International Journal of Pharmaceutics 422 (2012) 516–522

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

# Comparison of different methods for preparation of a stable riccardin D formulation via nano-technology

Guangpu Liu[a], Dianrui Zhang[a,*], Yang Jiao[b], Dandan Zheng[a], Yue Liu[a], Cunxian Duan[a], Lejiao Jia[a], Qiang Zhang[c], Hongxiang Lou[b]

[a] Department of Pharmaceutics, School of Pharmaceutical Sciences, Shandong University, 44 Wenhua Xilu, Jinan 250012, PR China
[b] Department of Natural Products Chemistry, School of Pharmaceutical Sciences, Shandong University, 44 Wenhua Xilu, Jinan 250012, PR China
[c] State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, 38 Bei Xueyuan Road, Beijing 100083, PR China

ARTICLE INFO

*Article history:*
Received 30 September 2011
Received in revised form 7 November 2011
Accepted 13 November 2011
Available online 22 November 2011

*Keywords:*
Riccardin D
Nanocrystal
Evaporative precipitation into aqueous solution
Microfluidisation
Dissolution rate
Stable

ABSTRACT

Riccardin D is a new compound extracted from liverwort *Marchantia polymorpha* L. It has been proved to be useful in antifungal therapy and reversing the resistance of *Candida albicans* against fluconazole. However, the poor solubility leads to the poor bioavailability and limits its development. In this study, nanocrystals were prepared in the evaporative precipitation into aqueous solution (EPAS) and the microfluidisation process. The characterizations of nanocrystals were compared by transmission electron microscope, size distribution, and zeta potential. In the EPAS method, the drug was dissolved in the organic phase and F68, HPMC, PVP K30 were dissolved in water with the mass ratio of 2:1:2:1. In the microfluidisation process, two key factors – pressure and number of cycles were screened and 8 cycles at 2000 bar was the most efficient parameter. The nanocrystals made in EPAS process were smaller, more uniform and had a narrower distribution than the microfluidisation nanocrystals. Differential scanning calorimetry (DSC) and X-ray diffraction confirmed the crystalline states that were both reserved. The solubility was greatly improved by the two methods and the EPAS nanocrystals were more soluble due to the smaller size. An enhanced dissolution was obvious in vitro. And the stable nanocrystals were successfully achieved by the two methods.



## 1. Introduction

Riccardin D (RD, Fig. 1) is a macrocyclic bisbibenzyl compound extracted from liverwort *Marchantia polymorpha* L. (Wu et al., 2009). It is a new substance and the study on pharmacology is ongoing. RD has shown its various biological activities and pharmacological actions, such as effectively reversing the multidrug resistance (Shi et al., 2008), its antifungal activity (Wu et al., 2009) and inducing apoptosis (Wu et al., 2010). However, its poor solubility greatly limits its further development.

Active entities with high permeability and low aqueous solubility are classified as Class II APIs by the Biopharmaceutics Classification System (BCS) (Amidon et al., 1995). About 40% of potential new drugs belong to Class II and the poor solubility greatly hinders their clinical translation (Chen et al., 2011). Low solubility is always leading to low oral bioavailability (Branchu et al., 2007). Due to the poor solubility, a soluble formulation is in urgent need to improve its bioavailability. Nano-sized drug particles have been confirmed to be a potential candidate for improving the solubility of poor water soluble drugs (Ali et al., 2009). Nanocrystal suspension is a carrier-free colloidal drug system that contains pure drug in the form of nanocrystals with the particle size between 100 nm and 1000 nm, stabilizers such as surfactants and polymers (Patravale et al., 2004). According to the Noyes–Whitney and Ostwald–Freundlich equations, decreasing particle size will increase the surface area of the diffusion layer and the saturated solubility (Bohm and Muller, 1999). The nanocrystals with decreased particle size and increased surface area can improve the dissolution rate and develop the bioavailability (Ali et al., 2009). In addition, nanocrystal suspension can be injected parenterally, especially intravenously due to the small size. The intravenous injection will lead to a 100% bioavailability (Keck and Muller, 2006). Sometimes nanocrystals can also present the passive targeting features like colloidal drug carriers (Peters et al., 2000).

The existing strategies for nanosizing can be divided into "bottom-up" method and "top-down" method. The bottom-up method means that the nanocrystals are constructed from molecules while the top-down method intends that the nanocrystals are comminuted from coarse powders to fine powders. The bottom-up methods based on precipitation include evaporative precipitation into aqueous solution (EPAS) (Chen et al., 2002; Rasenack and Muller, 2004) process, the liquid solvent change

* Corresponding author. Tel.: +86 531 88382015; fax: +86 531 88382015.
*E-mail address:* zhangdianrui2006@163.com (D. Zhang).

0378-5173/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2011.11.025

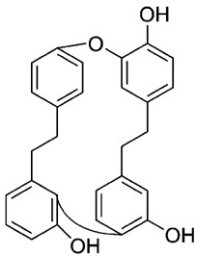


**Fig. 1.** Chemical structure of riccardin D.

process (Rasenack and Muller, 2004), spray drying, supercritical antisolvent process (Kim et al., 2008) and spray freeze drying into liquid (Kondo et al., 2009). The top-down methods based on mechanical comminution consist of milling technology, high pressure technology (Keck and Muller, 2006) and microfuidisation technology (Ali et al., 2009). Milling technology consists of jet milling, pearl milling, spiral jet milling and media milling (Merisko-Liversidge et al., 2003). Nowadays, two methods are always combined such as the combination technology precipitation and homogenization (NANOEDGE) (Keck and Muller, 2006).

The EPAS method relies on the nucleation and the growth of drug crystals to form the crystalline or semi-crystalline drug nanoparticles. In general, the hydrophobic drugs are dissolved in an appropriate organic solvent such as methanol, ethanol, acetone, tetrahydrofuran or N-methyl-2-pyrrolidone at a supersaturation concentration to obtain the nucleation of drug seeds. The stabilizers including F68, lecithin, polyvinylpyrrolidone (PVP), Tween 80, hydroxypropyl methylcellulose (HPMC) are dissolved in the antisolvent. The stabilizers are used to inhibit excess crystal growth or particle aggregation (Chen et al., 2008). The drug nanocrystals are formed by adding the drug solution into the antisolvent (Chen et al., 2011). The crucial factors of controlling the size and stability are the mixing process, solvents and stabilizers. The mixing step is vital to produce a uniform and rapid supersaturated solution which is favorable for the formation of uniform and small nanocrystals. The stabilizers are always combined for optimal effect.

In the microfluidisation process, firstly mechanical energy is transferred to fluid particles under high pressure. It is a jet stream principle. The solution is pumped and accelerated with a high speed to an interaction chamber. In the "Z" type chamber, the suspension changes directions of its flow leading to shear forces and particle collision. And in the second type chamber, "Y" type, the suspension stream is divided into two microstreams which then collide against each other (Keck and Muller, 2006).

Although the nanocrystal formulation process is simple, keeping the nanosize is a key challenge (Hu et al., 2011). As small particles are more soluble than the large ones, material transfer occurs from the fines to the coarse particles. This is called "Ostwald ripening" which causes the coarse particles to grow at the cost of fine particles re-dissolving (Horn and Rieger, 2001). Some surfactants can only act as short-term stabilizers. Immediate drying such as spray-drying and freeze-drying can be useful for long-term stability. During the two methods, crystalline state may change. Molecules in the amorphous state are unstable compared with the crystalline state. During processing or storage of the amorphous dispersion, uncontrolled crystallization of drug or other materials may occur. So the DSC and the XRPD will be carried out to examine the crystalline state transformation (de Waard et al., 2008).

In this study, as the bulk drug is rare, we need a simple and material-saving way. So we choose the EPAS process as the bottom-up process and the microfluidisation as the top-down process. The two methods were both optimized and compared. Stabilizers were screened and the physicochemical characterizations including the particle morphology, size distribution, DSC and XRPD were assessed. Of course, the key properties-solubility and dissolution rate were all evaluated. The RD was first studied in terms of pharmaceutics. Due to its poor solubility, LC–MS and HPLC were both used to examine the content of the drug.

**Table 1**
Composition of RD nanosuspensions by the method of EPAS.

| Formulation | RD (mg) | F68 (mg) | HPMC (mg) | PVP K30 (mg) | Ethanol (μL) |
|---|---|---|---|---|---|
| 1 | 10 | 20 | 20 | 5 | 300 |
| 2 | 10 | 10 | 10 | 20 | 300 |
| 3 | 10 | 5 | 10 | 5 | 300 |
| 4 | 10 | 0 | 5 | 20 | 250 |
| 5 | 10 | 20 | 0 | 5 | 250 |
| 6 | 10 | 10 | 0 | 20 | 250 |
| 7 | 10 | 5 | 20 | 0 | 200 |
| 8 | 10 | 0 | 20 | 10 | 200 |
| 9 | 10 | 20 | 10 | 0 | 200 |
| 10 | 10 | 10 | 5 | 10 | 150 |
| 11 | 10 | 5 | 5 | 0 | 150 |
| 12 | 10 | 0 | 0 | 10 | 150 |

## 2. Materials and methods

### 2.1. Materials

Riccardin D was isolated from liverwort *M. polymorpha* L. (Marchantiaceae) and was kindly donated by Professor Lou. Polaxamer 188 (F68) was purchased from Sigma (St. Louis, MO, USA). Polyvinylpyrrolidone K30 (PVP K30) was supplied by Hangzhou Nanhang Chemical CO., Ltd., China. Hydroxypropyl methyl cellulose (HPMC) was provided by Zhengzhou Tianying Chemical CO., Ltd. Mannitol (analytical grade) was obtained from Tianjin Guangcheng Chemical Agent CO., Ltd., China. Methanol (HPLC grade) and ethanol (analytical grade) were bought from Tianjing Siyou Fine Chemicals CO., Ltd., China. All the other chemicals and solvents were of chromatographic and analytical grade.

### 2.2. Preparation of two riccardin D nanocrystal suspensions

#### 2.2.1. EPAS process (bottom-up method)

RD nanocrystal suspension was prepared by an antisolvent precipitation technique. 10 mg RD was completely dissolved in ethanol (60 °C) as an organic phase. The organic phase was added slowly in to 8 mL water (0 °C) containing different concentrations of F68, PVP K30, HPMC (Table 1) with a magnetic stirrer (RCT Basic, IKA, Staufen, Germany) at 800 rpm. After the mixing procedure the suspension was stirred at 300 rpm for 2 h at room temperature to decrease the ethanol content. The nanosuspension in the bottom-up method was obtained.

#### 2.2.2. Microfluidisation process (top-down method)

The same content of RD, F68, PVP K30, HPMC as the optimal prescription in Section 2.2.1 were dispersed in 50 mL water and treated with an ultrasound machine for 10 min. Then the suspension was in comminution process using an IKA homogenizer machine at 15,000 rpm for 1 min. The resultant suspension was performed on the microfluidizer model M-110P (Microfluidics, American) at different pressures and cycles to obtain stable nanocrystal suspension. A cold water bath system was used during the microfluidisation process. The two preparation methods are shown in Fig. 2.

### 2.3. Lyophilization

The nanosuspensions were immediately dried with a freeze drier. 3 mL of the nanosuspension in a 15 mL Cillin-glass bottle was pre-frozen at −80 °C Ultra-low Temperature Freezer (DW-86L,

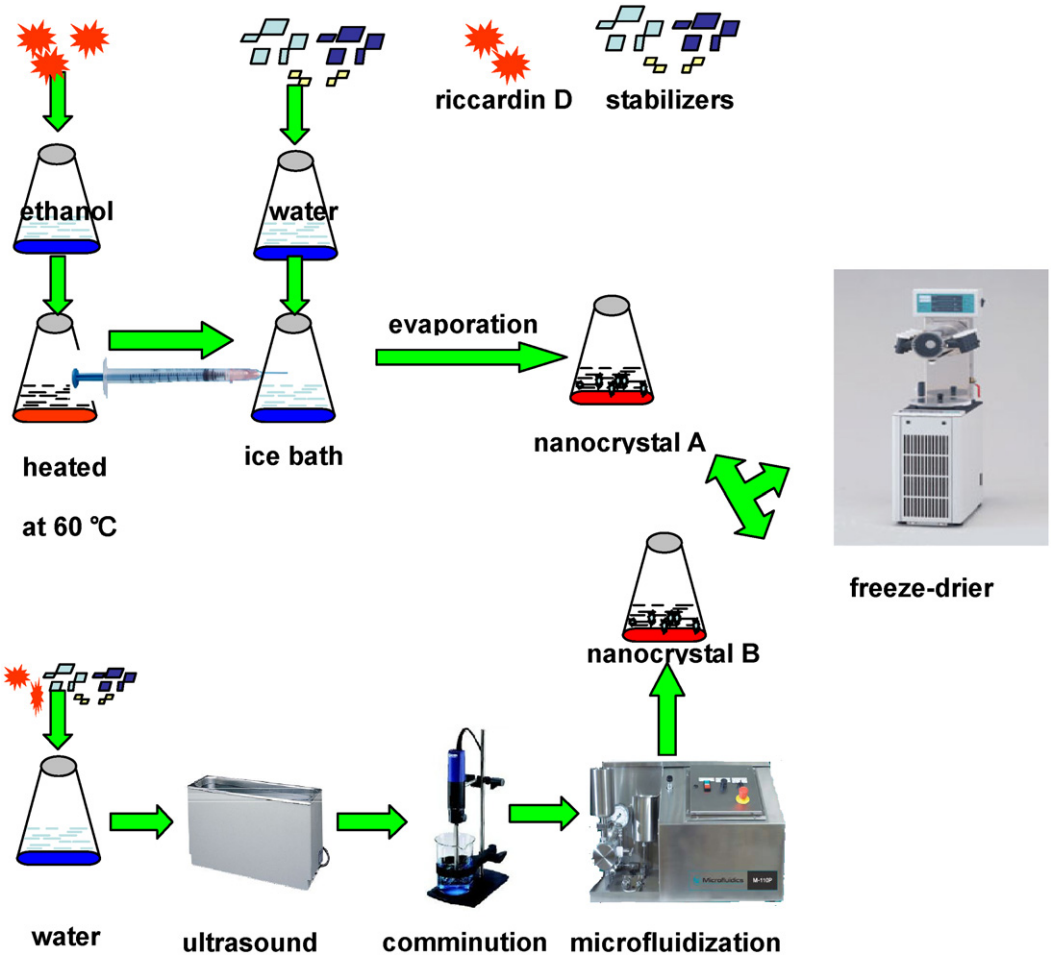


**Fig. 2.** The preparation routes for nanocrystals.

Haier, China) for 48 h. The frozen nanocrystals were freeze dried for 48 h with freeze drier (FD-1000, EYELA, Japan). The nanocrystal powder made in the EPAS process was called NC-A and the other was NC-B for short.

### 2.4. Characterization of nanosuspension

#### 2.4.1. Particle size and zeta potential of riccardin D nanocrystal suspension

Size evaluation of the nanoparticles followed by the different size reduction processes was assessed via laser diffraction (LD), with the machine MasterSizer 3000 (Malvern Instruments, Worcestershire, UK). The surface charge of the nanoparticles was evaluated by zeta potential measurements using the Delsa$^{TM}$ Nano C Particle Analyzer (Beckman Coulter, Inc.). All measurements were made at least in triplicate.

#### 2.4.2. Morphology observation by transmission electron microscope (TEM)

The morphology of the two different nanocrystals was observed by TEM (H-7000, Hitachi, Japan). One droplet of the nanocrystals suspension was placed on the 200-mesh copper grid and then negatively stained with 2% phosphotungstic acid for 30 s. The grid was dried under luminous heat bulb and was examined with the TEM.

#### 2.4.3. Differential scanning calorimetry (DSC) measurement

The thermal properties of the lyophilized powder samples were investigated with a DSC-41 apparatus (Shimadzu, Japan). The scanning temperature for each lyophilized powder sample was set from 25 to 400 °C with a heating rate of 10 °C/min. 10 mg of each sample was analyzed in an open aluminium pan and magnesia was used as reference. In order to evaluate the internal structure modifications after nanosizing process, thermal analysis was performed on riccardin D, the excipients (PVP K30, HPMC, Pluronic F68), their physical mixtures, mannitol and the freeze-dried powder (NC-A and NC-B).

#### 2.4.4. X-ray powder diffraction (XRPD) analysis

The crystalline state of the samples, including the drug, the excipients, the physical mixtures, cryoprotector and freeze-dried powders were performed with an X-ray diffractometer (Digaku, Japan). XRPD was carried out in symmetrical reflection mode using Cu Ka line as the source of radiation and the wavelength was set at 1.5405 Å. Standard runs using a 40 kV and 100 mA in this process. Samples were performed with a scanning rate of 0.02°/min and the scanning range of the $2\theta$ from the initial angle 3° to the final angle 50°.

### 2.5. LC–MS analysis

As the low solubility of RD, common HPLC cannot detect its saturated concentration in water. So the liquid chromatography–tandem mass spectrometry assay was necessary to be used. The Agilent 1100 system (PaloAlto, CA, USA) was equipped with a vacuum degasser unit, a binary pump and an autosampler. The HPLC system was coupled to an API 4000 quadrupole mass spectrometer

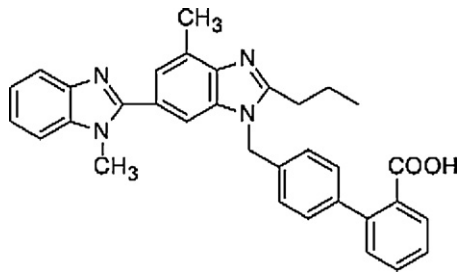


**Fig. 3.** Chemical structure of telmisartan.

(Applied Biosystem Sciex, Canada) via a TurbolonSpray ionization (ESI) interface.

Telmisartan (Fig. 3) was used as internal standard (IS). The ionization voltage was −4 kV. The capillary temperature was 400 °C. The heater gas temperature was set at 400 °C. Ultrapure nitrogen was used as heater, curtain and nebulizer gas. The curtain gas, nebulizer gas and the auxiliary gas were separately set at 20 psi, 35 psi and 30 psi. The collision–induced dissociation (CAD) voltage for RD and IS was −40 V and −30 V. The fragmentation transition for the multiple reaction monitoring (MRM) was *m/z* 423.3–405.2 for RD, and *m/z* 514.3–470.3 for the IS. Chromatographic separation was obtained on a Venusil XBP $C_{18}$ column (50 mm × 2.1 mm, 5 μm; Agela, USA). The chromatography was performed at 35 °C. The mobile phase consisted of acetonitrile–water containing 0.2% acetic acid (85:15, v/v), delivered at a flow rate of 0.2 mL/min for quantitative study. Each sample was injected by 2 μL (Xing et al., 2008) (Fig. 4).

Working solution of RD (1, 5, 25, 50, 100, and 250 ng/mL) and IS (20 ng/mL) were prepared by diluting with 50% methanol solution. The calibration curve and the RD concentration could be obtained.

### 2.6. HPLC analysis

HPLC analysis of RD was performed on an Agilent 1260 HPLC system (Agilent, USA) with a variable wavelength UV detector and an autosampler system. The detection wavelength was set at 210 nm. An Eclipse XDB-$C_{18}$ column (5 μm, 4.6 mm × 150 mm; Agilent, USA) at 25 °C and a mobile phase of methanol/water (70:30, v/v) were used for chromatographic separation. The flow rate was 1.0 mL/min.

### 2.7. Drug solubility

One of the goals to make nanocrystal suspension is raising the drug solubility. To confirm the change of the solubility between the pure RD and the nanosizing drug, a magnetic stirrer (RCT Basic, IKA, Staufen, Germany) was used. The physical mixture powder was used as reference. Samples containing equivalent RD (10 mg) were dispersed into 10 mL phosphate buffered solution (PBS, pH 7.4), the temperature and the stirring rate were set at 25 °C and 100 rpm, respectively. 72 h later 1 mL of medium was withdrawn, placed in a centrifuge tube and centrifuged at 14,000 rpm for 15 min with Zonkia HC-2062 high speed centrifuge (Anhui USTC Zonkia Scientific Instruments Co., Ltd.). The RD content was measured by the LC–MS mentioned in Section 2.5 and HPLC in Section 2.6. The experiment was made in triplicate.

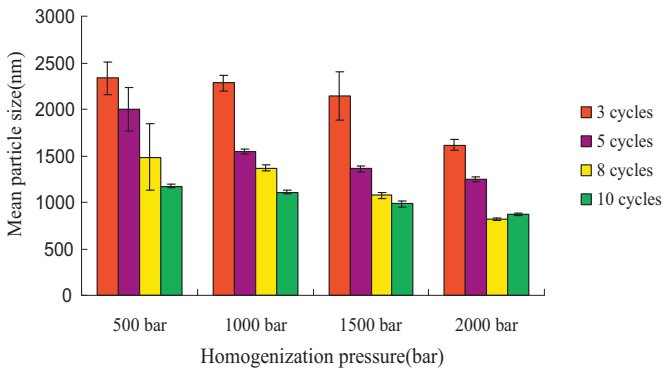


**Fig. 4.** Mean particle size of RD suspensions obtained with various homogenization pressures and cycles.

### 2.8. Dissolution

As the dissolution of the drug has an influence on the bioavailability, the dissolution behaviors of the RD suspension dried powders in vitro were conducted below. Powders containing equivalent of RD (10 mg) were dispersed into 300 mL PBS (pH 7.4, containing 1% Tween 80) and stirred with a magnetic stirrer (RCT Basic, IKA, Staufen, Germany). The temperature and the rate were set to be 37 °C and 100 rpm. PBS (pH 7.4) with 1% Tween 80 had been screened as the dissolution media to maintain the sink condition. At predetermined intervals, 1 mL medium was withdrawn and 1 mL blank medium was immediately added to obtain the constant volume. The 1 mL withdrawn medium was placed in centrifuge tube and centrifuged at 14,000 rpm for 15 min with Zonkia HC-2062 high speed centrifuge (Anhui USTC Zonkia Scientific Instruments Co., Ltd.). To compare the dissolution behavior of the pure drug and the nanosuspension dried powders, four groups are used including RD, physical mixture, NC-A and NC-B. The RD content was analyzed in the way mentioned in Sections 2.5 and 2.6.

## 3. Results and discussion

### 3.1. Screening study of polymer and surfactant

Different types and concentrations of surfactants and polymers were designed to screen for the optimal formulation which could have long term stability. The polymer screening was the content of the PVP K30 and the HPMC and the surfactant screening was the content of the F68 and lecithin. The addition of the adjuvant is necessary for the stability of the nanoparticles. However, the excess of the surfactants and polymers may cause side effects (such as anaphylactic response and irritability), so the optimal formulation is necessary. The particle size, zeta potential and stability were all taken into consideration to get the optimal prescription. After single-factor test, we found lecithin was not suitable as a surfactant. In this study, 12 formulations were tried in the EPAS process as shown in Table 1. After the homogeneous design, we found formula 3 was the most stable one and the particle size was the smallest (mean particle size about 184.1 nm). Based on this screening, prescription 3 turned out to be the best combination for nanocrystal suspensions.

### 3.2. Screening study of pressure and cycles

Formula 3 mentioned in Section 3.1 (without ethanol) was also adopted in this section. Nanosuspensions were prepared by the method of Section 2.2.2. Suspensions were fed to the microfluidiser and the homogenization pressure and cycle numbers were set to 500 bar, 1000 bar, 1500 bar, 2000 bar and 3, 5, 8, 10. According to the stability and particle size, we found the microfluidiser working with the homogenization pressure at 2000 bar in 8 cycles was the most effective combination (815.37 nm).

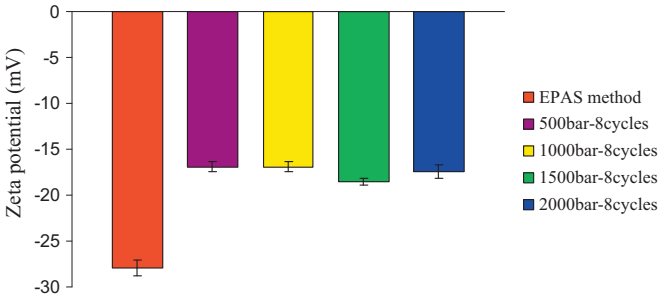


**Fig. 5.** Zeta potential of RD suspensions obtained with EPAS and microfluidisation methods.

### 3.3. Zeta potential of RD suspension

Fig. 5 compares the zeta potential of the RD nanosuspensions prepared by the EPAS and microfluidisation methods. All the stabilizers were non-ionic in nature and the range of zeta potentials were exhibited from −16 to −23 mV. Different methods made comparable zeta potential values. While by the microfluidisation method of different pressures zeta potentials were almost the same. According to the literature, particle aggregation is easy to occur if particle does not possess enough zeta potential to provide electric repulsion or enough steric barrier between each other. In the electrostatic stabilized system and sterical stabilized system, −30 mV and −20 mV of the zeta potential were desired to obtain each stable system. In our study this was a sterical system, so the nanocrystal suspensions both NC-A and NC-B we got were considered stable.

### 3.4. Morphology observation by TEM

The morphologies of the two nanosuspensions prepared by two methods were examined by TEM. The morphological characterization of them was shown in Fig. 6. By the method of EPAS (Fig. 6a), the particle size was relatively small, uniform and clearly in encapsulation. While the particles made in the microfluidisation way (Fig. 6b) were shaped irregularly and inhomogeneous in particle size distribution. As we all know, the inhomogeneous particles had the tendency of aggregation or adhesion to be larger. So the particles made by the EPAS method could be more stable.

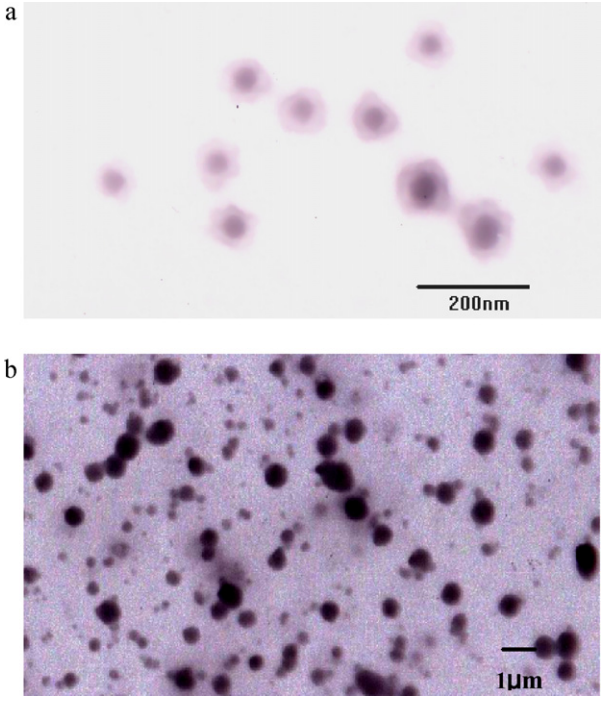


**Fig. 6.** TEM images of the EPAS nanocrystals (a) and microfluidisation nanocrystals (b).

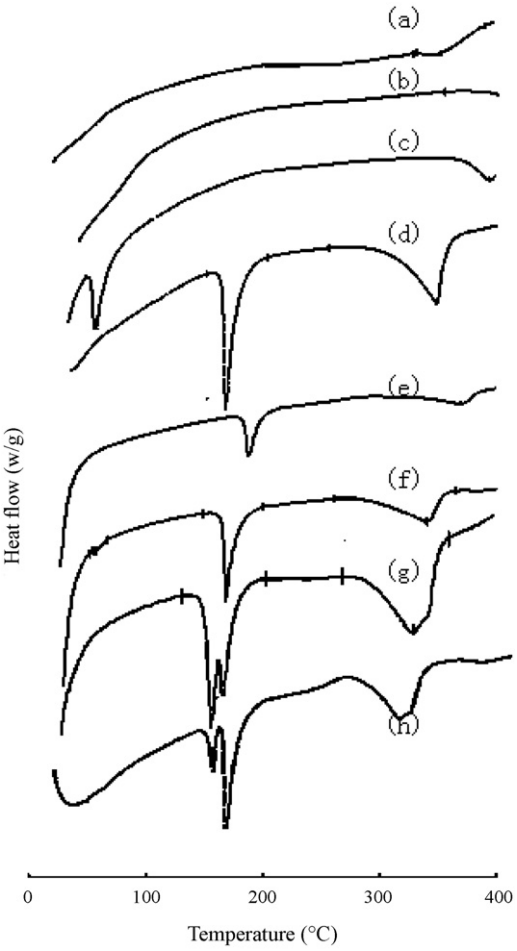


**Fig. 7.** DSC spectra of (a) HPMC, (b) PVP K30, (c) F68, (d) RD, (e) mannitol, (f) physical mixture, (g) NC-A and (h) NC-B powders.

### 3.5. DSC measurement

After the two methods, DSC study was carried out. DSC is commonly used to find out the differences in crystalline state for the physical or chemical changes during the preparation process. As shown in Fig. 7, DSC thermograms of (a) HPMC, (b) PVP K30, (c) F68, (d) RD, (e) mannitol, (f) physical mixture, (g) NC-A and (h) NC-B powders were obtained to show the crystalline potential changes and assess the interaction among the materials via two different methods. The melting point of RD was exactly 190.2 °C and mannitol was 168.33 °C. The nanocrystal freeze-dried powders of the two methods clearly displayed the same melting point mentioned above. However, the physical mixture only has one peak instead of two peaks. One explanation on this was the peak of RD was close to that of mannitol. And in the physical mixture mannitol was much more than RD. So it might lead to the fact that the peak of mannitol covered the peak of RD. The unchanged melting point of two methods revealed there is no transformation to an amorphous state in the two ways. This phenomenon is necessary for the long term stability (Wang et al., 2010).

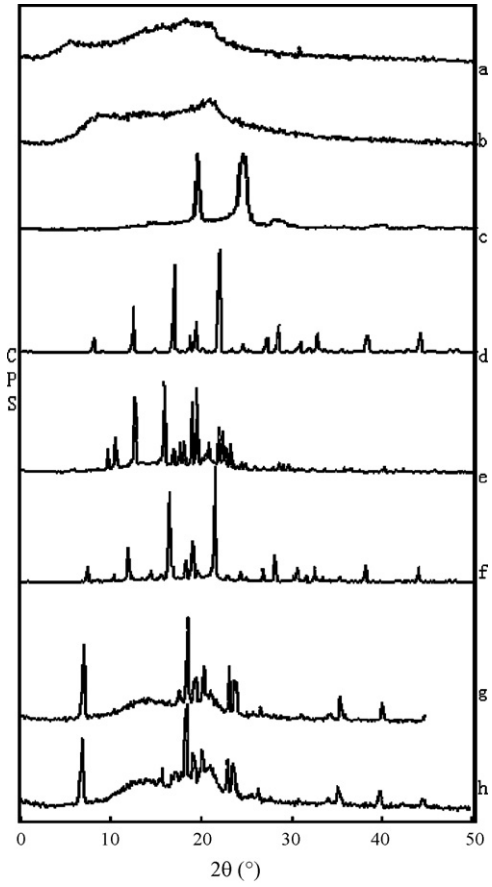


**Fig. 8.** XRPD spectra of (a) HPMC, (b) PVP K30, (c) F68, (d) RD, (e) mannitol, (f) physical mixture, (g) NC-A and (h) NC-B powders.

### 3.6. XRPD analysis

XRPD was further studied for examining the changes of RD crystalline state. The crystalline form is a key factor to influence the drug solubility. An enhancement of solubility may be caused by changing to a more amorphous crystalline state. Amorphous nature with no intense peaks was got in other nanocrystal experiment (Gao et al., 2011). However, for a long process experiment especially in developing a new drug, an amorphous state is not desired (Muller et al., 2001). Therefore, the diffraction pattern preserved would declare that the crystalline state was unchanged during the two methods. Fig. 8 exhibited the diffraction patterns of (a) HPMC, (b) PVP K30, (c) F68, (d) RD, (e) mannitol, (f) physical mixture, (g) NC-A and (h) NC-B powders. Characteristic peaks of RD were found at the angle of 18.8°, 20.5°, 21.1°, and 23.4°. In the physical mixture, SN-A and SN-B freeze-dried powder diffraction graph, peaks at the angle of 20.5° and 21.1° were also found. So we can conclude that the characteristic peaks were still preserved indicating the crystalline state was not changed. This is in accordance with the DSC analysis. However, compared with the RD, some peaks had slight migration (23.4°) and decreased peak area (21.1°). This may be explained by the dilution effect exerted by mannitol. Another reason is that the nanocrystal surface was encapsulated with excipients such as HPMC, PVP K30, and F68. This may lead to the deviation (Wang et al., 2010).

Combined the DSC and XRPD studies, we can conclude the crystallinity was preserved during the two methods. The increased solubility benefits from the small particle size instead of the changing crystalline state.

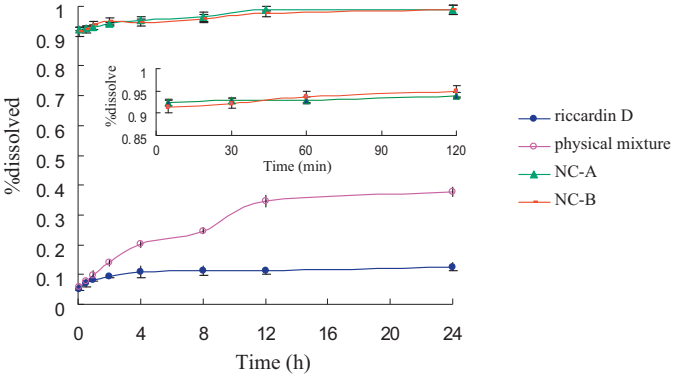


**Fig. 9.** Release profiles of RD from the pure RD, physical mixture, NC-A and NC-B (The inserted illustration was the dissolution profile of the NC-A and NC-B within the first 2 h).

### 3.7. Solubility studies

With the help of LC–MS, the equation of the calibration curve was as follows: $y = 0.0422x + 0.00831$, $r = 0.991$, where $y$ represents the peak area ratios of RD to that of IS, and $x$ represents the concentration ratios of RD to that of IS. And the linearity range is between 1 and 250 ng/mL. According to the calibration curve, the solubility of RD in pure water is $619.2 \pm 24.5$ ng/mL while the physical mixture powder is $3178.7 \pm 11.6$ ng/mL. The surprising news is that the solubility of NC-A is $30.5 \pm 2.1$ μg/mL and the solubility of NC-B is $222.084 \pm 10.5$ μg/mL. So the two methods can greatly increase the solubility of RD. The solubility of NC-B is much higher than NC-A for the smaller particle size. This result is closely related to the Ostwald–Freundlich equation: the smaller particle size will lead to the higher solubility.

### 3.8. Dissolution studies

In order to confirm whether the rate of dissolution of RD was improved, the drug release study in vitro was carried out. The dissolution studies of physical mixture powder, NC-A, NC-B were compared with pure RD in Fig. 9.

In the figure we can see the dissolution rate was significantly improved after preparing nanocrystal powders. F68 is a kind of surfactant which can help indissolvable drug improve its solubility. So the physical mixture can release more and faster than pure RD. Compared with the NC-A and NC-B, the effect of the surfactant is very limited. After the first minute, more than 90% RD can be released in NC-A and NC-B. However, in the time point of 24 h, 12.6% drug was released in pure RD and 37.7% drug was released in physical mixture. According to Noyes–Whitney equation, the releasing rate and solubility would be improved when the particle size is decreased. The equation is described by $dc/dt = D \times A \times (C_s - C_x)/h$. In this equation, $dc/dt$ represents the releasing rate, $D$ is the diffusion coefficient, $A$ is the surface area, $C_s$ is the saturation solubility, $C_x$ is the bulk concentration, and $h$ is the effective boundary layer thickness. As NC-A and NC-B are nanosizing, the releasing rate is too fast. So there is no distinction between them. In the study of DSC and XRPD, we found that there was no crystal form transition. The two key reasons for raising solubility are the crystal form and the particle size. Considering the excipients have little effect, the nanoparticle size is the mainly factor in developing the solubility and dissolution rate.

## 4. Conclusions

This is an experiment designed for a practical problem in developing a new drug. And we hope to solve a series of problems about drugs with poor solubility in a systematic way. In this study, uniform design was adopted for high efficiency. The content of the stabilizers and operation parameters of the microfluidiser were screened on the basis of TEM, size distribution and zeta potential. An optimized recipe was got and the solubility of RD was greatly improved. Two different methods were used in the research to prepare the nanocrystal suspension. We first employed emulsification process (bottom-up method) to get the ideal NC-A powder. Then we used the same prescription with microfluidiser (top-down method). After screening the pressure and cycles, desirable NC-B powder was obtained. Two methods were separately carried out in this experiment. Combining the two methods could be tried for a further study. And the two kinds of nanocrystal powder would be injected into the animals' body for tissue distribution and pharmacokinetics study soon. Two kinds of nanocrystal freeze-dried powder were successfully prepared and the physicochemical properties were systematically studied. We found NC-A was more soluble than NC-B for the smaller particle size and both of them could release more than 90% in 1 min. The two processes are simple and adequate to make nanocrystal, and the products were very stable. The crystalline states were not altered according to the DSC and XRPD analysis. Sustaining the initial crystalline state was important to its stability. And the improved solubility owed to the small particle size. To prolong the stability of the nanocrystal powder, the freshly prepared nanocrystal suspension was quickly freeze dried.

RD was found in the year 2004 and it was proved to have antifungal activities and reversal effects on multidrug resistance. However, its development was limited by the poor solubility. In this experiment, we succeeded in improving its solubility and dissolution rate. Many new chemical entities were abandoned for its low solubility. So raising solubility with the pharmaceutical knowledge instead of using organic solvents such as DMSO will help medicinal chemistry and natural pharmaceutical chemistry complete their jobs. It will benefit for the whole pharmacy development. For the abundant BSC class II new chemical entities, a systematical study appears to be essential and nanocrystal presenting a simple approach to tackle the problem must have a bright future.

## Acknowledgements

This work was supported by the Ministry of Science and Technology of the PR China (ZX09102-127) and the National Basic Research Program of China (973 Program), No. 2009CB930300.

## References

Ali, H.S.M., York, P., Blagden, N., 2009. Preparation of hydrocortisone nanosuspension through a bottom-up nanoprecipitation technique using microfluidic reactors. Int. J. Pharm. 375, 107–113.

Amidon, G.L., Lennernas, H., Shah, V.P., Crison, J.R., 1995. A theoretical basis for a biopharmaceutic drug classification: the correlation of in vitro drug product dissolution and in vivo bioavailability. Pharm. Res. 12, 413–420.

Bohm, B.H., Muller, R.H., 1999. Lab-scale production unit design for nanosuspensions of sparingly soluble cytotoxic drugs. Pharm. Sci. Technol. Today 2, 336–339.

Branchu, S., Rogueda, P.G., Plumb, A.P., Cook, W.G., 2007. A decision-support tool for the formulation of orally active, poorly soluble compounds. Eur. J. Pharm. Sci. 32, 128–139.

Chen, H., Khemtong, C., Yang, X., Chang, X., Gao, J., 2011. Nanonization strategies for poorly water-soluble drugs. Drug Discov. Today 16, 354–360.

Chen, H., Wan, J., Wang, Y., Mou, D., Liu, H., Xu, H., Yang, X., 2008. A facile nanoaggregation strategy for oral delivery of hydrophobic drugs by utilizing acid–base neutralization reactions. Nanotechnology 19, 375104.

Chen, X., Young, T.J., Sarkari, M., Williams 3rd, R.O., Johnston, K.P., 2002. Preparation of cyclosporine A nanoparticles by evaporative precipitation into aqueous solution. Int. J. Pharm. 242, 3–14.

de Waard, H., Hinrichs, W.L., Frijlink, H.W., 2008. A novel bottom-up process to produce drug nanocrystals: controlled crystallization during freeze-drying. J. Control. Release 128, 179–183.

Gao, L., Liu, G., Wang, X., Liu, F., Xu, Y., Ma, J., 2011. Preparation of a chemically stable quercetin formulation using nanosuspension technology. Int. J. Pharm. 404, 231–237.

Horn, D., Rieger, J., 2001. Organic nanoparticles in the aqueous phase—theory, experiment, and use. Angew. Chem. Int. Ed. Engl. 40, 4330–4361.

Hu, J., Ng, W.K., Dong, Y., Shen, S., Tan, R.B.H., 2011. Continuous and scalable process for water-redispersible nanoformulation of poorly aqueous soluble APIs by antisolvent precipitation and spray-drying. Int. J. Pharm. 404, 198–204.

Keck, C., Muller, R., 2006. Drug nanocrystals of poorly soluble drugs produced by high pressure homogenisation. Eur. J. Pharm. Biopharm. 62, 3–16.

Kim, M.S., Jin, S.J., Kim, J.S., Park, H.J., Song, H.S., Neubert, R.H., Hwang, S.J., 2008. Preparation, characterization and in vivo evaluation of amorphous atorvastatin calcium nanoparticles using supercritical antisolvent (SAS) process. Eur. J. Pharm. Biopharm. 69, 454–465.

Kondo, M., Niwa, T., Okamoto, H., Danjo, K., 2009. Particle characterization of poorly water-soluble drugs using a spray freeze drying technique. Chem. Pharm. Bull. (Tokyo) 57, 657–662.

Merisko-Liversidge, E., Liversidge, G.G., Cooper, E.R., 2003. Nanosizing: a formulation approach for poorly-water-soluble compounds. Eur. J. Pharm. Sci. 18, 113–120.

Muller, R.H., Jacobs, C., Kayser, O., 2001. Nanosuspensions as particulate drug formulations in therapy Rationale for development and what we can expect for the future. Adv. Drug Deliv. Rev. 47, 3–19.

Patravale, V.B., Date, A.A., Kulkarni, R.M., 2004. Nanosuspensions: a promising drug delivery strategy. J. Pharm. Pharmacol. 56, 827–840.

Peters, K., Leitzke, S., Diederichs, J.E., Borner, K., Hahn, H., Muller, R.H., Ehlers, S., 2000. Preparation of a clofazimine nanosuspension for intravenous use and evaluation of its therapeutic efficacy in murine *Mycobacterium avium* infection. J. Antimicrob. Chemother. 45, 77–83.

Rasenack, N., Muller, B.W., 2004. Micron-size drug particles: common and novel micronization techniques. Pharm. Dev. Technol. 9, 1–13.

Shi, Y.-q., Qu, X.-j., Liao, Y.-x., Xie, C.-f., Cheng, Y.-n., Li, S., Lou, H.-x., 2008. Reversal effect of a macrocyclic bisbibenzyl plagiochin E on multidrug resistance in adriamycin-resistant K562/A02 cells. Eur. J. Pharmacol. 584, 66–71.

Wang, Y., Zhang, D., Liu, Z., Liu, G., Duan, C., Jia, L., Feng, F., Zhang, X., Shi, Y., Zhang, Q., 2010. In vitroandin vivoevaluation of silybin nanosuspensions for oral and intravenous delivery. Nanotechnology 21, 155104.

Wu, X.-Z., Chang, W.-Q., Cheng, A.-X., Sun, L.-M., Lou, H.-X., 2010. Plagiochin E, an antifungal active macrocyclic bis(bibenzyl), induced apoptosis in *Candida albicans* through a metacaspase-dependent apoptotic pathway. Biochim. Biophys. Acta Gen. Subjects 1800, 439–447.

Wu, X.-Z., Cheng, A.-X., Sun, L.-M., Sun, S.-J., Lou, H.-X., 2009. Plagiochin E, an antifungal bis(bibenzyl), exerts its antifungal activity through mitochondrial dysfunction-induced reactive oxygen species accumulation in *Candida albicans*. Biochim. Biophys. Acta Gen. Subjects 1790, 770–777.

Xing, J., Lv, B., Xie, C., Qu, J., Lou, H., 2008. Liquid chromatography–tandem mass spectrometry assay for the quantitation of plagiochin E and its main metabolite plagiochin E glucuronides in rat plasma. J. Pharm. Biomed. Anal. 47, 949–953.