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Preparation and characterization of freeze-dried 2-methoxyestradiol nanoparticle powders

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Poorly water-soluble compounds are difficult to develop as drug products using conventional formulation techniques and are frequently abandoned early in discovery. In the present study, a nanoprecipitation-high-frequency ultrasonication technique was adapted to produce drug nanosuspensions. The formulation of 2-methoxyestradiol (2-ME) as nanosuspension, either in the form of lyophilized powder or granules, was very successful in enhancing dissolution rate, more 45 times than bulk 2-ME being dissolved in the first 10 min. The increase *in vitro* dissolution rate may favourably affect bioavailability. The nanosuspension produced was then characterized using particle size determination, zeta potential measurement, scanning electron microscopy (SEM), differential scanning calorimetry (DSC) and X-ray analysis. Results showed that freeze-dried nanosuspension composed of amorphous particles with a mean particle size of 244 ± 10.6 nm (polydispersity index of 0.21 ± 0.02) was obtained. Physical stability studies showed that 2-ME nanosuspension remained homogeneous with slight increase in mean particle size and polydispersity index over a 3-month period.

1. Introduction

A significant proportion of drugs on the market are poorly soluble in water and it is expected that this will be even more pronounced in the future (Lipinski 2001, 2002). Formulations of poorly water-soluble compounds are a resource demanding challenge. During the discovery phase, new compounds are evaluated both *in vitro* and *in vivo*, in which liquid formulations are frequently used. Poorly soluble compounds can be formulated, e.g., as aqueous pH-shifted solutions, provided the molecules are ionizable, in mixtures of water and organic cosolvents, or by solubilization in cyclodextrin (Stella and Rajewski 1997; Loftsson and Brewster 1996; Akers 2002) or using emulsions (Floyd 1999; Lawrence and Rees 2000; Nakano 2000). Recently, formulations containing nano-sized drug particles have been found to be promising candidates for enhancing the solubility of poorly water-soluble drugs (Kesisoglou et al. 2007). A pharmaceutical nanosuspension is a submicron colloidal dispersion of drug particles which is stabilized by suitable stabilizers (surfactants and/or polymers) (Patravale et al. 2004). One of the main advantages of nanosuspensions is their small particle size and increased surface area which can lead to the increased dissolution rate and improved bioavailability (Rabinow 2004). Applications of nanosuspensions in drug delivery (i.e., oral and pulmonary routes) have been reported (Chingunpituk 2007). For intravenous delivery, nanosuspensions of drugs have been shown to enhance drug targeting and increase the duration of drug action (Srinivas et al. 2009). To obtain an amorphous nanosuspension, the drug is first dissolved in an organic water-miscible solvent and the resulting solution is then rapidly mixed with an aqueous stabilizer solution. The mechanism of particle formation by precipitation after

a solvent quench has been reported in several recent papers (Lindfors et al. 2006a, b; Lannibois et al. 1997; Brick et al. 2003; Vitale and Katz 2003).

2-Methoxyestradiol (2-ME), an endogenous metabolite of estrogen, has a very low water solubility ($2 \mu\text{g/ml}$) and high melting point ($182\text{--}185^\circ\text{C}$). It is currently in phase II clinical trials both as a chemopreventative and chemotherapeutic agent and has been orally administered (James et al. 2006) to cancer patients. However, poor oral absorption of the compound is one of the major obstacles of 2-ME development. Given 2-ME molecular features, nanosuspensions can be considered as an attractive formulation approach (Rabinow 2004).

The aim of this study was to investigate the possibility of producing a nanosuspension of 2-ME, a practically water-insoluble drug by nanoprecipitation-high-frequency ultrasonication technique. The present study intended to determine whether lyophilization powder with trehalose-mannitol could be stabilized for a long time. In addition, in order to confirm whether the solubility of freeze-dried 2-ME nanoparticles was really improved, dissolution for this preparation was tested according to the Chinese Pharmacopoeia.

2. Investigations, results and discussion

2.1. Preparation of 2-ME nanosuspension

A simple technique using nanoprecipitation-high-frequency ultrasonication technique for the formulation of 2-ME nanosuspension was developed and optimized, which produced a stable nanosuspension. Nanoprecipitation has been applied for many years in the preparation of small particles. Typically, the drug

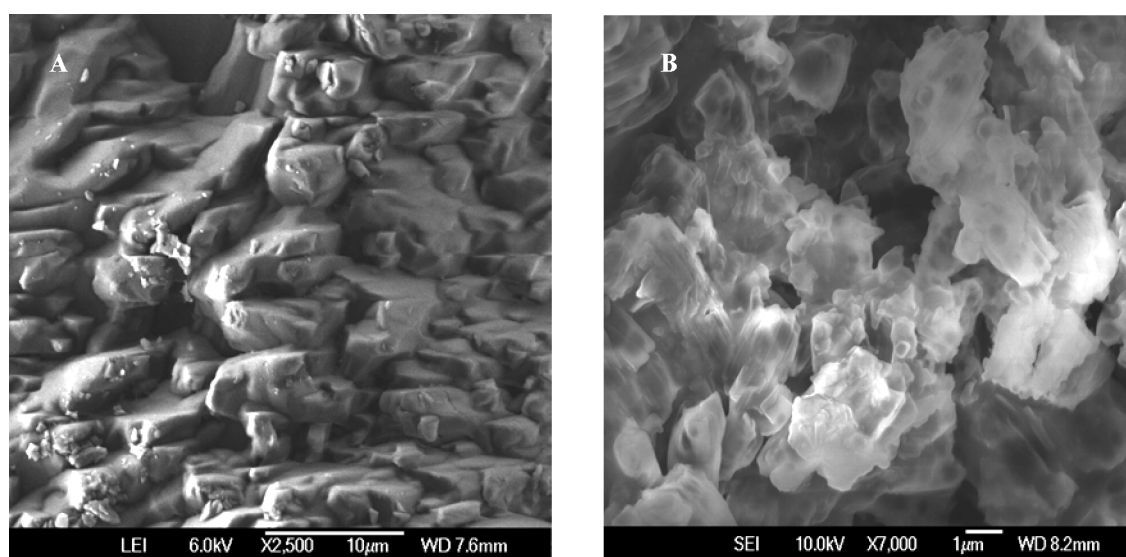


Fig. 1: The morphology of bulk 2-ME (A) and lyophilized 2-ME nanosuspensions (B) observed using SEM

is first dissolved in a solvent, and this solution is mixed with a miscible antisolvent. 2-ME is very poorly-soluble in water (10^{-3} – 10^{-4} mol/l) and well soluble in methanol and ethanol, but ethanol is less toxic, which was therefore selected for the preparation of nanosuspensions. High-frequency ultrasonication for the formulation of 2-ME nanosuspension was optimized. The cavitation and shear force produced during the exposure to ultrasound waves break the particles into distinct nanoparticles in suspension.

Formulation of nanosuspension requires a careful selection of stabilizers. Steric stabilization is often combined with electrostatic stabilization for additional repulsive contribution. The surfactant stabilizers can be non-ionic, such as Tween 80, poloxamer188, or anionic, such as SLS. Electrostatic stabilization is obtained by adsorbing charged molecules, which can be ionic surfactants or charged polymers, onto the particle surface (Filippos et al. 2007). Common pharmaceutical excipients that are suitable for use as polymeric stabilizers include the cellulose, such as CMC-Na, HPMC and HPC (Hecq et al. 2006). A mixture of stabilizers (3% lecithins + 3% poloxamer188 + 0.6% CMC-Na + 0.1% SLS) which reduced the free energy of the system resulted in a smaller average particle size using ethanol of the organic solvents. The aqueous nanosuspension produced by 2-ME had a mean particle diameter of 168 ± 2.5 nm.

2.2. Freeze-drying of the nanosuspension

Freeze-drying of the nanosuspensions with trehalose-mannitol yields a solid white powder. Particle size after redispersion of the freeze-dried products is measured. Average results (the particle size of each batch is measured in triplicate) compared to the results before freeze-drying are given in the Table. The results confirm the protective effect of trehalose-mannitol on nanoparticle agglomeration.

2.3. Morphology

The morphology of particle in nanosuspensions depended on the stabilizer used and drug concentration, which had been already confirmed by other authors (Zhang et al. 2006; Sinswat et al. 2007). In this study, nanoparticles were amorphous in shape (Fig. 1). The nanoparticle size observed by SEM was in

Table: Particle size and zeta potential of 2-ME nanosuspension and redispersion of the freeze-dried products (mean \pm SD, $n = 3$)

Batch No.	Mean particle size (nm)	Average zeta potential (mV)
20090510 (before freeze-drying)	171.2 ± 5.3	-28.51 ± 1.35
20090512 (before freeze-drying)	169.0 ± 1.5	-32.27 ± 2.37
20090515 (before freeze-drying)	165.1 ± 2.8	-28.59 ± 1.95
20090510 (after freeze-drying)	259.3 ± 7.5	-33.75 ± 2.51
20090512 (after freeze-drying)	239.0 ± 4.1	-35.43 ± 1.32
20090515 (after freeze-drying)	235.2 ± 5.9	-34.08 ± 2.74

good agreement with that determined by Zetasizer-Nano-ZS90, which was almost below 300 nm.

2.4. X-Ray diffraction (XRD) measurements

Figure. 2 shows the XRD patterns of the physical mixture of 2-ME and blank excipients, bulk 2-ME, excipients, lyophilized 2-ME nanosuspensions. The diffraction patterns of bulk 2-ME coarse powders showed characteristic high-energy diffraction peaks at 2θ values between 15° and 30° , indicating the crystalline structure of 2-ME. In the physical mixture, the diffraction patterns of 2-ME were well consistent with that of the bulk 2-ME, indicating that 2-ME also existed in the crystalline form. In the lyophilized nanosuspensions, the sharp peaks of pure 2-ME were not observed, indicating the disappearance of crystalline structure in 2-ME nanosuspensions. This suggests that 2-ME may be present in an amorphous state in the lyophilized 2-ME nanosuspensions.

2.5. Differential scanning calorimetry (DSC)

The DSC study investigation (Fig. 3) gave similar results to the XRD study. The bulk 2-ME coarse powders exhibited a sharp melt process, with an onset temperature of 187.06°C and a peak of 189.06°C . In the physical mixture, the endothermic peak of 2-ME drifted 5.0°C (181.98°C) to the left, due to the mixing with excipients and a little of 2-ME. Only two melting processes of excipients (about 53°C , 144°C) were observed

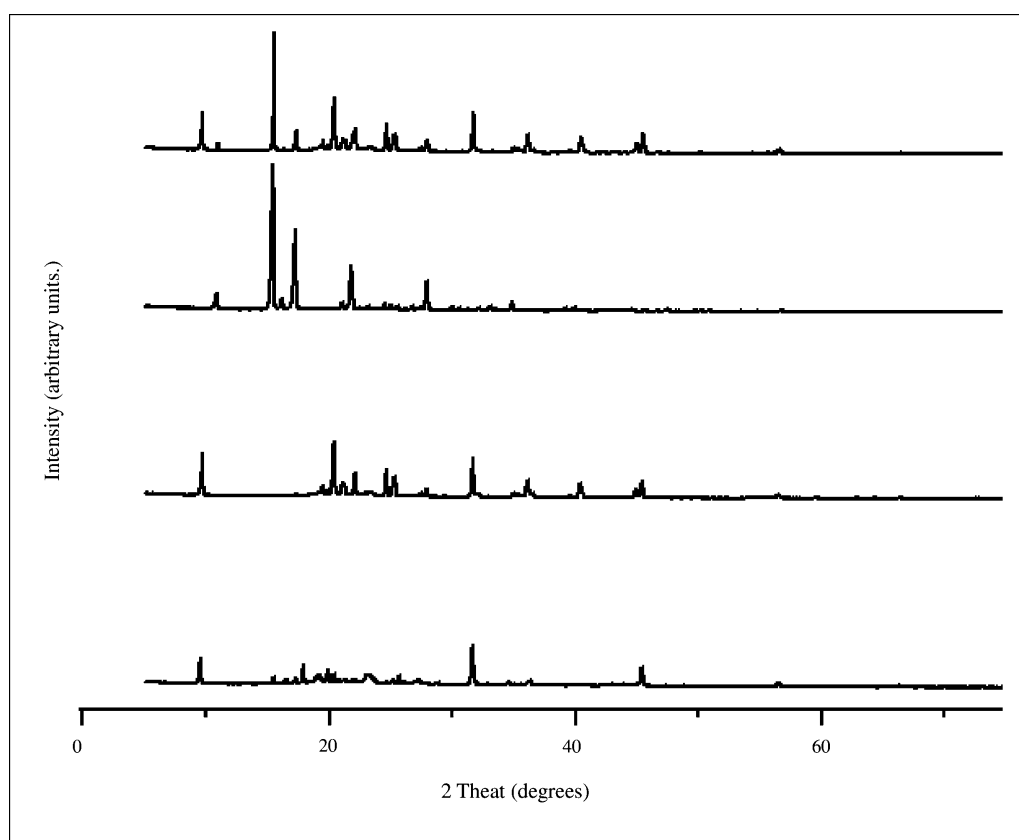


Fig. 2: XRD-spectra: from top to bottom: the physical mixture of 2-ME and excipients, bulk 2-ME, excipients, lyophilized 2-ME nanosuspensions

for the lyophilized 2-ME nanosuspensions, indicating that there was no crystalline 2-ME in the lyophilized nanosuspensions and the transition of crystalline state to amorphous state may have taken place during the micro-precipitation process. The formation of a high energy amorphous drug can increase C_{sat} compared to crystalline, coarse drug, so the faster dissolution profiles were expected.

2.6. In vitro dissolution studies

2-ME is a very weak organic acid with low aqueous solubility. The micronization of poorly soluble drugs could increase

the dissolution rate due to the increase in surface area but did not change the saturation solubility. When the particle size is reduced to the nanometer range, the solubility of drugs will increase significantly (Fig. 4). The increase in solubility can be explained by the Ostwald-Freundlich equation.

$$\log \frac{C_s}{C_\infty} = \frac{2v\sigma}{2.303 RT \rho r} \quad (1)$$

where C_s is the solubility, C_∞ is the solubility of the solid consisting of large particles, σ is the interfacial tension, v is the molar volume of the particle material, R is the gas constant, T is absolute temperature, ρ is the density of the solid, and r is

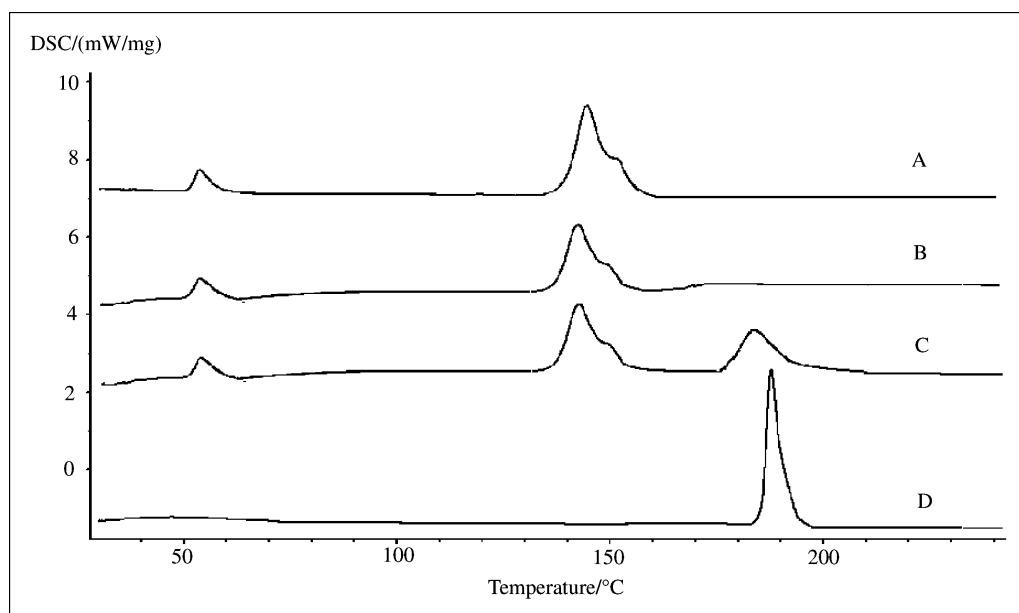


Fig. 3: Differential scanning calorimetry curves of the excipients (A), lyophilized 2-ME nanosuspensions (B), physical mixture of 2-ME and excipients (C) and bulk 2-ME (D)

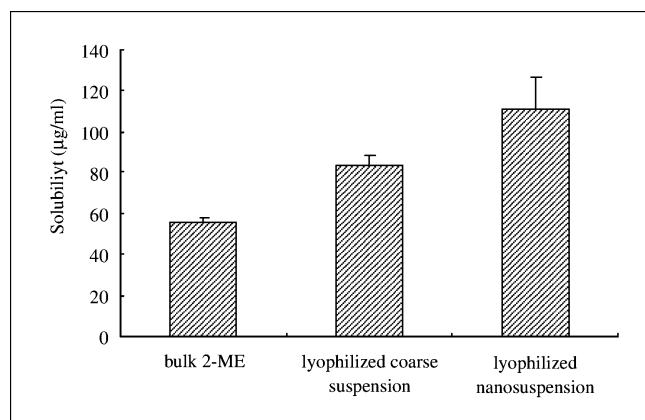


Fig. 4: Solubility as different particle size for 2-ME, data are mean \pm SD, $n=3$

the radius of particles (Muller and Peters 1998). According to Noyes–Whitney equation (Eq. 2), the dissolution rate of a drug into an aqueous solution depends on the diffusion coefficient (D), the surface area (S), the equilibrium solubility of the drug (C_s), and the thickness of the diffuse layer (h):

$$\frac{dC}{dt} = \frac{DS}{h}(C_s - C) \quad (2)$$

where C is the concentration of the drug in bulk solution. So, an increase in C_s and surface area due to the reduction of particle size (large surface area, S) would result in an increased dissolution rate.

In this study, the dissolution rate of 2-ME nanosuspensions, in comparison with the 2-ME coarse suspensions, was investigated. Typical cumulative dissolution profiles are shown in Fig. 5. In particular, Fig. 5 shows dissolution behaviour of 2-ME from freeze-dried coarse suspension (CS), freeze-dried nanosuspension (FN) and bulk drug in PBS with 1% Tween 80. The dissolution rate was markedly enhanced in the nanosuspensions, and nearly 50% of the drug dissolved within 1 h, 20% for the coarse 2-ME suspensions (5 µm), only 0.9% for the bulk

2-ME. This could be attributed to the increased surface area and enhanced saturation solubility of 2-ME in the nanosuspensions. As can be seen, for each 2-ME formulation both dissolution efficiency and dissolution percentage values increased in the following order: bulk 2-ME < coarse suspension < nanosuspension, while the time needed to dissolve 50% of drug decreased in the same order. Therefore, formulating the poorly water-soluble 2-ME as nanometer-size nanosuspensions had a dramatic effect on the drug solubility and dissolution rate.

2.7. Stability of nanosuspension

The physical stability of the lyophilized 2-ME nanosuspension was evaluated over 3 months at 4 °C and 25 °C. During this storage period, the particle size did not change, and the stability was maintained, with more than 99% of 2-ME remaining in the nanosuspension, indicating that the lyophilized product has a shelf-life of at least 3 months.

In conclusion, the nanoprecipitation–high-frequency ultrasonication technique was employed successfully to fabricate the 2-ME nanosuspensions. The particle size of nanoparticles is highly dependent on the stabilizers. By employing optimized conditions, nanosuspensions with mean diameters below 300 nm and with very low polydispersity could be prepared. This novel delivery system has a promising potential as an alternative formulation for 2-ME. *In vivo* evaluation of 2-ME release from nanosuspensions is underway and will be reported in a subsequent manuscript.

3. Experimental

3.1. Materials

2-ME (99.5% in purity) was home-made. Phosphatidylcholine (PC, injection grade) was purchased from Siwei (Zhengzhou, China). Poloxamer 188 (P188) and sodium carboxymethylcellulose (CMC-Na) was supplied by Shenyang Jiqi Pharmaceutical Co., Ltd. (China). Tween 80 and sodium laurylsulfate (SLS) were obtained from Sigma–Aldrich (USA). All reagents for high performance liquid chromatography (HPLC) analysis were of HPLC grade. Other chemicals used were of analytical grade.

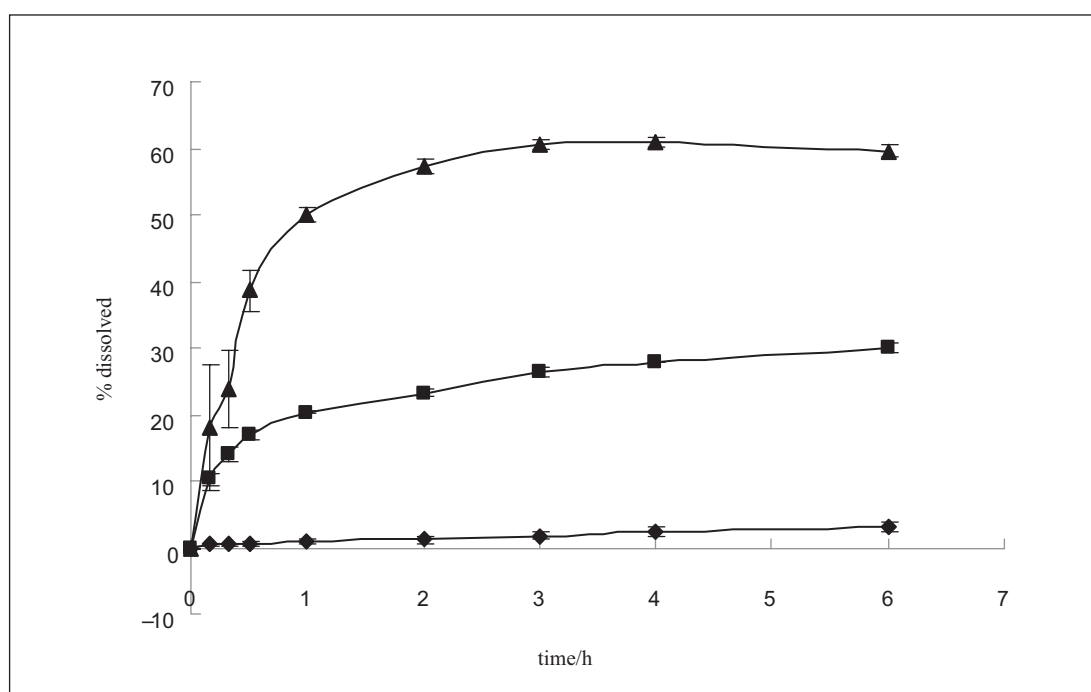


Fig. 5: Dissolution profiles of 2-ME: bulk 2-ME (◆), lyophilized coarse suspension (■), lyophilized nanosuspension (▲), data are mean \pm SD, $n=3$

3.2. Preparation of nanosuspensions

The 2-ME nanosuspensions were prepared by nanoprecipitation–high-frequency ultrasonication technique (Hany et al. 2009). In brief, the required amount of 2-ME, and phosphatidylcholine (3%, w/v) were dissolved in absolute alcohol. Poloxamer 188 (3%, w/v), CMC–Na (0.6%, w/v) and SLS (0.1%, w/v) were dissolved in 20 ml of phosphate buffered saline (PBS, pH7.4) to obtain the aqueous surfactant solution. The oil phase (10 ml) was dropped into the aqueous phase under rapidly stirring at 1000 rpm for 30 min at 50 °C. This slurry was dispersed by a high speed stirrer (ultraturax T25, Germany) at 15,000 rpm for 5 min. The organic solvent was removed in the rotary evaporator at 30 °C and 100 rpm under vacuum pressure (about 10 mm Hg). The resultant nanosuspension was subjected to probe-ultrasonicator (400 w, 40 cycles/3 sec) (LTD JY92–II, Scientz Biotechnology Co., China) in an ice-bath. Homogeneous and slightly 2-ME nanosuspensions were produced (Srinivas et al. 2009).

3.3. Freeze-drying and reconstitution

3.3.1. Freeze-drying

Suspensions (20 ml) containing 100 mg 2-ME were suspended in PBS containing cryo-protectant 0.5% trehalose–4.5% mannitol (w/v). In a freeze-dryer (Labconco, Corporation, USA), the samples were frozen at –80 °C for 12 h followed by drying at –50 °C for 24 h.

3.3.2. Reconstitution

20 ml of PBS with 5% glucose filtered through a membrane filter (0.22 µm) was added to a vial and shaken by vortex agitation to rehydrate the freeze-dried sample immediately before using.

3.4. Particle size analysis and zeta potential measurement

The particle size and zeta potentials of the dispersed systems were determined using a Zetasizer–Nano–ZS90 (Malvern Instruments, Malvern, UK). Analysis ($n=3$) was carried out for 100 s at room temperature by keeping angle of detection at 90°. The measured parameters are the average particle size, the polydispersity index (PI) and zeta potentials.

3.5. Scanning electron microscopy (SEM) of freeze-drying specimen

Morphological evaluation of 2-ME freeze-drying and unprocessed 2-ME were conducted by scanning electron microscopy (SEM). The lyophilized nanosuspension samples were placed on a carbon specimen holder, and then coated with platinum in a sputter coater (Polaron SC 7640), and then observed with a JSM–6700F scanning electron microscope (JEOL, Japan).

3.6. X-ray diffractometry (XRD)

X-ray diffraction (XRD) is a powerful and widely used tool for crystalline state evaluation. XRD diffractograms of 2-ME and other excipients in the nanosuspension were recorded using a Bruker AXS diffractometer (Model: D8 Advance) with Cu line as the source of radiation. Standard runs using a 40 kV voltage, a 40 mA current and a scanning rate of 0.02 °C/min over a 2θ range of 5–60 °C were used.

3.7. Differential scanning calorimetry (DSC)

The DSC measurements were performed using a Perkin–Elmer Diamond DSC (Perkin Elmer Instruments, USA) equipped with an intercooler. Data were treated mathematically using the resident PYRIS Software. The samples were analyzed in open aluminium pans and scanned under a nitrogen purge at 10 °C/min from 30 to 240 °C. The experiments were performed in triplicate on each batch of nanopowder. Identical experiments were conducted on the excipients, lyophilized 2-ME nanosuspensions, physical mixture of 2-ME and excipients and bulk 2-ME.

3.8. Solubility and *in vitro* dissolution studies

The saturation solubility evaluation of 2-ME in nanosuspension was carried out in phosphate buffered saline (PBS, pH7.4) with 1% Tween 80 at 37 °C. Lyophilized powder was dispersed into these media to obtain 2 mg/ml of drug suspension (excess) and placed on a shaking water bath (100 rpm, 37 °C \pm 0.1 °C) for 48 h. Samples were centrifuged, the resulting supernatant was diluted in a mobile phase and 20 µl volume was injected into the HPLC for analysis.

In vitro dissolution studies were performed in a ZRS–8G drug dissolution apparatus (Tianjin University Radio Factory, Tianjin, China) by the paddle method of Chinese Pharmacopoeia (2005). The dissolution medium used was 100 ml of pH 7.4 PBS solution with 1% Tween 80, 37 °C \pm 0.1 °C at a rotation speed of 100 rpm. At preselected time intervals, 2 ml samples were withdrawn, filtered through polycarbonate membranes (0.45 µm,

Millipore), and replaced with 2 ml of prethermo–stated fresh dissolution medium. Quantitative determination of 2-ME was performed by HPLC. Dissolution tests were performed in triplicate. The HPLC conditions were as follows: ODS column (4.6 mm \times 150 mm, 5 µm) (Agilent, USA) was used at room temperature. A mobile phase consisting of methanol–water (70:30, v/v) was at a flow rate of 1.0 ml/min, detection wavelength 285 nm, injection volume 20 µl. Standard solutions of 2-ME were prepared by diluting the appropriate volume of stock solution of 2-ME in methanol to give a final concentration of 0.1, 0.2, 0.5, 1, 2, 5 and 10 µg/ml.

3.9. Stability studies of formulations

Storage stability was studied by storing the lyophilized nanosuspension samples at 4 °C and 25 °C for up to 3 months. Periodically, samples were removed and the particle size was measured. In addition, 2-ME stability in the nanosuspension was examined by determining (HPLC assay) the amount of parent drug remained after specific storage periods.

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