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Freeze-dried nifedipine-lipid nanoparticles with long-term nano-dispersion stability after reconstitution

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ABSTRACT

Nifedipine (NI) is a poorly water-soluble drug and its oral bioavailability is very low. To improve the water solubility, NI-lipid nanoparticle suspensions were prepared by a combination of co-grinding by a roll mill and high-pressure homogenization without any organic solvent. The mean particle size and zeta potential of the NI-lipid nanoparticle suspensions were about 52.6 nm and −61.8 mV, respectively, and each parameter remained extremely constant during a period of 4 months under 6 °C and dark conditions, suggesting that the negative charge of the phospholipid, dipalmitoyl phosphatidylglycerol, is very effective in preventing coagulation of the particles. In order to assure the nano-order particle size of the suspensions in view of long-term stability, a freeze-drying technique was applied to the NI-lipid nanoparticle suspensions. The mean particle size of freeze-dried NI-lipid nanoparticles after reconstitution was significantly increased in comparison to that of the preparations before freeze-drying. It was found, however, that the addition of sugars (glucose, fructose, maltose or sucrose) to the suspensions before freeze-drying inhibited the aggregation of nanoparticles, suggesting that the long-term stability storage of freeze-dried NI-lipid nanoparticles after reconstitution would be overcome. In addition, freeze-dried nanoparticles with 100 mg sugar (glucose, fructose, maltose or sucrose) showed excellent solubility (>80%), whereas without sugar, as a control, showed low solubility (<20%). It was found that negatively charged phospholipids and sugars prevent coagulation of NI nanoparticle suspensions, and reproduce the nanoparticle dispersion after reconstitution; and remarkably increase the apparent solubility of nifedipine.

1. Introduction

Improvement of the aqueous solubility of poorly water-soluble drugs is one of the important factors for the enhancement of absorption and obtaining adequate oral bioavailability. Recently, nanotechnology has been applied to form pharmaceutical dosage forms such as lipid emulsions (Seki et al., 2004) or solid-lipid nanoparticles (Li et al., 2009). A reduction of particle size not only improves the dissolution rate of drug due to an increase in the drug particles surface area (Law et al., 2003), but also enhances the water solubility according to the "Ostwald–Freundlich's Equation" (Jinno et al., 2006). In addition, nano-ordered micronized particle may also be directly delivered through the intestinal Payer's patches (Desai et al., 1996).

The methods for preparation of nanoparticles are roughly categorized into break-down and build-up methods (Henzie et al., 2006; Rothemund, 2006). The former micronizes particles to a nano-order size by a dry (Carrasquillo et al., 2001; Moribe et al., 2005; Young and Li, 2005) or wet (Jacobs et al., 2000; Gao and Yao, 2004) process. Co-grinding methods with polymers, such as polyvinyl pyrrolidone and microcrystalline cellulose, and water-soluble substances, such as sugars, sugar alcohols, and amino acids, have been applied to improve water solubility of drugs (Adesogan et al., 2004; Williams et al., 2005), whereas this process is difficult to prepare nano-ordered micronized particles and, accordingly, has limited capability in water solubilization of drug. On the other hands, the wet process such as high-pressure homogenization is good for preparing the nanoparticles (Pupo et al., 2005; Uner et al., 2005). However, one of the disadvantages of the wet process is that organic solvents are often required in the process (Govender et al., 2000; Sakuma et al., 2002; Santhi et al., 2002; Kamiya et al., 2006). Consequently, the potential toxicity of residual solvents in the body and environmental pollution by liquid wastes are matters of concern. Therefore, the development of efficient and safe methods to prepare nanoparticles is desired for pharmaceutical industries.

Recently, using the combination of co-grinding by a roll milling and subsequent high-pressure homogenization, we succeeded in

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preparing nifedipine (NI), a poorly water-soluble compound, -lipid nanoparticle suspensions without any organic solvent at any stage of the process (Kamiya et al., 2008). The study showed that the mean particle size of the NI-lipid nanoparticle suspensions was about 55 nm, and incorporation of gelatin to the nanoparticle suspensions allowed reproduction of the nano-particle size after reconstitution with water up to 24 h storage at 6 °C. However, it was difficult to maintain the mean particle size of the NI-lipid nanoparticles for a longer time. A crucial challenge in the development of nanoparticles is how to assure their long-term physical stability upon storage. Freeze-drying is a commonly used technique and numerous studies have been done to improve the dispersebility of nanoparticles (Konan et al., 2002; Anhorn et al., 2008). In addition, in order to ensure adequate tonicity and reconstitution, suitable additives are required. Recently, Zhang et al. reported that sugars such as glucose and trehalose proved to be the very effective in preventing particle aggregation and inhibiting leakage of an active ingredient during the solid lipid microparticle freeze-drying process (Zhang et al., 2008).

The present study intended to determine whether preparations of NI-lipid nanoparticles could be stabilized by negatively charged phospholipids and lyophilization with sugar, such as glucose, fructose, maltose and sucrose, for a long time. In addition, in order to confirm whether the solubility of freeze-dried NI-lipid nanoparticles was really improved, dissolution test was performed for this preparation according to the Japanese Pharmacopoeia (Puddle method, JPXV).

2. Materials and methods

2.1. Materials

Hydrogenated soybean phosphatidylcholine (COATSOME® NC-21 (HSPC)) and dipalmitoyl phosphatidylglycerol (COATSOME® MGLS-6060 (DPPG)) were purchased from Nippon Oil and Fats Co., Ltd. (Tokyo, Japan). Nifedipine (JPXV, NI) was provided by Nippon Fine Chemical Co., Ltd. (Osaka, Japan). Ethanol (high-performance chromatography grade) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Glucose, fructose, maltose and sucrose were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The membrane filters (pore size: 0.20 and 0.45 μm) were purchased from Toyo Roshi Kaisha Ltd. (Tokyo, Japan). All reagents were of the highest grade commercially available, and all solutions were prepared using deionized distilled water.

2.2. Preparation of NI-lipid nanoparticle suspensions

NI-lipid nanoparticle suspensions were prepared as described previously (Kamiya et al., 2008). Briefly, 20 or 40 mg of NI and 1000 mg of lipid (HSPC:DPPG = 5:1 molar ratio) were added to a mortar, and physically mixed. The mixture was then co-ground by a roll mill (Model: R3-1R, Make: Kodaira Seisakusho Co., Ltd.). Its grinding part is consisted of three rollers, and the rotating velocity ratios for each roller were fixed as 1:2.5:5.8. Grinding was carried out for 5 min. The sample mostly adhered to the rollers, but partially fell from the rollers. Therefore, the mill was stopped every 30 s to collect the fallen samples. The co-grinding cycle was repeated 10 times.

The resultant roll mixture was dispersed in 200 ml of deionized distilled water, and premixed using a Speed Stabilizer (10,000 rpm, Kinematica Co.) at 9000 rpm for 10 min and subjected to a high-pressure homogenization (max pressure: 200 MPa, Nanomizer, NM2-L200-D10; Yoshida Kikaikogyo, Co.) with the pass cycle of 20, 40, 60, 80 or 100.

2.3. Measurement of mean particle size and zeta potential

The mean particle size and zeta potential of NI-lipid nanoparticle suspensions were measured by using an electrophoretic light scattering (ELS) photometer (Zetasizer, Sysmex Co., Ltd.) at room temperature. The NI-lipid nanoparticle suspensions were diluted 1/20 for the measurements. The particle sizes were analyzed based on weight distribution.

2.4. Stability studies of NI-lipid nanoparticle suspensions

The physical stability of the lipid nanoparticle suspensions containing 40 mg NI was evaluated as previously described (Kamiya et al., 2006). Briefly, the NI-lipid nanoparticle suspensions were kept in a closed clear glass beaker and stored at 6 °C for 150 days. At 10, 20, 30, 60, 120, and 150 days after preparation, aliquots of suspensions were collected from each beaker to measure the mean particle size and zeta potential by ELS as noted above.

2.5. Freeze-drying and reconstitution

Freeze-drying: 2 ml of the lipid nanoparticle suspensions containing 20 mg NI was collected into a vial which already contained 20, 40, 60 and 100 mg of glucose or 100 mg of fructose, maltose and sucrose. Each vial was frozen at −40 °C for 3 h and then the frozen sample was freeze-dried in a glass chamber for 24 h using a vacuum pump accompanied by a vapor condenser (−20 °C, 0.0225 Torr). After that, secondary drying as the final step was done at 20 °C for 3 h.

Reconstitution: 2 ml of deionized distilled water filtered through a membrane filter (0.20 μm) was added to the vial and shaken by vortex agitation to rehydrate the freeze-dried sample immediately after the freeze-drying process was completed. The mean particle size and zeta potential were determined by ELS mentioned above.

2.6. Dissolution test

The dissolution behavior of the freeze-dried lipid nanoparticles containing 20 mg NI was examined in accordance with the paddle method listed in the Japanese Pharmacopoeia (15th edition). The test solution was 900 ml water at 37.0 ± 0.5 °C and paddle rotation speed was 50 rpm. At 1, 3, 5, 10, 15, and 20 min, the samples (3 ml) were withdrawn with replacement of equal volume of dissolution media. The solution was then filtered through a membrane filter (0.45 μm). The amount of NI dissolved was monitored by high-performance liquid chromatography (HPLC). HPLC analysis was performed using Prominence UFLC (Shimadzu, Japan). The HPLC conditions were as follows: mobile phase, water/methanol 1:5 (v/v) mixture; flow rate, 1 ml/min; reverse-phase column (Cadenza 5CD-C18, 4 mm I.D. × 150 mm; Imtakt) at 40 °C; detection wavelength, λ = 230 nm. In this study, "dissolved" means that the sample solution filtered by 0.45 nm filter is clear and no precipitant can be visually observed.

3. Results and discussion

3.1. Determination of pass numbers of high-pressure homogenization in preparing NI-lipid nanoparticle suspensions

Generally, high-pressure homogenization in a wet process immediately generates high energy. As a result, temperature of the sample is gradually raised to about 40–50 °C which influences the stability of thermolabile drugs. In order to apply for various thermolabile drugs and proteins, the authors used a novel machine (Nanomizer, NM2-L200-D10; Yoshida Kikaikogyo, Co.) which was equipped with cooling system during homogenization. First of all,

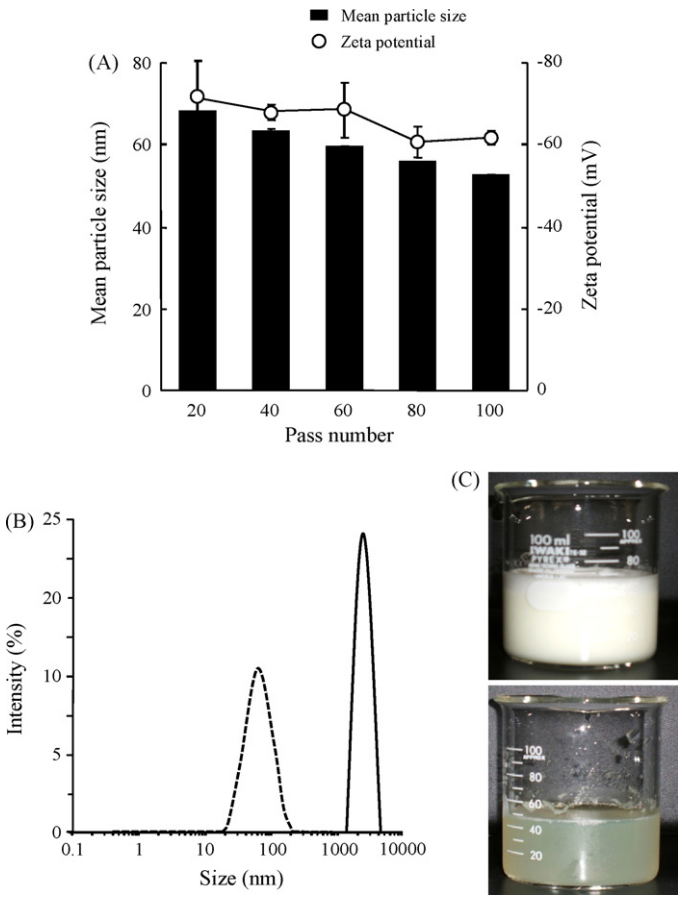

Fig. 1. Physical and visual features of the NI-lipid nanoparticle suspensions. (A) Pass number of high-pressure homogenization and the zeta potential versus the mean particle sizes of the NI-lipid nanoparticle suspensions. Each column and point represent the mean particle size and zeta potential, respectively. These data are the average values obtained from three determinations (±S.D.). (B) Particle size distribution of NI-lipid nanoparticle suspensions with high-pressure homogenization. The dotted and full curves represent the particle size distributions at 100 and 0 times of high-pressure homogenization, respectively. The curves are averages of three determinations. (C) Representative photographs of NI-lipid nanoparticle suspensions with high-pressure homogenization. Upper and lower photograph represent 0 and 100 times of high-pressure homogenization, respectively.

the authors examined the optimal pass numbers of high-pressure homogenization by measuring the mean particle size and zeta potential of the lipid nanoparticle suspensions containing 20 mg NI (Fig. 1A). The average mean particle size and zeta potential of NI-lipid nanoparticle suspensions at 20 times of pass number are 68.1 nm and −71.6 mV ($n = 3$), respectively. The average mean particle sizes decreased in a pass number-dependent manner and similar tendencies were observed up to 80 passes with a level off zeta potential of NI-lipid nanoparticle suspensions. In particular, the preparations treated with 100 times of homogenization provided a narrow particle size distribution (Fig. 1B) and less turbid solution (Fig. 1C, bottom) than the untreated samples (Fig. 1C, top). According to these results, the optimal pass numbers of homogenization was determined as 100 times in the present study.

3.2. Nano-dispersion stability of NI-lipid nanoparticle suspensions

In order to examine nano-dispersion stability of NI-lipid nanoparticle suspensions in cool and dark conditions, the authors measured the mean particle size and zeta potential of the suspensions containing 40 mg NI preserved at 6 °C in a dark place for 150 days (Fig. 2). The average mean particle size and the zeta potential

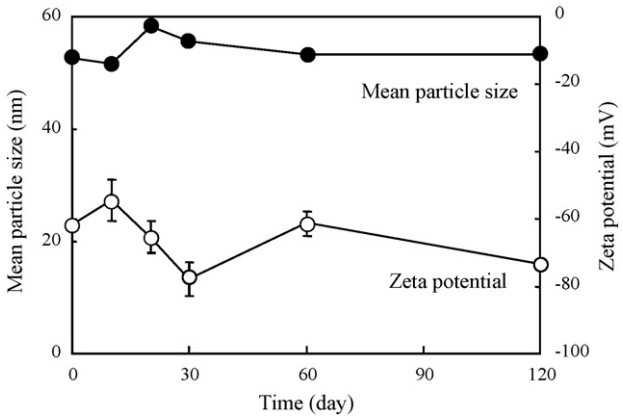

Fig. 2. The mean particle size and the zeta potential of NI-lipid nanoparticle suspensions as a function of time stored at 6 °C in dark conditions. Each point represents an average value obtained from three experiments (±S.D.).

remained extremely constant throughout the storage period of 120 days. The reasons why the nanoparticle suspensions could be stable for 120 days can partly be explained by the ionic repulsion force at the surfaces of nanoparticles by negatively charged phospholipid such as DPPG. Generally, fine particles in an aqueous solution easily aggregate together and form large particles or precipitation to become thermodynamically stable. Nanoparticles prepared by high-pressure homogenization had the high intrinsic surface energy which potentially caused them to aggregate. However, in the present study, the zeta potential decreased with a decrease of the particle size, i.e. an increase of surface area of the nanoparticles, as shown in Fig. 1A, which supports negatively charged DPPG could increase the electrostatic repulsion among nanoparticles. In fact, phospholipid such as DPPG is widely used for liposomes to help to stabilize or maintain the mean particle size (Huang and MacDonald, 2004; Sadzuka et al., 2008). In addition, although the authors also examined the long-term nano-dispersion stability of the nanoparticle suspensions containing 20 mg NI preserved at 6 °C in a dark place, the average mean particle size remained extremely constant for 120 days (data not shown), indicating that the differences of drug content in nanoparticle suspensions did not affect the long-term nano-dispersion stability.

At 150 days, white- and aggregated-lipids were observed in the suspensions and the authors could not measure each parameter. In addition, although the authors also checked nano-dispersion stability of the NI-lipid nanoparticle suspensions at room temperature (25 °C), the suspensions formed aggregations in 90 days. These data suggested that the nano-dispersion stability of the nanoparticle suspensions is temperature dependent and maintained about 4 months in cool and dark conditions.

3.3. Nano-dispersion stability of freeze-dried NI-lipid nanoparticles

Although the nano-dispersion stability of the NI-lipid nanoparticle suspensions was maintained 4 months, further long-term storage stability and practical convenience is required. Therefore, the NI-lipid nanoparticle suspensions were freeze-dried with a sugar such as glucose, fructose, maltose or sucrose, and the mean particles size and zeta potential were measured after reconstitution (Figs. 3 and 4). After reconstitution of the freeze-dried NI-lipid nanoparticles without the sugar, the average mean particle size was significantly higher than that before freeze-drying (control), suggesting that freeze-drying process caused the aggregation of the nanoparticles (Fig. 3A). However, interestingly, when various amounts of glucose were added to the nanoparticle sus-

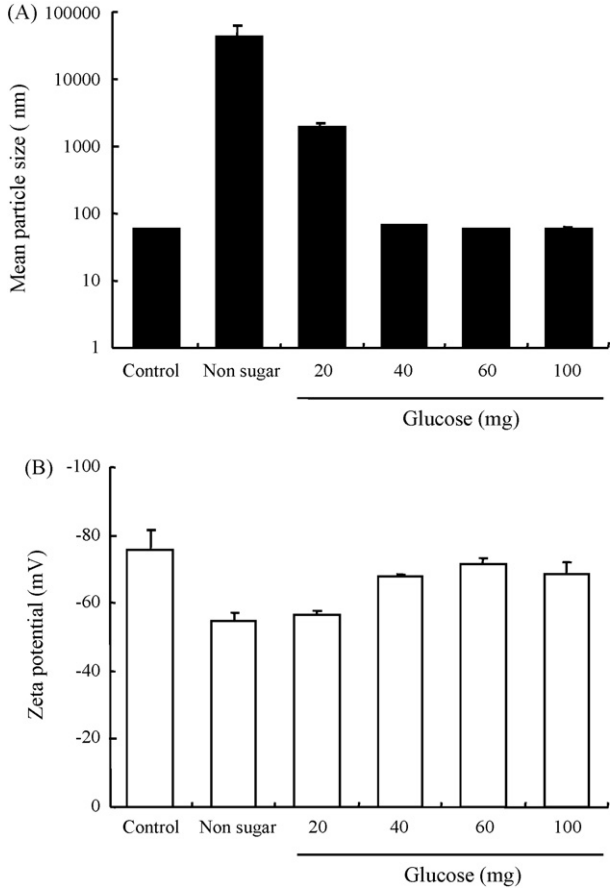

Fig. 3. Effect of glucose on the mean particle size and zeta potential of freeze-dried NI-lipid nanoparticles after reconstitution. Closed and open columns represent the mean particle size (A) and zeta potential (B), respectively. Each column represents the average of three determinations (±S.D.). Control: NI-lipid nanoparticle suspensions. Non-sugar: freeze-dried NI-lipid nanoparticles without sugar.

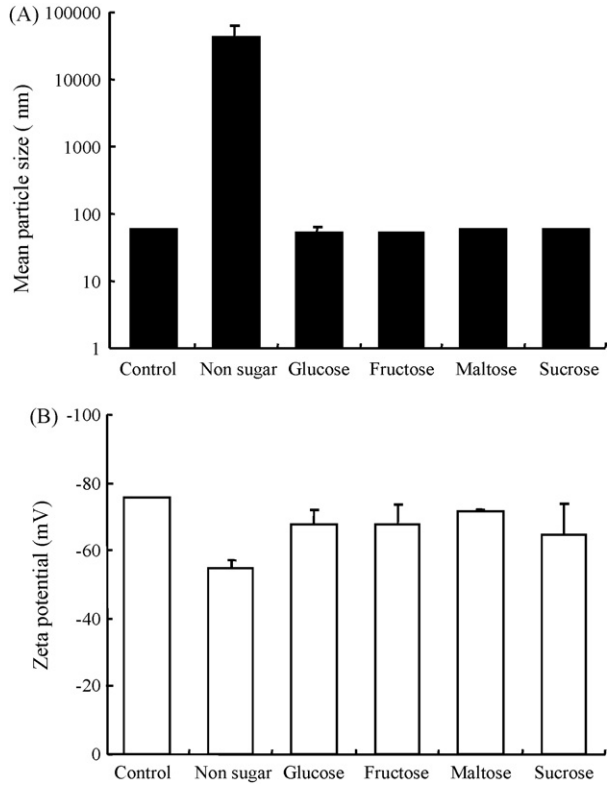

Fig. 4. Effect of various sugars on the mean particle size and zeta potential of freeze-dried NI-lipid nanoparticles after reconstitution. Closed and open columns represent the mean particle size (A) and zeta potential (B), respectively. Each column represents the average value obtained from three experiments (±S.D.). Control: NI-lipid nanoparticle suspensions. Non-sugar: freeze-dried NI-lipid nanoparticles without sugar. Glucose: freeze-dried NI-lipid nanoparticles with 100 mg glucose. Fructose: freeze-dried NI-lipid nanoparticles with 100 mg fructose. Maltose: freeze-dried NI-lipid nanoparticles with 100 mg maltose. Sucrose: freeze-dried NI-lipid nanoparticles with 100 mg sucrose.

pensions before freeze-drying, it was found that incorporation of more than 40 mg glucose reproduced the nearly same mean particle sizes of freeze-dried NI-lipid nanoparticles after reconstitution (Fig. 3A). The zeta potential was not significantly changed before/after freeze-drying (Fig. 3B). As preceding studies have shown the lyoprotective effect of sugars to prevent particle aggregation during the freeze-drying process (Konan et al., 2002; Zhang et al., 2008), the authors speculated that the addition of sugar may therefore facilitate the reconstitution of nanoparticle suspensions from freeze-dried product. In addition, the effects of other sugars (fructose, maltose and sucrose) were also examined during this process. As expected, all three sugars (100 mg) also suppressed the increases of the mean particle size of these freeze-dried nanoparticles (Fig. 4A). These results suggest that the freeze-dried NI-lipid nanoparticles with the sugars could overcome problems associated with long-term stability storage.

3.4. Solubility characteristics of freeze-dried NI-lipid nanoparticles with the sugars

Finally, to examine whether the freeze-dried NI-lipid nanoparticles with sugars would exhibit good solubility, the authors performed a dissolution test according to the Japanese pharmacopoeia (Puddle method, JPXV). As seen in Fig. 5, all four freeze-dried nanoparticles with 100 mg sugar (glucose, fructose, maltose or sucrose) showed excellent solubility (>80%), whereas without sugar, as a control, showed low solubility (<20%). Previously, Hecq et al. reported that commercial NI shows a water

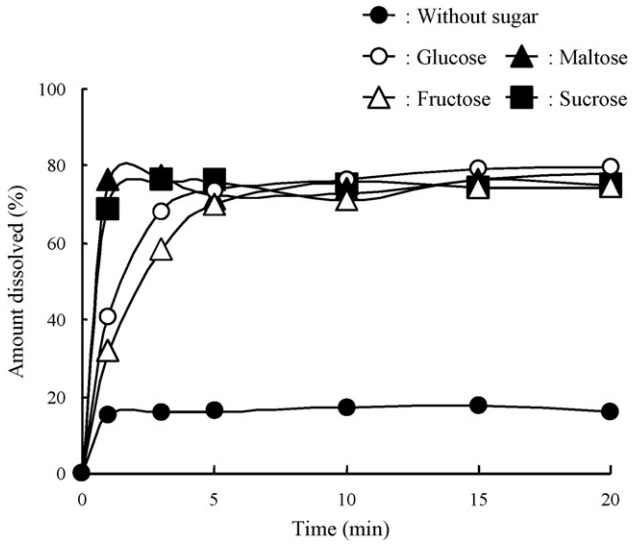

Fig. 5. Dissolution profiles of freeze-dried NI-lipid nanoparticles. Without sugar: nifedipine alone as a control. Glucose: freeze-dried NI-lipid nanoparticles with 100 mg glucose. Fructose: freeze-dried NI-lipid nanoparticles with 100 mg fructose. Maltose: freeze-dried NI-lipid nanoparticles with 100 mg maltose. Sucrose: freeze-dried NI-lipid nanoparticles with 100 mg sucrose.

solubility of 19.5 μg/ml (Hecq et al., 2005). In this study, the amount of NI dissolved (%) from freeze-dried nanoparticles with sugar is 4 times higher than that of control, we speculated the apparent solubility of NI in the lipid nanosuspension form to be about 78 μg/ml. For two formulations with disaccharides, such as maltose or sucrose, more rapid solubility was shown in the initial phase than that of other formulations with monosaccharides, such as glucose and fructose. The slightly higher solubility found for disaccharides and the slightly lower solubility for monosaccharides are probably due to the differences of the polymorphs. Previous reports demonstrated that the freeze-drying technique often changed the crystal form of disaccharides from a crystalloid state to an amorphous state, resulting in porous powdery freeze-dried preparations (Chang et al., 2005). Since a solvent like water could penetrate into the porous powdery preparations, the two formulations with disaccharide showed the rapid solubility in this experiment. In contrast, it was assumed that monosaccharides were maintained in the crystalloid state even after freeze-drying, and they dissolved to form paste like matrix inhibiting water penetration into them.

4. Conclusions

Nifedipine, one of the poorly water-soluble drugs, nanoparticle suspensions were formulated with negatively charged phospholipid, dipalmitoyl phosphatidylglycerol. They were prepared by a combination of dry roll milling followed by high-pressure homogenization in aqueous medium. The mean particle size of the nanoparticle suspensions was 52.6 nm and stayed with it for 4 months at 6 °C. It was suggested that the negatively charged phospholipid effectively inhibited aggregation of the nanoparticles by virtue of the electric repulsion. It was found that addition of 100 mg glucose, fructose, maltose, or sucrose before lyophilization of the nanoparticle suspensions inhibited the aggregation of the nanoparticles after reconstitution and increased apparent solubility of nifedipine more than 4 times.

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