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Research Article

Preparation and Optimization of Resveratrol Nanosuspensions by Antisolvent Precipitation Using Box-Behnken Design

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Abstract. Resveratrol, a natural polyphenolic component, has inspired considerable interest for its extensive physiological activities. However, the poor solubility of resveratrol circumscribes its therapeutic applications. The purpose of this study was to optimize and prepare resveratrol nanosuspensions using the antisolvent precipitation method. The effects of crucial formulation and process variables (drug concentration, stabilizer, and surfactant contents) on particle size were investigated by utilizing a three-factor three-level Box-Behnken design (BBD) to perform this experiment. Different mathematical polynomial models were used to identify the impact of selected parameters and to evaluate their interrelationship for predictive formulation purposes. The optimal formulation consisted of drug 29.2 (mg/ml), polyvinylpyrrolidone (PVP) K17 0.38%, and F188 3.63%, respectively. The morphology of nanosuspensions was found to be near-spherical shaped by scanning electron microscopy (SEM) observation. The X-ray powder diffraction (XRPD) and differential scanning calorimetry (DSC) analysis confirmed that the nanoparticles were in the amorphous state. Furthermore, in comparison to raw material, resveratrol nanosuspensions showed significantly enhanced saturation solubility and accelerated dissolution rate resulting from the decrease in particle size and the amorphous status of nanoparticles. Meanwhile, resveratrol nanosuspensions exhibited the similar antioxidant potency to that of raw resveratrol. The *in vivo* pharmacokinetic study revealed that the C_{max} and $AUC_{0\rightarrow\infty}$ values of nanosuspension were approximately 3.35- and 1.27-fold greater than those of reference preparation, respectively. Taken together, these results suggest that this study provides a beneficial approach to address the poor solubility issue of the resveratrol and affords a rational strategy to widen the application range of this interesting substance.

KEY WORDS: antisolvent precipitation; Box-Behnken design; oral bioavailability; resveratrol nanosuspensions; saturation solubility and dissolution rate.

INTRODUCTION

Resveratrol (3,5,4′-trihydroxystilbene) is a naturally occurring polyphenolic compound present in grapes, peanuts, and other foods that are usually consumed as part of human diet (1). In terms of chemical configuration, resveratrol exists as two structural isomers, *cis* (*Z*) and *trans* (*E*), which may exhibit different biological and pharmacological effects. The *trans*-resveratrol is the more normally used isomeric form than *cis*-isomer, primarily due to its potential biological activity. Recently, efforts to widen the range of rational utilization of this intriguing compound have been explored in some research fields, and many scientific studies have demonstrated that the resveratrol harbors broadly desirable bioactivities in such areas as antioxidant, antitumor, anti-inflammatory, and cardioprotective actions (2,3). Although resveratrol shows diverse valuable advantages for human health, therapeutic efficacies of resveratrol have not fully exerted for the reason that resveratrol presents low aqueous solubility and slow dissolution rate, which will inevitably impact its bioavailability. Therefore, in order to address these aforementioned problems, it is necessary to work out a reasonable strategy to improve its water solubility and increase its dissolution rate.

It is generally accepted that reducing drug particle size is an effective and widely adopted approach to increase solubility and speed up dissolution by enlarging the effective surface area (4). Especially when particle size is reduced to the nanometric region, the dissolution rate is proportional to the surface area available for dissolution as described by the Noyes-Whitney equation. In addition to the dissolution rate enhancement, the absolute solubility of nanosized active ingredient is also evidently altered in the light of Ostwald-Freundlich equation (5). According to this opinion, there is a great interest in developing alternative formulation strategies to diminish the drug particle size further down to the submicron range. Recently, one of the most promising methods is to tailor nanosized particles of these poorly soluble drugs.

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Nanosuspensions, submicron colloidal dispersions of drug particles stabilized with stabilizers, surfactants, or a mixture of both, highlight an increase in saturation solubility and very fast dissolution rate, owing to the increased surface to a volume ratio of the nanoparticles, especially for particle size below 1 μm (6). Top-down and bottom-up are the two primary technical approaches for drug nanosuspension production (7). The application of these different strategies varies from molecule to molecule, depending on their physicochemical characters and hardness of the compound (8). Most of the top-down production methods are high energy consumption processes where drug particles are crushed to diminish size associated with various technologies such as wet milling, microfluidization, and high pressure homogenization (9). On the other hand, the bottom-up process is generally designated as a precipitation process, because the principle here is to precipitate drug particles from a supersaturated drug solution. Briefly, the drug is first dissolved in a solvent, which is then rapidly introduced into the antisolvent to precipitate the crystals with the aid of polymer and/or surfactant to control ultrafine particles (10,11).

In recent years, several studies have focused on novel formulation approaching to increase the solubility of resveratrol in water. Complexation of resveratrol with β-cyclodextrin (β-CD) or hydroxypropyl-β-CD (HP-β-CD) has been explored to improve the water solubility (12). The encapsulation of resveratrol into polymeric matrix represents a powerful strategy. Compared with those resveratrol-loaded nanotechnological formulations, a pronounced benefit of resveratrol nanosuspensions is their capability to offer a high ratio of carried drug to excipient which raises the probability of obtaining higher therapeutic concentration producing desired pharmacological action and makes them superior to other colloidal drug delivery systems (13).

Nowadays, despite of the resveratrol nanosuspensions produced by antisolvent precipitation and reported in literature (14,15), a systematic assessment of multiple formulation variables on the characteristics of the produced nanosuspensions has not yet been undertaken in a quantitative manner. Therefore, the objective of the present study was to evaluate the antisolvent precipitation method for the production of resveratrol nanosuspensions using a statistical Box-Behnken design (BBD) as a means of adequately optimizing the various parameters. To evaluate the main and interaction variables that affect particle size, a three-factor three-level BBD was employed to schedule and perform the experiments. The effects of variable formulation parameters, such as the concentration of resveratrol in the organic phase and the contents of surfactant and stabilizer, were investigated systematically. The corresponding physical properties of the prepared resveratrol nanosuspensions were characterized by scanning electronic microscopy (SEM), X-ray powder diffraction (XRPD), and differential scanning calorimetry (DSC). The dissolution rate and saturation solubility of resveratrol nanosuspensions were also determined. The antioxidant activity of nanosuspensions was evaluated *in vitro*. Finally, *in vivo* pharmacokinetics study was also carried out to compare oral bioavailability of the nanosuspensions with that of the resveratrol coarse suspensions.

MATERIALS AND METHODS

Materials

The commercial *trans*-resveratrol (98.9% pure) was purchased from Tianjin Jianfeng Nature Product R&D Co., Ltd. (Tianjin China). Polyvinylpyrrolidone 17 PF (PVP K17) and Poloxamer 188 (F188) were kindly supplied by BASF (China) Co., Ltd. (Shanghai, China). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All other chemicals and reagents used were of analytical grade or better.

Preparation of Resveratrol Nanosuspensions

Resveratrol nanosuspensions were prepared in accordance with the liquid antisolvent precipitation technique (10). Briefly, raw resveratrol and surfactants were dissolved in ethanol at definite concentration to form the organic phase and the solution was then filtrated through a 0.22-μm microfilter to exclude the possible impurities. Meanwhile, the antisolvent phase was prepared by dispersing the specific concentration of stabilizer in distilled water. Then, the ethanol solution was rapidly introduced into antisolvent solution (below 3°C) under a vigorous stirring speed at 9000 rpm. A bluish opalescence suspension appeared immediately, and resveratrol nanosuspensions formed in the process of mixing. For long-term stability of the final product, the freshly prepared nanosuspensions were lyophilized with 3% (*w*/*v*) mannitol as cryoprotectant. After being rapidly frozen in liquid nitrogen, the nanosuspensions were freeze-dried using a lyophilizer (Eyela FDU-1200, Kyoto, Japan) at a vacuum degree of 20 Pa for 48 h to obtain dry powder.

Experimental Design

As shown in preliminary screening studies for developing resveratrol nanosuspensions by liquid antisolvent precipitation method, the critical variables, such as the resveratrol concentration (X_1) in the organic solution, the amount of stabilizer (PVP K17, X_2), and the surfactant (F188, X_3) in aqueous phase, significantly affect the physicochemical properties and stability profiles of the produced nanosuspensions. When it comes to multidimensional and combinational factors that determine target quality, it is reasonable to establish a mathematical approach for the development and optimization of pharmaceutical formulation, allowing an extraction of maximal information out of few well-designed experiments. Therefore, considering the selected factors and levels, a three-factor, three-level BBD, generated by Design-Expert software, was explored to probe the optimal variables and to investigate the influence of these three independent factors (X_1, X_2, and X_3) on dependent responses (particle size) because this design facilitates a more efficient alternative to the full three-level factorial. The parameters of the experiments can be observed in Table I.

Characterization of Nanosuspensions

After statistical analysis, optimum formulation was achieved in light of the predetermined constraints in which

Table I. Variables and Their Levels in the Box-Behnken Design

Independent variables	Levels		
	−1	0	1
X_1=Concentration of resveratrol (mg/ml)	20	30	40
X_2=Amount of stabilizer (%)	0.2	0.3	0.4
X_3=Level of surfactant (%)	2	3	4
Dependent variables			Constraints
Y_1=Particle size			Minimize

the particle size was in their minimum levels. The picked resveratrol nanosuspensions with desired particle size and narrow distribution were prepared for the evaluation of physicochemical characterization as well as the saturation solubility and dissolution kinetics.

Particle Size and Morphology

Particle size and size distribution of nanosuspensions were analyzed by laser diffraction using a Coulter LS 230 Analyzer (Beckman-Coulter Co., Ltd., USA) at room temperature. All samples were diluted with double-distilled water to reach a suitable concentration before measurement. The surface morphology was studied by scanning electron microscopy (Inspect F50, FEI, USA). A drop of the sample was deposited on a silicon wafer, dried, and sputtered for 20 s with gold before observation.

X-ray Diffraction Studies

The crystalline state of resveratrol in different samples was elucidated with an X-ray diffractometer (Rigaku, Japan). X-ray diffraction (XRD) was carried out in symmetrical reflection mode using Cu Kα line as the source of radiation, and the wavelength was set at 1.5405 Å. Standard runs using 40 kV and 100 mA in this process. Samples of pure resveratrol, poloxamer 188, physical mixture, and lyophilized nanosuspensions were analyzed, respectively. The instrument was operated with a scanning rate of 0.02°/min and a scanning range of the 2θ from the initial angle 3° to the final angle 50°.

Differential Scanning Calorimetry Assays

Thermal analysis was conducted for the pure resveratrol powder, their physical mixtures, lyophilized powder, cryoprotector, and other excipients. Five milligrams of each sample was accurately weighed and placed into aluminum crucible and sealed using an aluminum lid by a sealing machine. The thermograms were obtained by differential scanning calorimetry (DSC Q10, TA, USA) at a heating rate of 10°C/min. This was done in an inert atmosphere flushed with nitrogen at a rate of 30 ml/min, and Al_2O_3 was used as a reference.

Saturation Solubility Test

The saturation solubility of coarse resveratrol, lyophilized resveratrol nanosuspensions, and physical mixtures was determined in phosphate buffer saline (PBS) solution (pH 7.4). Excess amounts of samples were added into 10 ml PBS (pH 7.4) solution in a capped vial which was then placed in a controlled temperature shaking water bath (SHZ-82, Jintan Experimental Instrument Factory, Jiangsu, China) at 37°C, leaving them to dissolve for 48 h. Then, samples (1 ml) were withdrawn and centrifuged at 18,894×*g* for 1 h. The obtained supernatant sample was assayed using a UV/Vis spectrophotometer (UV-2450, Shimadzu, Kyoto, Japan) at 306 nm. The experiment was conducted in triplicate.

Dissolution Rate Study

The dissolution rate experiment was performed according to the Ch.P.2010 Edition Apparatus II (paddle) method (ZRS-8A, Tianda Tianfa Technology Co., Ltd., Tianjin, China). PBS solution (pH 7.4) was selected as dissolution medium. The temperature was set to 37±0.5°C, and the stirring rate was at 100 rpm. Accurately weighed samples containing the equivalent of 10 mg resveratrol were dispersed in the 900 ml dissolution medium. Then, samples, each of 4 ml, were withdrawn at different times and passed through a 0.1-μm syringe filter. Quantification of the samples was determined with a UV spectrophotometer at 306 nm. The measurements were repeated three times.

2,2-Diphenyl-1-picrylhydrazyl Free Radical Scavenging Activity

Scavenging activity on DPPH was determined according to the method reported with some modification (16). For the procedure, an ethanol solution of the radical DPPH was prepared and stored in a dark and cool environment. One hundred-milliliter different samples diluted in a series of concentration in ethanol were added to aliquots (3.9 ml) of a solution with a concentration of 50 mmol/l DPPH. A control sample was prepared by mixing DPPH ethanolic solution with Tris buffer (17). All the samples were incubated in the dark for 1 h at room temperature. Then, the absorbance was analyzed at 517 nm. In this assay, the SC50 value, which meant a concentration of sample being required to scavenge 50% of DPPH free radicals, was adopted to quantify the free radical scavenging effect of different samples. The lower SC50, the higher the antioxidant power of a compound is.

Pharmacokinetic Study in Rats

The pharmacokinetic study was performed in accordance with the University Ethics Committee for the use of experimental animals. Male Wistar rats (body weight 200±20 g) were supplied by the Experimental Animal Center of Taishan Medical University (Taian, China). All the rats were divided randomly into two groups comprising six animals in each and were housed in cages for at least 3 days prior to the beginning of the study and had free access to food and water. Two types of resveratrol formulations at a dose of 120 mg/kg weight were orally administrated to two groups of rats by gavage, i.e., resveratrol nanosuspensions of optimized formulation and resveratrol coarse suspensions were prepared by adding the same excipients as the optimal formulation and sonicated for 15 min.

Blood samples (0.5 ml) of each animal were obtained by the retro-orbital puncture at 0, 0.083, 0.25, 0.5, 1, 2, 3, 4, 6, and 8 h after administration. Plasma was separated from the whole blood in a heparinized tube by centrifugation at 3000 rpm for 15 min and was stored at −20°C until analysis.

Plasma samples were processed as follows: a 100-μl aliquot of plasma sample, 100 μl of the chlorzoxazone internal standard solution (23 μg/ml in methanol), and 200 μl methanol were added and vortexed for 3 min in a 2.0-ml polypropylene tube. The mixture was centrifuged at 10,000 rpm for 10 min, and then, the supernatant was transferred to a clean tube and evaporated to dryness under a nitrogen gas stream in a 35°C water bath. The dried residue was then redissolved in 100 μl of mobile phase and centrifuged at 10,000 rpm for 10 min, and the aliquot of the supernatant was injected into the HPLC system for analysis.

The content of resveratrol in plasma was assayed by HPLC method. The HPLC system was composed of a model LC-10A pump (Shimadzu, Kyoto, Japan) equipped with a 20-μl loop and a UV visible detector. A Kromasil C18 (250× 4.6 mm) analytical column was used with the mobile phase of acetonitrile and 0.1% (*v*/*v*) aqueous phosphoric acid (30:70) at the detection wavelength of 306 nm. The flow rate was 1.0 ml/min at room temperature.

The plasma concentration *versus* time profile was analyzed by WinNonlin Professional version 3.1 (Pharsight Corporation, Mountain View, CA, USA). Non-compartmental model was chosen to calculate the various pharmacokinetic parameters including the area under the plasma concentration-time curve from zero to infinity ($AUC_{0\to\infty}$) and mean residence time (MRT). The peak plasma concentration (C_{max}) was obtained directly from the experimental data. All data were expressed as the mean±standard deviation (SD).

RESULTS AND DISCUSSION

Statistical Analysis of Experimental Data by Design-Expert Software

Considering that the selected formulation parameters affect the properties of nanosuspensions, a statistically mathematical polynomial was fitted among the linear, two-factor (2F) interaction and quadratic model based on the analysis of multiple correlation coefficient r^2, predicted r^2, and adjusted r^2 which served as quality criteria to identify the optimal model and to evaluate the contributions of the selected independent variables on the responses. The Y_1 (particle size) data were calculated as input for the construction of its respective statistical mathematic polynomial model. As shown in Table II, when compared with other models, a good match for each of the responses Y_1 was suggested to select the quadratic model due to the largest r^2 values for all responses. Therefore, the quadratic polynomial incorporating interactional and quadratic terms was opted to describe the effects of the variables.

Table II. Results of Model Summary Statistics Analysis for Responses

	Y_1 (particle size)		
Model	r^2	Adjusted r^2	Predicted r^2
Linear	0.2811	0.1152	−0.4359
2F	0.5571	0.2914	−0.9891
Quadratic	0.8728	0.7092	−1.0214

The results of the experimental design showed that the pivotal influence parameters, such as drug concentration and the levels of stabilizer and surfactant in the antisolvent phase, led to small particle sizes in the process of resveratrol nanosuspension production. Each experimental response was described by the following quadratic Eq. (1) of the response surface:

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_4X_1X_2 + A_5X_2X_3 + A_6X_1X_3 + A_7{X_1}^2 + A_8{X_2}^2 + A_9{X_3}^2 \quad (1)$$

Analysis of variance (ANOVA) was implemented to designate the important factors of the quadratic models on the responses and their quantitative effect. Table III summarized the effects of the model terms and associated *p* values for all three responses. Only variables with *p* values <0.05 were considered to be statistically significant. The symbol and value of the quantitative effect suggested the tendency and magnitude of the term's influence on the response, respectively. A positive value in the regression equation exhibited an effect that favors the optimization due to synergistic effect, while a negative value indicated an inverse relationship or antagonistic effect between the factor and the response.

In addition, a series of the three-dimensional (3D) frameworks for optimization of resveratrol nanosuspensions with desirable physicochemical properties were further depicted to investigate the interaction and quadratic effects of these variables on the responses. The 3D response surface illustrations for particle size were presented in Fig. 1, respectively.

According to the results obtained from the 17 experimental runs, the various independent factors resulted in particle size of resveratrol nanosuspensions varying from 126 to 277 nm. In this case, the drug concentration (X_1), the amount of PVP K17 (X_2), their interactive term (X_1X_2, X_1X_3), and polynomial model of drug concentration (X_1^2) were identified

Table III. The Quantitative Factor Effects and Associated *p* Value for the Responses

	Y_1 (particle size)	
Parameters	Effect	*p* value
X_1	19.06	0.0033*
X_2	13.89	0.0074*
X_3	0.30	0.5994
X_1X_2	13.90	0.0074*
X_1X_3	14.00	0.0073*
X_2X_3	0.21	0.6586
X_1^2	13.01	0.0087*
X_2^2	0.58	0.4720
X_3^2	0.02	0.8950

*$p<0.05$, significant values

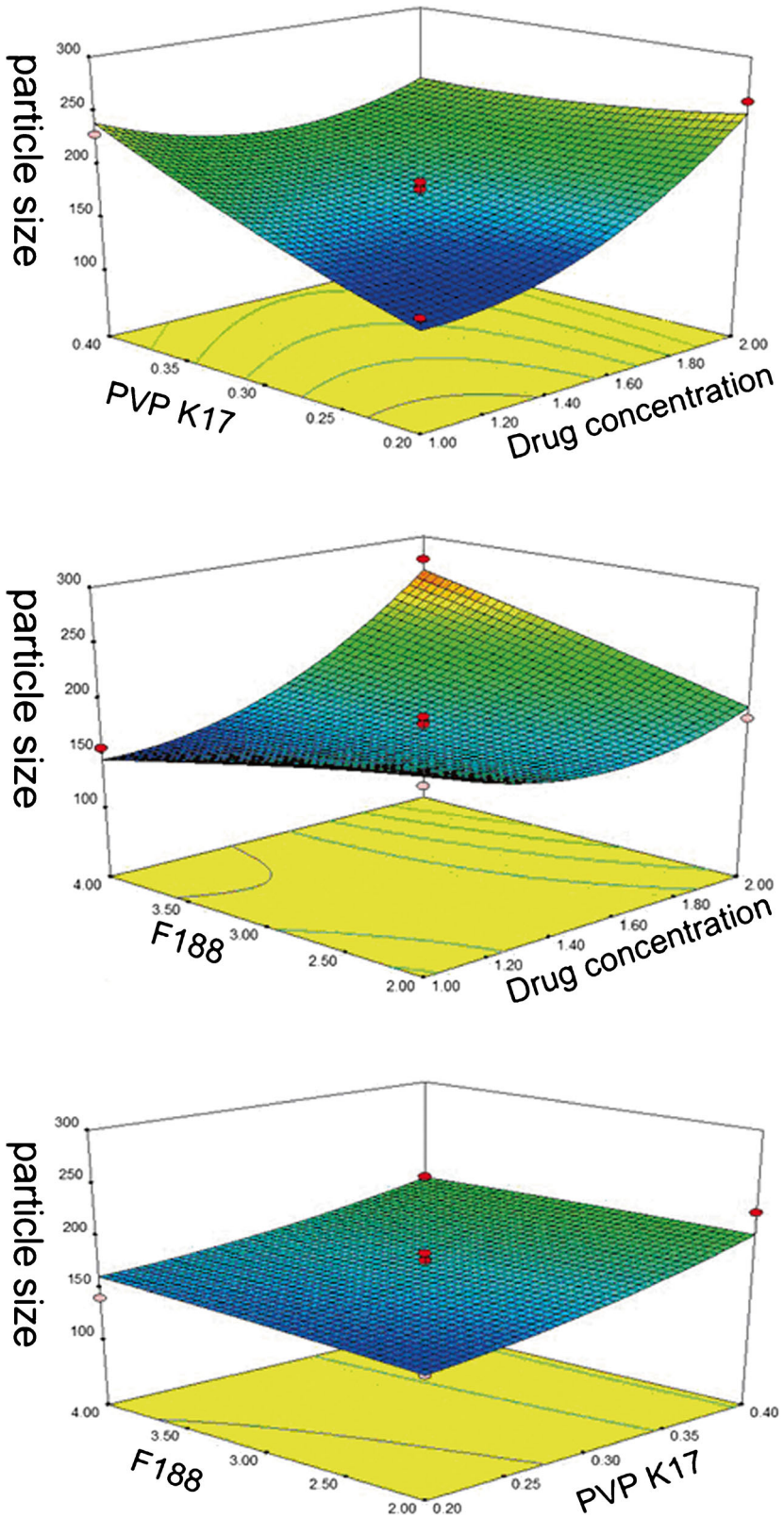

Fig. 1. Response surface model (RSM) showing the influence of the independent variables on the particle size

as significant model terms in light of the p value <0.05. Quantitative estimation of the significant models indicated that drug concentration and the amount of PVP K17 had the prime influence on particle size for its large positive coefficient (27.72 and 23.66, respectively), suggesting that increasing these two variables in the formulation can lead to larger

particle size. The final mathematical Eq. (2) of the fitted model was given below for particle size (Y_1).

$$Y_1 = 172.54 + 27.72X_1 + 23.66X_2 + 3.49X_3 - 33.48X_1X_2 + 33.59X_1X_3 - 4.14X_2X_3 + 31.57{X_1}^2 + 6.65{X_2}^2 - 1.19 \quad (2)$$

The correlation coefficient value (r^2) of this equation was found to be 0.9149, indicating that 91.49% of the sample variation in particle size was ascribed to the experimental variables studied. Therefore, this fitted equation can be used to predict the best formulation for resveratrol nanosuspensions.

The process of nanosuspension fabrication implies the creation of additional surface area, which hence results in a thermodynamically unstable system. Therefore, the presence of excipients, such as stabilizers and (or) surfactants in the nanoparticle formulation, is essential to maintain a physically stable state. The main functions of the stabilizer are to wet the hydrophobic drug particle surface completely and to provide steric barriers for the prevention of Ostwald's ripening and agglomeration of nanosuspensions (18). Consequently, adding an appropriate stabilizer can lower the excessive high surface energy of nanoparticles generated from transferring coarse particle size to nanometer scale in the system.

As shown in Fig. 1, mean particle size increased with the enhancement of the drug concentration in a nonlinear mode. In this study, the size of the newly formed nanosuspensions at both a low drug concentration (20 mg/ml) and a high drug concentration (40 mg/ml) was determined. At the low drug concentration, the initial particle size was approximately 126 nm, while higher concentrations of resveratrol in the suspension produce suspensions with larger particle size (277 nm). It is apparent that a smaller particle size can be obtained at relatively lower concentrations of resveratrol. The classical crystallization theory can explain the impact of drug concentration on particle size: During the process of nanosuspensions formation, the precipitation of nanoparticles involves in a series steps of nucleation, molecular growth, and growth by coagulation and condensation, followed by agglomeration. Furthermore, the rate of each step governs the final particle size and particle size distribution. Supersaturation is the crucial driving force of this process, which determines not only the nucleation rate but also the diffusion-controlled growth rate. The nucleation and growth of particles occur simultaneously and both compete for consumption of supersaturation (19). At higher drug concentration, due to greater supersaturation, the fact that the rapid diffusion controlled growth and agglomeration rate was more dominant than nucleation rate gave rise to larger crystals (20,21). Consequently, a critical high particle concentration without compromising the particle size significantly should be explored in the optimal screening process.

It was observed in Table III that the PVP K17 concentration in antisolvent had also a significant effect ($p<0.05$) on the particle size. The positive value of the coefficient suggested that the increase in the concentration of PVP K17 resulted in an increased mean particle size. Polyvinylpyrrolidone, a nonionic polymer, can be liable to anchor on the drug particle surface and to furnish a mechanical barrier against crystallization by occupying the adsorption sites and inhibiting the incorporation of drug molecules from solution into crystal lattice (22). Theoretically, a complete adsorption and a full coverage of polymer on the surface of the newly formed crystals are necessary to provide enough steric repulsion between the crystals. Thus, adequate stabilizers present in the precipitation system can satisfy the demand for inhibition of crystal growth and consequent prevention of crystal agglomeration. However, the concentration of stabilizer in the mixture of solvent/antisolvent affects the precipitated particle sizes in a paradoxical manner. Insufficient surface coverage of stabilizer could result in rapid crystal growth and agglomeration, while with a continuously increased concentration of polymer, an excessive amount of polymer would increase the particle size by thickening the cloak and inhibit the diffusion between the solvent and the antisolvent during precipitation. On the other hand, the occurrence of an increase in osmotic pressure by increasing the polymer concentration conduces to enhanced attraction among colloidal particles, leading to larger size (23,24).

After analyzing the polynomial equations associated with the dependent and independent parameters, a further optimization and validation process by means of the Design-Expert

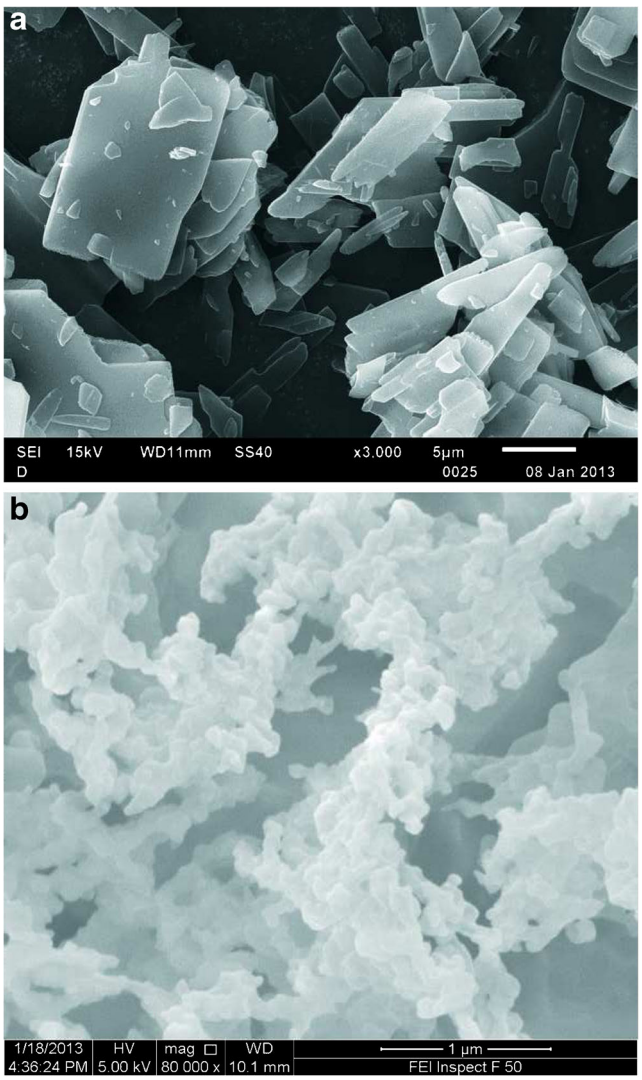

Fig. 2. SEM images of **a** raw resveratrol and **b** lyophilized resveratrol nanosuspensions

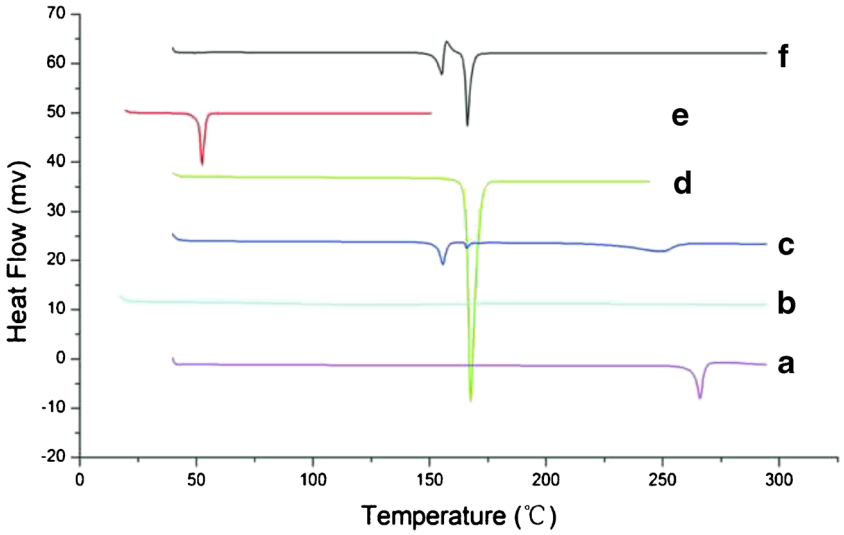

Fig. 3. DSC patterns of *a* raw resveratrol, *b* PVP K17, *c* physical mixture, *d* mannitol, *e* F188, and *f* lyophilized resveratrol nanosuspensions

software was to probe the optimal formulation of resveratrol nanosuspensions, which depended on the predefined criteria of minimum particle size. The composition of optimum formulation was determined as follows: drug 29.2 (mg/ml), PVP K17 0.38%, and F188 3.63%, which meet the requirements for optimization. At these levels, the predicted value of Y_1 (particle size) was 134.6 nm. Therefore, in order to confirm the predicted model, a new batch of nanosuspensions prepared with the optimized formulation factor levels yielded a particle size of 145.7±7.07 nm, which was in good agreement with the predicted values. A comparison between these observed results and theoretical predictions indicates the reliability and feasibility of BBD used in predicting a desirable formulation.

Physicochemical Characterization of Nanosuspensions

The optimized resveratrol nanosuspensions were successfully prepared and then subjected to physicochemical characterization examination. Morphology of raw resveratrol and precipitated drug particles after freeze-drying is shown in Fig. 2. The SEM studies revealed that raw drug particles exhibit irregular flaky structure with a broad particle size

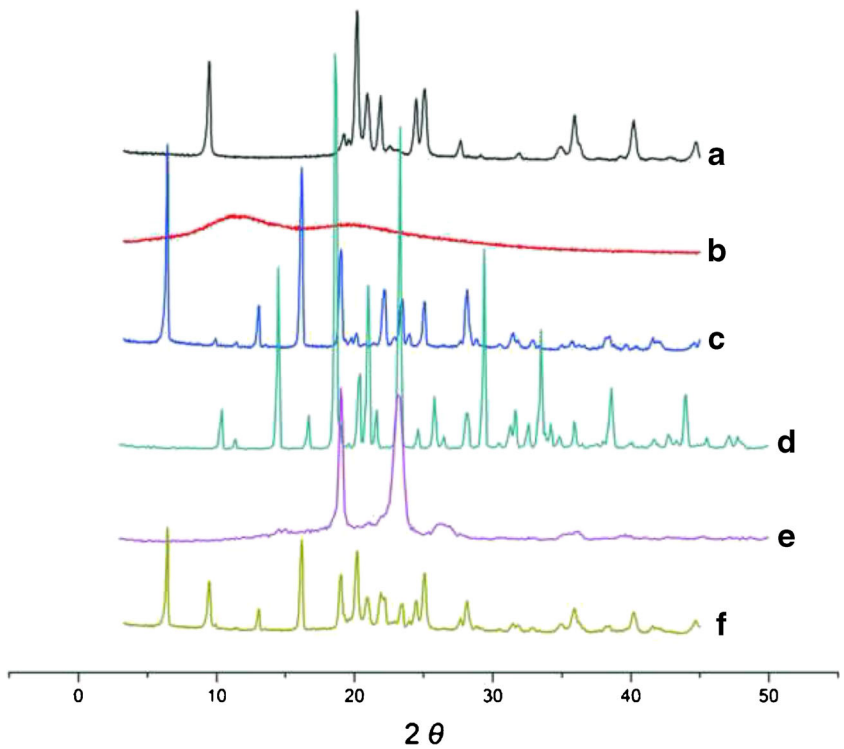

Fig. 4. XRPD patterns of *a* lyophilized resveratrol nanosuspensions, *b* PVP K17, *c* raw resveratrol, *d* mannitol, *e* F188, and *f* physical mixture

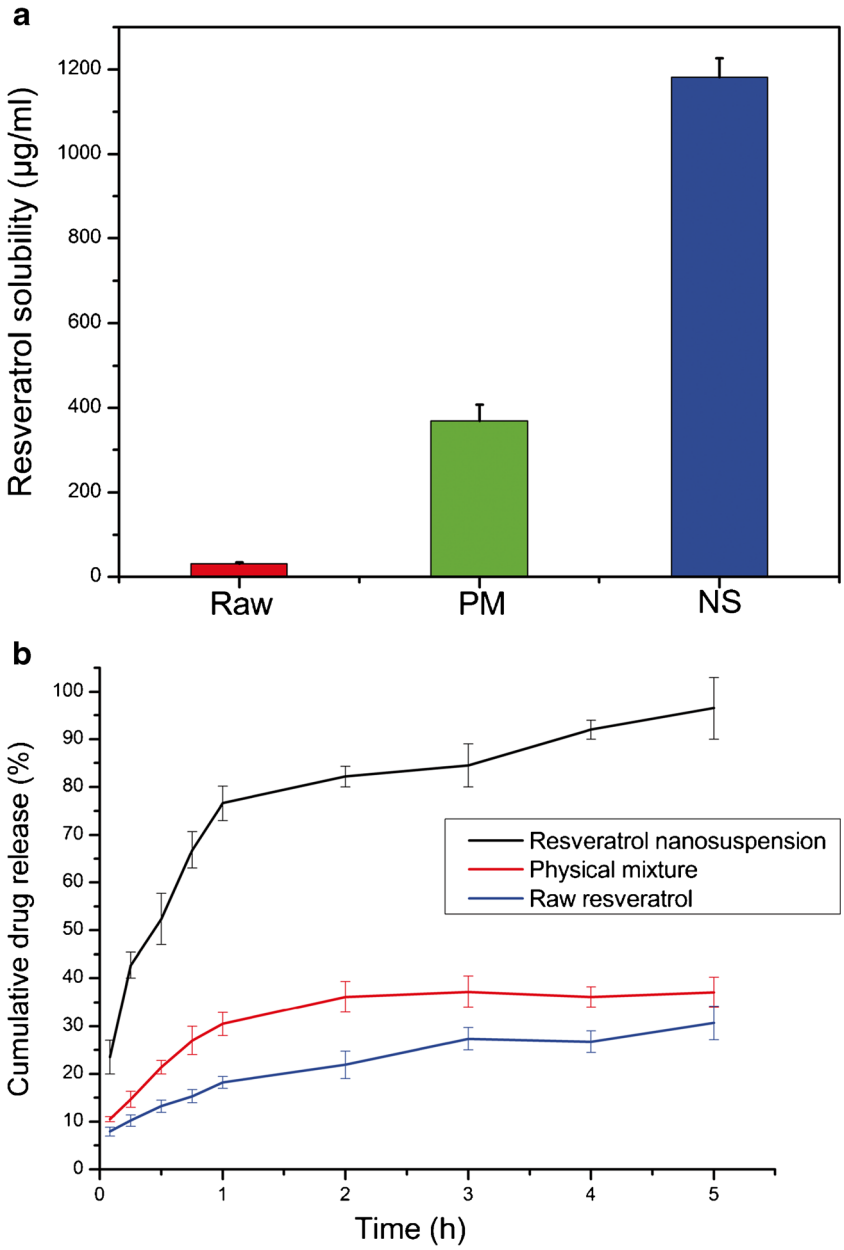

Fig. 5. Saturation solubility (**a**) and dissolution profiles (**b**) of lyophilized resveratrol nanosuspensions (*NS*), physical mixture (*PM*), and raw resveratrol (*Raw*)

distribution ranging from most above 1 to 5 μm. While resveratrol nanosuspensions appeared as an aggregation of individual near-spherical-shaped submicron particles with a mean particle size of approximately 140 nm.

These results indicate that the utilization of antisolvent precipitation approach is feasible to produce nanoparticles, and the selected PVP and F188 may be as effective stabilizers to control the particle size and size distribution by hindering particle growth.

DSC and XRPD Analysis

The characterization of the degree of crystallinity of resveratrol is critical because the crystallinity significantly affects saturation solubility and dissolution rate in the delivery process (14). To probe whether the changes of the crystalline state of resveratrol during the nanosuspension preparation process would occur or not, the physical state of diverse samples was confirmed by DSC and XRPD analysis. Samples used for measurement were pure resveratrol, PVP K17, F188, cryoprotectant mannitol, their physical mixture, and lyophilized nanosuspension powder of optimized formulation.

The DSC thermograms of different samples are depicted in Fig. 3. For raw resveratrol, the melting process occurred with a maximum peak at 265.78°C. The DSC profiles of F188 and cryoprotectant mannitol exhibited sharp melting peaks at 52.53°C and 167.7°C, respectively.

In the physical mixture, the melting peaks all appeared but drifted slightly because of mixing. In contrast, the endothermic peaks of resveratrol disappeared completely in the

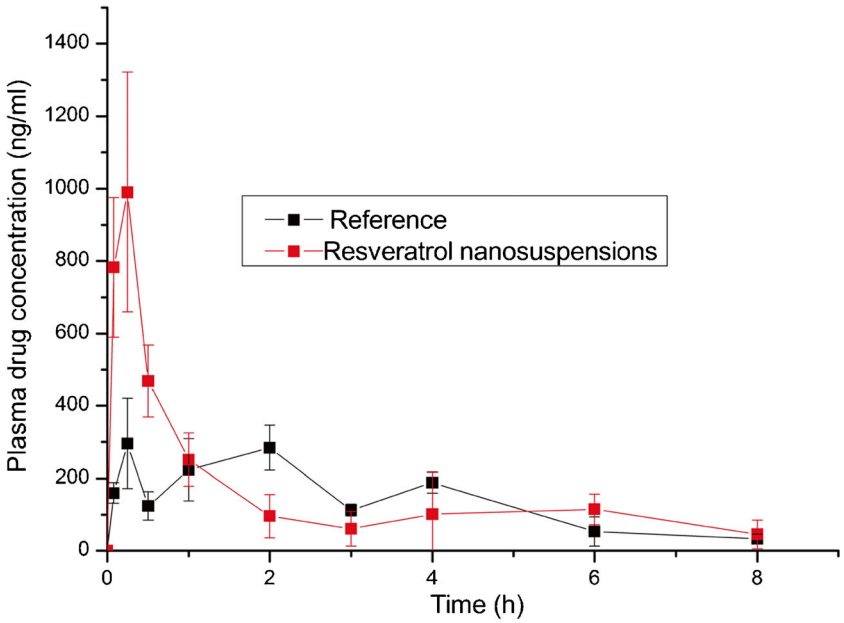

Fig. 6. Average plasma drug concentration *versus* time profiles after oral administration of resveratrol nanosuspensions and reference formulation (means±SD, n=6)

profiles of the nanosuspensions, suggesting that a change from the crystalline state to the amorphous state took place during precipitation. This phenomenon could be attributed to the fact that at a fast nucleation rate, the drug solute lacks sufficient time to incorporate into the growing crystal lattice accurately to form perfect crystals (24,25).

The changes in the crystalline state of resveratrol in the nanosuspensions were also verified by XRPD. As shown in Fig. 4, the diffractogram of the raw resveratrol exhibited intense crystalline peaks between 3° and 50°, indicating that the raw resveratrol is in crystalline form. However, some intense peaks disappeared in the profile of the nanosuspension powder. Absence of the peaks obviously distinct to the raw resveratrol diffraction pattern suggested a phase transition occurrence from crystalline form to the amorphous form in the precipitation process (26). This reduced crystallinity would bring about a higher surface disorder, resulting in higher saturation solubility and rapider dissolution rate than those of crystalline materials (27,28).

Phase Solubility and Dissolution Rate

The saturation solubility of resveratrol nanosuspensions was compared with that of raw resveratrol and physical mixture in PBS solution (pH 7.4). The results showed that the saturation solubility of resveratrol nanoparticles was much higher than that of the others. As illustrated in Fig. 5a, the saturation solubility of resveratrol nanosuspensions reaches approximately 38 times than that of raw resveratrol and 3.2 times than that of physical mixture, respectively.

This enhanced equilibrium solubility can be attributed to not only the solubilization of the surfactants in the presence of nanoparticle preparation but also the reduction of particle size and amorphous state already demonstrated by XRPD and DSC data which effectively increased the saturation solubility. The saturation solubility is generally regarded as a physicochemical constant. However, the saturation solubility of drug increases below a size of about 1 μm. That the dissolution pressure increases with the increase in particle curvature resulting from decreasing particle size, as described by Kelvin equation, can account for the dependence of equilibrium solubility in solutions on the nanoparticle size. In addition, the basis for this is the Freundlich-Ostwald equation describing solubility increase with decrease in particle size (29).

The profiles presented in Fig. 5b illustrated the dissolution rates of raw resveratrol, physical mixture, and resveratrol nanosuspensions. The results exhibited that resveratrol nanoparticles showed the fastest dissolution rate. After 30 min, 55% of the resveratrol in nanosuspensions dissolved in the phosphate buffer. However, only 15% of the bulk resveratrol and 23% of the physical mixture dissolved in the medium, respectively. The rate and extent of drug dissolution elevated apparently, owing to the significant reduction in particle size corresponding to increased surface area, enhanced absolute solubility, and amorphous nature of the drug in the preparation process (5).

Table IV. Pharmacokinetic Parameters of Resveratrol Nanosuspensions and the Reference Formulation After Oral Administration in Rats (n=6)

Parameter	Nanosuspensions	Reference
C_{max} (kg×ng/ml/mg)	8.2536±0.0120*	2.4663±0.095
MRT (h)	2.437±0.7286	2.642±0.97
$AUMC_{0\to\infty}$ (h×h×ng/ml)	4767.67±482.72*	4172.93±436.21
$AUC_{0\to\infty}$ (h×kg×ng/ml/mg)	11.67±2.39*	9.15±2.01
V_d (l/kg)	295.55±45.8*	444.89±36.6
CL (ml/h/kg)	87,444.4±672.1*	109,293±984.7

Data were represented as the mean±SD
*p<0.05, statistical significance compared with reference

Antioxidant Activity of Resveratrol

The practical health activities of resveratrol are thought to exert their antioxidant effects. Therefore, it is important to confirm that the formulation of resveratrol in nanoparticle delivery systems does not interfere with its innate nature. The antioxidant capacity of RSV was performed by the *in vitro* DPPH free radical scavenging assay. DPPH assay depended on the theory that a hydrogen donor is an antioxidant. The radical captures the odd electron of DPPH forming paired with hydrogen and reduces in the presence of an antioxidant molecule. Then, the violet color of DPPH solution declined, and the degree of this discoloration, indicating the scavenging capability of the added samples, leads to a decrease or loss of absorbance (17). The outcome of DPPH free radical scavenging test showed that the SC_{50} of nanosuspensions (226.06 μg/ml) was approximately the same as that of raw resveratrol (233.17 μg/ml) and physical mixture (228.36 μg/ml). It is noteworthy that resveratrol retains potent antioxidant activity during the preparation process, and such activity is not interfered with whatever the formulation composition.

Pharmacokinetics Study

To evaluate whether the fabrication of nanoparticulate drug delivery system could enhance its oral bioavailability, an *in vivo* test experiment was executed in rats to compare the pharmacokinetic parameters of the nanosuspensions and the coarse suspension of resveratrol as a reference formulation after gavage administration. The developed and validated HPLC method was established to determine resveratrol concentration in plasma. The plasma concentration-time profiles and the main PK parameters were shown in Fig. 6 and Table IV, respectively. After per oral administration of these two different formulations, the pharmacokinetics parameters of resveratrol nanosuspensions are expected to behave significantly different in comparison with the coarse suspension dispersion. The highest concentration of resveratrol following oral nanosuspension administration appeared at the second sampling time (15 min), suggesting that the absorption of resveratrol was rapid, which was in good agreement with previous reports (30). Comparison of C_{max}, $AUMC_{0\rightarrow\infty}$, and $AUC_{0\rightarrow\infty}$ values of nanosuspensions in Table IV revealed that there were approximately 3.35-, 1.14-, and 1.27-fold greater than those of reference preparation ($p<0.05$), respectively. For orally administrated drugs, dissolution is deemed as a critical rate decision step for absorption. When the nanosuspensions enter the gastrointestinal tract, nanoparticles provide a larger surface area for dissolution and molecular dispersion of resveratrol obtained from solubilized nanoparticles, forming an increased concentration gradient on the surface of nanocrystals according with the classical passive diffusion theory. These findings were consistent with the results obtained from the dissolution tests, indicating that obvious distinctions of pharmacokinetics properties between resveratrol nanosuspensions and coarse suspensions primarily stem from significantly improved dissolution kinetics by the diminished particle size with increased surface area and reduced diffusion layer thickness, thereby enhancing drug absorption. The poor solubility of resveratrol in gastrointestinal juice of resveratrol coarse suspensions than that of resveratrol nanosuspensions can account for the lower bioavailability. Meanwhile, an increase in adhesion surface area between nanoparticles and intestinal epithelium of villi involving a direct contact with the absorbing membranes of the gut and immediate release of drug available at the site of absorption might be another possible reason for the improved absorption of the nanosized drug particles (31). In addition, the lower parameters, such as volume of distribution (V_d) and clearance rate (CL) of the nanosuspension formulation, may lead to an enhancement in the chance of the drug escaping from the liver metabolism, also increasing bioavailability (32).

CONCLUSION

The resveratrol nanoparticles were successfully prepared by antisolvent precipitation with relatively narrow particle size distribution. BBD screening design helped in identifying the significant parameters that affected the response variables. The saturation solubility and dissolution rate of the optimized formulation were significantly enhanced as compared with raw material. The resveratrol nanosuspensions had the same antioxidant as raw resveratrol. The *in vivo* test revealed that the C_{max} and $AUC_{0\rightarrow\infty}$ values of nanosuspensions were approximately 3.35- and 1.27-fold greater than those of reference preparation, respectively. The improved formulation could offer a better drug delivery strategy for poorly water-soluble ingredients. In the future, pharmacodynamics studies are necessary to be performed to validate more therapeutic proofs and to evaluate whether nanoparticulate resveratrol may improve the current clinical effectiveness.

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