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Formation, Physical Stability and In Vitro Antimalarial Activity of Dihydroartemisinin Nanosuspensions Obtained by Co-grinding Method

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The purpose of this study was to investigate the formation of drug nanoparticles from binary and ternary mixtures, consisting of dihydroartemisinin (DHA), a poorly water-soluble antimalarial drug, with water-soluble polymer and/or surfactant. Binary mixtures of drug/polyvinyl pyrrolidone K30 (PVP K30), binary mixtures of drug/sodium deoxycholate (NaDC), and ternary mixtures of drug/PVP K30/NaDC were prepared at different weight ratios and then ground by vibrating rod mill to obtain ground mixtures. Nanosuspension was successfully formed after dispersing ternary ground mixtures or DHA/NaDC ground mixtures in water. The ternary ground mixtures did not give superior nanosuspension in terms of particle size reduction and recovery of drug nanoparticles, but they provided more physically stable nanosuspensions than DHA/NaDC ground mixtures. The size of drug nanoparticles was decreased with increasing grinding time and lowering amount of PVP K30 and NaDC. About 95% of drug nanoparticles were found in the nanosuspension from ternary ground mixtures. Zeta potential measurement suggested that stable nanosuspension was attributable to adsorption of NaDC and PVP K30 onto surface of drug particles. Atomic force microscopy and transmission electron microscopy with selected area diffraction indicated that DHA in nanosuspension was existed as nanocrystals. The obtained nanosuspensions had higher in vitro antimalarial acitivity against *Plasmodium falciparum* than microsuspensions. The results suggest that co-grinding of DHA with PVP K30 and NaDC seems to be a promising method to prepare DHA nanosuspension.

Keywords nanosuspension; nanoparticles; dihydroartemisinin; co-grinding

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INTRODUCTION

Human malaria caused by protozoa parasite *Plasmodium falciparum* is the most deadly type of malaria infection and is one of the major health problems in developing countries (World Health Organization, 2002). Artemisinin is an active antimalarial drug which is isolated from the Chinese medicinal herb *Artemisia annua* L (Qinghao). To date, artemisinin and its derivatives—including arteether, artemether, artelinic acid, dihydroartemisinin (DHA) and sodium artesunate—have received much attention as potent antimalarial drugs. Excellent clinical results have been reported in the treatment of *P. vivax* and *P. falciparum* infections, including the cerebral form, with both cloroquine-sensitive and chloroquine-resistant strains (Luo & Shen, 1987; Myint & Shwe, 1987; Myint et al., 1989; Titulaer, Zuidema, & Lugt, 1991). Artemisinin and derivatives are transformed in vivo to an active metabolite DHA. DHA itself can be used effectively in the treatment of malaria infection, especially against the tolerant species of *P. falciparum* (Wilairatana et al., 1998). It is an endoperoxide containing

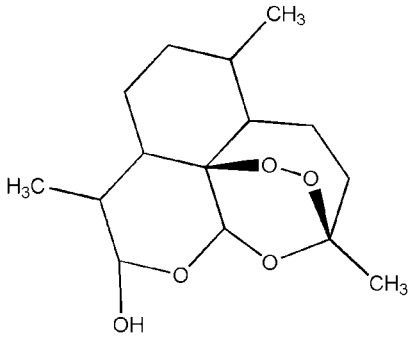

FIGURE 1. Chemical structure of dihydroartemisinin.

sesquiterpene lactone structure (Figure 1), which can be synthesized from artemisinin in fewer steps and lower cost than other artemisinin derivatives. Thus, it is of interest to develop DHA into a good quality drug product for malaria treatment. However, DHA has poor water solubility (0.168 $mg.mL^{-1}$ at 30ºC) (Sethabouppha, 1999), leading to a problem in formulation.

Particle size reduction is a widely employed process in the pharmaceutical industry to improve bioavailability of drugs with poor water solubility. Reduction of particle size into nanometer range can enhance bioavailability of drugs with poor water solubility due to increased solution velocity and saturated solubility because of the vapor pressure effect (Müller, Jacobs, & Kayer, 2000). Recently, nanosuspension has gained much interest as a promising dosage form for efficient delivery of hydrophobic drugs (Müller et al., 2000; Patravale, Date, & Kulkarni, 2004; Rabinow, 2004). In nanosuspension, the drug is maintained in required crystalline state with reduced particle size, leading to an increased dissolution rate and, therefore, improved bioavailability. Nanosuspension engineering processes currently used are precipitation (Kipp, Wong, Doty, & Rebbeck, 2003; Trotta, Gallarete, Pattarino, & Morel, 2001; Zili, Sfar, & Fessi, 2005), high pressure homogenization (Hecq et al., 2005; Jacobs, Kayser & Müller, 2001; Kocbek, Baumgartner, & Kristl, 2006; Möschwitzer et al., 2004), lipid emulsion (Patravale et al., 2004; Trotta, Gallarete, Carlotti, & Morel et al., 2005), and media milling (Liversidge & Conzentino, 1995; Liversidge, Cundy, Bishop, & Czekai, 1992). Nanosuspensions prepared by high pressure homogenization and media milling using pearl-ball mill are wet-grinding processes. Dry grinding of a drug with additives (i.e., co-grinding) has some advantages for size reduction of solid active pharmaceutical ingredients due to the simple, organic, solvent free preparation process. For effective size reduction of drug particles, water-soluble polymers and surfactants have been used as additives to inhibit particle agglomeration and improve dissolution of drugs (Kondo et al., 1993; Sugimoto et al., 1998; Suzuki et al., 2001). Formation of drug nanoparticles has taken place when the ground mixtures of drug with polymer and/or surfactant are dispersed in water. Such phenomena have been demonstrated in the ground mixtures systems consisting of drug and β-cyclodextrin (Tozuka et al., 2004; Wongmekiat, Tozuka, Oguchi, & Yamamoto, 2002; Wongmekiat, Tozuka, Oguchi, & Yamamoto, 2003); drug, polyvinylpyrrolidone (PVP); and sodium dodecyl sulfate (SDS) (Itoh et al., 2003; Pongpeerapat et al., 2004); and drug, hydroxypropyl methyl cellulose, and SDS (Moribe et al., 2006).

In this study, vibrating rod mill (Figure 2), which is operated based on the principle of attrition induced by an aluminum oxide ball in the mill body, was used. The advantage of this mill is that fine particles could be obtained within a short period due to high frequency and strong force of attrition. Co-grinding of DHA, poorly water-soluble drug, with PVP and sodium deoxycholate (NaDC) has been attempted as a method for preparing nanosuspension. The ternary system of drug with poor water solubility with PVP and NaDC has not been investigated previously. This work has been presented in the international conferences by Puttipipatkhachorn and co-workers since 2004 (Chingunpitak et al., 2004; Chingunpitak et al., 2005; Puttipipatkhachorn et al., 2006). In 2006, nanoparticulate formulations of some drugs with PVP K12 and NaDC using a wet-grinding NanoMill system were patented (Liversidge, Jenkins, & Liversidge, 2006a; Liversidge & Jenkins, 2006b) but the detail investigation was not performed. PVP, a water-soluble polymer, is a commonly used excipient in various pharmaceutical dosage forms due to its low toxicity and chemical stability. NaDC, a naturally occurring anionic bile salt, was used instead of synthetic surfactants like SDS in previous study because of its lower toxicity. It has been used as solubilizer and absorption enhancer of drugs. The ternary system of DHA with PVP K30 and NaDC was investigated in comparison with the binary systems of DHA with PVP K30 and DHA with NaDC. Parameters affecting nanoparticle formation from these ground mixtures, including additive ratio and grinding time were investigated. Drug nanoparticles obtained were characterized by atomic force microscopy (AFM) and transmission electron microscopy (TEM) with selected area diffraction method. Moreover, physical stability and in vitro antimalarial activity against *P. falciparum* of the obtained DHA nanosuspension were evaluated.

A **B**

FIGURE 2. Photographs of the apparatus (A) and grinding cell (B) of vibrating rod mill (TI-200, Heiko Seisakusho, Tokyo, Japan).

MATERIALS AND METHODS

Materials

DHA (Knoll AG, Liestal, Switzerland) was ground by jet pulverizer (Micron-Master® Model 08–506, The Jet Pulverizer Co., Ltd., Palmyra, NJ) prior to use. PVP K30 (Plasdone K29/32, ISP Technologies, Japan) and NaDC (Wako Pure Chemical Industries, Osaka, Japan) were used as received. All other chemicals were of analytical grade.

Preparation of DHA Nanosuspensions

In binary systems DHA was mixed with PVP K30 or NaDC; in ternary systems DHA was mixed with PVP K30 and NaDC at different weight ratios. The physical mixtures were prepared by mixing the mixtures in glass vials using a vortex mixer for 3 min. The ground mixtures were prepared by grinding the physical mixtures by a vibrating rod mill (TI-200, Heiko Seisakusho, Tokyo, Japan) at room temperature for 3–45 min. Then, the suspensions were prepared by dispersing the ground mixtures in distilled water and gently sonicating for 3 min. Finally, the nanosuspensions were obtained by filtering the suspensions through a 0.8 μm membrane filter (Millipore, Bedford, MA).

Particle Size Analysis

Particle size of the suspensions obtained by dispersion of the physical and ground mixtures was determined by the light-scattering method using Microtrac FRA® (Nikkiso, Tokyo, Japan; measurement range, 0.1–700 μm) or dynamic light scattering method using a Microtrac UPA® 150 (Nikkiso, Tokyo, Japan; measurement range, 0.003–6 μm). The Microtrac UPA® 150 was used to determine particle size of the nanosuspension, which was prepared by cutting off particle size larger than 0.8 μm. Mean of three determinations was reported. Particle size distribution was calculated from an equation; (d84% – d16%)/2, where d84% and d16% represent diameters at 84 and 16 cumulative percent frequency undersize, respectively.

Recovery of DHA Nanoparticles

The obtained nanosuspensions were diluted with a mixture of acetronitrile and water (1:1 v/v). Content of DHA in the nanosuspensions was determined by HPLC analytical system (Shimadzu, Kyoto, Japan) consisting of a pump, a UV detector at wavelength of 216 nm, and Intersil-ODS column. The mobile phase of acetronitrile and water (50:50 v/v), was delivered at a flow rate of 1 $mL.min^{-1}$ and injection volume of 20 μL. The amount of DHA was reported as percentage of recovery, which was determined from the total amount of analyzed DHA in the obtained nanosuspension relative to the added amount of DHA in the related ground mixtures.

Zeta Potential Measurement

Zeta potential of the nanosuspension was measured by a zeta meter (Zetasizer, Nanoseries, Malvern Instruments, Worcestershire, UK). The measurement was performed after dispersion of the samples in water. Mean and standard deviation of six determinations were reported.

Atomic Force Microscopy (AFM)

The samples were prepared by dropping colloidal suspension on a clean glass slide that was glued to the sample holder. The samples were air-dried at room temperature for a few minutes. DHA particles were observed using AFM unit (SPA 400-SPI 3800N, Seiko Instruments, Chiba, Japan). Imaging was operated in air at room temperature in the tapping mode with an intermittent contact technique using silicon probe. Both height and phase images were collected and analyzed with software.

Transmission Electron Microscopy (TEM)

The nanosuspension was dropped on a coated carbon grid, stained with 2% uranyl acetate solution for 10–15 min and then washed with distilled water several times. The test samples were dried in a vacuum desiccator. DHA particles were observed by TEM (H-7000, Hitachi, Ibaraki, Japan). The crystalline characteristics of the DHA particles observed by TEM were determined using selected area diffraction (SAD) technique. The measurement conditions were $\lambda = 0.0251$ Aº radiation generated at 200 kV as X-ray source with camera length of 100 cm. Two dimensions of X-ray patterns were photographed.

Physical Stability Study

Physical stability of the obtained DHA nanosuspension was investigated at 25ºC. The changes in appearance, particle size and DHA nanoparticles recovery after storage for two weeks were evaluated at predetermined time interval of 0 h (after filtration), 4 h, 12 h, 1 week, and 2 weeks.

In Vitro Antimalarial Activity Against *P. falciparum* K1 Strain

According to the method described by Target and Jensen (Trager & Jensen, 1976), *P. falciparum* K1 strain was cultivated in RPMI 1640 medium containing 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 32 mM NaHCO3, and 10% heat activated human serum with 3% erythrocytes, and was subsequently incubated at 37ºC in an incubator with 3% CO_2. Quantitative assessment of antimalarial activity in vitro was determined using microculture radioisotope techniques by monitoring [^{3}H] hypoxanthine uptake (Desjarardins, Canfield, Haynes, & Chulay, 1979). The level of incorporated radioactive, labeled hypoxanthine, indicates parasite growth. The drug concentration that inhibited 50% parasite growth (IC_{50}) was determined and reported as mean of duplicate tests.

RESULTS AND DISCUSSION

Preliminary Study on Nanopartcle Formation

In a preliminary study, the dispersions of different systems (e.g., drug in water, drug in aqueous PVP K30/NaDC solution, drug/PVP K30 mixtures in water, drug/PVP K30 mixtures in aqueous NaDC solution, drug/NaDC mixtures in water, drug/NaDC mixtures in aqueous PVP K30 solution, and drug/PVP K30/NaDC mixtures in water) were investigated. All systems were studied at 1:1:1 weight ratio of DHA/PVP K30/NaDC. DHA powder itself could not be dispersed in water whereas colloidal dispersions were obtained after dispersing the ternary ground mixtures of DHA/PVP K30/NaDC in water (Figure 3). Particle size of the suspensions obtained from these systems is shown in Table 1 and Figure 4. Both intact and ground DHA gave suspensions with micrometer-sized particles (microsuspension) after dispersing in water or PVP K30/NaDC solution. All physical mixtures, both binary and ternary systems, also formed microsuspensions. The microsuspensions also were obtained from ground mixtures of DHA/PVP K30 in water or NaDC solution. Furthermore, suspensions with nanometer-sized particles (nanosuspension) were obtained from dispersing ground mixture of DHA/NaDC in water or PVP K30 solution and from DHA/PVP K30/NaDC ground mixtures in water. The results revealed that nanosuspension could not be obtained from physical mixtures. Co-grinding of drug with PVP K30 was not able to form nanosuspension whereas nanosuspension with particle size of 170 nm was obtained from drug/NaDC ground mixtures. For the ternary drug/PVP K30/NaDC ground mixtures, the suspension obtained consisted of drug particles with mean diameter of 90 nm. The results indicated that effective particle size reduction of drug was achieved when both PVP K30 and NaDC were present. These preliminary results indicated that the binary drug/NaDC ground mixtures and the ternary drug/PVP K30/NaDC ground mixtures could be used as a platform for preparing nanosuspension and more investigation was further performed.

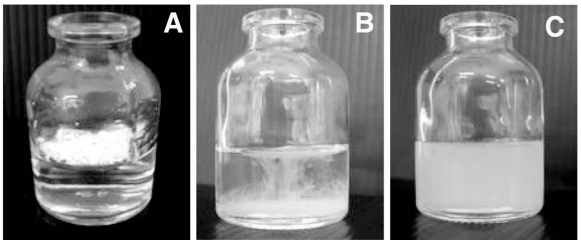

FIGURE 3. Appearance of suspensions obtained by aqueous dispersion of DHA (A), and ternary ground mixture of DHA/PVP K30/NaDC (weight ratio, 1:1:1) before sonication (B) and after sonication (C).

Effect of Additive Ratio on Size and Recovery of Drug Nanoparticles

Mean particle size and recovery of DHA nanoparticles of the suspensions obtained from dispersing ground mixtures in water are demonstrated in Figures 5 and 6, respectively. The results indicated that nanoparticle formation was dependent on

TABLE 1
Particle Size of the Suspensions Obtained from Different DHA Mixtures

		Particle Size (nm)			
		Physical Mixtures		15-Min Ground Mixtures	
DHA Mixtures	Aqueous Dispersion Media	*M*	Size Distribution	*M*	Size Distribution
DHA	Water	7350	3700	7200	4540
DHA	PVP/NaDC	8370	5000	18950	14800
DHA/PVP K30; weight ratio, 1:1	Water	5980	3780	10610	5510
DHA/PVP K30; weight ratio, 1:1	NaDC	6460	3850	14380	7390
DHA/NaDC; weight ratio, 1:1	Water	3280	2180	170	50
DHA/NaDC; weight ratio, 1:1	PVP	7410	4550	260	100
DHA/PVP K30/NaDC; weight ratio, 1:1:1	Water	9790	6730	90	30

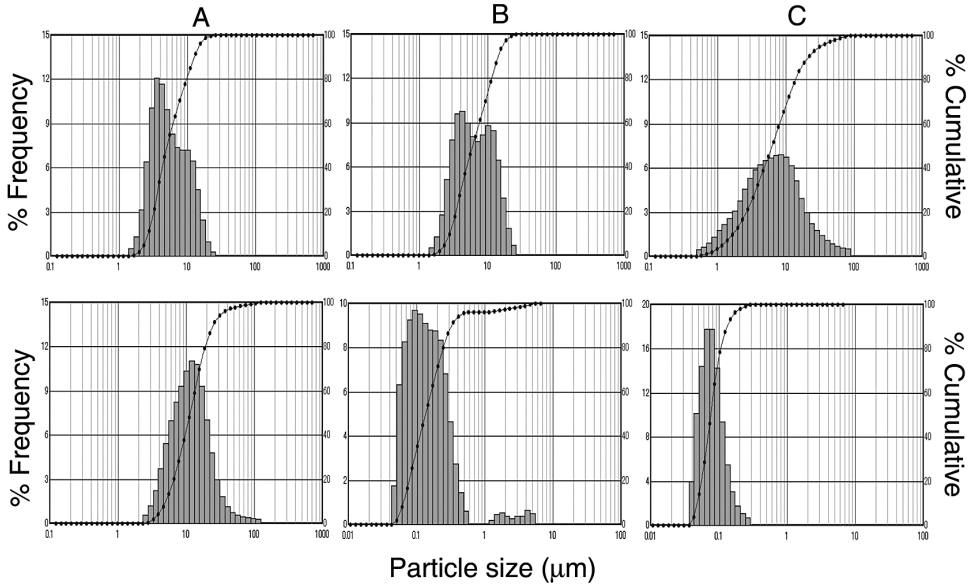

FIGURE 4. Particle size distribution of the suspension obtained by dispersion of DHA mixtures in different aqueous solutions. Row: upper, physical mixtures; lower, 15-min ground mixtures. Column: (A) DHA/PVP K30 in NaDC solution; (B) DHA/NaDC in PVP K30 solution; (C) DHA/PVP K30/NaDC in water.

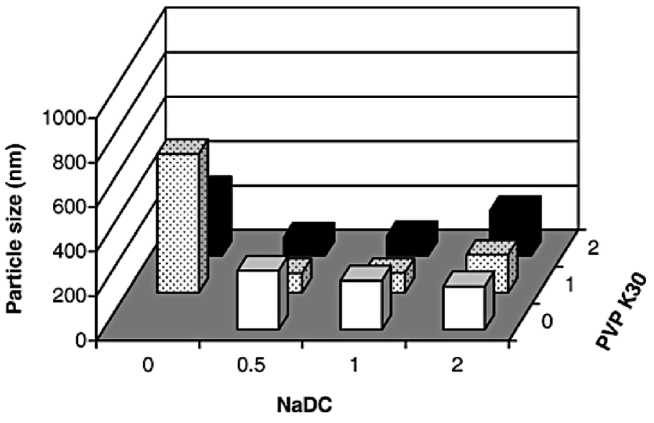

FIGURE 5. Mean particle size of the suspensions obtained by aqueous dispersion of 15-min ground mixtures of DHA with PVP K30 and NaDC at different weight ratios (n = 3).

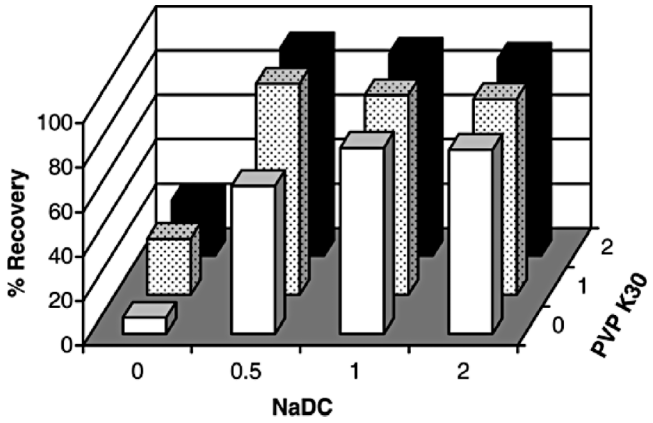

FIGURE 6. Percent recovery of DHA (< 0.8 μm) in the suspensions obtained by aqueous dispersion of 15-min ground mixtures of DHA with PVP K30 and NaDC at different weight ratios (n = 3).

addition of PVP K30 and NaDC in the mixtures. Increasing weight ratio of PVP K30 in binary ground mixture of DHA/PVP K30 gradually reduced particle size of the suspensions. Similarly, an increase in weight ratio of NaDC tended to reduce particle size of the suspensions obtained from binary ground mixtures of DHA/NaDC. The presence of NaDC in binary ground mixtures seemed to have greater effect on particle size reduction than PVP K30. In ternary ground mixtures of DHA/PVP K30/NaDC, lowering amounts of PVP K30 and NaDC led to formation of nanosuspension with smaller particle size. The nanosuspension obtained from binary ground mixtures of DHA/NaDC and ternary ground mixtures of DHA/PVP K30/NaDC had the mean particle size in the range of 190–260 nm and 80–200 nm, respectively. Co-grinding of DHA with PVP K30 or NaDC increased recovery of DHA nanoparticles in the suspensions obtained from binary ground mixtures (Figure 6). The results revealed that the presence of NaDC in binary ground mixtures gave remarkably higher recovery of nanoparticles than PVP K30 did. A higher amount of nanoparticles was obtained from ternary ground mixture of DHA/PVP K30/NaDC when compared to binary ground mixtures. Amount of PVP K30 and NaDC in ternary ground mixtures did not seem to have significant effect on percent recovery of nanoparticles. About 95% of nanoparticles were

formed from ternary ground mixture of DHA/PVP K 30/NaDC at weight ratio of 1:1:0.5. These results indicated that highest recovery of nanoparticles could be obtained from the ternary ground mixtures of DHA/PVP K30/NaDC.

Effect of Grinding Time on Size and Recovery of Drug Nanoparticles

Effect of grinding time in the range of 3–45 min on mean particle size and recovery of DHA nanoparticles of the suspensions obtained from ground mixtures is shown in Figure 7. Increasing grinding time tended to reduce size of DHA particles in the suspensions. It was indicated that suspensions obtained from ternary ground mixtures had smaller particle size and higher recovery of nanoparticles than those obtained from binary ground mixtures. Grinding for 3 min could provide nanosuspensions from binary mixtures of DHA/NaDC and ternary mixture of DHA/PVP K30/NaDC, but not from binary mixture of DHA/PVP K30. In binary mixtures of DHA/PVP K30, nanoparticle formation was not observed when ground for less than 30 min while nanosuspensions containing drug particles of 300–340 nm in size were obtained when ground for 30–45 min. Colloidal suspensions with drug particles of 150–380 nm in size were obtained from binary mixtures of DHA/NaDC which were ground for 3–45 min. Nanosuspensions obtained from the ternary ground mixtures had the smallest particle size when compared to those obtained from the binary ground mixtures. Particle size of DHA was reduced to 80–90 nm as grinding of the ternary mixtures took place for 15–45 min. It was observed that increasing grinding time from 15 to 45 min did not significantly reduce the size of drug particles in the nanosuspensions. Recovery of DHA nanoparticles formed from ground mixtures tended to increase with increasing grinding time. The ternary ground mixtures gave higher recovery of nanoparticles than the binary ground mixtures. The maximum percent recovery of drug nanoparticles obtained from binary ground mixtures of DHA/PVP K30 (grinding time, 45 min) and DHA/NaDC (grinding time, 30 min) was 34% and 82%, respectively. Moreover, the maximum percent recovery of nanoparticles obtained from ternary ground mixtures (grinding time, 10 min) was 97%. Amount of nanoparticles obtained from ternary ground mixtures increased with increasing grinding time up to 10 min and then slightly decreased with extended grinding time to 45 min. The results revealed that appropriate grinding time was required to obtain ground mixtures that were able to form nanosuspension with smaller particle size and higher recovery of nanoparticles.

Characterization of Drug Nanoparticles

Microstructure of the drug particles isolated from the suspensions was imaged using AFM technique. The result demonstrated that DHA particles were present in the form of rod-shaped particles in nanometer size range (Figure 8). Further investigation using TEM with selected area diffraction mode indicated that the particles isolated from nanosuspension obtained from ground mixtures of DHA/NaDC (Figure 9C) or DHA/PVP K30/NaDC (Figure 9B) had similar characteristic diffraction patterns to DHA (Figure 9A). The results from microscopic studies revealed the presence of nanocrystalline drug particles in the suspensions obtained from these coground mixtures.

Mechanism of Nanoparticle Formation

Mechanisms of dispersion of solid particles were determined by investigating the effects of PVP K30 and NaDC on surface charge of suspended particles using zeta potential measurement. The results of zeta potential measurement are shown in Table 2. With no additives, DHA exhibited its own negative charge with the magnitude of zeta potential between -7 to -14 mV. When DHA was ground with PVP K30, the magnitude of negative charge slightly increased. A dramatic increase in magnitude

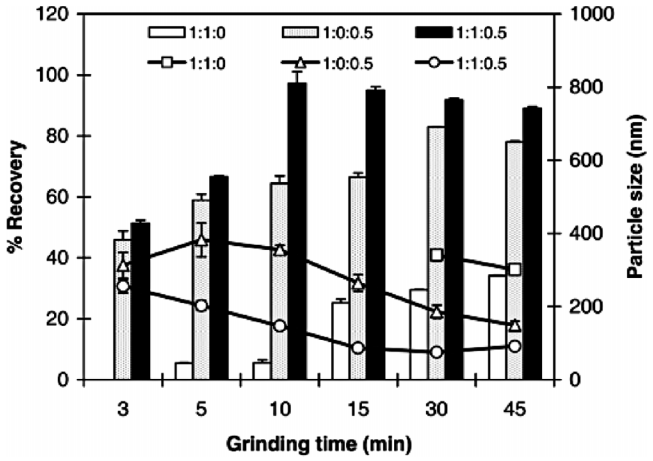

FIGURE 7. Effect of grinding time on mean particle size (solid line) and percent DHA recovery (bar chart) of the suspensions obtained by aqueous dispersion of the ternary ground mixtures of DHA/PVP K30/NaDC at different weight ratios ($M \pm SD$, $n = 3$).

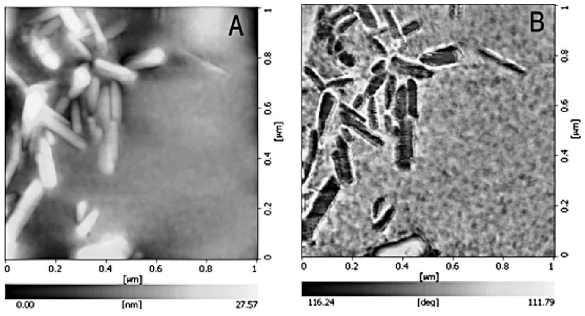

FIGURE 8. Topographical AFM amplitude image (A) and corresponding phase response on surface (B) of DHA nanoparticles isolated from nanosuspension obtained by aqueous dispersion of DHA/PVP K30/NaDC ternary ground mixtures (weight ratio, 1:1:1).

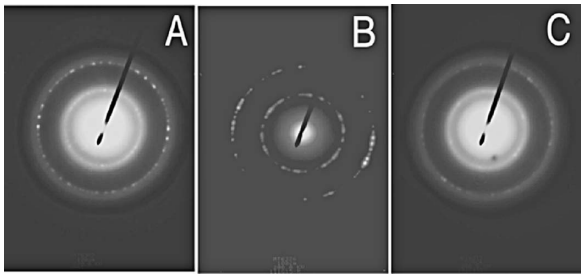

FIGURE 9. 2-D X-ray patterns of DHA nanoparticles isolated from nanosuspension obtained by aqueous dispersion of ground mixtures. (A) DHA; (B) 15-min ground mixture of DHA/PVP K30/NaDC (weight ratio,1:1:1); (C) 15-min ground mixture of DHA/NaDC (weight ratio,1:1).

TABLE 2
Zeta Potential of the Suspensions Obtained by Aqueous Dispersion of 15-Min Ground Mixtures of DHA ($M \pm SD$, $n = 6$)

Ground Mixtures	Zeta Potential (mV)
DHA	−10.5 ± 3.5
DHA/PVP K30; weight ratio, 1:1	−19.1 ± 0.6
DHA/NaDC; weight ratio, 1:1	−55.1 ± 3.3
DHA/PVP K30/NaDC; weight ratio, 1:1:1	−39.8 ± 0.9
DHA/PVP K30/NaDC; weight ratio, 1:2:1	−42.6 ± 0.8
DHA/PVP K30/NaDC; weight ratio, 1:3:1	−36.5 ± 0.5
DHA/PVP K30/NaDC; weight ratio, 1:1:2	−39.4 ± 1.1

of negative charge was observed when DHA was ground with NaDC. For ternary system, co-grinding of DHA with PVP K30 and NaDC also caused a pronounced increase in the magnitude of negative charge. The magnitude of negative charge of suspension obtained from DHA/PVP K30/NaDC ternary ground mixture was lower than that of DHA/NaDC binary ground mixture. Additionally, the zeta potential values of nanosuspension obtained from the ternary ground systems did not significantly alter as the amount of both PVP K30 and NaDC increased. The results indicate the interaction between PVP K30 and NaDC and suggest that adsorption of PVP occurs through interactions between adsorbed PVP moieties and NaDC monomers (Burry, Desmaziéres, & Treiner, 1997). Thus, the stabilization of DHA suspension from ternary ground mixtures was a result of the adsorption of both PVP K30 and NaDC onto drug particles. The increased negative charge was mainly attributable to an adsorption of NaDC on DHA particles. PVP K30 might act as a protective colloid, as a consequence of high steric hindrance between the adsorbed polymer layers, and NaDC controlled the nucleation of drug and caused electrostatic repulsion force between drug particles (Terayama et al., 2001). The role of PVP K30 and NaDC on drug nanoparticle formation from ternary ground system would be similar to that observed in the ternary ground mixtures consisting of drug, PVP, and SDS (Itoh et al., 2003; Pongpeerapat et al., 2004). The results obtained further suggest that with no added polymer, nanosuspension from binary ground mixture of drug with NaDC was obtained. Thus, NaDC adsorbed onto drug particles played a dominant role in controlling nucleation of drug and causing electrostatic repulsion force between drug particles.

Physical Stability of DHA Nanosuspension

Physical stability of DHA nanosuspensions obtained from co-ground mixtures after dispersing in water was studied at 25ºC. Changes in particle size and recovery of DHA nanoparticles after storage for 2 weeks are shown in Figure 10. A gradual increase in particle size was found in nanosuspensions obtained from DHA/NaDC ground mixtures after storage for 4 h, and subsequent precipitation was observed on day 14 of storage. This result suggests aggregation and/or growth of particles in these nanosuspensions. On the other hand, particle size of nanosuspension obtained from ternary ground mixture slightly increased with increasing storage time. Mean particle sizes of the suspensions obtained from ternary ground mixtures after storage for 4 h, 12 h, 1 week, and 2 weeks were about 90, 120, 160, and 180 nm, respectively. Amount of drug nanoparticles in the nanosuspensions obtained from DHA/NaDC ground mixtures decreased from 60% to 50% after storage for 12 h and then gradually decreased to 38% after storage for 14 days. In the nanosuspensions obtained from ternary mixtures, significant changes in amount of drug nanoparticles were not observed and amount of DHA nanoparticles was still relatively high (about 95%) after 14 days of storage. These results indicated that the nanosuspension obtained from ternary ground mixtures was more stable than those obtained from DHA/

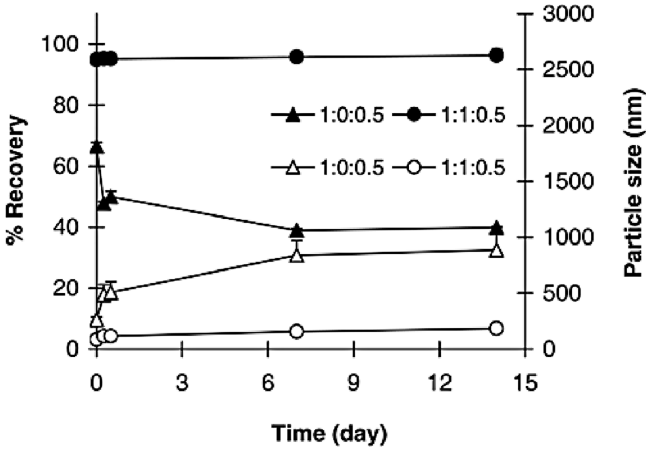

FIGURE 10. Changes in particle size (open symbol) and percent DHA recovery (solid symbol) of the nanosuspensions obtained by aqueous dispersion of 15-min ground mixtures of DHA/PVP K30/NaDC at different weight ratios at 25°C. ($M \pm SD$, $n = 3$).

NaDC ground mixtures. This finding was in good agreement with that found in zeta potential measurement as the magnitude of negative charge of the suspension obtained from ternary ground mixture was lower than that of DHA/NaDC ground mixture. The physical stability of the DHA nanosuspension obtained from ternary ground mixtures could be explained by the homogeneous size with narrow particle size distribution, electrostratic repulsion resulting in negative charge of NaDC adsorbed particles, and steric stabilization by PVP K30.

In Vitro Antimalarial Activity Against *P. falciparum* K1 Strain

Table 3 demonstrates in vitro antimalarial activity of DHA suspension using microculture radioisotope technique by monitoring [^{3}H] hypoxanthine uptake. The observation via microscope indicated that asexual *P. falciparum* parasites in red blood cells were cultured in the presence of solution and suspension of DHA with no histological changes in red blood cells. The results indicated that PVP K30 and NaDC had no activity against *P. falciparum* whereas inhibition of parasite growth was observed in the presence of DHA suspensions. It was suggested that DHA was absorbed by the erythrocyte cells, leading to inhibit the growth of *P. falciparum* in red blood cells. The DHA nanosuspension obtained from ternary ground mixture (mean particle size, 90 nm) exhibited higher antimalarial activity than the DHA microsuspension obtained from physical mixture of DHA/PVP K30/NaDC (mean particle size, 9790 nm). The colloid obtained from ground mixture of DHA/NaDC gave the highest activity when compared to the microsuspension and DHA solution. It might be that the protein solubilization effect of NaDC on red blood cell membrane (Jones, 1999; Kirkpatrick, Gordesky, & Marinetti, 1974) enhanced the permeation of DHA across red blood cell membrane to attach with plasmodia. Furthermore, DHA nanoparticles obtained from ternary ground mixture had lower activity than those obtained from binary ground mixture of DHA/NaDC. The presence of PVP K30 seemed to reduce the activity of drug particles obtained from ternary ground mixture. Mao and coworkers reported that the uptake of PEG-graft-trimethyl-chitosan copolymer-insulin nanocomplexes was influenced by a combination of polymer molecular weight, viscosity, and positive charge density (Mao et al., 2005). Sahoo and coworkers (2002) reported that the lower intracellular uptake of poly (D,L-lactide-co-glycolide) nanoparticles was related to the higher hydrophilicity of the nanoparticle surface caused by residual polyvinyl alcohol. Therefore, addition of PVP K30 might increase viscosity of the nanosuspension and/or increase hydrophilicity of DHA particles, which consequently might reduce the uptake of DHA into red blood cells, especially when high amount of PVP K30 is used. However, presence of PVP K30 in ground mixture improved the physical stability of the nanosuspensions obtained.

TABLE 3
In Vitro Antimalarial Activity Against *P. Falciparum* K1 Strain Using Microculture Radioisotope Technique of Suspensions Obtained by Aqueous Dispersion of 15-Min Ground Mixtures of DHA

Test Samples	IC_{50} x 10^4 (μg. mL^{-1})
DHA in DMSO	3.90
PVP K30 in water	Inactive
NaDC in water	Inactive
Microsuspension	
DHA/PVP K30/NaDC ; weight ratio, 1:1:1	7.33
Nanosuspension	
DHA/PVP K30/NaDC; weight ratio, 1:1:1	4.80
DHA:NaDC; weight ratio, 1:1	1.35

CONCLUSION

DHA nanosuspensions could be obtained by aqueous dispersion of ternary ground mixtures of DHA/PVP K30/NaDC and binary ground mixtures of DHA/NaDC. The size and recovery of drug nanoparticles between ternary and binary ground mixtures were not remarkably different. The results indicated that the ternary ground mixtures were superior systems as they gave more stable nanosuspension. The size of drug nanoparticles was dependent on amounts of PVP K30 and NaDC, and grinding time. Physical stabilization of DHA nanosuspensions obtained from ternary ground mixtures resulted from adsorption of PVP K30 and NaDC onto drug particles. The nanosuspension had higher antimalarial activity against *P. falciparum* in vitro than the micosuspension. Due to higher physical stability, co-grinding of DHA with PVP K30 and NaDC seems to be a promising method for preparing nanosuspension for further product development.

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REFERENCES

Burry, R., Desmazières, B., & Treiner, C. (1997). Interaction between poly(vinylpyrrolidone) and ionic surfactants at various solid/water interfaces: A calorimetric investigation. *Colloid. Surf. A: Physicochem. Eng. Aspects*, *127*, 113–124.

Chingunpitak, J., Puttipipatkhachorn, S., Petmitr, P., Tozuka Y., Moribe, K., & Yamamoto, K. (2005). Preparation and evaluation of dihydroartemisinin nanosuspension. The 4th Indochina Conference on Pharmaceutical Sciences. Ho Chi Minh City, Vietnam, 11–13 November 2005.

Chingunpitak, J., Puttipipatkhachorn, S., Tozuka, Y., Moribe, K., & Yamamoto, K. (2004). Production of micro- to nanoparticles of dihydroartemisinin. The 20th Asian Congress of Pharmaceutical Sciences. Bangkok, Thailand, 30 November–3 December 2004.

Desjarardins, R. E., Canfield, C. J., Haynes, J. D.,& Chulay, J. D. (1979). Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. *Antimicrob. Agents Chemother*., *16*, 710–718.

Hecq, J., Deleers, M., Fanara, D., Vranckx, H., & Amighi, K. (2005). Preparation and characterization of nanocrystals for solubility and dissolution rate enhancement of nifedipine. *Int. J. Pharm*., *299*, 167–177.

Itoh, K., Pongpeerapat, A., Tozuka, Y., Oguchi, T., & Yamamoto, K. (2003). Nanoparticle formation of poorly water soluble drugs from ternary ground mixtures with PVP and SDS. *Chem. Pharm. Bull*., *51*, 171–174.

Jacobs, C., Kayser, O., & Müller, R. H. (2001). Production and characterization of mucoadhesive nanosuspensions for the formulation of bupravaquone. *Int. J. Pharm*., *214*, 3–7.

Jones, M. N. (1999). Surfactants in membrane solubilization. *Int. J. Pharm*., *177*, 137–159.

Kipp, J. E., Wong, J. C. T., Doty, M. J., & Rebbeck, C. L. (2003). U.S. Patent No 6,607,784. Microprecipitation method for preparing submicron suspensions.

Kirkpatrick, F. H., Gordesky S. E., & Marinetti, G. V. (1974). Differential solubilization of proteins, phospholipids and cholesterol of erythrocyte membranes by detergents. *Biochim. Biophys. Acta.*, *345*, 154–161.

Kocbek, P., Baumgartner, S., & Kristl, J. (2006). Preparation and evaluation of nanosuspensions for enhancing the dissolution of poorly soluble drug. *Int. J. Pharm*., *312*, 179–186.

Kondo, N., Iwao, T., Masuda, H., Yamanouchi, K., Ishihara, Y., Yamada, N., Haga, T., Ogawa, Y., & Yokoyama, K. (1993). Improved oral absorption of a poorly water-soluble drug, HO-221, by wet-bead milling producing particles in submicron region. *Chem. Pharm. Bull*., *41*, 737–740.

Liversidge, G. G., & Conzentino, P. (1995). Drug particle size reduction for decreasing gastric irritancy and enhancing absorption of naproxen in rats. *Int. J. Pharm*., *125*, 309–313.

Liversidge, G. G., Cundy, K. C., Bishop, J. F., & Czekai, D. A. (1992). U.S. Patent No 5,145,684. Surface modified drug nanoparticles.

Liversidge, G., Jenkins, S., & Liversidge, E. (2006a). U.S. Patent No 2006188566. Nanoparticulate formulations of docetaxel and analogues.

Liversidge, G.G., & Jenkins, S. (2006b). U.S. Patent No 2006210638. Injectable compositions of nanoparticulate immunosuppressive compounds.

Luo, X. D., & Shen, C. C. (1987). The chemistry, pharmacology and clinical applications of qinghaosu (artemisinin) and its derivatives. *Med. Res. Rev*., *7*, 29–52.

Mao, S., Germershaus, O., Fischer, D., Linn, T., Schnepf, R., & Kissel, T. (2005). Uptake and transport of PEG-graft-trimethyl-chitosan copolymer-insulin nanocomplexes by epithelial cells. *Pharm. Res.*, *22*, 2058–2068.

Moribe, K., Pongpeerapat, A., Tozuka, Y., & Yamamoto, K. (2006). Drug nanoparticle formation from drug/HPMC/SDS ternary ground mixtures. *Pharmazie*., *61*, 97–101.

Möschwitzer, J., Achleitner, G., Pomper, H., & Müller, R. H. (2004). Development of an intravenously injectable chemically stable aqueous omeprazole formulation using nanosuspension technology. *Eur. J. Pharm. Biopharm*., *58*, 615–619.

Müller, R. H., Jacobs, C., & Kayer, O. (2000). Nanosuspensions for the formulation of poorly soluble drugs. In F. Nielloud & G. Marti-Mestres (Eds.), *Pharmaceutical emulsion and suspension* (pp. 383–407). New York: Marcel Dekker.

Myint, P. T., & Shwe, T. (1987). A controlled clinical trial of artemeter (qinghaosu derivative) versus quinine in complicated and severe falciparum malaria. *Tran. R. Soc. Trop. Med. Hyg*., *81*, 559–561.

Myint, P. T., Shwe, T., Soe, L., Htut, Y., & Myint, W. (1989). Clinical study of the treatment of cerebral malaria with artemeter (qinghaosu derivative). *Tran. R. Soc. Trop. Med. Hyg*., *83*, 72.

Patravale, V. B., Date, A. A., & Kulkarni, R. M. (2004). Nanosuspension: A promising drug delivery strategy. *J. Pharm. Pharmacol.*, *56*, 827–840.

Pongpeerapat, A., Itoh, K., Tozuka, Y., Moribe, K., Oguchi, T., & Yamamoto, K. (2004). Formation and stability of drug nanoparticles obtained from drug/PVP/SDS ternary ground mixture. *J. Drug Del. Sci. Tech*., *14*, 441–447.

Puttipipatkhachorn, S., Chingunpitak, J., Petmitr, P., Tozuka, Y., Moribe, K., & Yamamoto, K. (2006). Investigation of antimalarial dihydroartemisinin nanosuspension prepared by co-grinding method. Proceedings of the 5th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Geneva, 27–30 March 2006.

Rabinow, B. E. (2004). Nanosuspensions in drug delivery. *Nat. Rev. Drug Discov.*, *3*, 785–796.

Sahoo, S. K., Panyam, J., Prabha, S., & Labhasetwar, V. (2002). Residual polyvinyl alcohol associated with poly (D,L-lactide-co-glycolide) nanoparticles affects their physical properties and cellular uptake. *J. Controlled Release*, *82*(1), 105–114.

Sethabouppha, B. (1999). *Dissolution improvement of dihydroartemisinin capsules and tablets*, Master's thesis, Mahidol University, Bangkok, Thailand.

Sugimoto, M., Okagaki, T., Narisawa, S., Koida, Y., & Nakajima, K. (1998). Improvement of dissolution characteristics and bioavailability of poorly water-soluble drugs by novel cogrinding method using water-soluble polymer. *Int. J. Pharm*., *160*, 11–19.

Suzuki, H., Ogawa, M., Hironaka, K., Ito, K., & Sunada, H. (2001). A nifedipine coground mixture with sodium deoxycholate. II. Dissolution characteristics and stability. *Drug Dev. Ind. Pharm*., *27*, 951–958.

Terayama, H., Okumura, K., Sakai, K., Torigoe, K., & Esumi, K. (2001). Aqueous dispersion behavior of drug particles by addition of surfactant and polymer. *Colloid. Surf. B: Biointerfaces*, *20*, 73–77.

Titulaer, H. A. C., Zuidema, J., & Lugt, C. B. (1991). Formulation and pharmacokinetics of artemisinin and its derivatives. *Int. J. Pharm*., *69*, 83–92.

Tozuka, Y., Wongmekiat, A., Sakata, K., Moribe, K., Oguchi, T., & Yamamoto, K. (2004). Co-grinding with cyclodextrin as a nanoparticle preparation method of a poorly water soluble drug. *J. Incl. Phenom. Macrocycl. Chem*., *50*, 67–71.

Trager, W., & Jensen, J. B. (1976). Human malaria parasites in continuous culture. *Science.*, *193*, 673–675.

Trotta, M., Gallarate, M., Carlotti, M. E., & Morel, S. (2005). Preparation of griseofulvin nanoparticles from water dilutable microemulsions. *Int. J. Pharm*., *254*, 235–242.

Trotta, M., Gallarete, M., Pattarino, F., & Morel, S. (2001). Emulsions containing partially water-miscible solvents for the preparation of dry nanosuspensions. *J. Control. Release*., *76*, 119–128.

Wilairatana, P., Chanthavanich, P., Singhasivanon, P., Treeprasertsuk, S., Krudsook, S., Chalermrut, K., Phisalaphong, C., Kraisintu, K., & Looareesuwan, S. (1998). A comparison of three different dihydroartemisinin formulations for the treatment of acute uncomplicated falciparum malaria in Thailand. *Int. J. Parasitol*., *28*, 1213–1218.

Wongmekiat, A., Tozuka, Y., Oguchi, T., & Yamamoto, K. (2002). Formation of fine drug particles by co-grinding with cyclodextrin. I. The use of β-cyclodextrin anhydrate and hydrate. *Pharm. Res*., *19*, 1867–1872.

Wongmekiat, A., Tozuka, Y., Oguchi, T., & Yamamoto, K. (2003). Formation of fine drug particle by cogrinding with cyclodextrins. Part II. The influence of moisture condition during cogrinding process on fine particle formation. *Int. J. Pharm*., *265*, 85–93.

World Health Organization (2002). Annual report, Thailand.

Zili, Z., Sfar, S., & Fessi, H. (2005). Preparation and characterization of poly-ε-carprolactone nanoparticles containing griseofulvin. *Int. J. Pharm*., *294*, 261–267.