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Development of Cyclosporine A Nanosuspension using Experimental Design by Response Surface Methodology: *In Vitro* Evaluations

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ABSTRACT
Objectives: This study aims to develop nanosuspensions of cyclosporine A (CycA) using a top-down technology (high pressure homogenization - HPH) for oral admistration.
Materials and Methods: The formulations were prepared using different ratios of hydroxypropyl methylcellulose (HPMC) (1% and 0.5%) and sodium dodecyl sulfate (SDS) (1%) to improve the solubility of CycA. The HPH method was optimized by investigating the effects of critical formulation parameters (stabilizer ratio) and critical process parameters (number of homogenization cycles) on the particle size (PS), polydispersity index (PDI), and zeta potential (ZP) of nanosuspension using Design of Experiment (DoE). After lyophilization, differential scanning calorimetry (DSC), X-ray diffraction (XRD), Fourier transformed infrared (FTIR) spectroscopy, and morphological evaluation with scanning electron microscopy (SEM) were performed. The stability studies were performed at 4ºC and 25ºC storage conditions. The solubility of optimum CycA nanosuspension was investigated by comparing with coarse CycA powder and physical mixture (PM). In vitro dissolution studies were conducted in four media by using dissolution testing USP apparatus I.
Results: PS, PDI, and ZP values for nanosuspension were found about 250 nm, 0.6, -35 mV, respectively. Under storage conditions, CycA nanosuspension demonstrated significant physical stability at both 4ºC and 25ºC for 9 months. The solubility of CycA was improved 1.9- and 1.4-times by nanosuspension in accordance with CycA powder and PM, respectively. CycA nanosuspension exhibited higher dissolution than CycA coarse powder in 0.1 N HCl, FaSSIF, and FeSSIF medium.
Conclusion: CycA nanosuspension was successfully developed using the DoE approach with HPH method with HPMC: SDS combination in 1:0.5 ratio and solubility and dissolution of CycA was improved in nanosuspension.

Keywords: Cyclosporine A, Nanosuspension, High pressure homogenization, Solubility, Dissolution

INTRODUCTION

Currently, approximately 60% of drugs have low solubility. This circumstance affects the pharmacokinetics and pharmacodynamics of these drugs, resulting in low dissolution and low bioavailability when taken into the body. The low solubility of the active substance in water causes changes in the absorption of the drug in the gastric medium in both fasting and fed situations, causing variation in fasting-fed states. Also, low solubility leads to impaired dose-response proportionality of the drug, unexpected collapse after administration, decreased patient compliance, and as a result low bioavailability.[1]

Cyclosporine A (CycA), one of the above-mentioned low solubility active substances that have been used for many years, is a neutral cyclic non-ribosomal peptide that is composed of 11 amino acids and was first isolated from the fungal extract of *Tolypocladium inflatum* in 1973.[2] CycA, one of the calcineurin inhibitors, is a immunosuppressant widely used to prevent organ rejection after transplantation, just like the other calcineurin inhibitor tacrolimus.[3] CycA shows its immunosuppressant activity by forming a cyclosporine-cyclophilin complex after binding to cyclophilin, thereby inhibiting T-cell activation and calcineurin phosphatase that under normal circumstances is responsible for activating the transcription of interleukin-2 (IL-2).[4] The molecular formula of CycA is $C_{62}H_{111}N_{11}O_{12}$ and the unsaturated chain at position 1 and amino acids at positions 2, 3, 11 are responsible for the immunosuppressive effect.[5] The solubility of CycA in water at 25ºC is 0.04 mg/mL, and its solubility in n-hexane is 1.6 mg/g.[6] When CycA is evaluated in terms of solubility and permeability properties; it is classified as Class II (low solubility, high permeability) according to the Biopharmaceutical Classification System (BCS) created by Amidon et al. in 1995.[7] CycA was first introduced to the market as a conventional oil-based formulation under the name Sandimmun®, and then the microemulsion formulation was developed with the trade name Sandimmun Neoral® since the gastrointestinal system effect was evident in CycA pharmacokinetics.[3] Although partial improvement was achieved in pharmacokinetic parameters with the microemulsion formulation; studies with CycA are continuing to reduce side effects (an undesirable plasma peak above 1000 ng/ml is thought to cause nephrotoxicity), to prepare products at a lower cost, and to reduce inter- and intra-variation and fasting-fed variability. Many studies with current opinions and different drug delivery systems are included in the literature to improve the solubility and dissolution of CycA and provide higher blood concentration and decrease the toxicity.[3,8,9]

There are many opinions used to improve the solubility of drugs to improve oral bioavailability. Nanosuspension (NS) technology is an very attractive opinion that aims to improve the solubility, dissolution rate, and so bioavailability of BCS Class II and IV drugs by decreasing the particle size (PS) of drugs to nanometer sizes without any carriers.[10,11] When NSs are dried, they are called nanocrystals, but these nanocrystals do not mean that the structure is physicochemically crystalline.[12] When drugs were reduced to the nanometer size, the saturation solubility improves by the Noyes Whitney equation, therefore the nanocrystals/nanosuspensions can significantly improve the oral absorption and bioavailability of drugs.[13-16]

The techniques used for the preparation of NSs are classified into two main approaches; “top-down” and “bottom-up” technologies. In the bottom-up technology, the molecule starts by dissolving in a solvent, and then adding an insoluble solution forms a precipitate, resulting in

particles in nanometer sizes.[16] Top-down technologies include wet milling (WM) and high pressure homogenization (HPH) method, while bottom-up technology includes precipitation. In the wet milling or pearl milling/ball milling method, the drug macrosuspension is placed in a milling container and the container is rotated with the addition of beads prepared with special polymers such as glass, zirconium oxide, or hard polystyrene derivatives. Depending on the size, amount, or rotational speed of the beads in the container, NSs are obtained. The other most commonly used top-down method is the HPH method. Microfluidization and piston-gap homogenizer are two currently used homogenization principles. Microfluidization is a jet stream principle; the suspension is accelerated and passes at a high speed through the specially designed "Y" or "Z" type homogenization chamber. In the “Z” type chamber, the flow direction of the macrosuspension is changed several times while the particles are collided and cut; while in the “Y” type chamber, the macrosuspension is divided into two flows by the obstacle in front of it.[17] In the piston-gap homogenization method; the macrosuspension is forced to pass through a small size gap so that the particles can be reduced to smaller sizes. Top-down nanosuspension production methods have many advantages such as minimum solvent content, high drug loading, easy preparation methods, and rapid production. Due to these advantages of NSs; many studies have been performed with NS (or nanocrystal) formulations prepared by WM[9,18-22] and HPH[23-26] methods, which are top-down methods.

Nanosuspensions are prepared using surfactants such as sodium dodecyl sulfate (SDS), Tween 80, Vitamin E TPGS, poloxamers, and polymeric stabilizers such as hydroxypropyl methylcellulose (HPMC), polyvinyl pyrrolidone (PVP), polyvinyl alcohol (PVA). These stabilizers can be used individually or as a combination; furthermore, there are many studies in which both surfactants and polymeric stabilizers are used together to improve the stability of NSs.[23,27-29]

In the last 20 years, opinions aiming to ensure the quality of pharmaceutical products and to achieve the most proper formulation have been interesting in the pharmaceutical industry. The effects of critical formulation and/or process parameters can be determined by optimizing the formulation and/or process requirements with the Design of Experiment (DoE), which is one of these approaches. So, the effects of the independent variables, which are thought to be effective on the formulation, on the dependent variables can be successfully investigated experimentally.[30] In addition to many drug delivery system studies with DoE, studies on NS preparation have become common in the literature.[8,14,23,25,26] In Figure 1, the parameters that affect particle size, polydispersity index, and zeta potential in nanosuspension formulations obtained by the HPH technology are shown by the fishbone diagram.

As outlined above, there is still a need for a novel formulation of CycA developed for oral administration. The fact that the Sandimmun Neoral® microemulsion formulation is an expensive formulation that requires many excipients and Cremophor®RH40, which is included as a surfactant in its composition has a toxic effect; complicates its use in therapy. So the studies on increasing solubility and dissolution rate of CycA with nanoparticles, lipid nanoparticles, and liposome carrier systems that do not contain Cremophor®RH40 are available in the literature; however, studies on the preparation of CycA NS are limited. This research article covers the preparation of CycA NS by HPH technology with the DoE approach. One of the unique values of this article is to improve the product quality as a result of the developed formulation requiring fewer process parameters and providing a nanotechnology-based, innovative and creative approach suitable for scale-up in a shorter time with the DoE approach in the formulation development process.

Many results have been obtained in our previously studies on CycA nanosuspensions. In the first of these studies, CycA was prepared using the HPH method with HPMC and Soluplus®, and after DoE, the ratio of CycA:HPMC:Soluplus® 1:1:0.5 (w/w) was found to be the most

appropriate ratio. Characterization studies showed that the solubility improved by 2.1-times compared to the coarse powder.[8] In order to further this study, CycA NSs were prepared with the wet milling method in the second step, which is another NS preparation method. The ratio of CycA:HPMC:SDS® 1:1:0.5 (w/w) was determined as the optimum ratio after DoE and formulation were examined in vivo as well. The solubility improved 4.5-times compared to the coarse powder and higher solubility in the in vitro FeSSIF medium than the trade product. The pharmacokinetic study indicated that AUC_{0-24} values of CycA NS were to be 2.09 and 5.51-times higher than coarse powder in fasted and fed situations, respectively.[18] When the permeability of this formulation from Caco-2 cells is examined, the NS showed improved CycA transport by 5 and 1.5 times, respectively, compared to coarse CycA powder and trade product (Sandimmun Neoral®).[19]

Based on this information, this study aimed to prepare CycA NSs with HPH technology by using a polymer (HPMC) and surfactant (SDS) combination based on previous knowledge and to decide the optimum ratio with the DoE approach. HPMC was selected because it proved previously to be an efficient stabilizer for NSs.[21,23] SDS with surfactant structure is an electrostatic stabilizer that allows high zeta potential loading on the surface of the particles and therefore is widely used.[13,20,23] SDS could migrate to the solid-liquid intersurface and provide an electrostatic barrier against aggregation of the nanometer size particles.[10] One of the main purposes of this study is to examine the effect of surfactant ratio on PS, PDI, and ZP in the formulation prepared when a polymeric stabilizer (HPMC) and a surfactant (SDS) are used together with an experimental design approach. The effects of formulation parameters (HPMC: SDS ratio) and process parameters (cycle number of homogenization) as independent variables on dependent variables (particle size, polydispersity index, and zeta potential) were evaluated with the Design of Experiment (DoE) approach. As a result of the DoE analysis, the optimum formulation was determined and characterization studies were performed with this formulation. The physical stability (particle size and zeta potential results) of the optimum formulation for 9 months at 4 and 25ºC was evaluated. The solubility of the optimum CycA NS was compared with coarse powder and physical mixture (PM). For CycA in dissolution medium, fasted simulated intestinal fluid (FaSSIF) and fed simulated intestinal fluid (FeSSIF) dissolution studies, NSs were compared with coarse powder, PM, and trade product (Sandimmun Neoral®).

MATERIALS AND METHODS

Materials

CycA was provided as a gift from Deva Drug Company (Turkey). Hydroxypropyl methylcellulose (HPMC) was obtained from Colorcon (USA). Sodium dodecyl sulfate (SDS) and D (-) mannitol were purchased from Merck (Germany). SIF® Powder was purchased from Biorelevant© (UK).

Preparation of CycA nanosuspension

HPH method (Microfluidization technique) involved 0.2 g (1%) HPMC and 0.1 g or 0.05 g (0.5% or 0.25%) SDS dissolved in distilled water. In second step, CycA powder (1% w/w) was dispersed in this solution using the magnetic stirrer at 1000 rpm for 20 minutes. To prevent high pressure homogenizer (Microfluidics LV1 with a Z-type 84 µm chamber) from chamber blockage, the UltraTurrax (Heidolph® Silent Crusher) was used at 15000 rpm for 10 minutes to reduce the particle size of this suspension. At last, this suspension was transported to Microfluidics LV1 (Microfluidizer®) and homogenized for different homogenization cycles at 30000 psi process pressure.

Different homogenization cycles (X_1) (5, 10, 15, and 30 cycles) and different amounts of surfactant (SDS) (X_2) (0.5% and 0.25%) were defined as critical process parameters (independent variables) in preformulation studies for HPH method and their impacts on dependent variables (particle size (Y_1), polydispersity index (Y_2), and zeta potential (Y_3))

were assessed using DoE. The process parameter was consisted of four levels with two replicates. After doing trials in random order obtained by the Design Expert 9.0 software, results were evaluated using this software. The interactions between independent variables were investigated using the DoE approach and the optimum CycA NS formulation was determined for characterization investigations.

Particle size, polydispersity index, and zeta potential studies

The PS, PDI, and ZP measurements, which are prominent results with regard to nano drug delivery systems, were conducted at 25ºC by dynamic light scattering (DLS) method using particle sizer (Malvern Instruments® ZetaSizer- Nano ZS). For particle size and particle size measurement, firstly 750 µL of NS was added into the sample measuring cup and topped up with up to 1500 µL of distilled water. At the end of this dilution, the diluted sample was taken into the particle size measurement cuvette and the zeta potential measurement cuvette. Each sample was measured at least three times and results were calculated as the mean±standard deviation (SD).

Preparation of the physical mixture

The PM was made by stirring the coarse CycA powder for approximately 5 minutes with the same HPMC ratio and the same SDS ratio used in the optimum NS formulation.

Lyophilization of CycA nanosuspension

Lyophilization of NS is important for long-term stability and obtaining solid dosage forms. Lyophilization was applied after the PS, PDI, and ZP measurements of NS were prepared by HPH. Mannitol was chosen as the cryoprotectant for formulations and CycA: mannitol ratio was decided as 1:1 (% w/w) after preformulation studies [8]. Approximately 2 g of the NS was frozen at -80°C for 2 hours and lyophilization was performed at -50°C under 0.021 mbar pressure for 48 hours with Christ Alpha® 1-2 LD Plus.

In vitro characterization studies

Morphology study

The surface morphology of the powder samples (CycA coarse powder, PM, and lyophilized NS) was conducted by scanning electron microscopy (SEM). The samples were placed on carbon specimen holders and air-dried. The samples were then covered with gold-palladium composition prior to experiments and the morphological images were monitored using a microscope (Quanta® 400F).

X-ray powder diffraction (XRD) study

The X-ray powder diffraction spectral analysis of CycA coarse powder, stabilizers, PM, and lyophilized NS was carried out by Rigaku Ultima® IV (Japan). The scan rate was adjusted at 1º per minute and the scan range was 2θ in the range of 3-90°.

Fourier transformed infrared (FTIR) study

The FTIR spectrums of CycA coarse powder, PM, and CycA NS were examined with spectrometer (Perkin Elmer® Spectrum 400 ATR-FTIR). The scanning range of 650-4000 cm^{-1} and the discrimination power of 1 cm^{-1} were selected for measurement.

Physical stability study

After the optimum CycA NS was prepared and PS, PDI, and ZP values were measured initially. The physical stability studies for optimum CycA NS were commenced by storing formulation at two temperatures (4 °C and 25 °C) and the measurements were repeated for 9 months after each month of storage terms. PS, PDI, and ZP values measurements at designated time point were performed using Malvern ZetaSizer- Nano ZS (Malvern Instruments®) with the same protocol mentioned in the preceding sections. Studies were performed in triplicate and results were determined as the mean±standard deviation (SD).

Solubility studies

For the solubility study, an excess amount of CycA coarse powder, PM, and lyophilized CycA NS was added to the flasks and dispersed in distilled water. The flasks were agitated for 48

hours at 37±0.5 ºC. The samples were filtered with 0.22 µm nylon filters and investigated using a UV spectrophotometer (Agilent Technologies® Cary 60 UV-VIS) at 207 nm. Analysis were done in three times and the mean results and the standard deviations (SD) were calculated.

In vitro dissolution studies

In vitro dissolution studies were performed with CycA coarse powder, the PM, and CycA NS. They were weighed equal to 10 mg CycA and put in hard gelatin capsules with number 00. The study was conducted using USP Apparatus I Basket (Agilent Technologies® 708-DS), rotating at 150 rpm and a temperature of 37±0.5 ºC, according to the USP dissolution method.[31] The USP dissolution medium was 1000 mL of 0.1 N HCl containing 0.5% SDS and also the study was repeated with 1000 mL of 0.1 N HCl without SDS to investigate the efficacy of SDS on dissolution rate. Besides, the dissolution studies were performed in 500 mL of FaSSIF and FeSSIF media which were including several amounts of sodium taurocholate and phospholipids to simulate the *in vivo* fasted and fed state, respectively. Samples were withdrawn from the dissolution medium at predetermined times (5, 10, 20, 30, 45, 60, 90, and 120 min), and then the same amount of fresh medium was added to the dissolution medium (for sink condition). The experiment was repeated three times. The samples were filtered with a 0.22 µm membrane filter and the quantitative analysis of CycA was performed by a validated HPLC method at 205 nm. After analysis, the mean results and the standard deviations (SD) were determined.

Analytical methods

For the purpose of quantification in solubility and dissolution studies, analyzes were made with both UV spectrophotometric method and HPLC chromatographic method.

UV spectrophotometric method was used to determine the CycA concentration in the solubility study. The method was validated according to validation parameters.

HPLC method was used to determine the CycA concentration in four dissolution media. Chromatographic separation was carried out using Agilent® 1220 Infinity LC HPLC with a C18 RP column (150 mm x 4.6 mm, 5µm). The mobile phase consisted of acetonitrile: water (75:25 v/v) and the flow rate was set to 1 mL per minute and the column temperature was at 60°C. The injection volume of samples was 20 µL and the detection of the drug was conducted at 205 nm. The HPLC method was validated for validation parameters such as specificity, linearity, range, accuracy, precision, and robustness, etc.

Data analysis

The one-way ANOVA followed Tukey HSD post hoc test was used to analyze for statistical data at the significance level (α) of 0.05. All results were presented as mean±standard deviation (SD).

RESULTS

Preparation of CycA nanosuspension

HPH method for drug powders in the stabilizer solution is a proper method to prepare NS formulations. While the process parameters determining the final dispersion are homogenization pressure and the number of homogenization cycles (pass number), the formulation parameters are stabilizer types and ratios. Stabilizers have an influence on the long-term physical stability but have no influence on the form of the produced NSs.

The determination of the appropriate stabilization in the preparation of NSs is due to trial and error depending on the active ingredient.[29,32] For NSs, it has been reported that the type of stabilizer, as well as its amount, is crucial, and stability problems may occur in the case of using an insufficient stabilizer.[29] Many studies are reporting the use of stabilizers in combinations to ensure and maintain thermodynamic stability in the preparation of NSs.[28,33-35]

In our study, HPMC (a polymer) and SDS (a surfactant) were used as combined stabilizers and their ratios were investigated on PS, PDI, and ZP values of NSs. PS, PDI, and ZP results of NSs are shown in Figure 2.

ANOVA and interaction for PS, PDI, and ZP results of CycA NSs prepared using HPH method, using HPMC: SDS combination in two ratios (1:0.5 and 1:0.25) with the experimental design are presented in Table 1 and the contour plots are given in Figure 3.

Table 1. ANOVA and interaction for PS, PDI, and ZP results of CycA: HPMC: SDS nanosuspension

The model was found to be significant (p:0.0133) as shown in Table 1, where the interaction of SDS ratio and homogenization cycle in terms of PS was examined. While the effects of SDS ratio (p:0.3175) and the homogenization cycle (p:0.0546) were not significant individually; SDS ratio*homogenization cycle interaction was found to be significant (p:0.0049).

The interaction equation for the model affecting the particle size (PS), A: SDS ratio, B: homogenization cycle, and AB: interaction of A and B, is shown in Eq. 1.

$$PS = +699.87 -82.59*A -217.67*B -351.56*AB \quad (1)$$

The model was found to be significant (p:0.0269) when the interaction of SDS ratio and homogenization cycle was evaluated in terms of PDI, as reported in Table 1. While the SDS ratio (p:0.1937) was not significant individually; the homogenization cycle and SDS ratio*homogenization cycle interaction were found to be significant (p:0.0273 and p:0.0284, respectively).

The interaction equation for the model affecting the polydispersity index (PDI), A: SDS ratio, B: homogenization cycle, and AB: interaction of A and B, is shown in Eq. 2.

$$PDI = +0.63+0.043*A -0.10*B +0.10*AB \quad (2)$$

The model was found to be significant (p<0.0001), when the interaction of SDS ratio and homogenization cycle was evaluated in terms of ZP, as reported in Table 1. SDS ratio, homogenization cycle and SDS ratio*homogenization cycle interaction were all found significant (p<0.0001, p:0.0174 and p:0.0152, respectively).

The interaction equation for the model affecting the zeta potential (ZP), A: SDS ratio, B: homogenization cycle, and AB: interaction of A and B, is shown in Eq. 3.

$$ZP = -32.41 -2.49*A -1.03*B -1.06*AB \quad (3)$$

According to the data and contour plots obtained, as the SDS ratio and homogenization cycle decrease, PS values increase; as the SDS ratio decreases and the homogenization cycle increases, PDI values decrease. In all NSs, ZP values were above -20 mV, which was acceptable, indicating the stability of NS. Hence, the ratio of CycA: HPMC: SDS was determined as 1:1:0.5, and homogenization cycles of 30 were found to be suitable as process parameters to achieve optimum NS with the HPH method.

In a study, ritonavir (RTV) NSs were prepared using the HPH method. The DoE approach was used to explain the impact of the critical formulation parameters. After DoE analysis, the optimum formulation was decided with 4% HPMC and 20 passes.[23] To enhance the water solubility of ziprasidone hydrochloride monohydrate (ZHM), which is one of a BCS Class II drug, the impacts of the formulation and process parameters in NSs prepared using microfluidization method were evaluated by DoE approach. NSs showed the lowest PS value ($p < 0.05$) after 30 homogenization cycles and optimum NSs were ZHM: Vitamin E TPGS 2:1 at 30 passes and ZHM: PVP K30 1:1 at 20 passes.[26] Our results and these studies have shown that increasing the number of passes through the homogenizer causes a decrease in the particle size of the NS.

Surface morphology study

After preparation of NS, CycA NSs obtained after 5 passes, 10 passes, 15 passes, and 30 passes of CycA: HPMC: SDS 1:1:0.5 formulation were lyophilized to examine the effects of homogenization cycle (pass number) on the internal structure (Figure 4).
Surface morphologies of CycA coarse powder, HPMC, SDS, mannitol, PM, and optimum CycA NS were investigated using SEM (Figure 5). SEM images showed that CycA coarse powder was in a crystalline state with sharp edges (Figure 5A). The PM consists of coarse powder, HPMC, SDS, and mannitol, and these components must be seen in the morphological examination of the PM after SEM. According to Figure 5E, HPMC was shown as long fibers, SDS was seen to be spherical and mannitol was found to be crystalline and spiky. CycA in the NS exhibited a sharp surface (Figure 5F).
When the morphological images of NSs are evaluated; it is thought that CycA coarse powder is covered with stabilizers all around and the high energy applied in the high pressure NS production process. The observed morphological results are similar to the literature.[8,26]

X-ray powder diffraction (XRD) study

XRD analysis is an analysis method that is frequently used to explain the crystalline or amorphous structure of substances. XRD studies were conducted to evaluate crystal properties of CycA, HPMC, SDS, mannitol, PM, and optimum CycA NS (Figure 6).
The XRD patterns of SDS and mannitol showed obvious diffraction peaks, although the peaks in SDS were weaker. This suggests that they were crystalline state. Also, the PM was found to be crystalline, this confirmed that the PM had the same properties of CycA, HPMC, SDS, and mannitol. As shown in **Figure 6**, CycA coarse powder, HPMC, and lyophilized CycA NS showed amorphous state. It has been shown by others[36,37] that CycA has amorphous state and our results also showed this situation. It is known that the amorphous structure of NSs can cause positive effects on the solubility and dissolution.[38]

Fourier transformed infrared (FTIR) study

FTIR measurements were done beside XRD analysis to evaluate possible changes in the CycA of the applied pressure during the preparation of the formulations by HPH method. FTIR spectroscopy results of samples are shown in Figure 7.
Figure 7 shows that the characteristic bands of the active substance were the amide carbonyl band at 1623 cm^{-1} and the amide N-H band at 3314 cm^{-1}. When the FTIR results of CycA coarse powder were examined, characteristic CycA bands similar to those in the study of Bertacche et al. were observed at 2960 cm^{-1}.[39] In the HPMC spectrum, the characteristic band was the band belonging to the C-O group at 1052 cm^{-1}; it was the band of RO-SO_2-OR sulfate at 1203 cm^{-1} in the SDS spectrum. The bands that were seen as characteristic of mannitol were O-H bands at 3277 cm^{-1}, C-O bands at 1077 cm^{-1}, and 1016 cm^{-1}. In the FTIR spectrum of the PM, the band at 1624 cm^{-1} belongs to CycA, while the bands at 1248 cm^{-1} and 1206 cm^{-1} belong to SDS. Since the bands seen around 1000 cm^{-1} were thought to belong to HPMC and mannitol, it has been proven that there is no interaction in the spectrum of the PM. In the FTIR spectrum of NSs prepared using the HPH method; characteristic bands of CycA at 1630 cm^{-1}, SDS at 1249 cm^{-1}, mannitol at 1083 cm^{-1}, and HPMC at 1021 cm^{-1} were seen. When this spectrum of NS is evaluated together with the XRD results; it proves that there is no polymorphic change between the active substance and the excipients and that the pressure applied while preparing the formulation and the lyophilization process does not change the physicochemical structure. In a study by Attari et al., characteristic bands of the active substance were observed in olmesartan medoxomil NSs and no interaction was found.[40]

Physical stability study

Stability issues, known as Ostwald ripening, that result in the growth of nano-sized particles is critical for NS formulations. It is important to maintain PS, PDI, and ZP results in the evaluation of physical stability in NSs. The small PDI value in stable NSs indicates that the particles are of similar size and have less tendency to coalesce and grow; also high ZP values

indicate that due to the high electrical charge in the stabilizers, the aggregation of the particles will be prevented.[41]

The stability evaluations of optimum CycA NS was carried out by measuring PS, PDI, and ZP values at 4°C and 25°C (Figure 8). There was no major alteration in PS and ZP results of CycA NSs for 9 months at 4ºC and 25ºC (Figure 8A-D). In accordance with the results of the all stability study, the ZP value was over -20 mV at 4°C and 25°C and indicated that the formulation was physically stable.

Solubility study

The saturation solubility studies were conducted in distilled water with CycA coarse powder, the PM, and lyophilized CycA NS at 37 °C. In Table 2, the water solubility was found to be 6.48 ± 0.88 µg/mL with CycA coarse powder and 8.78 ± 0.38 µg/mL with the PM.[8,18,19] These results demonstrated that the PM improved the water solubility of CycA at about 1.4-times. Also, the solubility of CycA NS was 12.61 ± 1.48 µg/mL and the solubility of CycA improved 1.9-times compared to coarse powder. According to Beauchesne et al.[6], the solubility of CycA in water at 25ºC is 0.04 mg/mL, but in our study the solubility was obtain to be 6.48 ± 0.88 µg/mL. The reason for this difference may be that the source of the active substance used in the studies was different or that the analysis was done in different ways. The results obtained prove that the purpose of increasing the solubility of CycA has been achieved by NS preparation. The reason for this improve is based on the Ostwald-Freundlich equation. According to the equation, the solubility and dissolution improve by increasing the surface area by reducing the particles to nano-size.[42] In a study, a 2.13-times improve in water solubility was found in CycA NSs prepared using HPH method using a combination of HPMC and Soluplus® stabilizer after 30 homogenization cycles.[8] In another study, the solubility of CycA was improved by 4.5-times by wet milled NSs.[18]

In vitro dissolution studies

Dissolution studies were done in various dissolution media with CycA coarse powder, PM, trade product (Sandimmun Neoral®), and CycA NS. In dissolution studies, firstly 0.1 N HCl medium containing 0.5% SLS, which suggested by FDA and USP for Sandimmun Neoral®, was used[31], and then to examine the effects of SLS in this medium on dissolution, the study was repeated with 0.1 N HCl medium without SLS.

In 0.1 N HCl medium containing 0.5% SLS; while CycA coarse powder, PM, and CycA NS showed dissolution over 70%, CycA coarse powder showed no dissolution and CycA NS showed 40% dissolution in the study performed in 0.1 N HCl medium. The trade product showed more than 90% dissolution in both media (Figure 9A and B).

Furthermore, the dissolution studies were performed with FaSSIF media that simulates fasted and FeSSIF media that simulates fed. The physiological conditions (such as bile salts and lecithin) can be simulated in vitro and in vivo prediction can be provided for drugs such as BCS Class II drugs owing to these biorelevant media. Figure 10 shows that the dissolution profiles of CycA coarse powder, trade product, PM, and CycA NS in FaSSIF and FeSSIF media.

CycA coarse powder and PM showed a 15% dissolution in 120 minutes in FaSSIF medium. While the NS showed 70% dissolution, the trade product showed higher dissolution of all samples with 80% dissolution at the and of the 120 minutes (Figure 10A).

While CycA coarse powder and PM showed almost 40% dissolution; the NS prepared using the HPH method showed a 60% dissolution in 120 minutes, in FeSSIF medium, in the other biorelevant media. Similar to the FaSSIF results, the trade product showed 80% dissolution in the FeSSIF medium (Figure 10B).

As a result of the dissolution studies, it was seen that the dissolution profile change between the two media was caused by SLS in these media. In the literature research, it was found that similar results were supporting this result.[26,43]

There are many studies with the idea that NSs can diminish the dissolution variation of drugs with low water solubility in fasting and fed states. It was found that ziprasidone hydrochloride monohydrate (ZHM) NS stabilized with PVP and Vitamin E TPGS showed >95% dissolution in FeSSIF medium and more than 80% in FaSSIF medium.[26] In the dissolution study conducted in FaSSIF medium with NSs prepared using HPH method of five active substances (albendazole, fenofibrate, itraconazole, probucol, and revaprazan hydrochloride) with low water solubility, it was found that NSs showed higher dissolution than microsuspensions in all five active substances. In the pharmacokinetic study with free access to food and water in Male Wistar rats with the same NS formulations; NSs were found to have higher AUC and C_{max} than microsuspensions (for albendazole approximately 2- and 3.2-times, for fenofibrate approximately 2.2- and 3.5-times, for itraconazole approximately 7- and 8.6-times, for probucol approximately 6.4- and 2.9-times, and for revaprazan hydrochloride approximately 1.4- and 2.1-times, respectively) at the end of administration at a dose of 100 mg/kg.[44]
In a study to investigate the impact of particle size on the absorption of aprepitant, a single-pass method of intestinal perfusion (SPIP) in the rat jejunum was used with phosphate buffer, fasted state simulated intestinal fluid (FaSSIF), and fed state simulated intestinal fluid (FeSSIF) as perfusion medium. The results showed that the absorption of aprepitant from the NSs was found equal to all perfusion media (phosphate buffer=FaSSIF=FeSSIF), but food had a noticeable impact on absorption from the microsuspensions (FeSSIF > FaSSIF > phosphate buffer).[45]
To sum up, dissolution studies performed in fasting, fed or both media are important studies to predict oral absorption of new formulations in the gastrointestinal fluids. Thanks to these studies, preliminary data for in vivo studies can be obtained and in vitro/in vivo correlations can be made as a result of supporting in vitro dissolution studies with fasting and fed in vivo studies.

CONCLUSION

In conclusion, CycA NS was successfully obtained by HPH method that one of the top-down production technologies and the DoE approach gave to take into account the impact of critical formulation and process parameters on the dependent variables. The optimum CycA NS was obtained with CycA: HPMC: SDS 1:1:0.5 ratio and 30 homogenization cycles after determining the interactions statistically. According to the stability results at 4ºC and 25ºC, it was found that the NSs remained physically stable for 9 months. CycA NSs improved water solubility of CycA with 1.9-times and 1.4-times compared to coarse powder and PM, respectively. Also, CycA NS showed higher dissolution than CycA coarse powder in 0.1 N HCl media, FaSSIF, and FeSSIF media. When characterization, solubility, and dissolution results were evaluated together, it was found that CycA NS prepared with HPH method was found to be successful and this study proved that the nanosuspension could be a encouraging strategy for improving the solubility and dissolution of CycA for oral administration.

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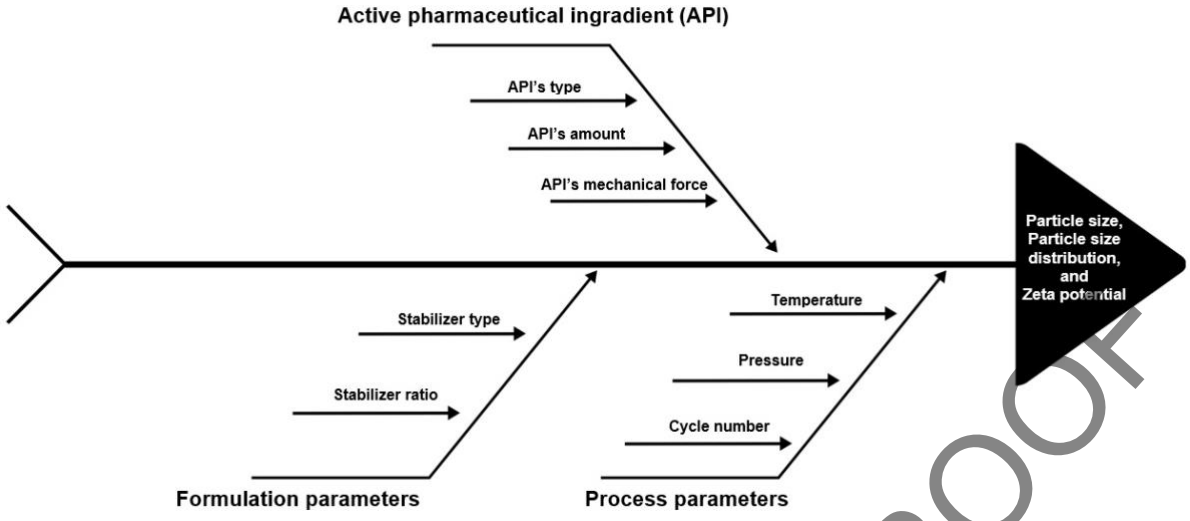


Figure 1. Fishbone diagram with process and formulation parameters for HPH technology

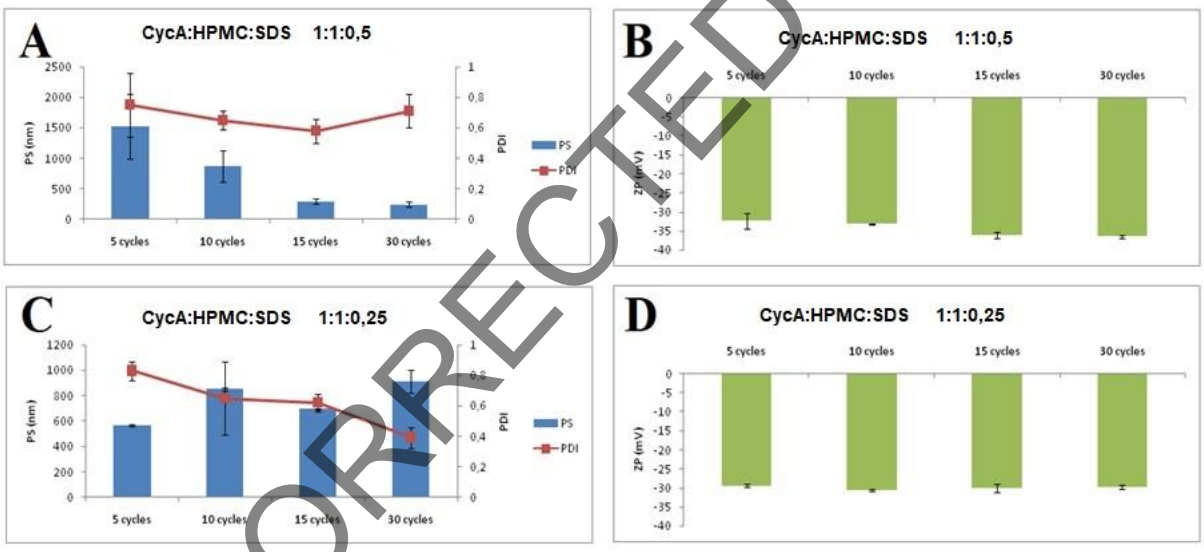


Figure 2. PS, PDI, and ZP results of CycA nanosuspension stabilized with HPMC: SDS (1:0.5 and 1:0.25) (mean±SD; n=3)

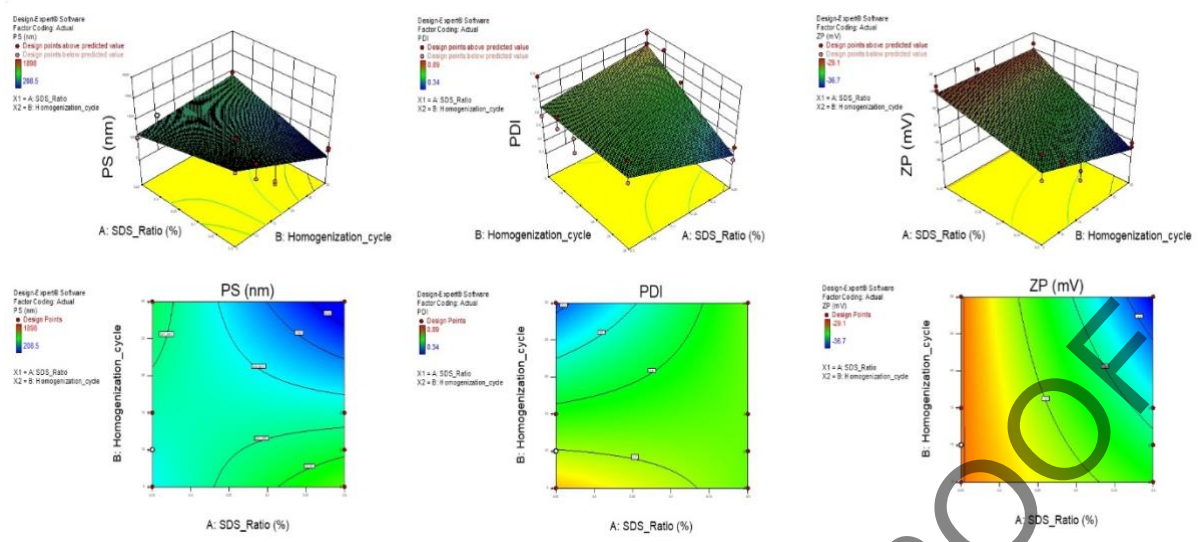


Figure 3. 3D surface (top) and contour (down) graphs demonstrating the effects of the SDS ratio and homogenization cycle on PS, PDI, and ZP values

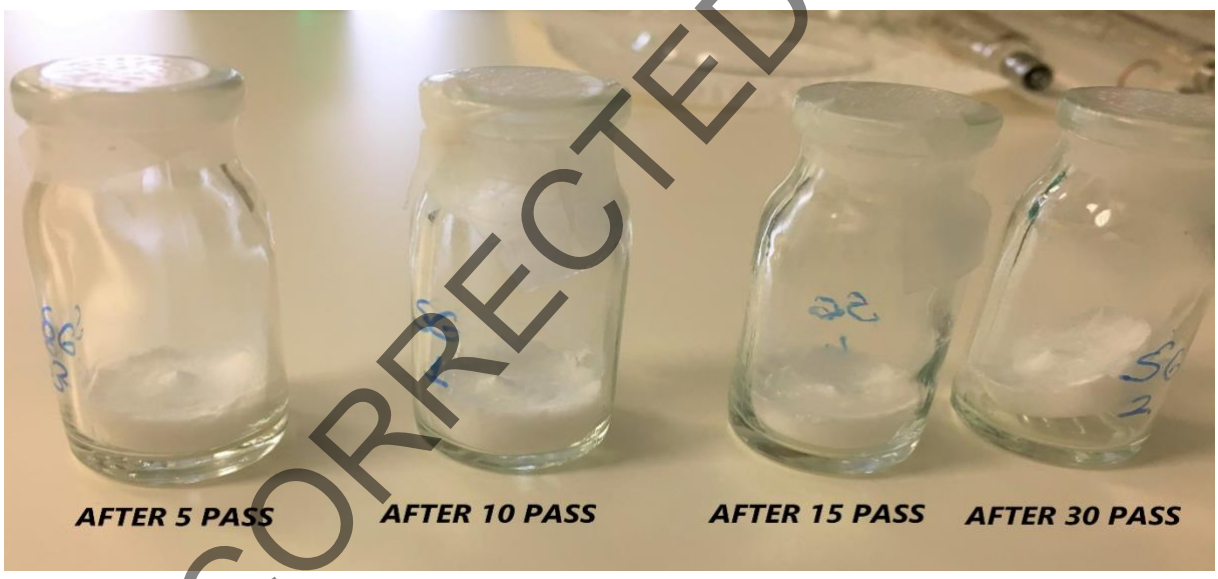
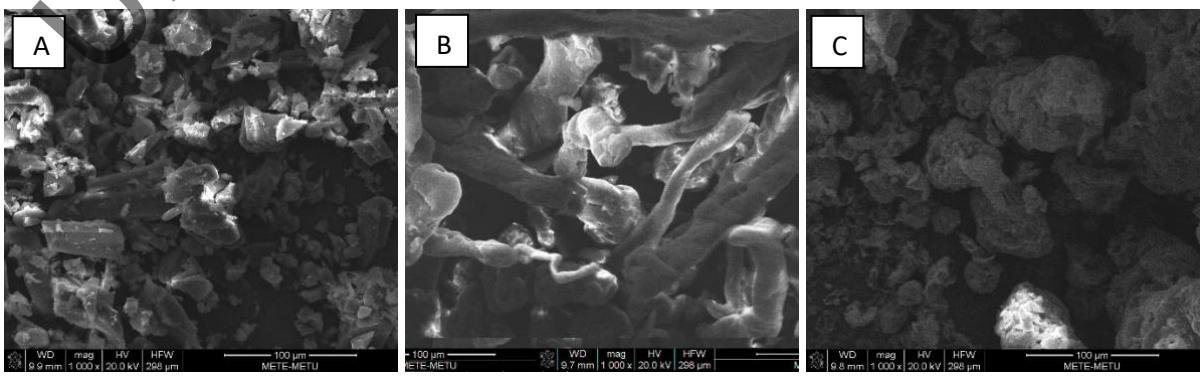


Figure 4. Lyophilized CycA nanosuspensions composed of CycA: HPMC: SDS (1:1:0.5) after different homogenization cycles (pass number)



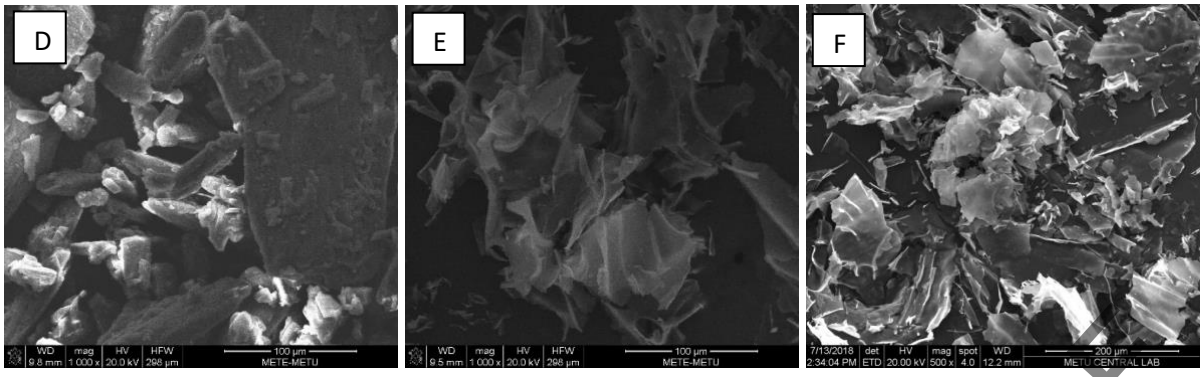


Figure 5. SEM images of (A) CycA coarse powder (mag. 1000x), (B) HPMC (mag. 1000x), (C) SDS (mag. 1000x), (D) mannitol (mag. 1000x), (E) the physical mixture (mag. 1000x), (F) CycA nanosuspension (mag. 500x)

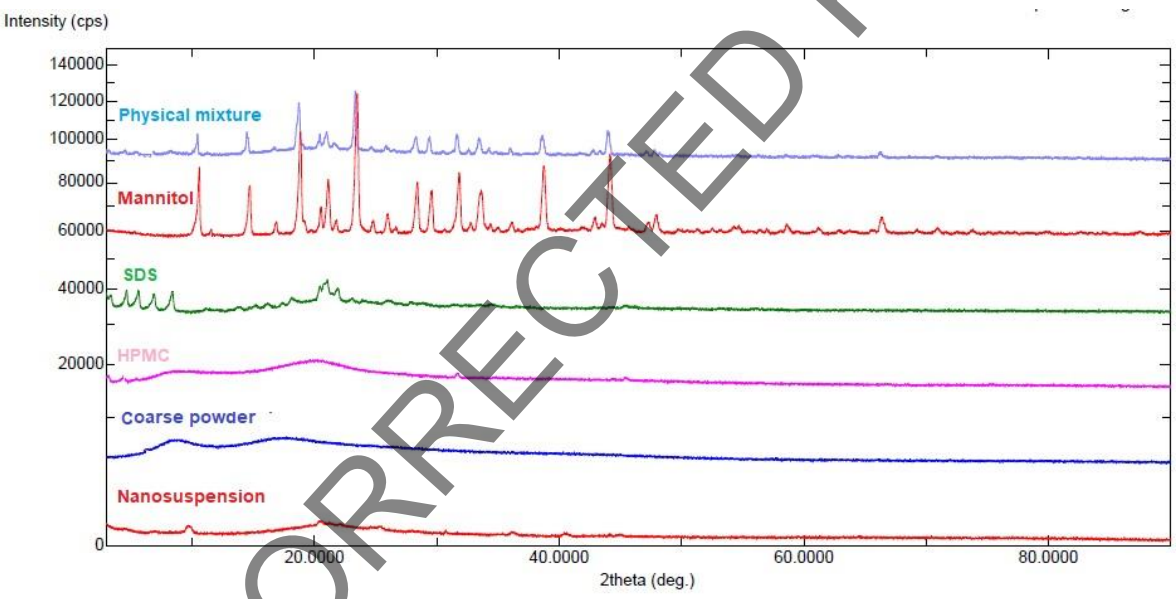


Figure 6. X-ray diffraction patterns of CycA nanosuspension, CycA coarse powder, HPMC, SDS, mannitol, and physical mixture

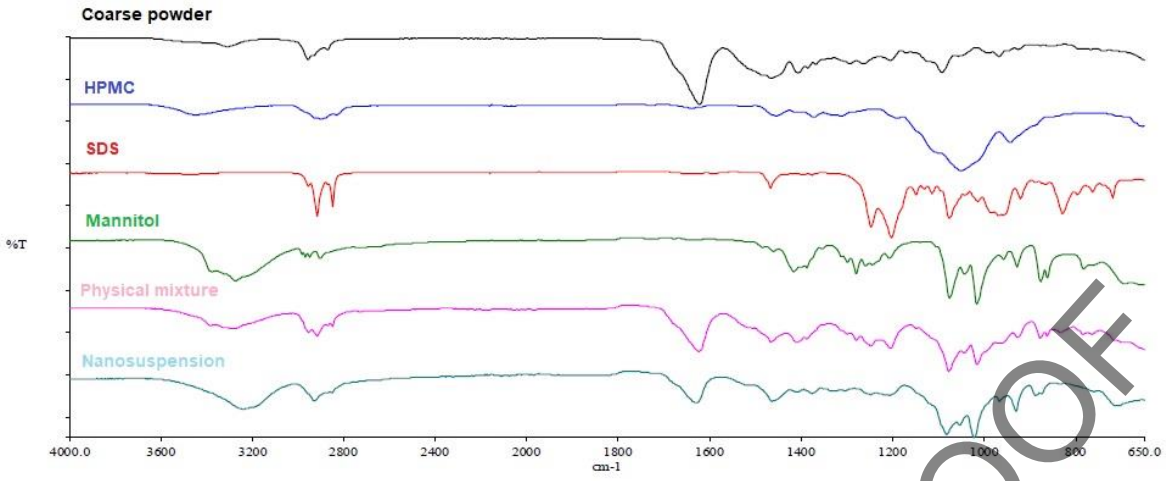


Figure 7. FTIR spectrums of CycA coarse powder, HPMC, SDS, mannitol, physical mixture, and CycA nanosuspension

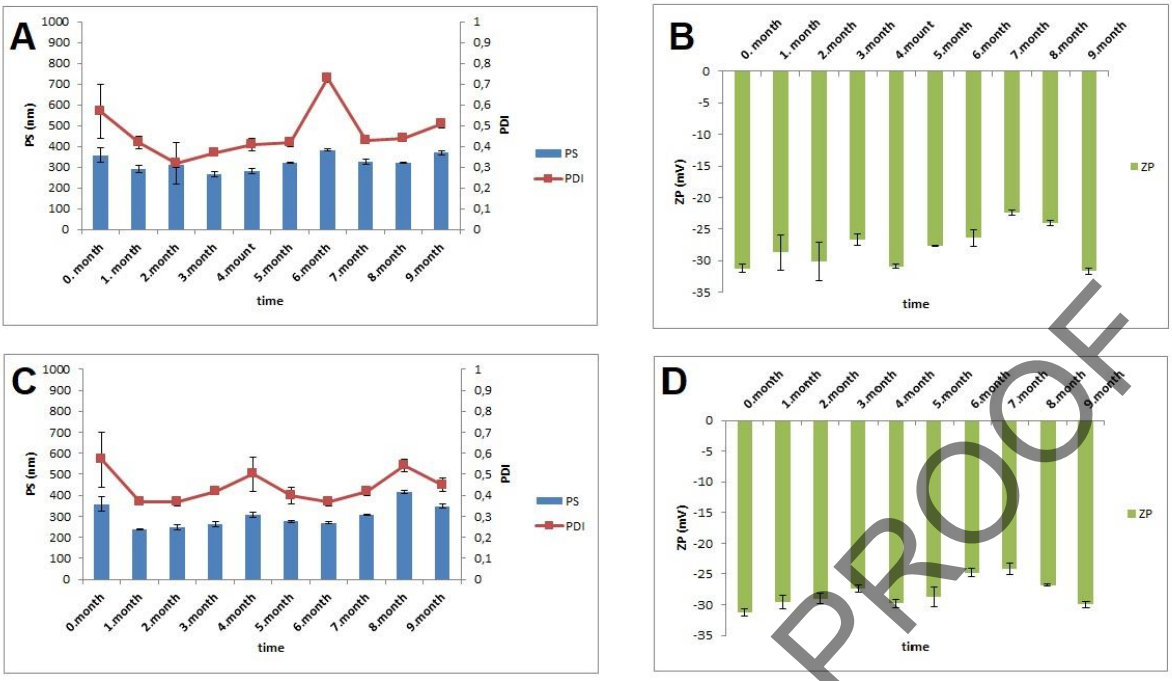


Figure 8. Physical stability results of CycA nanosuspension; 4ºC (A and B) and 25ºC (C and D) (not statistical significant)

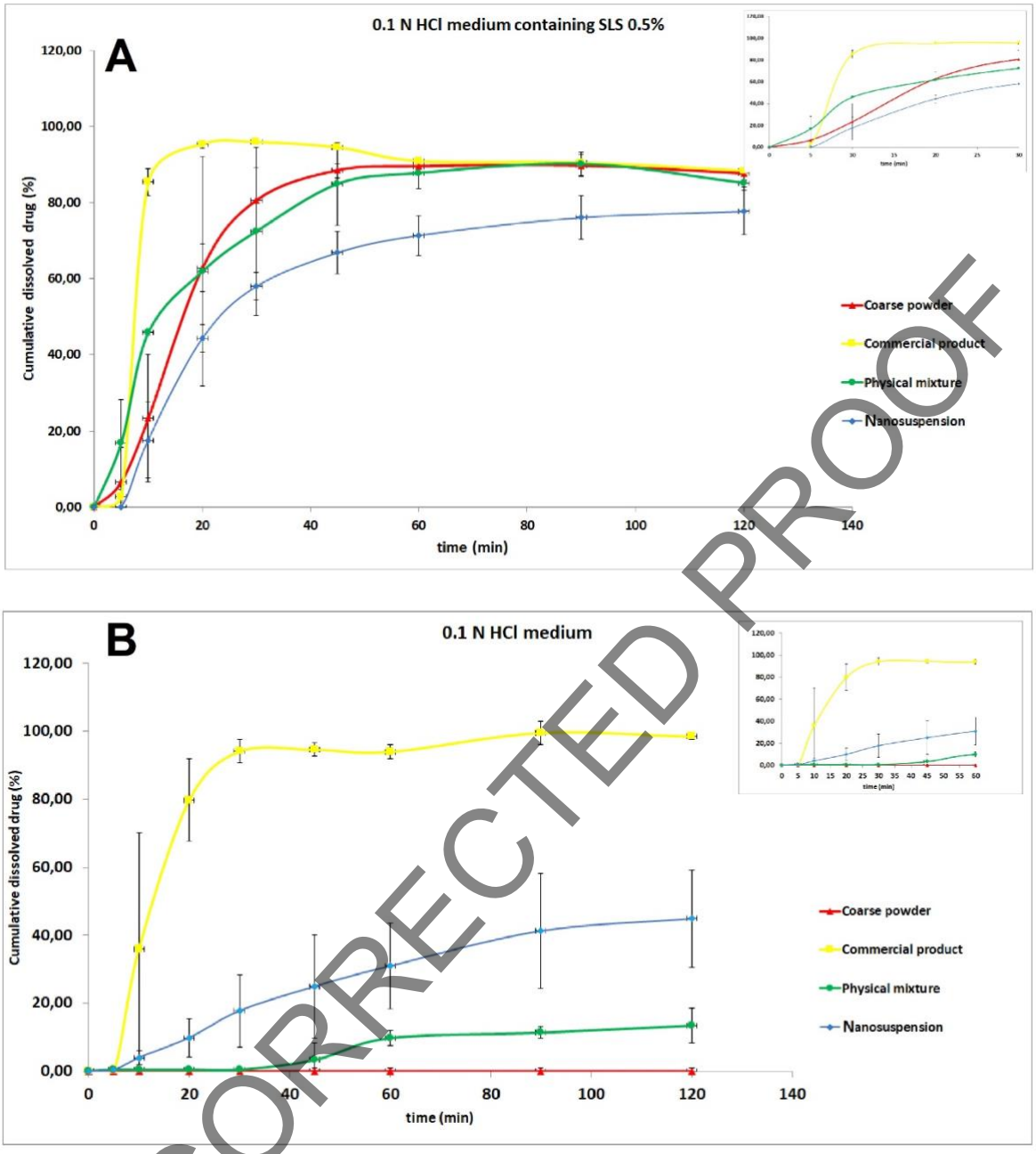


Figure 9. Dissolution profiles of CycA coarse powder, commercial product, physical mixture, CycA nanosuspension in (A) 0.1 N HCl medium containing 0.5% SDS and (B) 0.1 N HCl medium

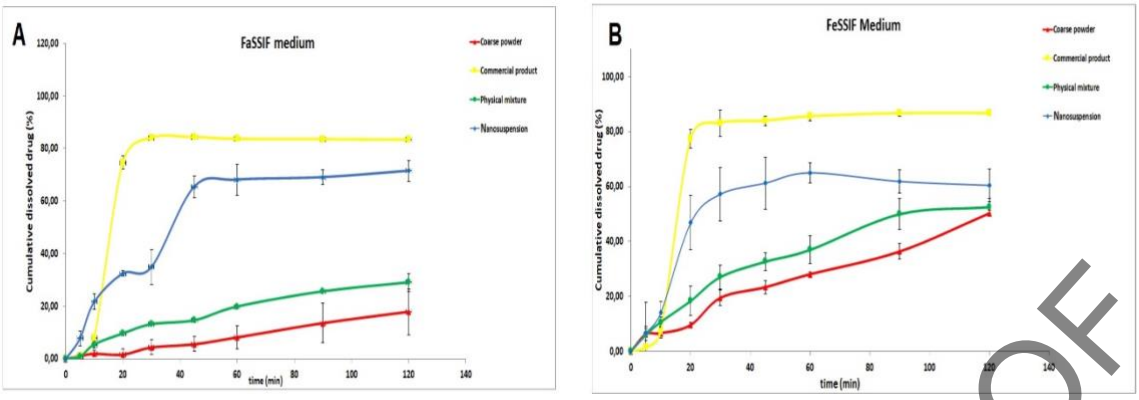


Figure 10. Dissolution profiles of CycA coarse powder, commercial product, physical mixture, CycA nanosuspension in (A) FaSSIF medium and (B) FeSSIF medium

Table 1. ANOVA and interaction for PS, PDI, and ZP results of CycA: HPMC: SDS nanosuspension

Source	**PS**		**PDI**		**ZP**	
	F value	**P-value**	**F value**	**P-value**	**F value**	**P-value**
Model	*5.46*	*0.0133*	*4.36*	*0.0269*	*27.21*	*< 0.0001*
A-SDS Ratio	*1.09*	*0.3175*	*1.90*	*0.1937*	*73.62*	*< 0.0001*
B-Homogenization cycle	*4.54*	*0.0546*	*6.31*	*0.0273*	*7.60*	*0.0174*
AB	*11.83*	*0.0049*	*6.20*	*0.0284*	*8.00*	*0.0152*

Table 2. Saturation solubility of CycA coarse powder, the physical mixture, and CycA nanosuspensions (mean ± SD; n=3)

Sample	**Saturation solubility (µg/mL)**
CycA coarse powder	*6.48 ± 0.88*
Physical mixture	*8.78 ± 0.38*
CycA nanosuspension	*12.61 ± 1.48*