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Research Article

PHYSICOCHEMICAL PROPERTIES OF HESPERIDIN NANOCRYSTAL

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ABSTRACT

Hesperidin nanocrystals was generated by high pressure homogenization. The physicochemical properties of a spray dried hesperidin nanocrystals were investigated including to re-dispersion, crystalline state, kinetic solubility, dissolution velocity. Hesperidin nanosuspension with concentration of 10% (w/v) was dried by a Mini Spray-dryer B-190. Photon correlation spectroscopy (PCS) and laser diffraction (LD) were employed to determine the particle size. DSC and X-ray diffraction were used to study crystalline state. The saturation solubility and dissolution were determined at certain time points at 25oC.

Spray-dried hesperidin nanocrystals could be redispersed in water completely. The size average and polydispersity index (PI) were 396 nm and 0.555 after redispersion. The d50% of nanocrystals was 0.370 µm. The crystalline state showed that the high pressure homogenization process did not change the crystaline state. Saturation solubility of spray-dried hesperidin nanocrystals in water was 87.2 µg/ml and higher than hesperidin raw material. Hesperidin nanocrystals was dissolved completely within 15 minutes in various media. Whereas hesperidin raw materials after 15 minutes was dissolved maximum only 37%. Spray-dried hesperidin nanocrystals could provide superior physicochemical properties. Spray-dried hesperidin nanocrystals increased the saturation solubility and especially the dissolution velocity in comparison to raw material, i.e. drug delivery improved in case dissolution velocity is the rate limiting step.

Keywords: Nanosuspension, Nanocrystals, Hesperidin, Spray drying, Re-dispersion, DSC, X-ray, Saturation solubility, Dissolution velocity

INTRODUCTION

The flavonoid hesperidin is a flavanone glycoside (glucoside) comprised of the flavanone (a class of flavonoids) hesperitin and the disaccharide rutinose. Hesperidin is the predominant flavonoid in lemons and oranges. The peel and membranous parts of these fruits have the highest hesperidin concentrations. Therefore, orange juice containing pulp is richer in the flavonoid than that without pulp. Sweet oranges *(Citrus sinensis)* and tangelos are the richest dietary sources of hesperidin. Hesperidin is classified as a citrus bioflavonoid [1, 2].

Hesperidin, in combination with a flavone glycoside called diosmin, is used in Europe for the treatment of venous insufficiency and hemorrhoids. Hesperidin, rutin and other flavonoids thought to reduce capillary permeability and to have anti-inflammatory action were collectively known as vitamin P. These substances, however, are not vitamins and are no longer referred to, except in older literature, as vitamin P [1, 2].

Hesperidin is a solid substance with low solubility in water. It is, however, much more soluble in water than its aglycone hesperetin. Hesperidin's molecular formula is $C_{28}H_{34}O_{15}$, and its molecular weight is 610.57 daltons. It, chemically (S)-7-[[6-O-(6-deoxy- alpha-L-mannopyranosyl)- beta-D-glucopyranosyl] oxy]- 2,3-dihydro-5-hydroxy- 2-(3-hydroxy-4-methoxyphenyl)-4H-1-Benzopyran-4-one, is a white to yellow crystalline powder melting at ca 260 C. Bioflavonoids are called phytochemicals, Vitamin P, and Vitamin C.

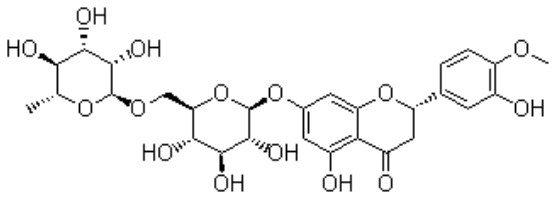


Fig. 1: Chemical structure of hesperidin

A lot of new pharmaceutical development substance is poor solubility in aqueous environment [3-5]. In the most case, it is associated to physicochemical problem such as slowly dissolution rate and very low solubility. In recently decade, particle size reduction of the poorly soluble substances is an interesting method to overcome the dissolution and solubility problem. Micronization offers a technology to increase dissolution, however does not change the saturation solubility [6, 7, 8]. Therefore, this method was not sufficient enough to overcome the problem because in many case, drugs are very low solubility and in combination with desired high blood level in the body. Therefore it is needed to shift particle size reduction to nanometer range. The nanoparticles improve the in vivo performance of poorly soluble drugs thus leading to an increased surface area and increased dissolution velocity. According to Kelvin-Gibbs and Ostwald-Freundlich equation, a drug nanoparticles provides also enhancement drug solubility in aqueous media [9-13].

Development nanotechnology was attempted by Sucker *et al.* and Auweter using precipitation technique [14-17]. Drug is dissolved in solvent media and subsequently added non-solvent to obtain drug nanoparticles. Presence stabilizer such as surfactant into the media can avoid particles growth or microparticles formation. Similar method of this technique was reported also by Kipp *et al*. using precipitation combined with high energy input to reduce particles growth and to maintain nanoparticles [18]. Applicability of both techniques is requisites limitation in pharmaceutical industry because drug must be dissolved at least in one solvent and it is required a solvent to be miscible with non-solvent. These prerequisites exclude the processing of drugs which are simultaneously poorly soluble in aqueous and in non aqueous media [11].

More efficient milling technology was published by Liversidge *et al.* to obtain drug nanoparticle. Pear/ball mills are employed by the élan company (Nanosystems) to produce a drug nanoparticle product called NanoCrystals [19, 20]. The drugs powder is dispersed in a surfactant solution and the obtained suspension is poured into a pearl mill containing glass pearl or zirconium oxide pearls. Alternatively, the drug microparticulate suspension can be milled by applying high pressure homogenization (HPH) process leading to a product referred to as a nanosuspension, the registered trade name is Dissocubes [21-23]. This process was developed and patented by Mueller *et al.* in 1999 and formerly owned by the Drug Delivery Service GmbH in Germany and is now owned by SkyePharma PLC [9, 10, 24].

In this research, hesperidin nanocrystals were generated by HPH. Physicochemical properties of hesperidin nanocrystals were carefully investigated. In previous research, HPH could generate submicron particulate of substances and drug nanosuspensions provided superior physicochemical properties compared to raw material.

MATERIAL AND METHODS

Materials

Hesperidin was purchased from Sigma Aldrich GmbH (Deisenhofen, Germany). As stabilizer sodium dodecyl sulfate (Fluka Chemie GmbH, Germany), Tween® 80 (Uniqema, Berlgium), Poloxamer®188 (BASF Aktiengesellschaft, Ludwighafen, Germany) and Polyvinyl Alcohol (PVA) Wt. 90.000 (Sigma Aldrich GmbH, Deisenhofen, Germany) were used. Double-distillated water was used as dispersion media and other chemicals were of analytical reagent grade.

Methods

Production of nanosuspension and particle size analyses

Hesperidin nanosuspension was stabilized by polyvinyl alcohol (PVA). Micronized hesperidin were dispersed into stabilizer solution and stirring using an Ultra-Turrax T25 (Janke & Kunkel GmbH, Staufen, Germany). A Micron LAB 40 (APV Homogenizers, Unna, Germany) was employed to produce nanosuspension at 5°C. Homogenization was performed at 1500 bar for 20 cycles. Mean particle sizes were determined using photon correlation spectroscopy (PCS) (Zetasizer Nano Z-S, Malvern Instrument, UK) yielding particle size average (z-ave) and polydispersity index (PI). Laser diffractometry (LD) (Coulter®LS 230, Beckmann-Coulter Electronics, Krefeld, Germany) with PIDS was used. The LD data obtained were evaluated using volume distribution as diameter (d) values of 50%, 90% and 99%. During storage time, particle sizes of the sample were also determined to evaluate physically stability of the nanosuspensions.

Spray drying (SD)

In general, spray drying was employed to obtain nanocrystal powder. An aqueous nanosuspension was transferred into nanocrystals powder by a Mini Spray-dryer B-190 (Büchi Labortechnik AG, Switzerland). Mini Spray-dryer B-190 was set regarding to temperature inlet (110-100°C), outlet (74-76°C), air volume (600 L/h). Spray-dried nanocrystals were directly collected after the process. The nanocrystals were used for different purposes, e.g. as reference for crystalline state investigation.

Crystalline status evaluation

a. Differential Scanning Calorimetry (DSC)

DSC analysis was performed using a Mettler DSC 822e1200 (Mettler Toledo, Germany). The instrument was calibrated with indium (calibration standard, purity 99.999%) for melting point and heat of fusion. Analysis was performed under a nitrogen purge (20 ml/min). Standard aluminum sample pans of 40 µl were used. About 2 mg drug nanocrystals were taken for analysis. A heating rate of 5°C/min was employed. An empty pan was used as reference.

b. Powder x-ray diffraction (PXRD)

A powder X-ray diffractometer (wide angle scattering-WAXD, Philips, Amedo, the Netherlands) was used for diffraction studies. PXRD studies were performed on the samples by exposing them to CuK_α radiation (40 kV, 25 mA) and scanned from 0.06° to 40°, 2θ at a step size of 0.04° and step time of 0.5s. Samples used for PXRD analysis were same as those of DSC analysis.

Redispersability and phase separation

The re-dispersability of hesperidin nanosuspensions was determined with shaking the vials by hand and until all of the sediment had been uniformly dispersed in the aqueous phase. After homogenously re-dispersion, the particle sizes were determined by LD and PCS. Phase separation was determined visually in all formulations during long-term storage.

Saturation solubility determination

Saturation solubility of hesperidin nanocrystals with mean particle size about 300 nm was determined using a shaker (Innova™ 4230, New Brunswick Scientific Edision, NJ-USA) at 25°C in a period of 1 week. The excess nanocrystals were placed into 40 ml capped vials containing dispersion medium and then sonicated in a water bath (Bandelin RK 514, Berlin, Germany) for 30 second. The suspensions were located in a thermostated storage at 25 ± 0.01°C, shielded from light to prevent any degradation of the molecules. Then shaker was moved at 100 rpm and at certain time samples were withdrawn. An aliquot was filtered using Sartorius® 0.1 µm filters (Sartorius AG, Goettingen Germany) and then transferred into 1.5 ml Eppendorf tube for drug contain determination with high performance liquid chromatography (HPLC) method. Experiments were carried out in triplicate, solubility data were averaged.

HPLC analysis of hesperidin

Concentration of dissolved hesperidin in collected medium was determined by HPLC. The HPLC conditions were as following: column, Eurospher 100-5 C18, 250 x 4 mm (B6Y535); mobile phase: isocratic 5 mM ammoniumacetate/ acetonitril = 75 : 25 (v/v) and adjusted until pH of 4.45 with acetic acid; flow rate, 1.0 mL/min; UV/Vis detector; λ max. at 285nm, temperature; 40°C [25].

Dissolution test hesperidin nanocrystals

The dissolution behavior of hesperidin nanocrystals was evaluated using a USP XXIII rotating paddle apparatus with a Pharmatest PTW SIII (Pharma Test, Hamburg, Germany) at 37 °C and a rotating speed of 100 rpm in 900 ml of water. 5 mg of dried hesperidin or hesperidin microcrystal (5 mg was chosen to maintain a Sink condition) were dispersed in the dissolution medium. Samples were withdrawn from the dissolution chamber at certain times. An aliquot was filtered through Sartorius® 0.1 µm filters (Sartorius AG, Goettingen Germany) and assayed by HPLC (HPLC, Kroma-System 2000, Kontron Instruments, Germany) to evaluate the amount of hesperidin dissolved. Dilution was intentionally avoided, to prevent any possible interference with the chemical equilibrium, particularly considering the presence of colloidal particles.

RESULT

Particle size characteristics of hesperidin nanosuspension

Particle size analysis was performed by laser diffractometry and by photon correlation spectroscopy (PCS). **Table 1** shows the particle size data of the hesperidin nanosuspensions stabilized by 4 different stabilizers. The particle size of the hesperidin nanosuspensions decreased with increasing number of cycle and increasing homogenization pressure. The effectiveness of the pre-milling was clearly revealed by reducing width of the particle size distribution. The width of the size distribution of the raw material was about 130 µm (d10% of 0.6 µm to d99% of 130 µm) and reduced by pre-milling to about 23 µm (d10% of 0.6 µm to d99% of 23 µm) after the pre milling process. A clear advantage was exhibited and pre-milling could increase the homogeneity of the suspension.

The particle size averages of all of the formulations are in the nanometer range. This means formulations E, F, G and H have fulfilled the requirements of a nanosuspension. Particle size of the formulation F could reach d50% of 0.277 µm and d99% of 2.057 µm. This means formulation F resulted in smaller particle sizes compared to the other formulations.

Table I: Mean particles size parameter of hesperidin nanosuspensions using 4 different stabilizers: (E) 2% of poloxamer 188 (F) 0.2% of SDS (G) 2% of Tween 80 (H) 2% of PVA.

Size parameters	Formulations			
	E	F	G	H
Z ave (nm)	309	293	449	309
PI	0.434	0.307	0.308	0.404
d50% (µm)	0.344	0.277	0.343	0.378
d90% (µm)	1.632	0.831	2.415	2.172
d99% (µm)	2.728	2.057	5.627	3.942

Spray-drying, and Redispersability

A spray drying process was accomplished to obtain dried hesperidin nanocrystals. Although this method was only moderately effective (low yield), the spray-dried nanocrystals had better flow characteristics compared to lyophilized nanocrystals [27, 28]. Therefore, spray-dried hesperidin nanocrystal was chosen for further investigation.

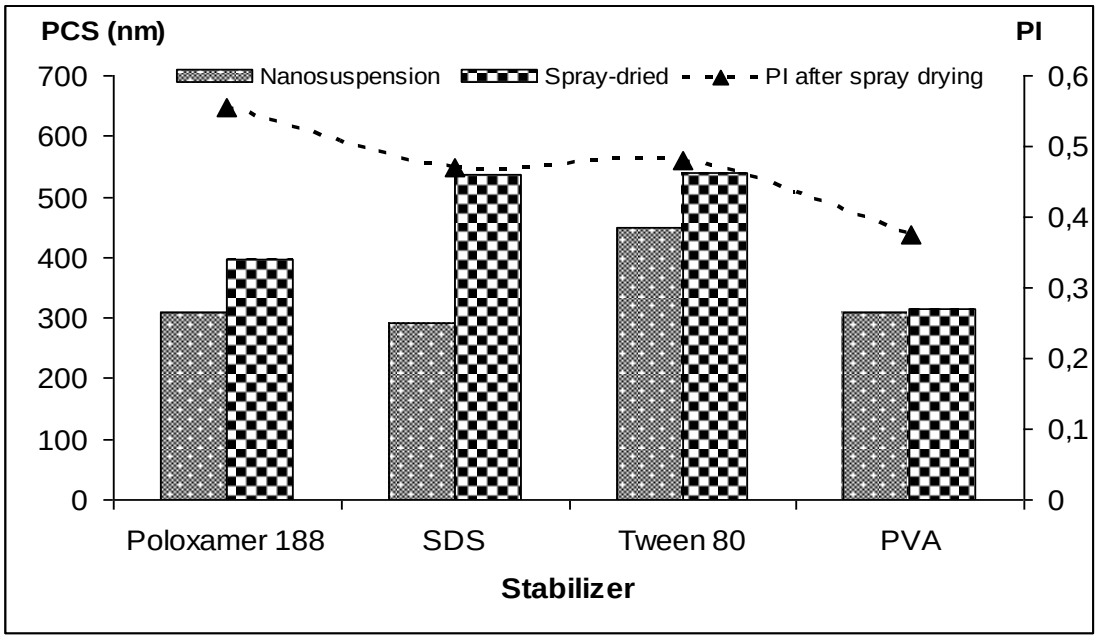


Fig. 2: Mean particle size by PCS of spray-dried hesperidin nanocrystals stabilized by poloxamer 188, SDS, Tween 80 and PVA after redispersion in water in comparison with original nanosuspension

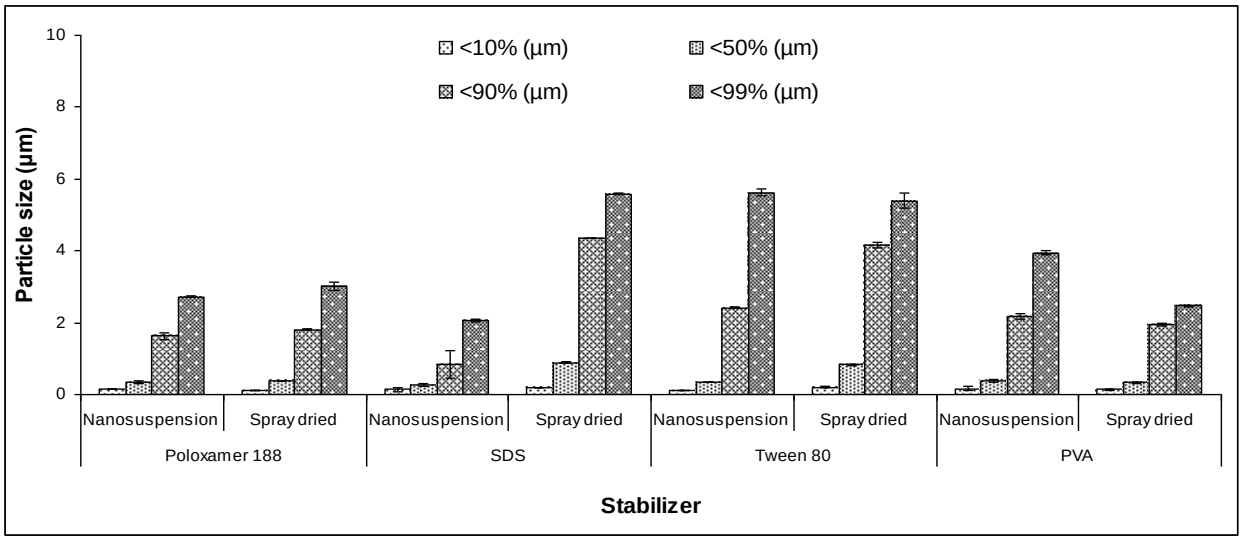


Fig. 3: Volume size distribution by LD of spray-dried hesperidin nanocrystals stabilized by poloxamer 188, SDS, Tween 80 and PVA after redispersion in water in comparison with original nanosuspension

As usual, the spray-dried nanocrystal was evaluated with respect to physicochemical properties e.g. redispersability, crystalline state, morphology, kinetic solubility and dissolution velocity.

Dried form of hesperidin nanocrystal has almost completely re-dispersed in water. Even without the addition of certain cryoprotectant, the spray-dried hesperidin could easily be redispersed. The particle size average by PCS differed little from the original hesperidin nanosuspensions. For example, spray-dried hesperidin nanocrystals stabilized by poloxamer 188 had an average size and polydispersity index (PI) of 396 nm and 0.555 after redispersion. The d50% of hesperidin nanocrystals was 0.370 µm. This particles size analysis was similar to the original nanosuspensions with the size average of 309 nm (**Fig. 2**).

III.3 Crystalline status

According to the DSC thermogram**,** fusion temperature peaks of the raw material and dried hesperidin nanocrystals were similar and showed no significant change. The peaks of fusion temperature indicate that dried hesperidin nanocrystals were in the same crystalline state with raw material. Again, the apparent modification in the hesperidin nanocrystals was only a minor shift of the drug melting points. Differences on the melting points due to stabilizers, but there was no modification of crystal form to an amorphous state.

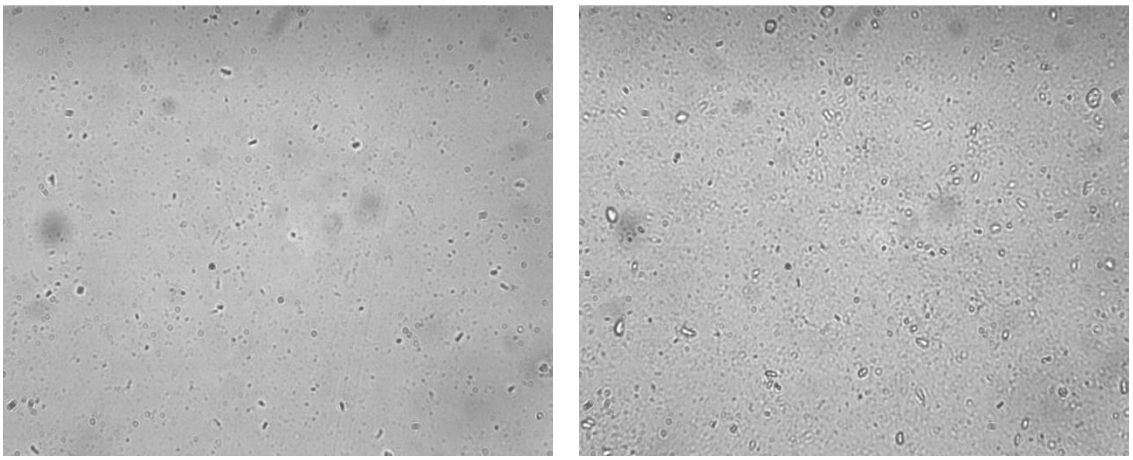
Crystal form of spray dried nanocrystals was also confirmed by powder x-ray diffraction. In this case the hesperidin raw material was proven already as being in a crystalline state. In addition, peak intensities were also studied to investigate crystal form of the hesperidin nanocrystals. Moreover, **Fig. 6** reveals all of the peaks of hesperidin nanocrystals similar to the raw material. These results confirm that there was no decreased crystal state on the hesperidin nanocrystals.

Kinetic saturation solubility and dissolution velocity

In water, hesperidin solubility was improved almost 5 fold. Saturation solubility of dried hesperidin nanocrystals in water

was 87.2 ± 0.8 µg/ml. This was definitely higher than the saturation solubility of hesperidin raw material (19.8 ± 0.4 µg/ml). In the literature mentioned that 1 g hesperidin dissolves in 50L water [1, 2]. Accordingly the solubility of hesperidin in water is 20 µg/ml, which is slightly differs from the obtained result only (**Fig. 7**).

Dissolution velocity evaluation showed superiority dissolution profile of hesperidin nanocrystals. Only in 15 minutes, all hesperidin nanocrystals were dissolved completely in water, buffer having a pH of 1.2 and 6.8. In contrast, only 37%, 34% and 36% of hesperidin raw materials were dissolved into water, buffer having a pH of 1.2 and 6.8 respectively (**Fig. 8**).



a **b**

Fig. 4: Light microscopic pictures of hesperidin nanosuspensions stabilized by PVA after production (a) and re-dispersed spray-dried hesperidin nanocrystals (b)

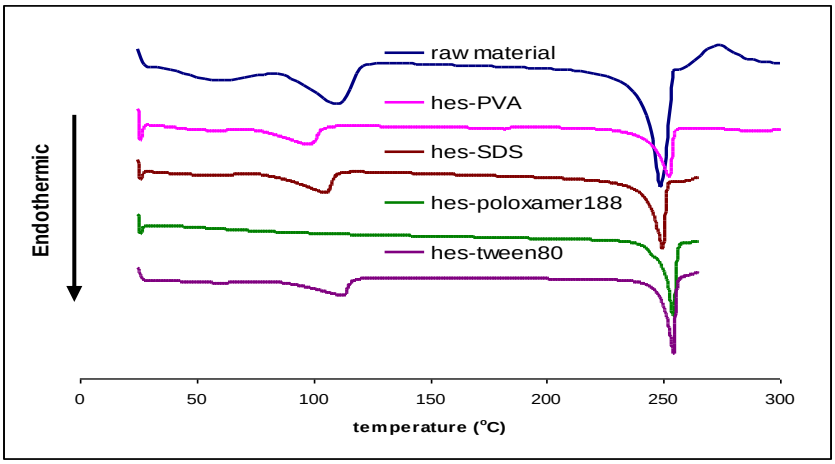


Fig. 5: Influence of stabilizers on fusion temperature and crystaline state of spray-dried hesperidin nanocrystals

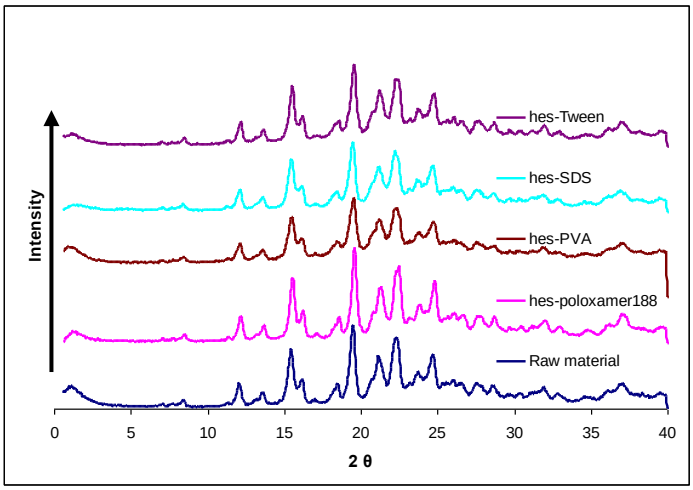


Fig. 6: X-ray diffractogram pattern of all lyophilized hesperidin nanocrystals and raw material

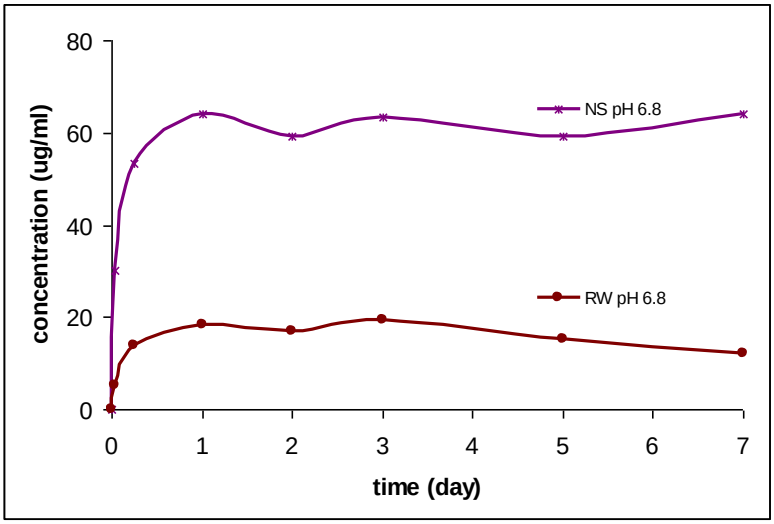


Fig. 7: Saturation solubility of heperidin nanocrystals and raw material in water at 25°C

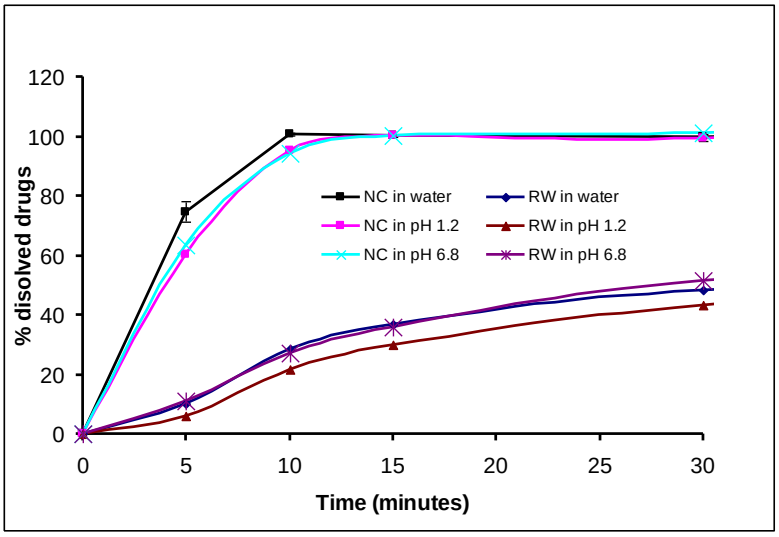


Fig. 8: Dissolution profile of hesperidin nanocrystals and raw materials in water and in buffer pH of 1.2 and 6.8

DISCUSSION

Particle size characteristics of hesperidin nanosuspension

Laser diffractometry (LD) yields a volume size distribution. As characterisation parameters the diameters 10, 50, 90 and 99% were used. For example d99% means that 99% of the particles are below the given size value.

PCS yields the mean diameter of the bulk population (*z*-average, measuring range: 3 nm – 6 µm) and a polydispersity index (PI) as measure for the width of the size distribution. The PI ranges from zero (monodisperse particles) to 0.500 (broad distribution), values above 0.5 do not allow allocation of a logarithmic normal distribution to the PI.

A homogenous nanosuspension is one of many advantages of the nanosuspensions produced by high pressure homogenization. This characteristic has been found to provide maximum physical stability (avoidance of Ostwald ripening). Therefore, the hesperidin nanosuspensions should be as homogeny as possible, meaning a reduction of the number of micrometer particles. When the product should be injected intravenously, a low content of micrometer particles is essential for nanosuspensions on parenteral injections to avoid capillary blockage. **Table 1** shows the diameters 50–99% was measured by laser diffractometry. Typically, after twenty cycles, d99% of the volume size distribution of the particles is maximum 5 µm (a part from formulation G). According to the USP Pharmacopeial Forum and British Pharmacopoeia in 1980, the diameter of particles (globules in emulsion) should not exceed 5 µm for parenteral application [26]. According to the resulted particle size, these hesperidin nanosuspensions can be officially used for parenteral administration.

The LD diameter d50% of the nanosuspensions was below 1 µm (**Table 1**). Formulation E, F, G and H of the hesperidin nanosuspensions have volume size distribution d50% by LD in the nanometer range.

Redispersability

The aqueous nanosuspensions can be processed for dispersability as dry powder for solid dosage forms such as tablets, capsules, pellets, effervescent or dried material for injectable products [22]. These dried powders from nanocrystals are designed to re-disperse into nanometer-sized particles when dispersed in water or an alternate water-based environment. Ideally the same size and size distribution should be similar to nanosuspension before drying. The ability of the dried nanocrystals to re-disperse into non aggregated/non agglomerated nano-particulate dispersion is critical to the development of a solid dosage form that maintains the benefits of this enabling drug delivery technology.

According to this result, the dried hesperidin nanocrystal is a promising powder for incorporation into solid dosage forms.

The particle size average by PCS revealed little difference among the different formulations of spray-dried hesperidin nanocrystals. The physical properties of spray-dried hesperidin were better than the lyophilized product. Flow ability of spray-dried nanocrystals was distinctly improved. This result is in agreement with Möschwitzer *et al.* who claims that spray drying can improve the flow properties of powder [27, 28].

Moreover, spray-dried hesperidin nanocrystals stabilized by PVA had the smallest average particle size after redispersion. The average particle size of this nanocrystal was 315 nm, practically identical to the original nanosuspension (particle size average of 309 nm) and only showing little growth. Therefore this hesperidin nanocrystal formulation was preferable for incorporating into a solid dosage form for oral administration. The particles size difference can be seen clearly on **Fig. 3**.

In addition observation of morphology of the nanocrystals in the PVA-stabilized nanosuspension was performed using light microscopy (**Fig. 4)**. On figure 4, all particles were almost distributed as single crystals and clearly homogeneous. No agglomerated or aggregated particles were detected. This result is confirmed by the PI value. In this case, the low PI (below of 0.5) indicates a relative narrow distribution of the particles.

Crystalline status

Some literature mentions the melting point and the crystalinity of hesperidin. Pure hesperidin exists as long hair-like needles, tan or pale yellow in colour, with melting point ranging from 258° to 262°C (softening at 250°C). The molecular formula for this compound is $C_{18}H_{34}O_{15}$ and molecular weight is 610.57 daltons [2].

As expected, the crystalline state of hesperidin was not influenced by the drying process. Exposure energy during homogenization and the subsequent drying process did not alter crystalline state.

The presence of different stabilizers in the hesperidin nanocrystal formulations also had no influence on the crystalline state. **Fig. 5** shows fusion temperatures of the dried hesperidin nanocrystals with various stabilizers. According to these thermograms, the melting points of the nanocrystals did not change significantly. The melting points occurred in the range of 252°C to 254°C. The presence of stabilizer could explain the slight shift in those melting points.

Water was also evaporated from dried nanocrystals and from hydrated form of hesperidin during heating process, as clearly shown by the peaks at around 100°C in **Fig. 5**. However, all peaks in the thermogram indicate the same crystaline state of the hesperidin nanocrystals and the raw material. This means that the dried hesperidin nanocrystals were not converted to an amorphous state and remained in crystalline form.

Dried hesperidin nanocrystal was investigated by powder x-ray diffractions. In this study, the peaks for each hesperidin on the difractogram pattern were compared which each other and with a diffractogram of the raw material as reference. Dried hesperidin nanocrystals have the same finger prints with the raw material. In addition, the intensity of the peaks was about the same. Again, this study found that applying energy during homogenization and drying process did not transform the crystalline state and dried nanocrystals were still in the same crystal form. No process led to an amorphous state.

Kinetic saturation solubility and dissolution velocity

One important physicochemical characteristic of drug nanosuspension is enhancement kinetics saturation solubility. According to Kelvin and Ostwald-Freundlich equation, submicron particulate can increase kinetic solubility until few fold. This postulate is proven by increasing kinetic solubility of hesperidin nanosuspension in comparison to saturation solubility of raw material (in this case, raw material of hesperidin was micronized) as revealed on **Fig. 7.**

This result shows the superiority of hesperidin nanocrystals on kinetic solubility is in agreement with the Kelvin and Ostwald-Freundlich equation and previous investigation by Mauludin *et. al. (*29).

Dissolution velocity of hesperidin nanocrystals was subjected to intensive evaluation in three different media. Dissolution has been performed in Sink condition. As expected, dissolution of hesperidin nanocrystals was superior to the raw material (microcrystals). According to Whitney-Noyes equation, particle size is reciprocal to particle size. Its means, particle size can affect dissolution velocity (**Fig. 8**). Decreasing of particle size will improve dissolution velocity. Increasing dissolution velocity can conduct to improve bioavailability especially to biopharmaceutical class system (BCS) II which dissolution velocity is the rate limiting step.

CONCLUSION

Spray-dried hesperidin nanocrystals could provide superior physicochemical properties. Spray-dried hesperidin nanocrystals increased the saturation solubility and especially the dissolution velocity in comparison to hesperidin microcrystals, i.e. drug delivery improved, in case dissolution velocity is the rate limiting step.

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