International Journal of Pharmaceutics 405 (2011) 218–227

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Pharmaceutical Nanotechnology

Universal wet-milling technique to prepare oral nanosuspension focused on discovery and preclinical animal studies – Development of particle design method

Toshiyuki Niwa*, Satoru Miura, Kazumi Danjo

Department of Industrial Pharmacy, Faculty of Pharmacy, Meijo University, 150 Yagotoyama, Tempaku, Nagoya, Aichi 468-8503, Japan

ARTICLE INFO

Article history:
Received 2 August 2010
Received in revised form
17 November 2010
Accepted 10 December 2010
Available online 16 December 2010

Keywords:
Wet-milling
Nanosuspension
Oscillating beads-mill
Oral formulation
Manufacturing scale
Discovery and preclinical researches

ABSTRACT

Simple and easy methods to prepare oral nanosuspension of a poorly water-soluble pharmaceutical candidate compound, called a candidate, have been developed to support the discovery and preclinical studies using animals. The different wet-milling processes in miniature, middle and large preparation scales have been established in order to cover the various types of studies with wide scale. The powder of phenytoin, a poorly water-soluble model drug candidate, was suspended in the aqueous medium, in which the appropriate dispersing agents were dissolved, and milled by agitating together with small hard beads made of zirconia. Three general-purpose equipments with stirring, oscillating and turbulent motions were applied instead of the specific milling machine with high power to avoid much investment at such early development stage. The operational condition and dispersing agents were optimized to obtain finer particles using the middle-scaled oscillating beads-milling apparatus in particular. It was found that the nanosuspension, which whole particle distribution was in the submicron range, was successfully produced within the running time around 10 min. By applying the newly developed dispersing medium, the nanoparticles with identical size distribution were also prepared using the stirring and turbulent methods on miniature and large scales, respectively; indicating only 50 mg to 30 g or more amount of candidate could be milled to nanosuspension using three equipments. The crystalline analysis indicated that the both crystal form and crystallinity of the original bulk drug completely remained after wet-milling process. The results demonstrated that the wet-milling methods developed in this research would be a fundamental technique to produce nanosuspension for poorly water-soluble and oral absorbable drug candidates.

1. Introduction

In the past decade, solubilization technologies has been strongly desired to develop the pharmaceutical products for poorly water-soluble drugs because more than 40% of the commercial pharmaceuticals or the promising compound, so-called "candidate", in the development pipeline are reported to be categorized as poorly soluble (Prentis et al., 1988; Lipinski, 2000, 2002; Schroter, 2006). To address this need, a variety of solubilization techniques have been developed, including those based on the following approaches: (1) self-emulsifying drug-delivery systems (SEDDS) (Pouton, 2000; Gursoy and Benita, 2004), (2) solid dispersions prepared by coprecipitation (Vasconcelos et al., 2007; Shanbhag et al., 2008), spray-drying (Friesen et al., 2008; Janssens et al., 2009), freeze-drying (Ahmed and Aboul-Einien, 2007) or hot melt extrusion (Gupta et al., 2002), (3) complex formation with water-soluble excipients (Brewster and Loftsson, 2007; Carrier et al., 2007), (4) particle size reduction including the use of attrition-milled nanocrystalline forms (Kesisoglou et al., 2007; Merisko-Liversidge and Liversidge, 2008), and so on. Some pharmaceutical products have already been launched using these specific technologies. Despite the availability of a multitude of these technologies, a universal approach that can be also applied to exploratory research activities during the early development stages, i.e. discovery and preclinical stages, could not be found at present because all technologies above usually require a specific machine and/or large amount of the active compound to investigate the feasibility. In fact, discovery researchers sometimes face the problems that the enough oral exposure could not be attained to evaluate the biological performance of candidates even at high dose due to their poor gastrointestinal dissolution and absorption properties. However, no easily accessible solubiliztion technique is available to data to execute their animal experiments using limited resources (time, investment, compound).

* Corresponding author. Tel.: +81 52 839 2662; fax: +81 52 839 2662.
E-mail address: niwat@meijo-u.ac.jp (T. Niwa).

0378-5173/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2010.12.013

In order to satisfy such requirement of the discovery researchers, e.g. medicinal chemists, pharmacologists, toxicologists and ADME researchers, it was investigated that a solubilization methodology and process, so-called "solubilization tool" fitted for in vivo screening and profiling researches, would be established. To attain this object, the micronization of drug candidate to submicron-sized particles has been proposed in the current research. Among this approach, the high-shear beads milling technique in aqueous medium was adopted since its preparation process is simple and the suspension to be orally administered to animals could be directly prepared. The drug particles were size-reduced in aqueous suspension by grinding with small hard beads. The advanced wet-milling technique, which is well-known as "NanoCrystal technology" (Merisko-Liversidge et al., 2003), has ever given four pharmaceutical products currently on market (Junghanns and Müller, 2008). The author previously developed the simple wet-milling technique using a rotation–revolution mixer to produce the aqueous suspension (Niwa and Hashimoto, 2008), but the target of the present investigation is to prepare the suspension with submicron-sized particles, called nanosuspension, to support the animal studies. In addition, it was also researched that the manufacturing scale would be downsized and upsized to widely cover the discovery and preclinical studies including early screening ones using around 10–100 mg of compound and late safety ones using around 10–100 g of compound, respectively.

In this research, the simple nano-milling technique using an oscillating beads-milling apparatus, which is widely utilized in pharmacological and physiological fields as a crushing device for biological tissues such as cartilage, organs and yeast (Katou et al., 2003; Takagi et al., 2010), was newly developed as a standard method to supply the nanosuspension of poorly water-soluble candidate to the animal studies. The operational procedure and the formulation in dispersing medium were optimized to obtain nanosuspension with higher recovery. The particle size distribution of the candidate was periodically monitored during treatment to check the milling progress. Phenytoin was mainly used as a model drug candidate and other two compounds with low aqueous solubility were additionally applied to confirm the robustness independent of drug properties. Further, smaller- and larger-scaled manufacturing techniques than the oscillating procedure were also established using stirrer and turbulence mixer, respectively, with different milling motion. Especially miniature method using a conventional laboratory stirrer is likely convenient for exploratory researches due to its simplicity and universality. Physicochemical properties of resultant suspended particles were fully characterized with emphasis on surface morphology, electrostatic charge, crystallinity and thermal behavior to reveal the pharmaceutical advantages.

2. Materials and methods

2.1. Chemicals

Phenytoin and nifedipine were purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan) and Daito Pharmaceutical Co., Ltd. (Toyama, Japan), respectively. Pranlukast hydrate was provided by Ono Pharmaceutical Co., Ltd. (Osaka, Japan). Several water-soluble polymers including polyvinylpyrrolidone (Kollidone 30), hydroxypropyl cellulose (HPC-L), hydroxypropyl methylcellulose (Metolose 60SH-50), polyvinyl alcohol (polymerization degree: 2000) were provided by BASF Japan Ltd. (Tokyo, Japan), Nippon Soda Co., Ltd. (Tokyo, Japan), Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan), Wako Pure Chemical Co., Ltd., respectively. Two surfactants of sodium lauryl sulfate and polysorbate 80 were purchased from Wako Pure Chemical Co., Ltd. Polyvinylpyrrolidone, hydroxypropyl cellulose, hydroxypropyl methylcellulose, polyvinyl alcohol, sodium lauryl sulfate and polysorbate 80 were abbreviated to PVP, HPC, HPMC, PVA, SLS and Tween 80 in this report, respectively. All other chemicals and solvents were of analytical reagent grade, and deionized-distilled water was used throughout the study.

2.2. Manufacturing instruments

The oscillating beads-milling apparatus (Multi-Beads Shocker, Yasui Kikai, Osaka, Japan), which is originally used to extract bioactive components, e.g. DNA, RNA, proteins from physiological tissues or cells such as bone, tooth, cartilage, yeast, was mainly used in this study. The laboratory magnetic stirrer (HS4-SP, AsOne, Osaka, Japan) and the shaking mixer with turbulent rotating motion (Turbula Mixer T2F, Willy Bachofen, Basel, Switzerland), described turbulence mixer hereafter, were also used to reduce and enlarge the manufacturing scale, respectively. Zirconia (zirconium oxide) hard small balls, called as "beads" hereafter, with 1 mm, 0.5 mm, 0.3 mm and 0.1 mm in diameter (YTZ-1, -0.5, -0.3, -0.1) were purchased from Nikkato Co., Ltd. (Osaka, Japan).

2.3. Preparation of milled particles

The wet-milling in aqueous phase was executed by three types of beads-milling equipment, which were newly developed, to prepare the drug nanosuspension. Among them, the oscillating beads-milling apparatus was mainly used, which is applied to the middle manufacturing scale in this research. The schematic diagram expressing its fundamental structure and milling process was illustrated in Fig. 1. 0.6 g of poorly water-soluble drug and 60 g of zirconia beads were weighed to a conical tube with 50-mL capacity, and suspended in 15 mL of aqueous dispersing medium. Then, the tube was put into the holder and oscillated at 2700 rpm for 12 min as standard. The holder was cooled at 0 °C by circulating a refrig-

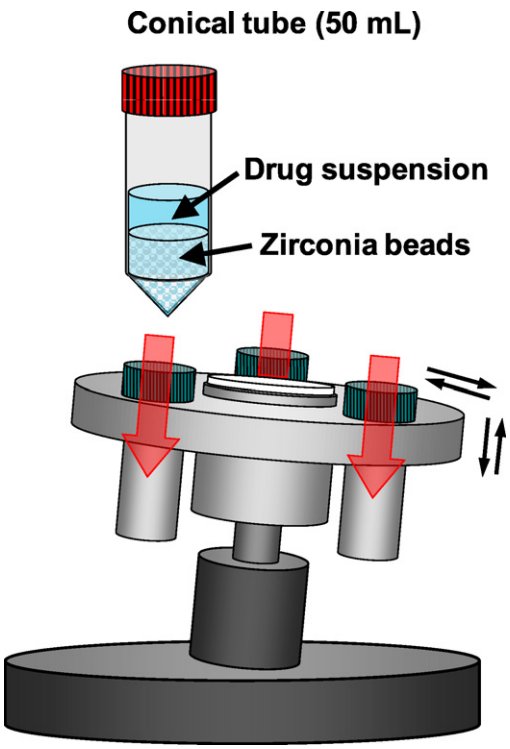

Fig. 1. Schematic diagram of oscillating beads-milling apparatus to prepare nanosuspension of the drug. Three tubes can be set in the holder and simultaneously treated.

erant. The drug loading was increased up to 2.4 g with keeping the volumes of beads and dispersing medium. As shown in Fig. 1, three sets of suspension in three tubes were simultaneously milled, and then mixed after treatment. Phenytoin and 0.5% PVP plus 0.1% SLS mixed aqueous solution were mainly used as a model drug and a standard dispersing medium, respectively. The beads with 0.3 mm in diameter were mainly used and the effect of beads diameter on milling power was also investigated.

For small manufacturing scale, the magnetic stirrer was selected instead of the oscillating beads apparatus. The glass vials with 10-mL or 50-mL capacities were filled with same quantity ratio of drug, beads, and dispersing medium as that in the oscillating system above, which amount were 0.1 or 0.6 g, 10 g or 60 g, and 2.5 mL or 15 mL, respectively. The suspension was ground with beads (0.3-mm in diameter) by stirring a magnetic bar at 700 rpm for 24 hr as standard. The drug loading was changed to 0.05–0.5 g or 0.6–3 g for 10-mL or 50-mL vials, respectively. Further, the turbulence mixer was applied to the large manufacturing scale. The suspension composed of 7.2–28.8 g of phenytoin, 720 g of 0.3-mm zirconia beads and 180 mL of aqueous medium were loaded in a steel bottle with 570-mL capacity and rotated at 96 rpm for 90 min as standard.

After milling process, the resultant nanosuspension of the drug was manually withdrawn by means of a pipette to separate from beads, and then supplied to analysis of particles.

2.4. Particle size distribution (PSD)

The particle size distributions of the original bulk particles and wet-milled particles in the prepared nanosuspension were measured by a laser diffraction scattering method using the diffractometer (LMS-30, Seishin Enterprise Co., Ltd., Tokyo, Japan). The original particles dispersed in 0.5% of PVP aqueous solution or small aliquot of nanosuspension were diluted with water appropriately in the batch-type cell. The obtained data were plotted as frequency distribution as a function of logarithmic particle size. The diameters at the 10%, 50%, and 90% of the cumulative volume distribution (D10, D50, and D90, respectively) were represented as size distribution. In some cases, the particle size distributions were also measured by a dynamic light scattering method using Zetasizer Nano-ZS (Sysmex, Kobe, Japan) to check the consistency between methods. For measurement, one drop of drug nanosuspension was diluted with ca. 4 mL of the water passed through a 0.22 μm disposable filter (Millex GV-13, Nihon Millipore, Tokyo, Japan) and dispersed homogeneously at 25.0 °C. A mean diameter (*Z*-Average) and a polydispersity index (PDI) were calculated based Cumulants fitting analysis. The PDI ranges from 0 for a perfectly monodispersed particle population to 1.0 for a very broad size distribution. All measurements of PSD were repeated in triplicate and the average sizes were reported.

2.5. Zeta potential

Electrophoretic mobility measurements were performed with Zetasizer Nano-ZS (Sysmex) at 25.0 °C. 750 μL of wet-milled nanosuspension diluted by the filtered water was set into the specific cuvette for the instrument. The zeta potentials were calculated by the instrument according to the Helmholts–Smoluchowski's equation. The measurements were performed in triplicate.

2.6. Morphology

The morphology of the milled particles was observed compared to the original bulk particles under a scanning electron microscope (SEM, JSM-6060, JEOL Ltd., Tokyo, Japan). The powdery particles lyophilized by freeze-dryer (PFR-1000/UT-2000, Tokyo Rikakiki, Tokyo, Japan) were fixed to the special sample stage and coated using a platinum sputtering equipment (JFC-1600, JEOL Ltd.).

2.7. Crystalline analysis

The suspension obtained after the oscillating beads-milling operation was ultracentrifuged at 40,000 rpm for 60 min (Optima XL-90, Beckman Instruments Inc., CA, USA) to separate the solid component from aqueous phase. Then, the settled wet mass, or concentrated slurry, was recovered and dried in the tray drier (DRA-630DA, Advantec Toyo Ltd., Tokyo, Japan) at 60 °C. In addition, the suspension was also frozen in liquid nitrogen and freeze-dried using the drier (RLE-52, Kyowa Vacuum Engineering Co., Ltd., Tokyo). The concentrated or dried wet-milled particles were provided to the following crystal analyses; X-ray powder diffraction (XRPD) analysis was conducted using a Geiger-Flex diffractometer (RAD-2VC, Rigaku Co., Tokyo, Japan) with Cu $K\alpha_1$ radiation and a Ni filter at a voltage of 30 kV and a current of 20 mA. Samples were scanned over 2θ range of 5–40° at a rate of 5°/min. Differential scanning calorimetry (DSC) was performed using DSC instrument (DSC-60, Shimadzu Co., Ltd., Kyoto, Japan). Around five milligram of each test sample was placed in an aluminum pan. The heating program was carried out using a modulated setting at 10 °C/min over 30–310 °C under nitrogen gas flow.

3. Results and discussion

3.1. Concept of present wet-milling to prepare nanosuspension

The simple and universal technique to prepare the oral suspension over wide scale has been required at the discovery and preclinical stages for pharmaceutical development. Especially, the miniature-scaled manufacturing applicable to much less than 1 g of candidates is indispensable to the initial biological screening researches in small animals, e.g. mice and rats. Three types of wet-milling technique have been developed in this study to provide the nanosuspension of poorly water-soluble compounds for the early development research. Each manufacturing technique is corresponding to the screening and regulatory studies approximately as follows:

(1) Miniature scale using a laboratory magnetic stirrer: pharmacological and pharmacokinetic researches at discovery stage.
(2) Middle scale using an oscillating beads-milling apparatus: toxicological (acute, sub-acute) research at preclinical stage.
(3) Large scale using turbulence mixer: toxicological (chronic, large animals) or preformulation researches at preclinical and clinical stages.

The milling manner is basically common among these techniques, in which the drug particles are size-reduced by grinding between beads although the collision impact is different in each technique. Here these techniques applicable to any amounts of compound could become reasonable tool to realize the nanosuspension in such early development stage, but it should be emphasized that the present research does not mention the scale-up on manufacturing. Additional investigation including scale-up manufacturing oriented to the industrial production would be required to develop the clinical and commercial dosage form as reported previously (Merisko-Liversidge et al., 1996, 2003; Kipp, 2004; Junghanns and Müller, 2008).

The amount of dispersing agents, which were required to promote the size reduction and stabilize nanometer-sized dispersion effectively, was investigated to be minimum in order to avoid their toxicity attributing to such surface active action especially at high

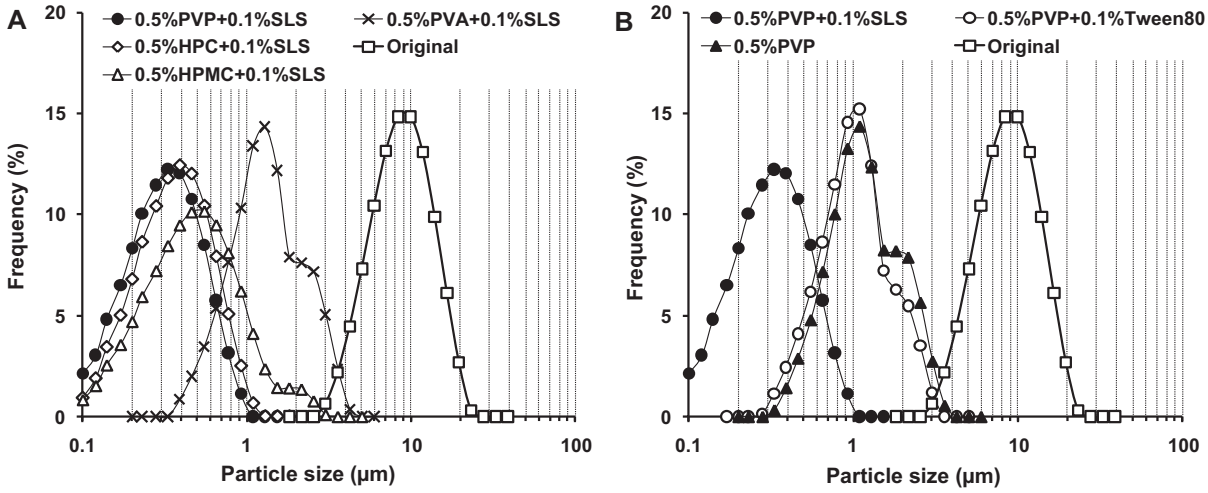

Fig. 2. Effect of dispersing agents dissolved in aqueous medium on particle size distribution of wet-milled phenytoin particles using oscillating beads-milling apparatus. As dispersing agents, the effects of (A) various water-soluble polymers and (B) various surfactants on milling were investigated. *Key*: (●) 0.5% PVP + 0.1% SLS, (◊) 0.5% HPC + 0.1% SLS, (△) 0.5% HPMC + 0.1% SLS, (×) 0.5% PVA + 0.1% SLS, (▲) 0.5% PVP, (○) 0.5% PVP + Tween 80, (□) original bulk particles of phenytoin.

dosing. In addition, the formulation in the dispersing medium, so-called "vehicle" in vivo studies, is strongly desired to be common on any scales because the vehicle formulation work should be minimum due to restricted amounts of compounds in exploratory stage.

3.2. Optimization of driving conditions and formulation for oscillating beads-milling apparatus

First, the loading of materials into the milling vessel, a 50-mL conical tube, were investigated using the oscillating beads-milling apparatus in order to produce the nanometer-sized particles of drugs. As a result of the preliminary manufacturing trials it was found that the loading volume of the zirconia beads should be approximately equal to that of dispersing solution to enhance the milling power. Therefore, the volume of solution and beads loading were fixed to 15 mL and 60 g, equivalent to 16 mL of packing volume, respectively, throughout the study irrespective of the drug loading. The drug loading was set to 0.6 g as standard, which is calculated to 40 mg/mL of suspension concentration, and increased with keeping the volumes of beads and dispersing medium. The driving time of the apparatus was fixed to 12 min unless mentioned specifically. Three sets of suspension tube were simultaneously milled to obtain the samples as much as possible although the driving with one or two tubes loading is practicable.

Secondly, the effect of components of dispersing agent dissolved in the aqueous medium on the particle size was investigated under the fixed condition just mentioned above. The particle size distributions are shown in Fig. 2, in which the left (A) and right (B) figures are displaying the effects of water-soluble polymers and surfactants, respectively. It was found that the combination of PVP and SLS could generate the smallest particles, which distribution was entirely shifted to submicron range (<1 μm). D10, D50, and D90 values of the wet-milled particles of phenytoin were 0.141, 0.292, and 0.550 μm, respectively, as listed in Table 1. By using water-soluble cellulose polymers such as HPC and HPMC instead of PVP, the nanonization was also achieved in combination with SLS. Whereas, the particles in single-micron size were still remained in case of PVA. Pongpeerapat et al. (2008) suggested that the PVP-based layering structure adsorbed around the drug particles, as steric barriers, effectively prevented the agglomeration of the primary particles prepared by ground mixture. According to their theory, it was assumed that the adsorption tendency of such hydrophilic polymers on the surface of the drug particles might contribute to the milling efficiency. The polymeric molecular having hydrophobic side chain such as pyrrolidinyl group (PVP), hydroxypropyl group (HPC and HPMC) might have strong interaction with the hydrophobic surface of the particles, resulting in effective size reduction (Fig. 2A). On the other hand, if surface active component was not formulated or changed from anionic SLS to nonionic Tween 80, the sufficient milling was not attained (Fig. 2B). The higher negative values of zeta potential than −30 mV, which is reported to be the minimum to stabilize the colloidal particles by electrostatic repulsion (Jacobs and Müller, 2002), were given by using SLS as a surfactant (Table 1). The insufficient milling in only PVP or PVP + Tween 80 systems might be probably derived from their low zeta potential level about −20 mV. These results show that the size reduction was successfully promoted through a combination of a steric stabilizer, PVP, and an electrostatic stabilizer, SLS. This combi-

Table 1
Representative particle sizes and zeta potential of original bulk and milled particles of phenytoin using various dispersing agents.

Dispersing media	D10 (μm)[a]	D50 (μm)[a]	D90 (μm)[a]	Zeta potential (mV)[b]
0.5%PVP + 0.1%SLS	0.141	0.292	0.550	−41.0
0.5%HPC + 0.1%SLS	0.160	0.334	0.623	−34.6
0.5%HPMC + 0.1%SLS	0.177	0.430	0.968	−32.0
0.5%PVA + 0.1%SLS	0.525	0.994	2.023	−25.0
0.5%PVP	0.558	1.030	2.092	−20.7
0.5%PVP + 0.1%Tween 80	0.496	0.930	1.819	−20.5
Original bulk particles	5.404	9.606	16.72	N.M.[c]

[a] D10, D50, D90 are diameters at the 10%, 50% and 90% of the cumulative volume distribution, respectively.
[b] Zeta potential of the particles was measured by electrophoretic mobility method.
[c] N.M.: not measured.

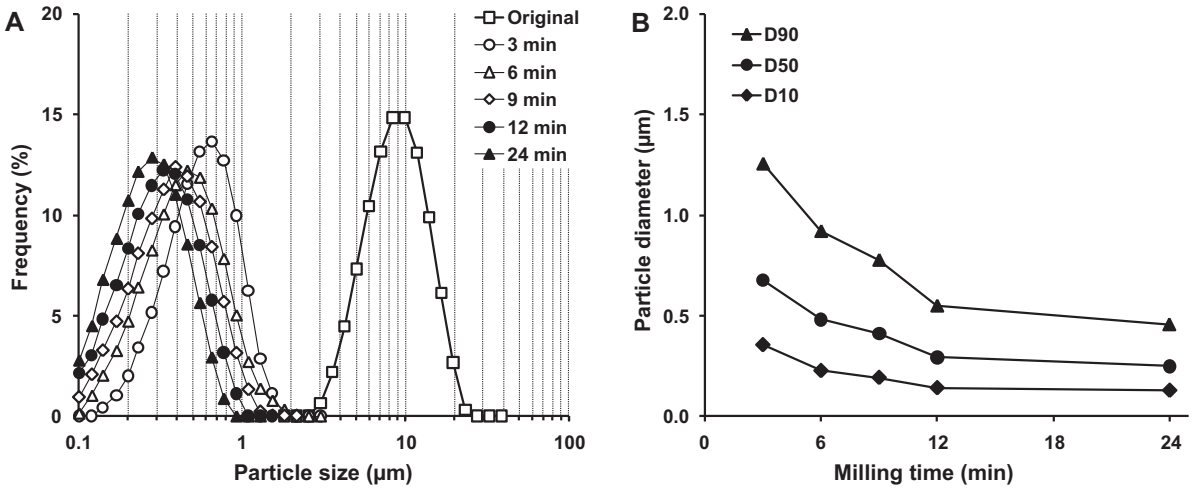

Fig. 3. (A) Transition of particle size distribution during wet-milling process and (B) representative particle diameters as a function of milling time using oscillating beads-milling apparatus. 600 mg of phenytoin was loaded in 15 mL of dispersing medium and collected at preset time in the milling process. D10, D50, and D90 indicate the 10%, 50%, and 90% diameter on the cumulative volume distribution, respectively.

nation also resulted in best milling performance on the miniature- and large-scaled preparation as mentioned later. Additionally, little size reduction happened in phenytoin particles without using the dispersing agents (data not shown), indicating that the wet-milling in aqueous medium is attributed to not only mechanical stress but also molecular interaction with dispersing agents.

The progress of size reduction during the oscillating beads-milling process was exhibited in the 0.5%PVP plus 0.1%SLS system using 0.3-mm beads (Fig. 3). The particles were drastically micronized at only 3 min-driving and the whole distribution was shifted to submicron size at 12 min, further asymptotically reaching to a plateau for 12–24 min (Fig. 3B). Such much shorter running time than that of the conventional planetary beads-milling, e.g. 24 h (Van Eerdenbrugh et al., 2008a, 2008b), is advantage of the current oscillating apparatus. The running time of this apparatus was fixed to 12 min throughout the study regardless of drug loading. The temperature of suspension could be controlled under 15 °C during the treatment while circulating a coolant in the vessel holders. This result indicates that the heat sensitive drug can be also applied.

The effect of beads diameter on the particle size distribution was investigated in the range of 0.1–1 mm at 2700 rpm using oscillating beads-milling apparatus. It was found that the smaller the diameter of zirconia beads became, the finer particles were prepared as shown in Fig. 4. Especially, the nanosuspension, which whole particle distribution was in the submicron range, was successfully produced using 0.1 and 0.3 mm beads. Takatsuka et al. reported that the total collision energy was a function of both the weight of a bead and the number of collisions between the beads and particles of compound (Takatsuka et al., 2009). However, the present results suggested that the number of collisions might be more predominant to pulverize the particles than the weight of a bead in the current oscillating system because the correlation between the bead diameter and the milled particle size was a monotone function and there was no local minimum of the milled size. The beads diameter was fixed to 0.3 mm as standard in this research because the 0.1-mm beads were somewhat difficult to separate from the nanosuspension by passing through a sieve or withdrawing through a pipette.

3.3. Application of other compounds using oscillating beads-milling

Other poorly water-soluble compounds were applied to the current oscillating beads-milling in order to expand its application. Nifedipine, anti-hypertensive agent, and pranlukast, anti-bronchial asthma agent, were milled in the standard condition optimized using phenytoin. The sizes of original particles of nifedipine and pranlukast were much larger and smaller than those of phenytoin, respectively, as shown in Fig. 5. The representative diameters measured by two instruments based on different spectroscopic analyses are also listed in Table 2. Irrespective of compounds and original particle sizes applied, three size distributions of the milled particles were highly equivalent each other and ranged below 1 μm. All average diameters of milled particles were around 0.3 μm and 180 nm measured by laser diffraction and dynamic light scattering methods, respectively. Although both mean values obtained by the different detection methods were not identical, their good inter-consistency of diameters might assure that the nanoparticles were formed in any cases without doubt. In addition, much smaller PDI values, which are close to 0, compared to those in the previous report (Jacobs and Müller, 2002) indicate that the distributions were quite narrow and nearly monodispersed particle population.

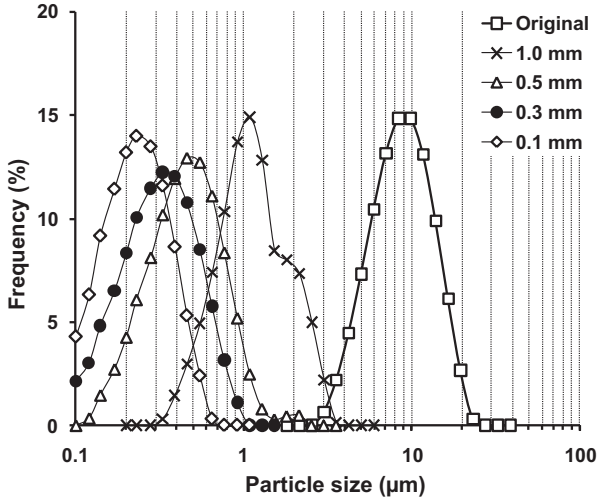

Fig. 4. Effect of zirconia beads diameter on particle size distribution of wet-milled phenytoin particles using oscillating beads-milling apparatus. The phenytoin particles were wet-milled under oscillating at 2700 rpm for 12 min using (×) 1 mm, (△) 0.5 mm, (●) 0.3 mm, and (◊) 0.1 mm-sized zirconia beads. The particle size distribution of the original bulk particles of phenytoin (□) was also shown.

Table 2
Representative particle sizes of various drugs before and after wet-milling using oscillating beads-milling apparatus measured by laser diffraction and dynamic light scattering methods.

Drugs	Particles	Laser diffraction			Dynamic light scattering	
		D10 (μm)[a]	D50 (μm)[a]	D90 (μm)[a]	*Z*-Average (nm)[b]	PDI[c]
Phenytoin	Original bulk	5.404	9.606	16.72	N.M.[d]	N.M.
	Wet-milled	0.141	0.292	0.550	160.0	0.167
Nifedipine	Original bulk	8.370	19.00	43.91	N.M.	N.M.
	Wet-milled	0.171	0.353	0.650	191.4	0.173
Pranlukast	Original bulk	1.819	3.232	6.496	N.M.	N.M.
	Wet-milled	0.169	0.334	0.598	186.3	0.147

[a] D10, D50, D90 are diameters at the 10%, 50% and 90% of the cumulative volume distribution.
[b] *Z*-Average is a mean diameter based on Cumulants fitting analysis.
[c] PDI is poly-dispersity index based on Cumulants fitting analysis.
[d] N.M.: not measured.

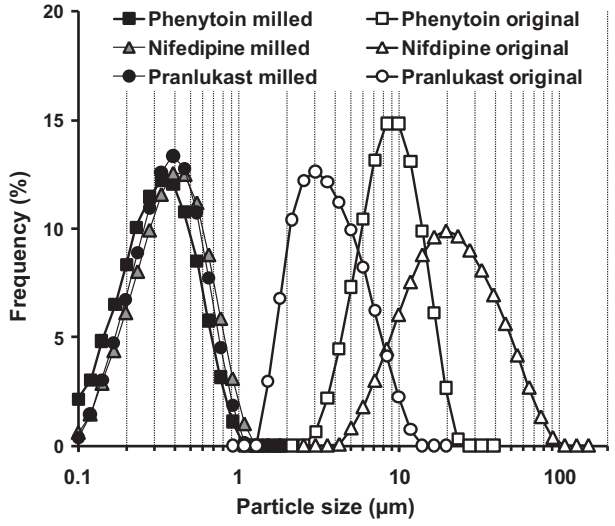

Fig. 5. Particle size distributions of various drugs before and after wet-milling using oscillating beads-milling apparatus. *Key*: (□,■) original and milled particles of phenytoin; (△,▲) original and milled particles of nifedipine; (○,●) original and milled particles of pranlukast.

3.4. Manufacturing line-ups corresponding to various loading amounts of drug

Taking the formulation supply to animal studies at discovery and preclinical stages into consideration, the widening of loading amount of the drug candidate was investigated. The amount of phenytoin loaded in 15 mL of dispersing medium was increased stepwise from 600 mg, which corresponds to 40 mg/mL of concentration, using oscillating beads-milling apparatus. The drug was milled in the standard condition mentioned above (0.3-mm zirconia beads, 2700 rpm, 12 min). The size distributions and their D10, D50, and D90 values were plotted as a function of drug concentration in Fig. 6. The identical submicron-sized distributions were obtained irrespective of drug loading until the concentration was increased up to 100 mg/mL, in which the phenytoin loading was 1500 mg per a vial. Whereas, the single-micron particles were still remained when the concentration was increased to 120 mg/mL or more. 12 min of the milling time was assumed to be too short to prepare entirely nanometer-sized suspension because it was found that the prolongation of driving time successfully resulted in complete shift to nanometer range up to 160 mg/mL (data not shown). Since the present oscillating apparatus has triplet holders and treat them simultaneously as shown in Fig. 1, 4.5 g of the drug can be nano-milled as a maximum through the standard operation.

The broadening of preparation scale is strongly required at discovery and preclinical stages. Their scales are generally spread from less than 100 mg to over 50 g, which is dependent on the types of

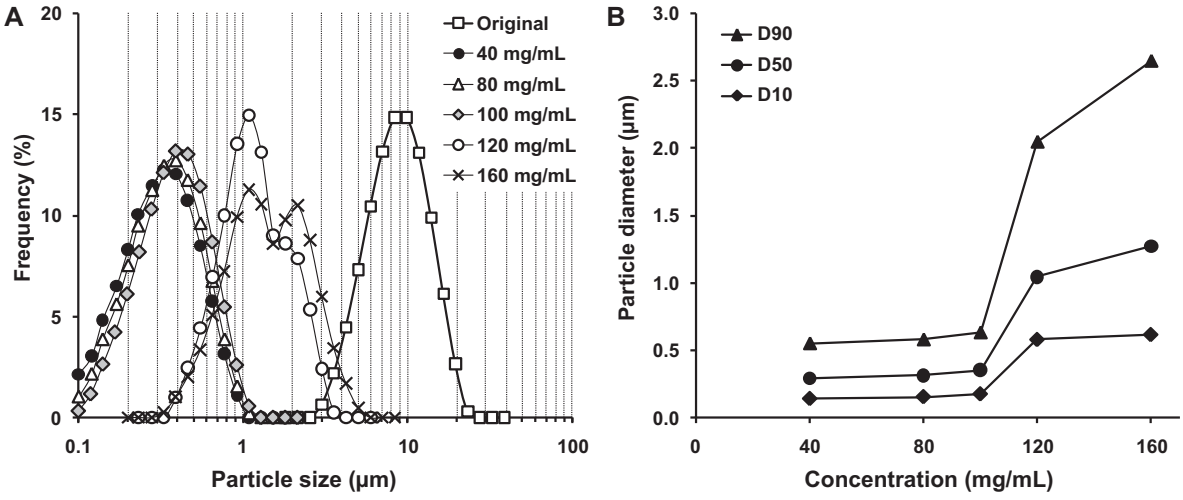

Fig. 6. (A) Effect of drug concentration in suspension on particle size distribution and (B) representative particle diameters as a function of drug concentration using oscillating beads-milling apparatus. 600–2400 mg of phenytoin were loaded in 15 mL of dispersing medium and wet-milled for 12 min. D10, D50, and D90 indicate the 10%, 50%, and 90% diameter on the cumulative volume distribution, respectively.

study and animal species, dose, and duration of the test. Further, the available compound for formulation design is strictly limited in general. The smaller- and larger-scaled manufacturing methods compared to the oscillating beads-milling apparatus were investigated to prepare oral nanosuspension of compounds oriented to the various types of in vivo study, such as early pharmacological screening study, preclinical safety study.

As a smallest manufacturing scale, the phenytoin and 0.3-mm zirconia beads were suspended in the optimized dispersing medium loaded in 10-mL vial and stirred by a magnetic stirrer for laboratory use. 50-mL vial was additionally used as a second smallest preparation. It was found that the size distributions in both vials were gradually decreased with time and reached to a plateau level for 24–48 h, and their sizes after 24-h milling were entirely distributed in submicron region (data not shown). The same milling effect was also achieved by using 0.5-mm glass beads instead of zirconia beads. The temperature of suspension was almost kept at ambient during milling due to its mild grinding. These results indicate that the specific milling machine with high power is not always required to prepare nanosuspensions if the dispersing agents in the medium were appropriately formulated. 0.5% PVP + 0.1% SLS was also the most effective medium to obtain the finer particles on the miniature scale in the same way as shown in Fig. 2. Such simple milling method using the laboratory stirrer is quite convenient to screen candidates because much investment cannot be paid at early development stage. The size distribution curves of the milled particles and their representative sizes were shown in Fig. 7 when the loading of phenytoin in 2.5 mL of aqueous medium was varied from 50 mg to 500 mg in stirrer method. The particles with equivalent submicron distributions to those in the oscillating apparatus were produced except 200 mg/mL of concentration, in which the drug loading was 500 mg in a vial. As also shown in the oscillating method (Fig. 6), the distribution was partially spread to single-micron range at higher concentration. Therefore, it is feasible to break 50–400 mg of the particles down to submicron-size, namely nano-mill, using the present miniature-scaled method. Further, 0.6–2.4 g of phenytoin was completely nano-milled in a same manner using 50-mL vial (data not shown).

On the other hand, the large-scaled manufacturing method was also developed to cover the preclinical studies required the largest amount of compound such as a long-term safety study in dogs. Assuming that the micronization would be promoted by making beads to collide each other in suspension frequently, the turbulence mixer was adopted. Fundamentally this equipment is used to mix the dry samples by rotating a container in the planetary and twisting motion, different from the agitation mixers with a settled container. Any sizes, shapes and materials of the container such as a polyethylene bottle and a glass vial can be attached to the mixer. The aqueous suspension having same drug concentration as that of the oscillating beads-milling apparatus was put into a steel bottle and rotated together with beads. The preliminary trials demonstrated that it took around 90 min to reduce the whole size distribution to submicron region using common dispersing medium (0.5% PVP + 0.1% SLS). The drug loading in the bottle was increased from 7.2 g as a standard using the turbulence mixer. The effect of suspension concentration on the milled particle size distribution was exhibited in Fig. 8 as contrasted with the oscillating and stirring methods (Figs. 6 and 7). These resultant similar distribution curves exhibited that a maximum concentration to be entirely nano-milled was around 160 mg/mL on any scales regardless of milling manner. The scale-up trials revealed that 28.8 g of phenytoin was effectively treated as a maximum amount using the turbulence mixer. Although the further scale-up experiments have not been done due to lack of the compound, 50 g or larger amount of drugs would be nano-milled if the bottle with larger capacity is used.

The milling results on each preparation scale were summarized in Fig. 9, in which the representative particle diameters (D10, D50, D90) were plotted as function of drug loading. This figure indicates that the nanometer-sized particles with 200–400 nm in average diameter were successfully prepared throughout the wide range of loading from 10-mg to 10-g orders independent of the equipments. The nanoparticles were successfully recovered with high yield (>95%) in any methods because there is little adhesion of particles to the beads and wall of vessel. The common dispersing medium, 0.5%PVP + 0.1%SLS aqueous solution, is applicable to any manufacturing scales and any drug loadings, that is advantageous to the animal studies at discovery and preclinical stages for the following reasons: (1) the extra amount of the candidate compounds is not available to optimize the formulation due to their restricted synthesis; and (2) the exposure to excipients can be set uniform among animals at any oral dose of the candidates. In addition, the present technique can provide the nanosuspension while keeping the quite low composition of PVP and SLS, ranging in 1/8–1/32 and 1/40–1/160 weight ratio against the candidates, respectively. In some previous report the much larger amount of excipients than

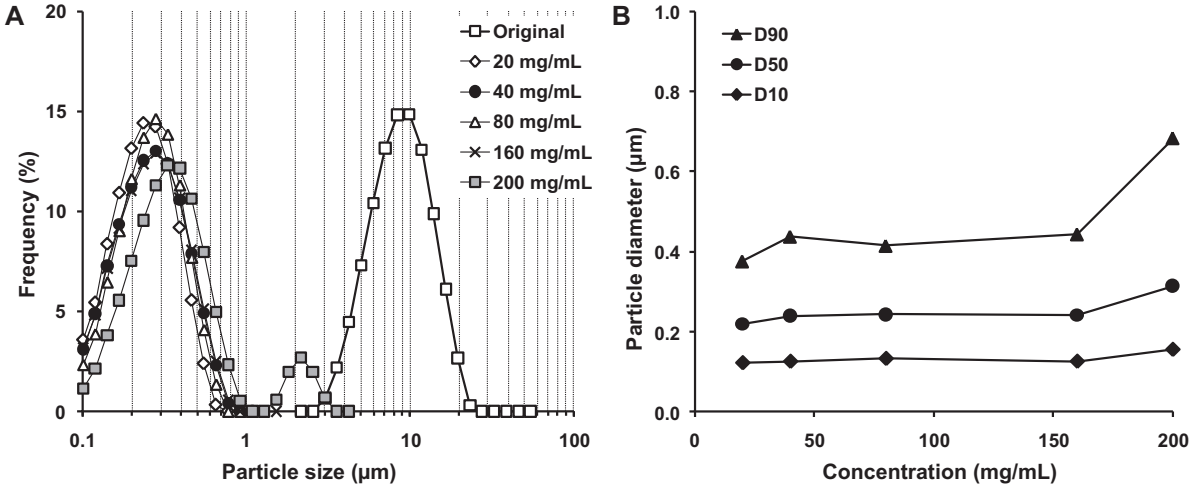

Fig. 7. (A) Effect of drug concentration in suspension on particle size distribution and (B) representative particle diameters as a function of drug concentration using magnetic stirrer. 50–500 mg of phenytoin were loaded in 2.5 mL of dispersing medium and wet-milled for 24 h. D10, D50, and D90 indicate the 10%, 50%, and 90% diameter on the cumulative volume distribution, respectively.

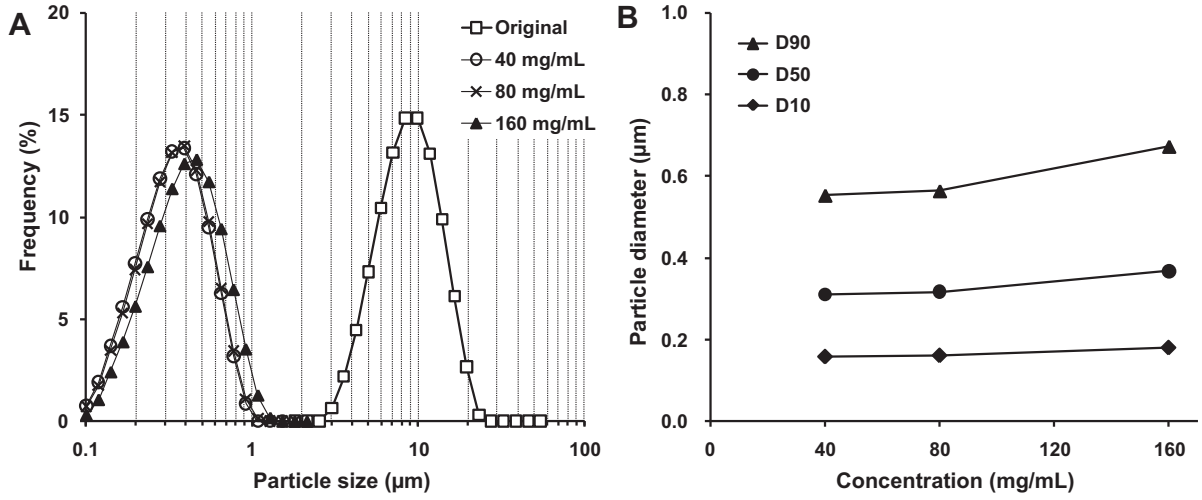

Fig. 8. (A) Effect of drug concentration in suspension on particle size distribution and (B) representative particle diameters as a function of drug concentration using turbulence mixer. 7.2–28.8 g of phenytoin were loaded in 180 mL of dispersing medium and wet-milled for 90 min. D10, D50, and D90 indicate the 10%, 50%, and 90% diameter on the cumulative volume distribution, respectively.

drug was formulated to mill the drug particles down to submicron (Moribe et al., 2008; Pongpeerapat et al., 2008; Kawabata et al., 2010). So, the toxic response of these excipients could be negligible because their administration dose can be reduced as minimum.

3.5. Physicochemical properties of nanoparticles

The wet-milled particles suspended in the aqueous dispersing medium were freeze-dried to be provided for microscopic observation. SEM photographs in Fig. 10 obviously demonstrated that the original bulk particles (A) were drastically broken down and the freeze-dried product appeared to be mosaic structure assembled from lots of tiny pieces (B-1 and B-2). Each piece was considered to be a milled particle of phenytoin because 93% weight in solidified mass was composed of phenytoin. The size of each milled particle was visually found to be 200–300 nm in diameter, which was highly consistent with the results measured by the laser diffraction method. Thus, the complete nano-milling through the current wet-milling technique was also confirmed by the visual observation.

The effect of milling operation on the crystalline property of phenytoin was investigated by X-ray powder diffraction (XRPD) and DCS. The nanosuspension was ultracentrifuged to separate the solid component from aqueous medium. Then, the paste-like precipitate was recovered and dehydrated by a tray-dryer. The ultracentrifuged product and its tray-dried product composed of the milled particles as well as the freeze-dried product mentioned above were provided for the analyses. As shown in Fig. 11, the positions of diffraction peaks and the onset temperature at endothermic peak (297 °C) of any treated particles were identical to those of the original bulk, suggesting that any polymorphic transformations might not occur during wet-milling process. The XRPD peak intensities of the tray-dried particles (C) were almost equivalent to those of the original bulk (A), proposing that the crystallinity of milled particles maintained same crystalline state as starting materials after nano-milling. The diffraction interference by water in wet sample or co-existing with PVP and SLS might weaken the peak intensities in the ultracentrifuged (B) or freeze-dried products (D), respectively. These results concluded that the current media milling process in aqueous phase might not induce a polymorphic transition and amorphization of the drug as other authors have been previously reported (Van Eerdenbrugh et al., 2008b; Kawabata et al., 2010; Tanaka et al., 2009). Consequently, the crystalline state was still kept even if the drug were broken down into nano-sized particles.

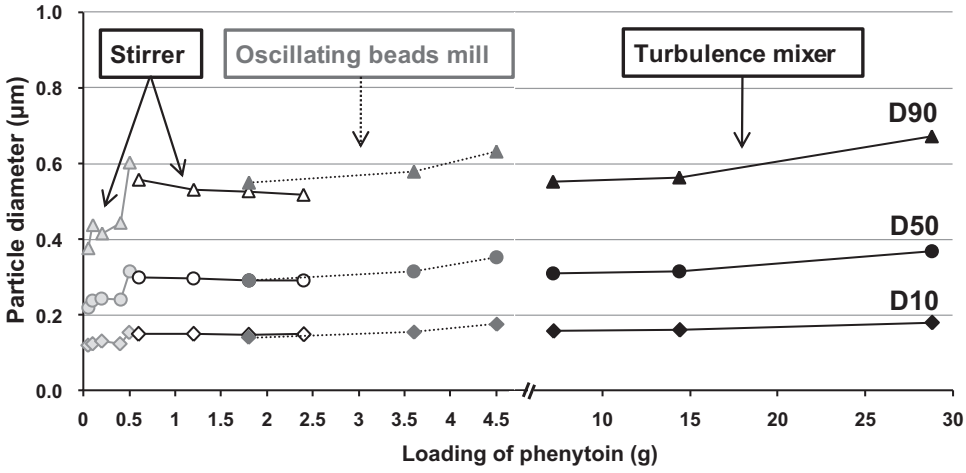

Fig. 9. Summary of representative particle diameters as a function of drug loading in the suspension using three types of milling apparatus. D10 (diamond), D50 (circle), and D90 (triangle) indicate the 10%, 50%, and 90% diameter on the cumulative volume distribution, respectively.

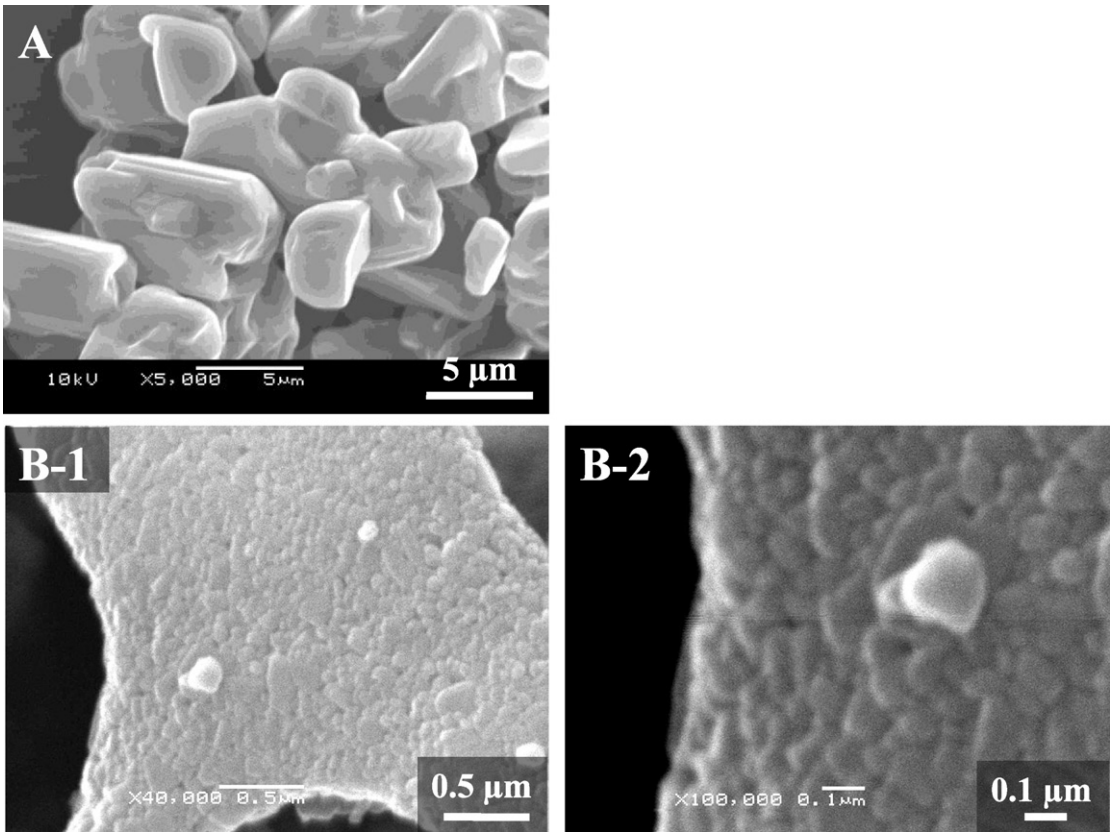

Fig. 10. Scanning electron microphotographs of phenytoin particles. *Key*: (A) original bulk particles; (B-1 and B-2) wet-milled particles obtained after freeze-drying process.

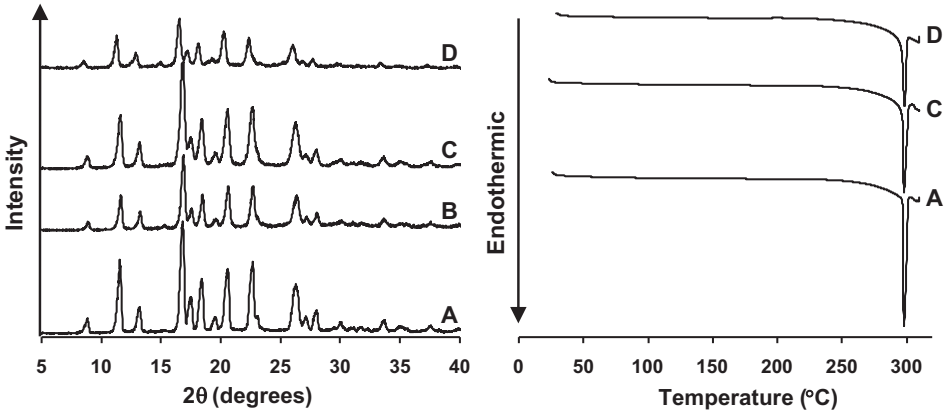

Fig. 11. X-ray powder diffraction patterns (left) and DSC profiles (right) of the original bulk and wet-milled particles of phenytoin. *Key*: (A) original bulk particles; (B) ultracentrifuged product of wet-milled particles; (C) tray-dried product of wet-milled particles; (D) freeze-dried product of wet-milled particles.

4. Conclusion

In the present study, the wet-milling technique in aqueous medium was developed to prepare the oral nanosupension focused on the discovery and preclinical animal studies for poorly water-soluble candidate compounds. The preparation scale was widened from 10-mg to 10-g orders to apply to various types of study such as initial screening study using smaller animals (mice and rats) and advanced profiling study using larger animals (dogs and monkeys). Three milling systems based on stirring, oscillating and turbulent motions were established to break drug particles down to submicron size, nanoparticles. It was found that the combination of two dispersing agents such as a steric stabilizer and an electrostatic stabilizer (0.5% PVP + 0.1% SLS) efficiently promoted the size reduction of drug particles even if the specific milling equipments were not used. Especially the nanosuspension was available even in such short time as 10 min or thereabouts by using the oscillating apparatus. The equivalent size distributions were obtained among three model drugs, possibly demonstrating that this technique could be applied to various physicochemical properties of the compounds. The crystalline analyses revealed that the process gave rise to no crystalline change, polymorphization or amorphization of the candidates, which is highly beneficial in the drug development aspect because the physicochemical stability issue might not be required to be discussed at later development stage. The gastrointestinal absorption would be overestimated at this stage if the commercial dosage composed of amorphous drug is difficult to develop. The authors believe that the nanosuspension prepared by

the present technique would be a good tool to evaluate the poorly water-soluble candidates from pharmacological, toxicological and pharmacokinetic perspectives at discovery stage. However, the potential risks for contamination derived from abrasion of beads should be addressed prior to clinical administration, which could be common issue in such beads milling technique. Finally, the investigation for drying of nanosuspension and its redispersion is now progressing to apply the present technique to development of the major pharmaceutical dosage forms such as tablets, capsules.

Acknowledgements

The authors wish to thank Shinmaru Enterprises Corporation (Sakai, Japan) and Ono Pharmaceutical Co., Ltd. (Osaka, Japan) for kindly providing Turbula Mixer and pranlukast hydrate, respectively. The authors are grateful to Mr. Isao Funahashi, Mr. Hidenori Nakano and Ms. Mayo Kondo for their excellent technical assistance throughout this work.

References

Ahmed, I.S., Aboul-Einien, M.H., 2007. In vitro and in vivo evaluation of a fast-disintegrating lyophilized dry emulsion tablet containing griseofulvin. Eur. J. Pharm. Sci. 32, 58–68.

Brewster, M.E., Loftsson, T., 2007. Cyclodextrins as pharmaceutical solubilizers. Adv. Drug Deliv. Rev. 59, 645–666.

Carrier, R.L., Miller, L.A., Ahmed, I., 2007. The utility of cyclodextrins for enhancing oral bioavailability. J. Control. Release 123, 78–99.

Friesen, D.T., Shanker, R., Crew, M., Smithey, D.T., Curatolo, W.J., Nightingale, J.A.S., 2008. Hydroxypropyl methylcellulose acetate succinate-based spray-dried dispersions: an overview. Mol. Pharm. 5, 1003–1019.

Gupta, M.K., Tseng, Y.C., Goldman, D., Bogner, R.H., 2002. Hydrogen bonding with adsorbent during storage governs drug dissolution from solid-dispersion granules. Pharm. Res. 19, 1663–1672.

Gursoy, R.N., Benita, S., 2004. Self emulsifying drug delivery systems (SEDDS) for improved oral delivery of lipophilic drugs. Biomed. Pharmacother. 58, 173–182.

Jacobs, C., Müller, R.H., 2002. Production and characterization of a budesonide nanosuspension for pulmonary administration. Pharm. Res. 19, 189–194.

Janssens, S., Anné, M., Rombaut, P., Van den Mooter, G., 2009. Spray drying from complex solvent systems broadens the applicability of Kollicoat IR as a carrier in the formulation of solid dispersions. Eur. J. Pharm. Sci. 35, 241–248.

Junghanns, J.A.H., Müller, R.H., 2008. Nanocrystal technology, drug delivery and clinical applications. Int. J. Nanomed. 3, 295–309.

Katou, Y., Kanoh, Y., Bando, M., Noguchi, H., Tanaka, H., Ashikari, T., Sugimoto, K., Shirahige, K., 2003. S-phase checkpoint proteins Tof1 and Mrc1 form a stable replication-pausing complex. Nature 424, 1078–1083.

Kawabata, Y., Yamamoto, K., Debari, K., Onoue, S., Yamada, S., 2010. Novel crystalline solid dispersion of tranilast with high photostability and improved oral bioavailability. Eur. J. Pharm. Sci. 39, 256–262.

Kesisoglou, F., Panmai, S., Wu, Y., 2007. Application of nanoparticles in oral delivery of immediate release formulations. Curr. Nanosci. 3, 183–190.

Kipp, J.E., 2004. The role of solid nanoparticle technology in the parenteral delivery of poorly water-soluble drugs. Int. J. Pharm. 284, 109–122.

Lipinski, C.A., 2000. Drug-like properties and the causes of poor solubility and poor permeability. J. Pharmacol. Toxicol. Meth. 44, 235–249.

Lipinski, C.A., 2002. Poor aqueous solubility – an industry wide problem in drug discovery. Am. Pharm. Rev. 5, 82–85.

Merisko-Liversidge, E., Sarpotdar, P., Bruno, J., Hajj, S., Wei, L., Peltier, N., Rake, J., Shaw, J.M., Pugh, S., Polin, L., Jones, J., Corbett, T., Cooper, E., Liversidge, G.G., 1996. Formulation and antitumor activity evaluation of nanocrystalline suspensions of poorly soluble anticancer drugs. Pharm. Res. 13, 272–278.

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.

Merisko-Liversidge, E.M., Liversidge, G.G., 2008. Drug nanoparticles: formulating poorly water soluble compounds. Toxicol. Pathol. 36, 43–48.

Moribe, K., Wanawongthai, C., Shudo, J., Higashi, K., Yamamoto, K., 2008. Morphology and surface states of colloidal probucol nanoparticles evaluated by atomic force microscopy. Chem. Pharm. Bull. 56, 878–880.

Niwa, T., Hashimoto, N., 2008. Novel technology to prepare oral formulations for preclinical safety studies. Int. J. Pharm. 250, 70–78.

Pongpeerapat, A., Wanawongthai, C., Tozuka, Y., Moribe, K., Yamamoto, K., 2008. Formulation mechanism of colloidal nanoparticles obtained from probucol/PVP/SDS ternary ground mixture. Int. J. Pharm. 352, 309–316.

Prentis, R.A., Lis, Y., Walker, S.R., 1988. Pharmaceutical innovation by seven UK-owned pharmaceutical companies. Br. J. Clin. Pharmacol. 25, 387–396.

Pouton, C.W., 2000. Lipid formulations for oral administration of drugs: non-emulsifying, self-emulsifying and "self-microemulsifying" drug delivery systems. Eur. J. Pharm. Sci. 11, S93–S98.

Shanbhag, A., Rabel, S., Nauka, E., Casadevall, G., Shivanand, P., Eichenbaum, G., Mansky, P., 2008. Method for screening of solid dispersion formulations of low-solubility compounds – Miniaturization and automation of solvent casting and dissolution testing. Int. J. Pharm. 351, 209–218.

Schroter, C., 2006. Prioritizing molecules based on physicochemical characteristics. Am. Pharm. Rev. 9, 60–67.

Takagi, M., Yoshioka, H., Wakitani, S., 2010. A mass separation of chondrocytes from cartilage tissue utilizing an automatic crushing device. J. Biosci. Bioeng. 109, 73–74.

Takatsuka, T., Endo, T., Jianguo, Y., Yuminoki, K., Hashimoto, N., 2009. Nanosizing of poorly water soluble compounds using rotaion/revolution mixer. Chem. Pharm. Bull. 57, 1061–1067.

Tanaka, Y., Inkyo, M., Yumoto, R., Nagai, J., Takano, M., Nagata, S., 2009. Nanoparticulation of poorly water soluble drugs using a wet-mill process and physicochemical properties of the nanopowders. Chem. Pharm. Bull. 57, 1050–1057.

Van Eerdenbrugh, B., Froyen, L., Van Humbeeck, J., Martens, J.A., Augustijns, P., Van den Mooter, G., 2008a. Drying of crystalline drug nanosuspensions – the importance of surface hydrophobicity on dissolution behavior upon redispersion. Eur. J. Pharm. Sci. 35, 127–135.

Van Eerdenbrugh, B., Froyen, L., Van Humbeeck, J., Martens, J.A., Augustijns, P., Van den Mooter, G., 2008b. Alternative matrix formers for nanosuspension solidification: dissolution performance and X-ray microanalysis as an evaluation tool for powder dispersion. Eur. J. Pharm. Sci. 35, 344–353.

Vasconcelos, T., Sarmento, B., Costa, P., 2007. Solid dispersions as a strategy to improve bioavailability of poorly water soluble drugs. Drug Discov. Today 12, 1068–1075.