size with a narrow PI, high equilibrium solubility, good solubilization capacity under an *in vitro* digestion condition, and rapid release characteristics. Desirable intestinal absorption of a bufalin SMEDDS was achieved. *In vivo* bioavailability was significantly improved with bufalin delivered *via* SMEDDS compared with in suspension. The experimental design used in this study may provide a valuable and efficient method to yield an optimized formulation for a SMEDDS.

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