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ORIGINAL ARTICLE

Development of Cyclosporine A Nanosuspension Using an Experimental Design Based on Response Surface Methodology: *In Vitro* Evaluations

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ABSTRACT

Objectives: This study aimed to develop nanosuspensions (NSs) of cyclosporine A (CycA) using a top-down technology [high-pressure homogenization-(HPH)] for oral administration.

Materials and Methods: Formulas 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 NS using the Design of Experiment (DoE). After lyophilization, differential scanning calorimetry, X-ray diffraction, fourier-transform infrared spectroscopy, and morphological evaluation with scanning electron microscopy were performed. Stability studies were conducted at 4 ˚C and 25 ˚C storage conditions. The solubility of the optimum CycA NS was investigated by comparing it with a coarse CycA powder and a physical mixture (PM). *In vitro* dissolution studies were conducted in four media using United States Pharmacopeia apparatus I.

Results: PS, PDI, and ZP values for the NS were approximately 250 nm, 0.6, and 35 mV, respectively. Under storage conditions, the CycA NS exhibited significant physical stability at both 4 ˚C and 25 ˚C for 9 months. The solubility of CycA was improved 1.9 and 1.4 times by NS in the presence of CycA powder and PM, respectively. CycA NS exhibited higher dissolution than CycA coarse powder in 0.1 N HCl, fasted simulated intestinal fluid, and fed simulated intestinal fluid.

Conclusion: CycA NS was successfully developed using the DoE approach with the HPH method with HPMC:SDS combination in a 1:0.5 ratio, and the solubility and dissolution of CycA in the NS were improved.

Keywords: Cyclosporine A, nanosuspension, high pressure homogenization, solubility, dissolution

INTRODUCTION

Approximately 60% of drugs have low solubility. This condition affects the pharmacokinetics and pharmacodynamics of these drugs, resulting in low dissolution and bioavailability when they are 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. Low solubility also 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 composed of 11 amino acids that was first isolated from the fungal extract of *Tolypocladium inflatum* in 1973.[2] CycA, one of the calcineurin inhibitors, is an 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, which, under normal circumstances,

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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, and 11 are responsible for the immunosuppressive effect.[5] The solubility of CycA in water at 25 ˚C 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.[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® because 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), prepare products at a lower cost, and reduce inter-and intravariation and fasting-fed variability. Many studies with current opinions and different drug delivery systems have been included in the literature to improve the solubility and dissolution of CycA, to provide higher blood concentrations, and to decrease toxicity.[3,8,9]

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

The techniques used for preparing NSs are classified into two main approaches; "top-down" and "bottom-up" technologies. In bottom-up technology, the molecule is first dissolved in a solvent, and then an insoluble solution is added to form a precipitate, resulting in particles in nanometer sizes.[16] Top-down technologies include wet milling (WM) and high-pressure homogenization (HPH), while bottom-up technologies include precipitation. In the WM or pearl milling/ball milling method, the drug macrosuspension is placed in a milling container, which 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 second most commonly used top-down method is the HPH method. Microfluidization and piston-gap homogenization are two homogenization principles currently used. Microfluidization is a jet stream principle; the suspension is accelerated and passes at high speed through a 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 NS 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.

NSs are prepared using surfactants such as sodium dodecyl sulfate (SDS), tween 80, vitamin ED-α-Tocopherol polyethylene glycol 1000 succinate, poloxamer, and polymeric stabilizers such as hydroxypropyl methylcellulose (HPMC), polyvinylpyrrolidone (PVP), and polyvinyl alcohol. 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 aimed at ensuring the quality of pharmaceutical products and achieving the most proper formulation have gained interest in the pharmaceutical industry. The effects of critical formulation and/or process parameters can be determined by optimizing the formulation and/or process requirements using the design of experiment (DoE), which is one of these approaches. Therefore, the effects of the independent variables, which are thought to be effective in the formulation, on the dependent variables can be successfully investigated experimentally.[30] In addition to many drug delivery system studies using DoE, studies on NS preparation have become common in the literature.[8,14,23,25,26] In Figure 1, the parameters that affect PS, polydispersity index (PDI), PDI and zeta potential (ZP) in NS formulations obtained by HPH technology are shown by the fishbone diagram.

As mentioned above, there remains a need for a novel formulation of CycA to be developed for oral administration. The Sandimmun Neoral® microemulsion formulation is expensive and requires many excipients, and Cremophor®RH40, which is included as a surfactant in its composition, has a toxic effect; complicates its use in therapy. Thus, studies on increasing the 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 describes the preparation of CycA NS by HPH using 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 using the DoE approach in the formulation development process.

Numerous results have been obtained in our previous studies on CycA NSs. 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

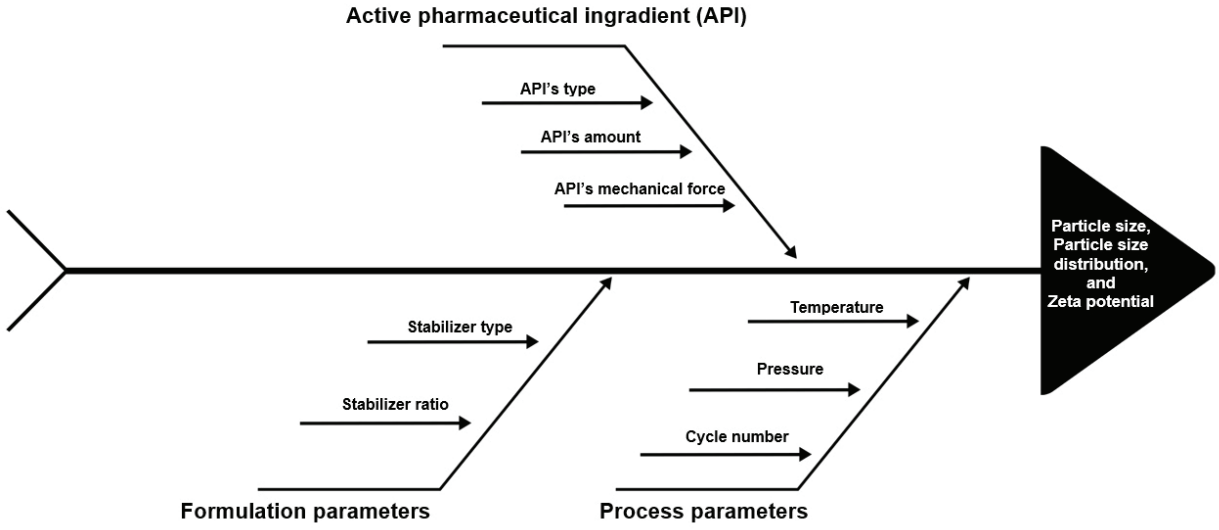

Figure 1. Fishbone diagram showing the process and formulation parameters of HPH
HPH: High-pressure homogenization

found to be the most appropriate ratio. Characterization studies showed that the solubility improved by 2.1-fold compared to the coarse powder.[8] In order to further this study, CycA NSs were prepared using the WM 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 optimal ratio after DoE and formulation were examined *in vivo*. The solubility improved 4.5-fold compared to the coarse powder and was higher in the *in vitro* fed simulated intestinal fluid (FeSSIF) medium than in the trade product. The pharmacokinetic study indicated that area under the curve $(AUC)_{0-24}$ values of CycA NS were 2.09- and 5.51-fold higher than those of coarse powder in fasted and fed situations, respectively.[18] When the permeability of this formulation in Caco-2 cells was examined, NS showed improved CycA transport by 5 and 1.5 times, respectively, compared with coarse CycA powder and trade product (Sandimmun Neoral®).[19]

Based on this information, this study aimed to prepare CycA NSs using HPH technology by using a polymer (HPMC) and surfactant (SDS) combination based on previous knowledge and to determine the optimum ratio using the DoE approach. HPMC was selected because it was previously proven to be an efficient stabilizer for NSs.[21,23] SDS with a surfactant structure is an electrostatic stabilizer that allows high ZP loading on the surface of particles and therefore is widely used.[13,20,23] SDS can migrate to the solid-liquid intersurface and provide an electrostatic barrier against the aggregation of nanometer-sized particles.[10] One of the main objectives of this study was to examine the effect of the surfactant ratio on PS, PDI, and ZP in formulations prepared when a polymeric stabilizer (HPMC) and surfactant (SDS) were 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 (PS, PDI, and ZP) were evaluated using the DoE approach. As a result of the DoE analysis, the optimum formulation was determined, and characterization studies were conducted using this formulation. The physical stability (PS and ZP results) of the optimum formulation for 9 months at 4 °C and 25 °C was evaluated. The solubility of the optimum CycA NS were was compared with those of coarse powder and physical mixture (PM). For CycA dissolution medium, fasted simulated intestinal fluid (FaSSIF) and FeSSIF dissolution studies, NSs were compared with coarse powder, PM, and a trade product (Sandimmun Neoral®).

MATERIALS AND METHODS

Materials

CycA was provided as a gift by the Deva Drug Company (Türkiye). HPMC was obtained from Colorcon (USA). SDS and D (-) mannitol were purchased from Merck (Germany). SIF® Powder was purchased from Biorelevant© (UK).

Preparation of the CycA NS

The HPH method (microfluidization technique) involved 0.2 g (1%) HPMC and 0.1 or 0.05 g (0.5% or 0.25%) SDS dissolved in distilled water. In the second step, CycA powder (1% *w/w*) was dispersed in this solution using a magnetic stirrer at 1000 rpm for 20 minutes. To prevent chamber blockage of the high-pressure homogenizer (Microfluidics LV1 with a Z-type 84 µm chamber), UltraTurrax (Heidolph® Silent Crusher) was used at 15000 rpm for 10 minutes to reduce the PS of this suspension. Finally, this suspension was transported to Microfluidics LV1 (Microfluidizer®) and homogenized for different homogenization cycles at 30,000 psi.

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 on the HPH method, and their impacts on dependent variables [PS (Y_1), PDI (Y_2), and ZP (Y_3)] were assessed using DoE. The process

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

PS, PDI, and ZP studies

PS, PDI, and ZP measurements, which are prominent results regarding nanodrug delivery systems, were conducted at 25 ˚C by dynamic light scattering method using a particle sizer (Malvern Instruments® ZetaSizer-Nano ZS). For PS and PS measurement, the first 750 µL of NS was added to the sample measuring cup and topped with up to 1500 µL of distilled water. At the end of this dilution, the diluted sample was placed in the PS and ZP measurement cuvettes. Each sample was measured at least three times, and the results were calculated as the mean ± standard deviation (SD).

Preparation of the PM

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

Lyophilization of the CycA NS

Lyophilization of NS is essential for long-term stability and the generation of solid dosage forms. Lyophilization was performed after the PS, PDI, and ZP measurements of the NS were prepared by HPH. Mannitol was chosen as the cryoprotectant for the formulations, and the CycA: mannitol ratio was 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 morphologies of the powder samples (CycA coarse powder, PM, and lyophilized NS) were observed 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 morphological images were monitored using a microscope (Quanta® 400F).

X-ray powder diffraction (XRD) study

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

Fourier transform infrared (FTIR) spectroscopy

The FTIR spectra of CycA coarse powder, PM, and CycA NS were examined using a spectrometer (Perkin Elmer® Spectrum 400 Attenuated Total Reflectance-FTIR). A scanning range of 650-4000 cm^{-1} and the discrimination power of 1 cm^{-1} were selected for the measurements.

Physical stability assessment

After preparing the optimal CycA NS, PS, PDI, and ZP values were initially measured. The physical stability studies for the optimal CycA NS were initiated by storing the formulation at two temperatures (4 ˚C and 25 ˚C), and the measurements were repeated for 9 months after each month of storage. PS, PDI, and ZP values at designated time points were measured using a Malvern ZetaSizer-Nano ZS (Malvern Instruments®) with the same protocol described in the preceding sections. Studies were conducted in triplicate, and results were determined as the mean ± 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 ˚C. The samples were filtered with 0.22 µm nylon filters and were investigated using an ultraviolet (UV) spectrophotometer (Agilent Technologies® Cary 60 UV-visible spectroscopy) at 207 nm. Analysis was performed thrice, and the mean results and SDs were calculated.

In vitro dissolution studies

In vitro dissolution studies were conducted using CycA coarse powder, PM, and NS. They were weighed to 10 mg and placed in hard gelatin capsules with number 00. The study was conducted using a USP Apparatus I Basket (Agilent Technologies® 708-DS) rotating at 150 rpm and a temperature of 37 ± 0.5 ˚C, according to the USP dissolution method.[31] The USP dissolution medium was 1000 mL of 0.1 N HCl containing 0.5% SDS, and the study was repeated with 1000 mL of 0.1 N HCl without SDS to investigate the efficacy of SDS on the dissolution rate. In addition, dissolution studies were performed in 500 mL of FaSSIF or FeSSIF media, which included several amounts of sodium taurocholate and phospholipids, to simulate the *in vivo* fasting and feeding state, respectively. Samples were withdrawn from the dissolution medium at predetermined intervals (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 using a validated HPLC method at 205 nm. The mean results and standard deviations (SD) were determined.

Analytical methods

For quantification in solubility and dissolution studies, analyses were performed using both UV spectrophotometric and HPLC chromatographic methods.

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

The HPLC method was used to determine the CycA concentrations in the four dissolution media.

Chromatographic separation was performed using an Agilent® 1220 Infinity LC HPLC system with a C18 RP column (150 mm x 4.6 mm, 5 µm). The mobile phase consisted of acetonitrile:

water (75:25 *v/v*), the flow rate was set to 1 mL per minute and the column temperature was 60°C. The injection volume of the samples was 20 µL and the detection of the drug was conducted at 205 nm. The HPLC method was validated for specificity, linearity, range, accuracy, precision, and robustness.

Statistical analysis

Statistical analysis was performed using SPSS software (version 22.0, IBM Corp., USA). A One-Way analysis of variance followed by Tukey's HSD post-hoc test was used to analyze statistical data at a significance level (α) of 0.05. All results are presented as mean ± standard deviation (SD).

RESULTS

Preparation of the CycA NS

The HPH method for preparing drug powders in stabilizer solutions is a suitable method for preparing NS formulations. While the process parameters determining the final dispersion are the homogenization pressure and the number of homogenization cycles (pass number), the formulation parameters are the stabilizer types and ratios. Stabilizers influence the long-term physical stability but do not influence the form of the produced NSs.

The appropriate stabilization of NSs is determined by 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 when using an insufficient stabilizer.[29] Many studies have reported the use of stabilizers in combination 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 to determine the PS, PDI, and ZP values of NSs. The PS, PDI, and ZP results of NSs are shown in Figure 2.

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

Surface morphology study

After preparation of the NS, CycA NSs obtained after 5 passes, 10 passes, 15 passes, and 30 passes of the CycA: HPMC: SDS 1:1:0.5 formulation were lyophilized to examine the effects of the homogenization cycle (pass number) on the internal structure (Figure 4).

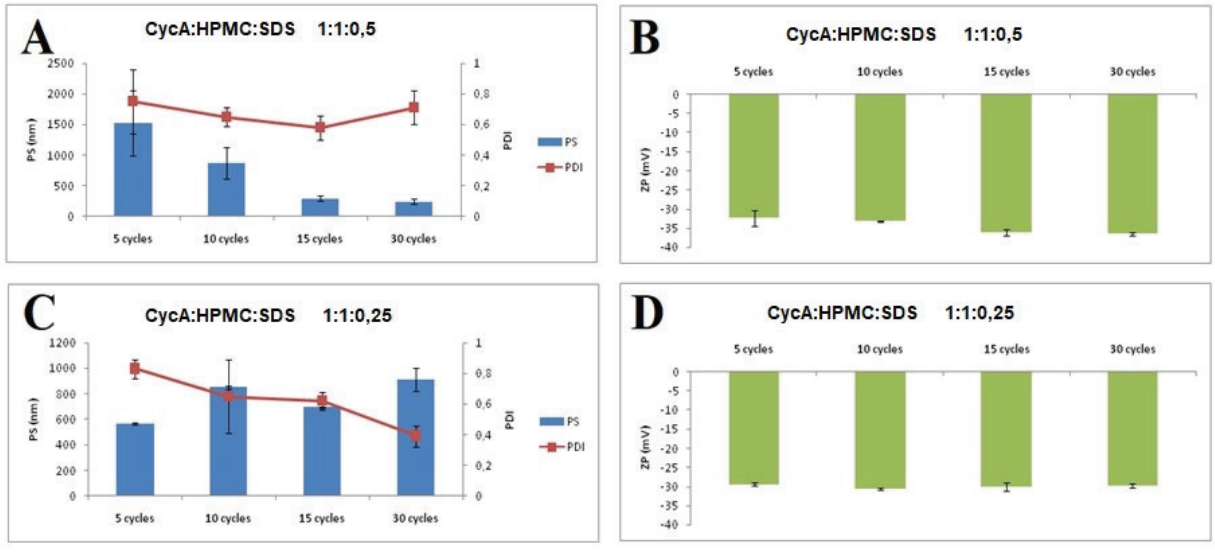

Figure 2. PS, PDI, and ZP results for the CycA NS stabilized with HPMC: SDS (1:0.5 and 1:0.25) (mean ± SD; n= 3)

PS: Particle size, PDI: Polydispersity index, ZP: Zeta potential, CycA: Cyclosporine A, NS: Nanosuspension, HPMC: Hydroxypropyl methylcellulose, SDS: Sodium dodecyl sulfate, SD: Standard deviation

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

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

ANOVA: Analysis of variance, CycA: Cyclosporine A, HPMC: Hydroxypropyl methylcellulose, SDS: Sodium dodecyl sulfate, NS: Nanosuspension, PS: Particle size, PDI: Polydispersity index

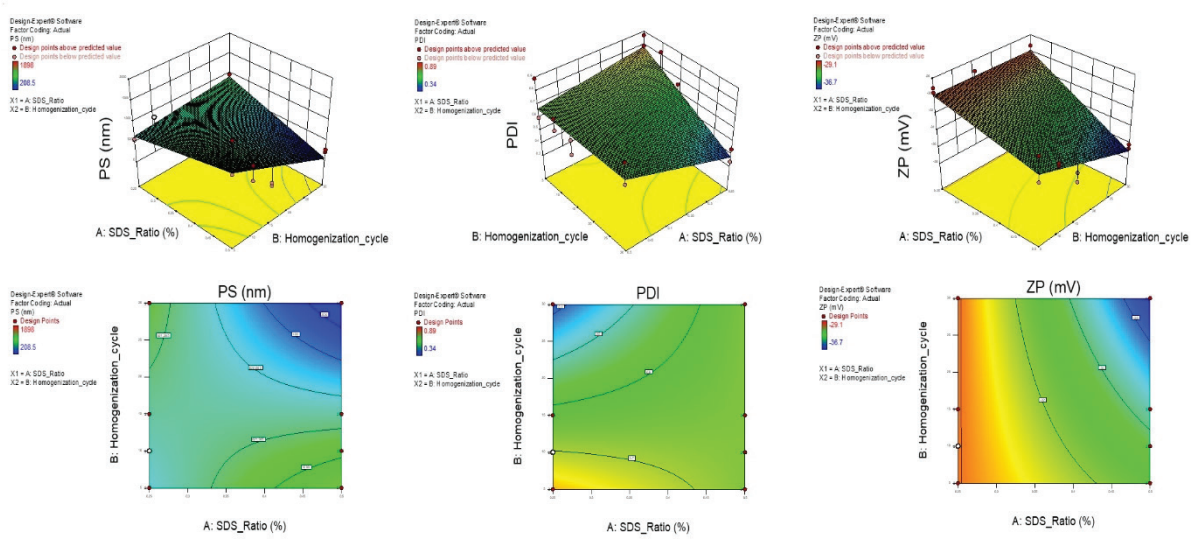

Figure 3. 3D surface (top) and contour (down) graphs illustrating the effects of the SDS ratio and homogenization cycle on PS, PDI, and ZP
PS: Particle size, PDI: Polydispersity index, ZP: Zeta potential, SDS: Sodium dodecyl sulfate

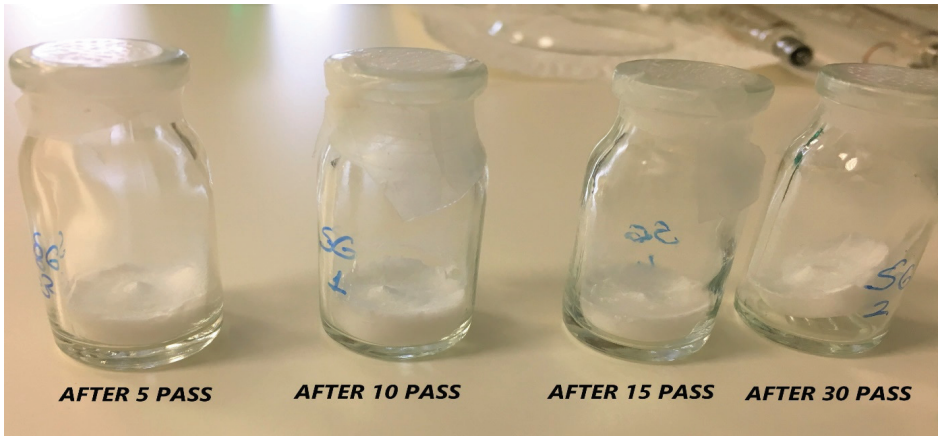

Figure 4. Lyophilized CycA NSs composed of CycA: HPMC: SDS (1:1:0.5) after different homogenization cycles (pass number)
CycA: Cyclosporine A, NSs: Nanosuspensions, HPMC: Hydroxypropyl methylcellulose, SDS: Sodium dodecyl sulfate

The surface morphologies of CycA coarse powder, HPMC, SDS, mannitol, PM, and the optimal CycA NS were investigated using SEM (Figure 5). The SEM images showed that the 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 should be observed 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).

XRD study

XRD analysis is a method that is frequently used to explain the crystalline or amorphous structure of substances. XRD studies were conducted to evaluate the crystal properties of CycA, HPMC, SDS, mannitol, PM, and the optimal CycA NS (Figure 6).

FTIR spectroscopy

FTIR measurements were performed in addition to XRD analysis to evaluate possible changes in CycA under the applied pressure during the preparation of the formulations by the HPH method. The FTIR spectroscopy results of the samples are shown in Figure 7.

Physical stability assessment

The stability evaluation of the optimal CycA NS was carried out by measuring the PS, PDI, and ZP at 4 °C and 25 °C (Figure 8). There were no major alterations in the PS and ZP results of CycA NSs for 9 months at 4 °C and 25 °C (Figure 8A-D). In accordance with the results of all stability studies, the ZP value was › 20 mV at 4 °C and 25 °C, indicating that the formulation was physically stable.

Solubility study

Saturation solubility studies were conducted in distilled water with CycA coarse powder, 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 demonstrate that PM improved the water solubility of CycA by approximately 1.4-fold. Furthermore, the

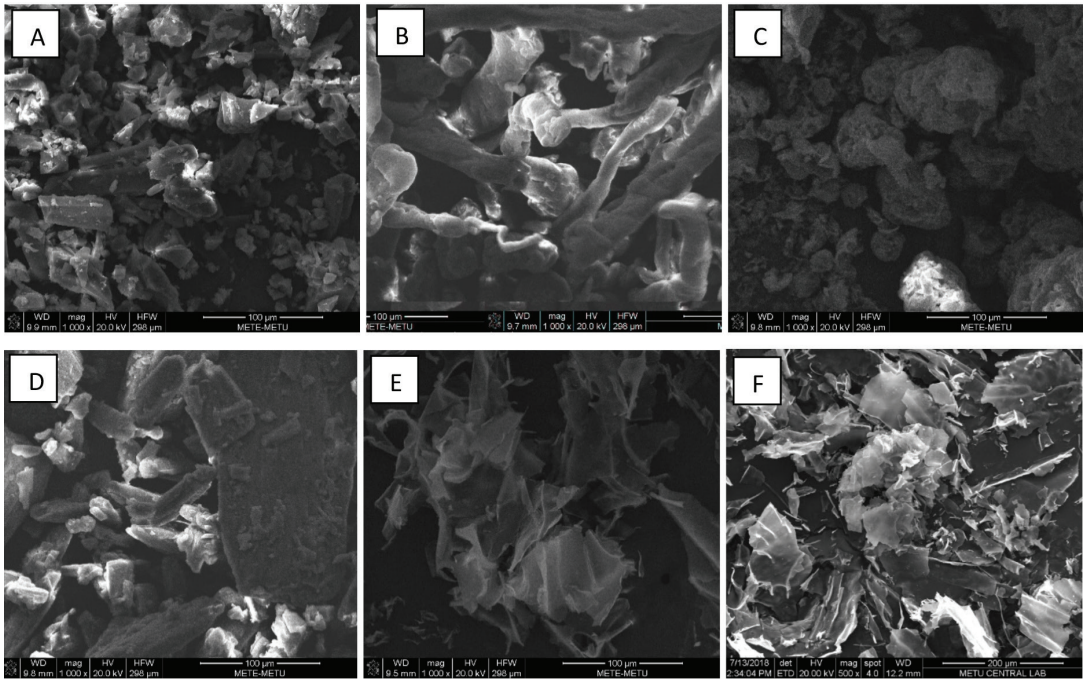

Figure 5. SEM images of (A) CycA coarse powder (mag. 1000x), (B) HPMC (mag. 1000x), (C) SDS (mag. 1000x), (D) mannitol (mag. 1000x), (E) the PM (mag. 1000x), (F) the CycA NS (mag. 500x)

CycA: Cyclosporine A, NSs: Nanosuspensions, HPMC: Hydroxypropyl methylcellulose, SDS: Sodium dodecyl sulfate, PM: Physical mixture, mag.: Magnification, SEM: Scanning electron microscopy

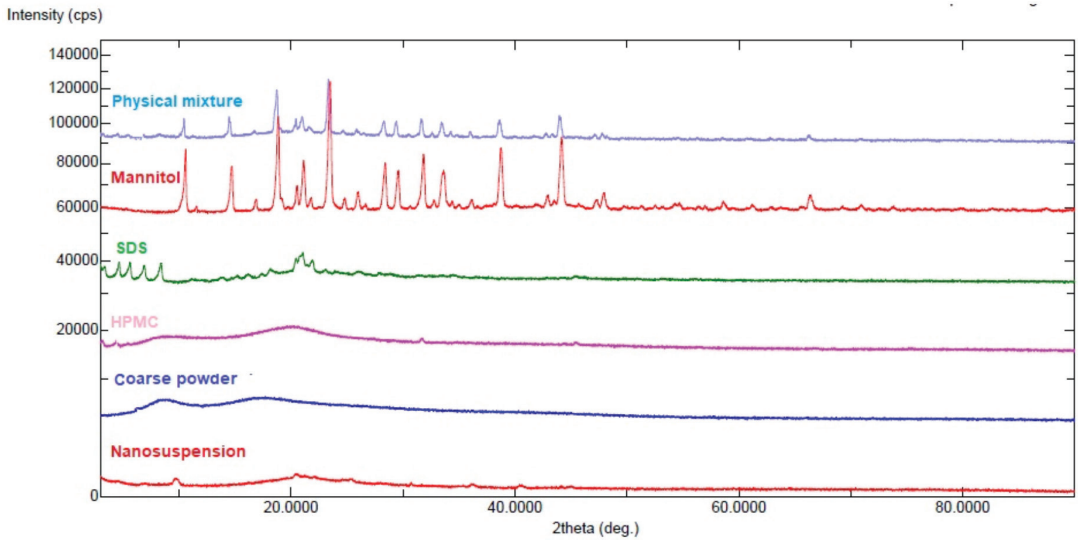

Figure 6. XRD patterns of CycA NS, CycA coarse powder, HPMC, SDS, mannitol, and the PM

XRD: X-ray powder diffraction, CycA: Cyclosporine A, NS: Nanosuspension, HPMC: Hydroxypropyl methylcellulose, SDS: Sodium dodecyl sulfate, PM: Physical mixture

solubility of CycA NS was 12.61 ± 1.48 µg/mL, and the solubility of CycA improved 1.9-fold compared to the coarse powder. According to Beauchesne et al.,[6] the solubility of CycA in water at 25 ˚C is 0.04 mg/mL, but in our study, the solubility was obtained to be 6.48 ± 0.88 µg/mL. The reason for this difference may be that the sources of the active substances used in the studies were different or that the analysis was performed in different ways.

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

Sample	Saturation solubility (µg/mL)
CycA coarse powder	6.48 ± 0.88
PM	8.78 ± 0.38
CycA NS	12.61 ± 1.48

CycA: Cyclosporine A, PM: Physical mixture, NS: Nanosuspension, SD: Standard deviation

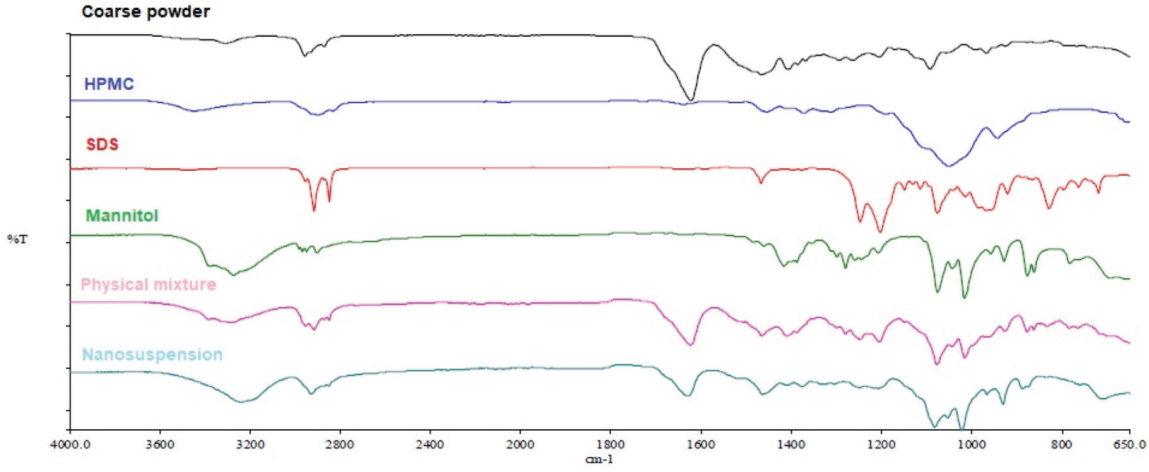

Figure 7. FTIR spectra of the CycA coarse powder, HPMC, SDS, mannitol, PM, and CycA NS

FTIR: Fourier transform infrared radiation, CycA: Cyclosporine A, HPMC: Hydroxypropyl methylcellulose, SDS: Sodium dodecyl sulfate, NS: Nanosuspension, PM: Physical mixture

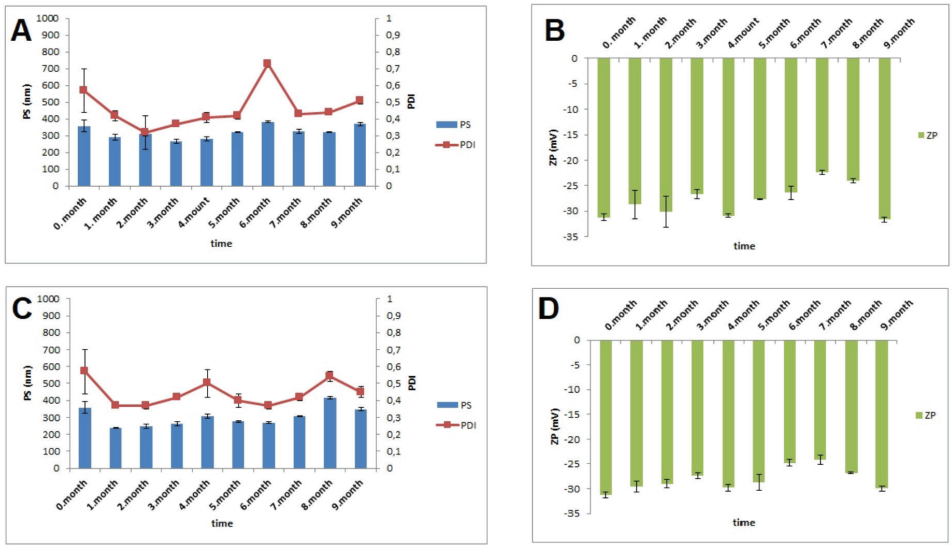

Figure 8. Physical stability results of CycA NS; 4 °C (A and B) and 25 °C (C and D) (not statistical significant)

CycA: Cyclosporine A, NS: Nanosuspension

In vitro dissolution studies

Dissolution studies were conducted in various dissolution media containing CycA coarse powder, PM, a trade product (Sandimmun Neoral®), and CycA NS. In dissolution studies, first, 0.1 N HCl medium containing 0.5% sodium lauryl sulfate, as suggested by the 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; 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, dissolution studies were conducted with FaSSIF and FeSSIF media that simulate fasting and feeding. The physiological conditions (such as bile salts and lecithin) can be simulated *in vitro*, and *in vivo* predictions can be provided for drugs such as BCS Class II drugs using these irrelevant media. Figure 10 shows the dissolution profiles of CycA coarse powder, the trade product PM, and CycA NS in the FaSSIF and FeSSIF media.

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

While CycA coarse powder and PM showed almost 40% dissolution; the NS prepared using the HPH method showed 60% dissolution in 120 minutes in FeSSIF medium and other

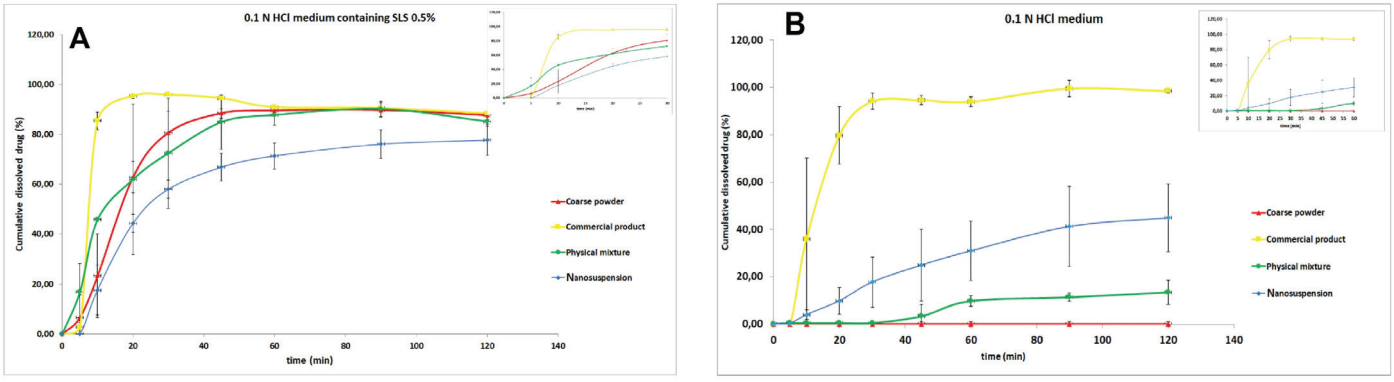

Figure 9. Dissolution profiles of CycA coarse powder, a commercial product, and a PM of CycA NS in (A) 0.1 N HCl containing 0.5% SDS and (B) 0.1 N HClCycA: Cyclosporine A, HPMC: Hydroxypropyl methylcellulose, SDS: Sodium dodecyl sulfate, NS: Nanosuspension, PM: Physical mixture, HCl: Hydrochloric acid

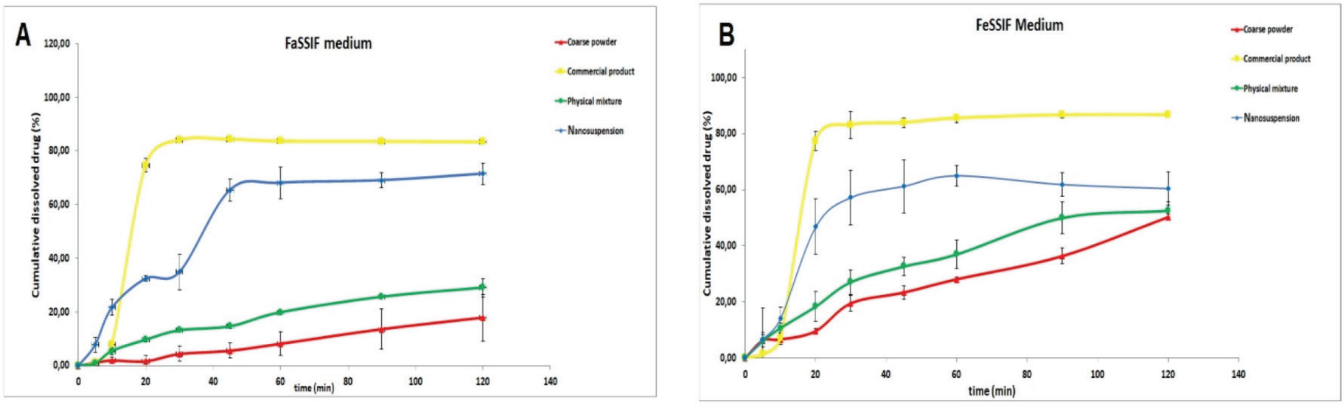

Figure 10. Dissolution profiles of the CycA coarse powder, commercial product, and PM as well as the CycA NS in (A) FaSSIF medium and (B) FeSSIF medium

CycA: Cyclosporine A, PM: Physical mixture, FaSSIF: Fasted simulated intestinal fluid, FeSSIF: Fed simulated intestinal fluid

irrelevant media. Similar to the FaSSIF results, the trade product exhibited 80% dissolution in the FeSSIF medium (Figure 10B).

DISCUSSION

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

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

PS = +699.87 82.59*A 217.67*B 351.56*AB (1)

The model was found to be significant (p= 0.0269) when the interaction of the SDS ratio and homogenization cycle was evaluated in terms of PDI, as reported in Table 1. Although 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 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 (2)

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

The interaction equation for the model affecting the 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 (3)

According to the obtained data and contour plots, 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 is acceptable, indicating the stability of the 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 process parameters to achieve the optimum NS with the HPH method.

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

When the morphological images of NSs are evaluated; it is thought that CycA coarse powder was covered with stabilizers and that high energy was applied in the high-pressure NS production process. The observed morphological results were similar to those reported in the literature.[8,26]

The XRD patterns of SDS and mannitol showed obvious diffraction peaks although the peaks of SDS were weaker. This suggests that they were in a crystalline state. Also, the PM was found to be crystalline, confirming that the PM possessed the same properties as CycA, HPMC, SDS, and mannitol. As shown in Figure 6, the CycA coarse powder, HPMC, and lyophilized CycA NS were in an amorphous state. It has been shown by others[36,37] that CycA exists in an amorphous state, and our results also showed this state. It is known that the amorphous structure of NSs can have positive effects on solubility and dissolution.[38]

In FTIR studies, 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 the CycA coarse powder were examined, characteristic CycA bands similar to those observed in the study of Bertacche et al.[39] were observed at 2960 cm^{-1}. 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 considered 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 observed around 1000 cm^{-1} were thought to belong to HPMC and mannitol, it has been proven that there is no interaction in the PM spectrum. 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 was evaluated together with the XRD results; it proved that there was no polymorphic change between the active substance and the excipients and that the pressure applied while preparing the formulation and the lyophilization process did not change the physicochemical structure. In a study by Attari et al.,[40] characteristic bands of the active substance were observed in olmesartan medoxomil NSs, and no interaction was found.

Stability issues, known as Ostwald ripening, that result in the growth of nanosized particles are critical for NS formulations. It is important to maintain the PS, PDI, and ZP values in the evaluation of the physical stability of NSs. The small PDI value in the 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 is prevented.[41]

The obtained results of solubility study prove that the purpose of increasing the solubility of CycA was achieved by the NS preparation. The reason for this improvement is based on the Ostwald-Freundlich equation. According to this equation, the solubility and dissolution of the NPs improve by increasing the surface area by reducing the PS to nano-size.[42] In a study, a 2.13-fold improvement in water solubility was found in CycA NSs prepared using the 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-fold by wet-milled NSs.[18]

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

Many studies have suggested that NSs can diminish the dissolution variation of drugs with low water solubility in fasting and feeding states. It was found that ZHM NS stabilized with PVP and vitamin E TPGS exhibited > 95% dissolution in FeSSIF medium and > 80% dissolution in FaSSIF medium.[26] In a dissolution study conducted in FaSSIF medium with NSs prepared using the HPH method for five active substances (albendazole, fenofibrate, itraconazole, probucol, and revaprazan hydrochloride) with low water solubility, it was found that NSs showed higher dissolution than microsuspensions for all five active substances. In a 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 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 PS on the absorption of aprepitant, a single-pass method of intestinal perfusion in the rat jejunum was used with phosphate buffer, FaSSIF, FeSSIF as perfusion medium. The results showed that the absorption of aprepitant from the NSs was equal to that from all perfusion media (phosphate buffer = FaSSIF = FeSSIF), but food had a noticeable impact on absorption from the microsuspensions (FeSSIF > FaSSIF > phosphate buffer).[45]

In summary, dissolution studies performed in fasting, feeding, or both media are important to predict the 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 determined 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 the HPH method, which is one of the top-down production technologies, and the DoE approach took into account the impact of critical formulation and process parameters on the dependent variables. The optimum CycA NS was obtained with a CycA: HPMC: SDS 1:1:0.5 ratio and 30 homogenization cycles after statistically determining the interactions. According to the stability results at 4 °C and 25 °C, it was found that the NSs remained physically stable for 9 months. CycA NSs improved the water solubility of CycA 1.9- and 1.4-fold compared to coarse powder and PM, respectively. CycA NS showed higher dissolution than CycA coarse powder in 0.1 N HCl, FaSSIF, and FeSSIF media. When the characterization, solubility, and dissolution results were evaluated together, it was found that CycA NS prepared using the HPH method was successful, and this study proved that the NS could be an encouraging strategy for improving the solubility and dissolution of CycA for oral administration.

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Ethics

Ethics Committee Approval: Not required.

Informed Consent: Not required.

Authorship Contributions

Concept: S.G.P., N.Ç., Design: S.G.P., N.Ç., Data Collection or Processing: S.G.P., Analysis or Interpretation: S.G.P., N.Ç., Literature Search: S.G.P., N.Ç., Writing: S.G.P., N.Ç.

Conflict of Interest: The authors have no conflicts of interest to declare.

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