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Quality by Design Approach for Understanding the Critical Quality Attributes of Cyclosporine Ophthalmic Emulsion

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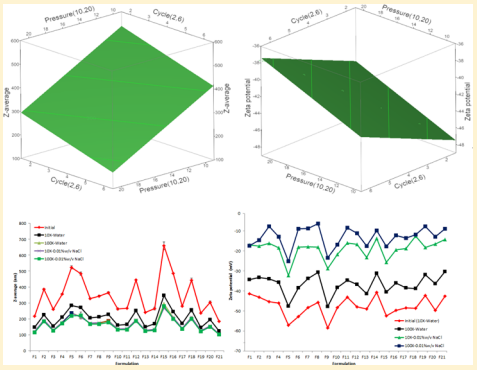
Supporting Information

ABSTRACT: Restasis is an ophthalmic cyclosporine emulsion used for the treatment of dry eye syndrome. There are no generic products for this product, probably because of the limitations on establishing in vivo bioequivalence methods and lack of alternative in vitro bioequivalence testing methods. The present investigation was carried out to understand and identify the appropriate in vitro methods that can discriminate the effect of formulation and process variables on critical quality attributes (CQA) of cyclosporine microemulsion formulations having the same qualitative (Q1) and quantitative (Q2) composition as that of Restasis. Quality by design (QbD) approach was used to understand the effect of formulation and process variables on critical quality attributes (CQA) of cyclosporine microemulsion. The formulation variables chosen were mixing order method, phase volume ratio, and pH adjustment method, while the process variables were temperature of primary and raw emulsion formation, microfluidizer pressure, and number of pressure cycles. The responses selected were particle size, turbidity, zeta potential, viscosity, osmolality, surface tension, contact angle, pH, and drug diffusion. The selected independent variables showed statistically significant ($p < 0.05$) effect on droplet size, zeta potential, viscosity, turbidity, and osmolality. However, the surface tension, contact angle, pH, and drug diffusion were not significantly affected by independent variables. In summary, in vitro methods can detect formulation and manufacturing changes and would thus be important for quality control or sameness of cyclosporine ophthalmic products.



KEYWORDS: *quality by design, cyclosporine, microemulsion, ophthalmic, critical quality attributes droplet size, zeta potential, viscosity, diffusion*

1. INTRODUCTION

Topical cyclosporine (cyclosporine A; Restasis) has been approved for the treatment of chronic dry eye associated with inflammation.[1] Cyclosporine is an immunomodulatory agent that inhibits activation of T-cells. Topical application of cyclosporine formulation to the eye is associated with decreased inflammatory markers in the lachrymal gland, increased tear production, and improved vision and comfort.[2,3] Cyclosporine is a highly lipophilic molecule with log *P* of 8.2 and has a poor aqueous solubility of 0.004 mg/mL at 25 °C.[4,5] Because of this reason, intraocular penetration of cyclosporine as an aqueous solution is poor. To overcome these challenges, various ocular delivery systems have been developed to optimize the bioavailability of cyclosporine in the eye, which include oil solution,[6] cationic emulsion,[7] liposome,[8] nanoparticles,[9] and micelles.[10] The microemulsion dosage form of cyclosporine (Restasis) is the one and only prescription formulation approved for the treatment of dry eye syndrome. The formulation is reported to be a microemulsion dosage form containing 0.05% w/w cyclosporine, and its inactive ingredients include glycerin, Tween 80, carbomer copolymer type A (CCA), water, and sodium hydroxide to adjust the pH.[11] There are no generic products for this product, probably because of the limitations on establishing in vivo bioequivalence methods and lack of alternative in vitro bioequivalence testing methods.

The generic pharmaceutical drug products have to conform to the same standards of quality, efficacy, and safety requirement of innovator drug products. Testing procedures are to be established to prove that generic pharmaceutical drug products are therapeutically equivalent and interchangeable with their associated innovator's product. The regulation 21 CFR 320.24(b) provides a list of in vivo and in vitro methods to establish bioequivalence in descending order of preference:

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(i) in vivo studies in humans comparing drug/metabolite concentrations in an accessible biological fluid, (ii) in vivo testing in humans of an acute pharmacological effect, (iii) controlled clinical trials in humans to establish safety and efficacy, (iv) in vitro methods, and (v) any other approach deemed adequate by FDA.[12,13] One or more of these approaches might be used to demonstrate bioequivalence (BE). For example, the bioequivalence of solid oral dosage forms intended for systemic delivery is established by in vivo pharmacokinetic (PK) studies with a support of comparative in vitro dissolution data. This approach has been successfully applied to a large number of drug products.[14] However, the conventional in vivo BE study with PK end points such as C_{max} and AUC is neither appropriate nor feasible for testing the potency of topically applied ophthalmic cyclosporine microemulsion. Determination of bioequivalence for locally acting drugs in the eye is more complicated as local drug concentrations cannot be measured directly. Therefore, it is a challenging issue for both the pharmaceutical industry and the regulatory agency to determine the BE of locally acting drugs in the eye. The guidance on bioavailability and bioequivalence drafted by Committee of Proprietary Medical Products (CPMP) of the European regulatory authorities stated "for medicinal products not intended to be delivered into the general circulation, the common systemic bioavailability approach cannot be applied."[15] Draft guidance documents on locally acting drug products have been developed by FDA over the last several years to provide recommendations to sponsors to meet statutory and regulatory requirements.[16−21] Generally, FDA addresses the issue on a case by case basis as outlined by the drug-specific guidance. Therefore, it is necessary to identify the key scientific principles for consistent and efficient identification of bioequivalence methods for locally acting drugs in the eye.

The blood concentration of cyclosporine was reported to be below 0.1 ng/mL even after 12 months of twice daily administration of Restasis.[11] This is considered as one of the difficulties associated in establishing in vivo bioequivalence of generic formulations of cyclosporine ophthalmic microemulsion. Accordingly, to demonstrate both the safety and efficacy of the generic products of cyclosporine ophthalmic microemulsion, either clinical studies or in vitro testing methods need to be established. Clinical studies require enrollment of a large number of Keratoconjuntivitis sicca (KCS) patients which can be very difficult, and can create enormous time and resource constraints. In vitro testing method may generate less variable results, easier to control, and more likely to detect differences between drug products if they do exist.[22] However, the clinical relevance of the in vitro testing methods is vital for their validity. Most importantly, the in vitro testing method should be able to detect any change in the critical quality attributes (CQA) due to manufacturing variations, as the quality (Q1) and quantity (Q2) of generic formulations would be similar to the reference listed drug product (RLD).

The present study was carried out to understand and identify the appropriate in vitro test methods that can discriminate the effect of formulation and process variables on CQA of cyclosporine microemulsion formulations having the same Q1/Q2 as that of Restasis. Quality by design (QbD) approach was used to study the effect of formulation and process variables on CQA of cyclosporine microemulsion formulations. Hunter screening design was chosen to study the formulation and process variables, and accordingly, 21 formulations were prepared and subjected to in vitro evaluation. The responses selected were particle size, zeta potential, viscosity, turbidity, osmolality, surface tension, contact angle, pH, and drug diffusion. The formulations were also subjected to accelerated stability testing conditions of dilution, electrolyte, temperature, and centrifugal force.

2. MATERIALS AND METHODS

2.1. Materials. Cyclosporine (>99%), castor oil USP, glycerin USP, Tween 80, acetonitrile (HPLC grade), sodium lauryl sulfate, phosphoric acid, and sodium hydroxide were purchased from Fisher Scientific Co. (Norcross, GA, USA) and were of analytical reagent grade. Carbomer copolymer type A (CCA, Pemulen TR-2 NF polymer) was obtained from Lubrizol Corp. Wickliffe, OH, USA.

2.2. Methods. *2.2.1. Design of Experiments (DoE).* Formulation and processing variables effect on the CQA of cyclosporine microemulsion was assessed using QbD approach. The qualitative (Q1) and quantitative (Q2) composition of the cyclosporine microemulsion formulation was kept constant and was similar to Restasis. The formulation and process variables likely to affect the CQA of the product were identified based on product/process understanding and preliminary results. The preparation involves three step sequential processes to disperse oily solution of cyclosporine in the aqueous phase containing emulsifying agent and other emulsion stabilizing agents. The first step involves the formation of primary emulsion with a portion of aqueous phase at elevated temperature, which is then dispersed in another portion of aqueous phase to form a raw emulsion. The raw emulsion is homogenized by means of a microfluidizer to form a microemulsion. The formulation variables chosen for the present study were mixing order method (X_1), phase volume ratio (X_2), and pH adjustment method (X_3), while the process variables were temperature (X_4) of primary and raw emulsion formation, microfluidizer pressure (X_5), and number of pressure cycles (X_6). The formulation ingredients mixing order was identified as an important variable. Here in this study, three commonly used methods were assessed (methods A, B and C). In method A, oil phase consisted of cyclosporine and castor oil, aqueous phase 1 consisted of Tween 80, water, and glycerin, and the aqueous phase 2 consisted of water and CCA. In method B, oil phase consisted of cyclosporine, castor oil, glycerin, and Tween 80 (1/3 of the total), while the aqueous phases 1 and 2 consisted of water and CCA without and with Tween 80 (2/3 of the total), respectively. Method C was almost identical to method A, with the only difference that glycerin was added into aqueous phase 2 rather than aqueous phase 1. Phase volume ratio has been reported to affect emulsion droplet size and stability.[23] Hence, the effect of phase volume ratio (1:5, 1:7.5, and 1:10) on CQA was studied. The pH of the microemulsion is to be in between 6.5 and 7.5 and hence requires the addition of a fixed percentage weight of 1 N sodium hydroxide solution. A preliminary study showed that pH adjustment method (whether sodium hydroxide solution was added to aqueous phase 2 or the final formulation) significantly impacted the formulation viscosity and droplet size. Accordingly, the effect of pH adjustment method on CQA of cyclosporine microemulsion was also studied. In pH adjustment method I, the pH of aqueous phase 2 was adjusted, while pH adjustment method II involved pH adjustment of final preparation. A fixed percentage weight of 1 N sodium hydroxide solution was added to either aqueous phase 2 (method I) or the final preparation

(method II) to maintain the pH between 6.5 and 7.5. Temperature of the primary and raw emulsion formation was selected as one of the parameters due to its reported effect on surfactant properties pertaining to emulsion formulation stability, micelles concentration, size, shape, and emulsification efficiency.[24,25] It has also been reported that microfluidizer pressure and number of cycles affected the droplet size of niosomes,[26] and hence the effect of these processing variables on CQA of cyclosporine microemulsion was investigated.

Hunter L18 screening design (JMP version 10, SAS, NC, USA) was used to study the effect of formulation and process variables (factors) with three center points. The investigated independent factors and their levels are given in Table 1, and

Table 1. Independent Formulation and Process Variables and Their Levels

independent factor	low level	high level
ingredient mixing order method (X_1)	A, B	C
phase ratio (X_2)	1:5	1:10
pH method (X_3)	I	II
temperature (X_4, °C)	40	70
pressure (X_5, Kpsi)	10	20
number of pressure cycles (X_6)	2	4

experimental matrix involving the preparation of 21 formulatios (coded from F1 to F21) is given in Table 2. The responses selected include primary emulsion and raw emulsion droplet size $D_{chord,90}$ (Y_1 and Y_2), microemulsion responses such as Z-average (Y_3), turbidity (Y_4), zeta potential (Y_5), viscosity (Y_6), osmolality (Y_7), surface tension (Y_8), contact angle (Y_9), pH (Y_{10}), and drug diffusion in 3 h (Y_{11}). All the measured responses were fitted to the following equation:

$$Y = B_0 + B_1X_1 + B_{2A}X_{2A} + B_{2B}X_{2B} + B_{2C}X_{2C} + B_3X_3 + B_4X_4 + B_5X_5 + B_{6A}X_{6A} + B_{6B}X_{6B}$$

where B_0 = intercept, B_1 to B_6 = coefficients of independent variables, X_1 to X_6 = independent variables, and Y = response.

2.2.2. Preparation of Cyclosporine Microemulsion Formulations. Cyclosporine microemulsion formulations were prepared in three sequential steps using an oily phase (cyclosporine + castor oil or cyclosporine + castor oil + glycerin + 1/3 Tween 80), aqueous phase 1 (water + glycerin + Tween 80 or water + CCA or water + Tween 80), and aqueous phase 2 (water + CCA or water + CCA + 2/3 Tween 80 or water + CCA + glycerin). The first step involved the formation of primary emulsion by homogenizing (mechanical homogenizer, RW 20 digital, Wilmington USA) oil phase and aqueous phase 1 at 1000 rpm for 10 min while keeping temperature constant (40, 55, or 70 °C). The ratios of oil to aqueous phase I evaluated as one of the formulation variables were 1:5, 1:7.5, and 1:10. The resultant primary emulsion was then dispersed mechanically at 1000 rpm for 10 min into aqueous phase 2 to form raw emulsion (second step), and at this stage, constant temperature was also maintained. The emulsion droplet size was monitored throughout the primary and raw emulsion formation stages with an online size measurement tool, i.e., focused beam reflectance measurements (FBRM, Mettler Toledo/Lasentec, Redmond, WA, USA). In the last step, the raw emulsion was further subjected to high shear forces by passing through a microfluidizer (M-110P, Microfluidics, MA, USA) at 10, 15, or 20 Kpsi pressure for 2, 4, or 6 cycle. Temperature elevation of the microemulsion was prevented by passing it through ice slurry during the homogenization process. A fixed percentage weight of 1 N sodium hydroxide solution was added to either aqueous phase 2 or final formulation to result in a pH of 6.5−7.5.

2.2.3. Physicochemical Characterization. The microemulsion formulations were characterized for pH, osmolality (Advanced Model 3320 Micro-Osmometer, Advanced Instruments Inc., Norwood MA), viscosity (DV-III ULTRA, Brookfield Engineering Laboratory, Moddleboro, MA), turbidity (diluted 5-times with deionized water; 2100AN Turbidim-

Table 2. Hunter Screening Design Matrix

formulation	ingredient mixing order method (X_1)	phase ratio (X_2)	pH method (X_3)	temperature (X_4, °C)	pressure (X_5, Kpsi)	number of pressure cycle (X_6)
1	A	5	I	40	20	6
2	B	5	II	40	10	6
3	B	10	I	70	20	2
4	B	5	II	70	10	6
5	A	10	II	70	10	2
6	C	5	I	40	10	2
7	A	7.5	I	55	15	4
8	B	10	I	40	10	6
9	A	7.5	II	55	15	4
10	C	10	II	70	20	6
11	B	7.5	I	55	15	4
12	C	5	I	70	10	2
13	C	10	II	70	20	6
14	B	10	I	40	20	2
15	A	10	II	40	10	2
16	C	5	I	40	10	2
17	B	5	II	70	20	2
18	A	10	I	70	10	6
19	C	10	II	40	20	6
20	A	5	II	40	20	2
21	A	5	I	70	20	6

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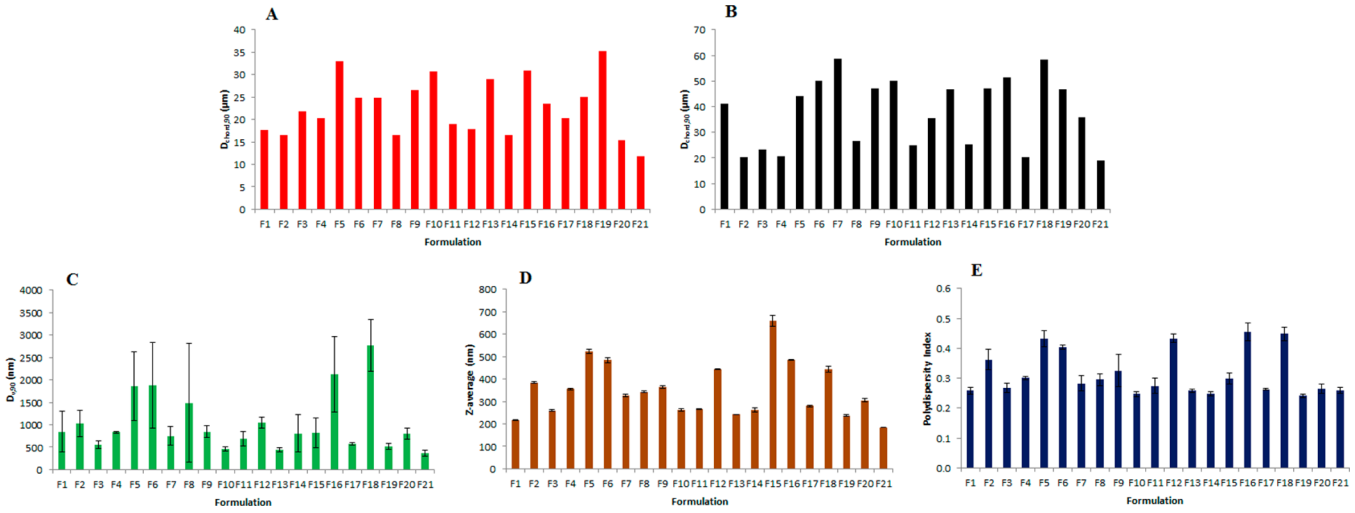

Figure 1. Droplet sizes of (A) primary emulsion $D_{chord,90}$, (B) raw emulsion $D_{chord,90}$, and microemulsion (C) $D_{v,90}$; (D) Z-average; (E) polydispersity index.

eter, Hach, Loveland, CO), surface tension, and contact angle (Pocket goniometer PGX+, Model 68−76, TMI, New Castle, DE, USA). The surface membrane used for contact angle measurement was polymethyl methacrylate (Goodfellow Corporation, PA, USA). All the instruments were calibrated with their respective standard before sample measurements, and measurements were made at room temperature (22 °C). Particle size and zeta potential were measured at 25 °C by dynamic light scattering (DLS) method using Zetasizer NanoZS instrument (Malvern Instruments Inc., Westborough, MA, USA). Undiluted samples were used for particle measurements, while 10-times diluted samples with deionized water were used for zeta potential measurement (n = 3).

2.2.4. In Vitro Drug Diffusion Study. The diffusion of cyclosporine from microemulsion across a dialysis membrane was studied using vertical Franz diffusion cells as well as dialysis tubes (1 mL of Float-A-Lyzer, 300 kD MWCO, Spectrum Lab). In studies with Franz diffusion cells, the receptor chamber contained 10 mL of 0.5% w/w sodium dodecyl sulfate (SDS) solution, while 1 mL of microemulsion formulation containing 0.5 mg of cyclosporine was placed in the donor chamber. The dialysis membrane (cellulose ester membrane with 300 kD MWCO, Spectrum Lab) was interspersed between donor and receptor chambers. The temperature of the diffusion cells was maintained at 37 °C. The membrane area available for drug diffusion was 1.77 cm^2. The magnetic bar in the receptor medium was stirred at 400 rpm, and sample was withdrawn at the end of 3 h. With respect to the studies with dialysis tubes, 0.6 mL of test formulation (F15 or F21) was placed into each of the dialysis tubes, which was then placed inside a screw cap centrifuge tube (capacity 50 mL) filled with 30 mL of receptor medium (0.5% w/v sodium dodecyl sulfate). The centrifuge tubes, in turn, were placed in a reciprocating shaker water bath (37 °C, Precision Model 50 Reciprocal Shaker-Water) and shaken at 200 oscillations per min. At appropriate time intervals, 1 mL samples were withdrawn from the centrifuge tubes and replaced with equal volume of fresh receptor medium. A control study was carried out to find the maximum amount of drug diffused from ethanolic (40% v/v ethanol−water) solution of cyclosporine across the dialysis membrane with 40% v/v ethanol−water as the receptor medium. All the samples of drug diffusion studies were analyzed for determining the concentration of cyclosporine by HPLC method described elsewhere.[27]

An Agilent 1100 Series High-Performance Liquid Chromatography (HPLC) system equipped with binary solvent pump, autosampler, photodiode array detector, thermostatted column compartment, and Chemstation chromatographic software was used for determining cyclosporine concentration. The stationary phase was a reversed phase Luna C8 (2), 4.6 mm × 150 mm (3 μm packing) column and 4.6 mm × 2.5 mm (5 μm packing) Luna C8 guard column (Phenomenex Torrance, CA, USA). The column temperature was maintained at 60 °C. The composition of the mobile phase was an isocratic mixture of water, acetonitrile, and phosphoric acid (250:750:1) pumped at a flow rate of 1 mL/min. Samples (100 μL) were injected, and eluted cyclosporine was detected at 210 nm. A good linear relationship was observed between the peak area of cyclosporine and its concentration (1−10 μg/mL) with a high correlation coefficient (r = 1.0000). The HPLC analytical method was validated according to USP ⟨1225⟩ validation of Compendial Methods.[28] The method was found to be precise (intra- and interday variation was <1.0%) and accurate (mean recovery 99.5%). The standard curve, constructed as described above, was used for quantifying cyclosporine concentration in the samples of in vitro drug diffusion studies.

2.2.5. Accelerated Stability Testing. The stability of cyclosporine microemulsion formulations was evaluated at accelerated conditions of dilution (10 and 100 times with deionized water and 0.01% w/v sodium chloride solution), centrifugation (4000 and 12000 rpm for 30 min), temperature (60 and 80 °C for 1 h), and freeze−thaw (two cycles at −20 °C). Droplet size and zeta potential were identified as the most critical characteristics of these systems and hence measured, as describe above, after exposure to these conditions.

2.2.6. Statistical Analysis. Data collected for responses in each run was analyzed using JMP software (version 10, SAS, NC, USA) and fitted into linear regression model. F ratio was calculated to measure the error in the model. Larger values of F ratio indicate the smaller error in the model, while smaller values of F ratio indicate larger error in the model. Model correctness was assessed by lack of fit test, with replicate experiments (pass if statistically insignificant). Analysis of variance (ANOVA) was used to compare the multiple means of

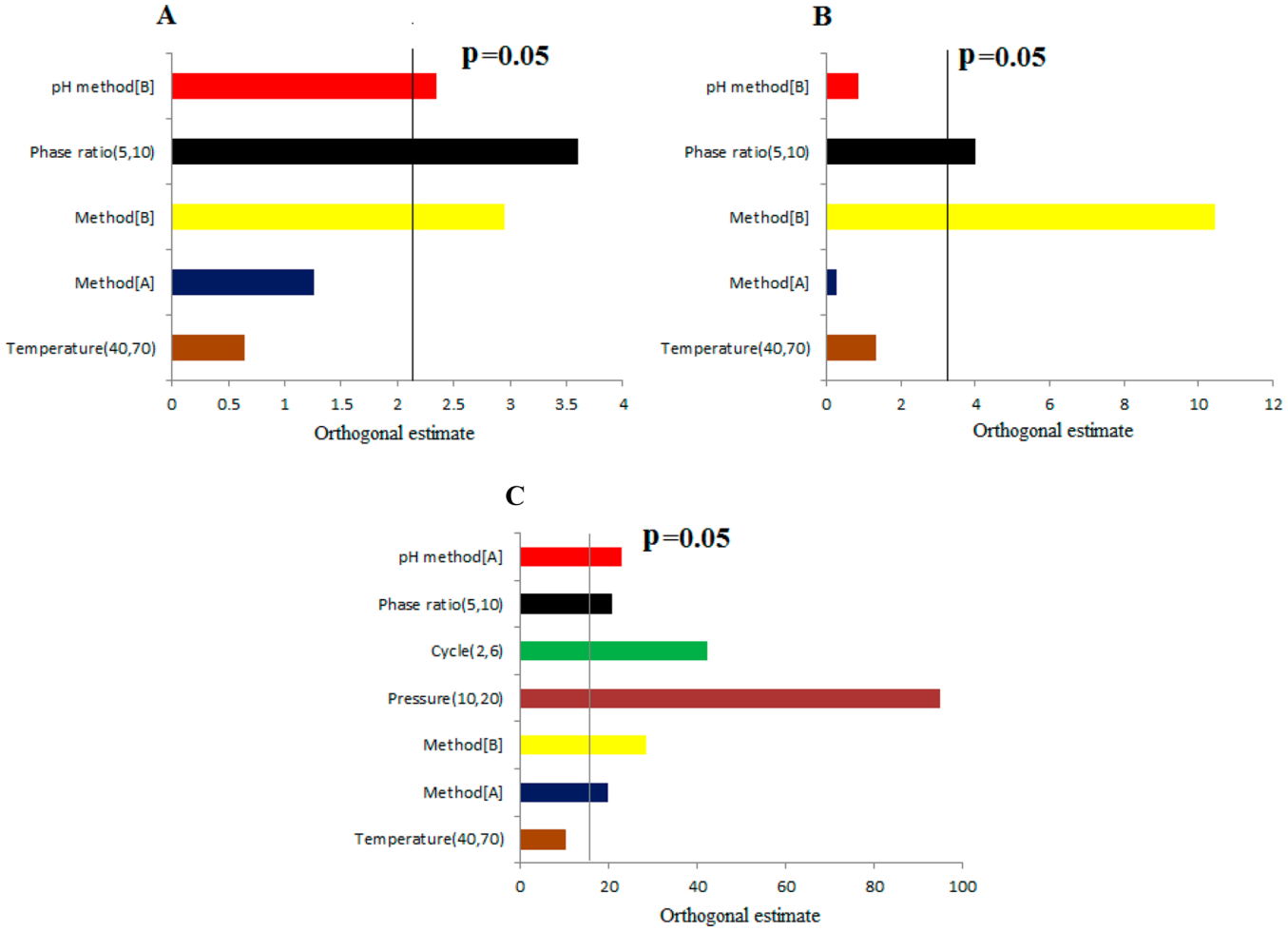

Figure 2. Pareto chart showing the effect of formulation and process variables on (A) primary emulsion, $D_{chord,90}$, (B) raw emulsion, $D_{chord,90}$, and (C) microemulsion Z-average.

the responses by analyzing the variance due to pure random error and differences between means. A value of $p < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

The CQA of a drug product are identified on the basis of their possible impact on clinical efficacy and safety. A systematic risk analysis is used after considering both material and process variables in the production of drug products. In this section, only the physicochemical parameters affected by formulation and process variables were discussed, and those that were insensitive to formulation and process variables were presented in supplementary section (surface tension, contact angle, pH, and in vitro drug diffusion).

3.1. Primary Emulsion, $D_{chord,90}$. First stage of cyclosporine microemulsion preparation involved the formation of primary emulsion by mixing the oil phase and aqueous phase 1. During this phase the oil phase dispersed into tiny droplets by the shearing action of the homogenizer. The sizes of droplets were monitored through online FBRM probe that measures droplet's chord length. The $D_{chord,90}$ (denotes the chord length of 90% of droplets) values were varying from 11.72 to 35.19 μm (Figure 1A) and the data best fitted to the following linear equation.

$$Y_1 = 23.00 + 0.12X_{1A} - 3.96X_{1B} + 3.68X_2 + 2.37X_{3I} + 0.02X_4$$

A positive sign for the coefficient of the independent factors (investigated variables) in the response equation suggests that they increase the response value, while a negative sign has the opposite meaning. The factors significantly ($p < 0.05$) affecting $D_{90,chord}$ were ingredients' mixing order method B (X_{1B}), phase volume ratio (X_2), and pH adjustment method I (X_{3I}). The mixing order methods A (X_{1A}) and B (X_{1B}) produced larger and smaller droplets, respectively, although X_{1A} effect was not statistically significant ($p > 0.05$) when compared to X_{1B} (Figure 2A). This is probably related to the difference in the composition of their oil phase and aqueous phase 1. In method A, oil phase consisted of castor oil and cyclosporine, and aqueous phase 1 consisted of water, Tween 80, and glycerin. However, the oil phase used in method B consisted of castor oil, cyclosporine, glycerin, and Tween 80 (1/3 of the total), and aqueous phase 1 was composed of CCA aqueous dispersion. The presence of Tween 80 in the oil phase of method B was responsible for the formation of smaller droplets. Secondly, it is possible that the polymer (CCA) in the aqueous phase 1 prevented droplets fusion to produce larger droplets. This was not the case in method A. Larger phase volume ratio (X_2) produced larger droplets, and increasing external phase volume reduced the agitation efficiency of the homogenizer with a

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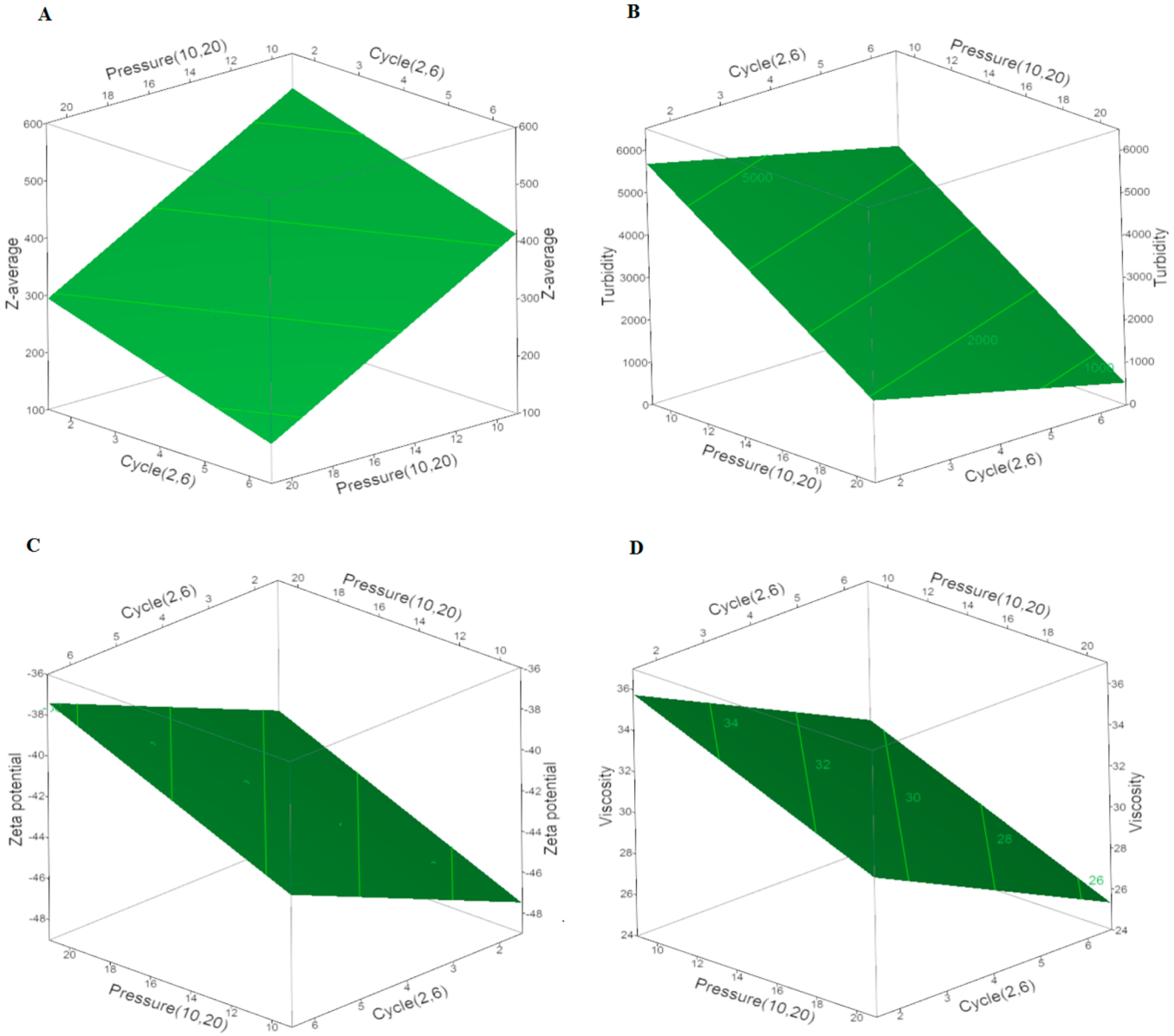

Figure 3. Effect of product and process variables on microemulsion: (A) turbidity, (B) zeta potential, (C) viscosity, and (D) osmolality.

subsequent reduction of shearing forces required for size reduction of the droplets.[29] Similarly, larger droplets were produced with the use of pH adjustment method II. The pH adjustment method II involved adding sodium hydroxide solution to CCA polymer solution to make it alkaline. This produced a very viscous polymer dispersion. When this viscous CCA polymer dispersion was used instead of the one without pH adjustment, larger droplets were produced. The increased viscosity of external phase reduced the shear force required to break emulsion into smaller droplets and hence resulted in larger droplets.

3.2. Raw Emulsion, $D_{chord,90}$. The second step of microemulsion formation involved homogenization of primary emulsion with aqueous phase 2 to produce raw emulsion. The raw emulsion droplet $D_{chord,90}$ ranged from 19.10 to 58.57 μm (Figure 1B), which is larger than that of primary emulsion droplet $D_{chord,90}$. The primary emulsion on adding to aqueous phase 2 results in fusion of droplets and reduces the strength of shearing forces with a resultant increase in droplet size. The data best fitted to the following equation:

$$Y_2 = 37.84 + 6.04X_{1A} - 14.89X_{1B} + 4.43X_2 - 0.88X_{3I} - 1.83X_4$$

The most significant ($p < 0.05$) independent factors influencing Y_2 were ingredients' mixing order methods A and B (X_1) and phase volume ratios (X_2) (Figure 2B). Method A produced larger droplets, whereas method B produced smaller droplets. Similarly, an increase in phase volume ratio (X_2) produced bigger droplets. The reasons for these observations were explained above under primary emulsion section.

3.3. Microemulsion Characterization. *3.3.1. Droplet Size.* The droplet size of the microemulsion is critically important as it relates to physical stability and clinical outcome. Therefore, it could be a good quality target product profile (QTPP). The smaller droplet size of the microemulsion has fewer tendencies to cream and coalesce and will be physically more stable in comparison to bigger size droplet micro-

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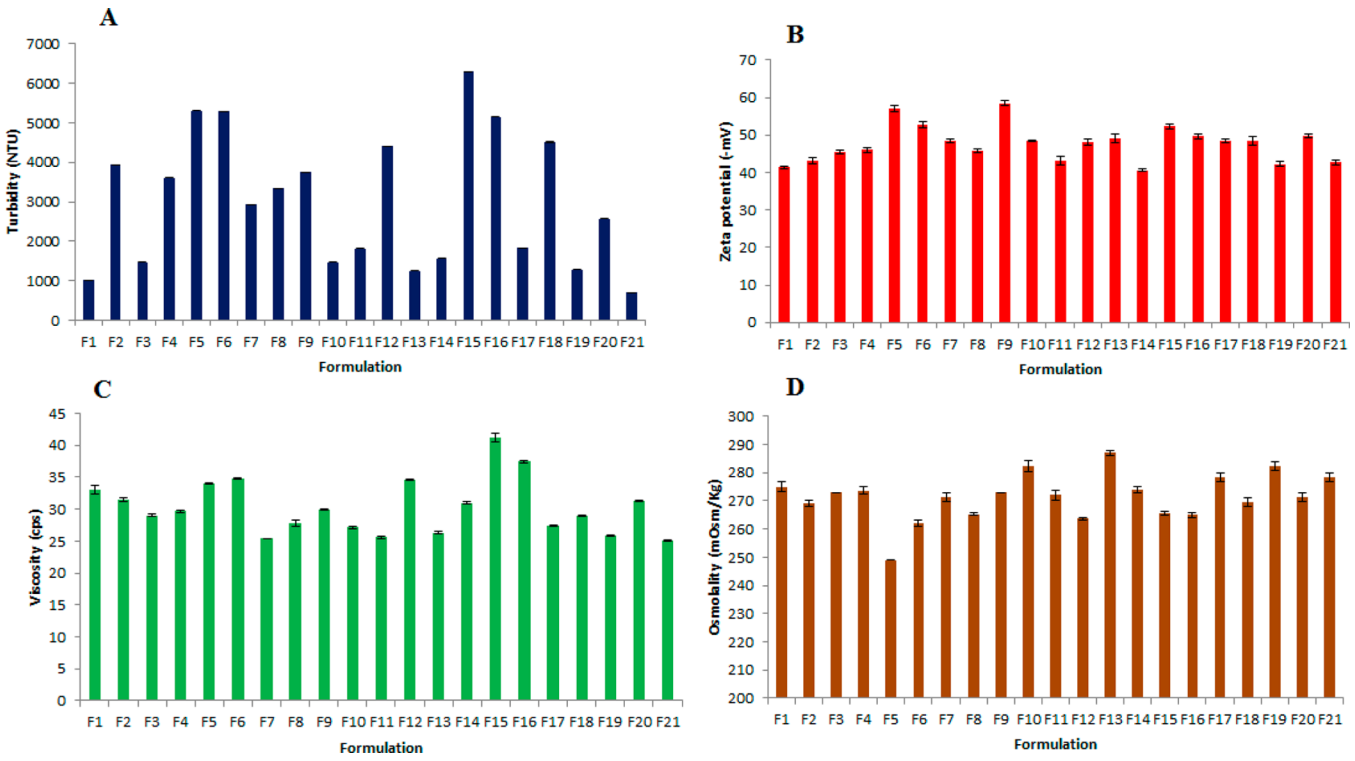

Figure 4. Pareto chart showing effect of formulation and process variables on the microemulsion: (A) turbidity, (B) zeta potential, (C) viscosity, and (D) osmolality.

emulsions.[30] It also provides higher ocular bioavailability because of more surface area to volume ratio available for diffusion and/or permeation across ocular tissues.[31] Furthermore, the impulse of the blinking may eject the drug out of the eye if the particle size is larger. The last step of microemulsion formation involved passing the raw emulsion through microfluidizer to reduce droplet size. The droplet size was measured by DLS without dilution that scatters light at 170° angle. In this study, volume weighted means were used for D_{90} analysis as it correlates with the mass distribution of the oil droplets better than number and intensity weighted means.[32] The droplet size of microemulsion formulations ($D_{v,90}$) varied from 371.67 ± 66.67 to 2763.33 ± 580.46 nm (Figure 1C). The droplet size of microemulsion obtained by DLS is often expressed as Z-average. It is a cumulated mean and intensity weighted harmonic mean size and offers two advantages. The data would be mathematically stable and resistant to experimental noise. It has been suggested to denote particle size as Z-average when used in quality control testing.[33] Furthermore, Z-average is a hydrodynamic size that reflects the particles in dispersed or solution state and very sensitive to small changes in sample, for example, during aggregation. The Z-average of DLS is considered comparable with other techniques such as laser diffraction and light obscuration when the particles are in monodispersed state.[31,32] The value of Z-average of microemulsion droplets varied from 183.93 ± 0.72 (F21) to 659.30 ± 25.08 nm (F15) (Figure 1D), and the data best fitted to the following equation:

$$Y_3 = 349.27 + 28.53X_{1A} - 38.21X_{1B} + 20.47X_2 - 23.30X_{3I} - 9.18X_4 - 102.53X_5 - 50.15X_6$$

The R^2 between predicted and empirical values of Z-average was 0.9460 indicating a good agreement between these two values. Similarly, R^2 and R^2-adjusted values were very close suggesting less error in the model. The factors significantly ($p < 0.05$) affecting Z-average were ingredients mixing order methods A and B (X_1), phase volume ratio (X_2), pH method I (X_{3I}), microfluidizer pressure (X_5), and number of microfluidizer pressure cycles (X_6) (Figure 2D). Effects of X_1, X_2, and X_{3I} factors have been explained under primary and raw emulsion sections. The influence of microfluidizer pressure (X_5) and its pressure cycles (X_6) on microemulsion droplet size (Z-average, Y_4) were shown in Figure 5A. Its value decreased with an increase in microfluidizer pressure, and a reverse trend was observed at low process pressure. The higher the pressure, the more intense the impact of shear forces responsible for droplet size reduction, and vice versa. A similar trend was reported regarding the effect of process pressure of microfluidizer on the niosomes size.[26]

The pH adjustment method I produced smaller droplets compared to pH adjustment method II. When the pH was adjusted before the formation of primary and raw emulsions (pH adjustment method II), it resulted in the formation of thick viscous polymer dispersion preventing the droplet size increase due to coalescence in primary and raw emulsion. It appears that the droplets are surrounded by unfolded larger polymer chains that ultimately fragmented into smaller polymer chains due to high pressure of the microfluidizer and thus prevented increase in droplet size. However, when sodium hydroxide was added to the final preparation (pH adjustment method II), it resulted in larger droplets. The impact of microfluidizer pressure and the number of pressure cycles would be less severe on the polymer fragmentation when the oil droplets are surrounded by folded polymer. This resulted in larger polymer fragments, which would then unfold upon pH adjustment method II and thus account for larger droplets. These effects were also observed on the viscosity.

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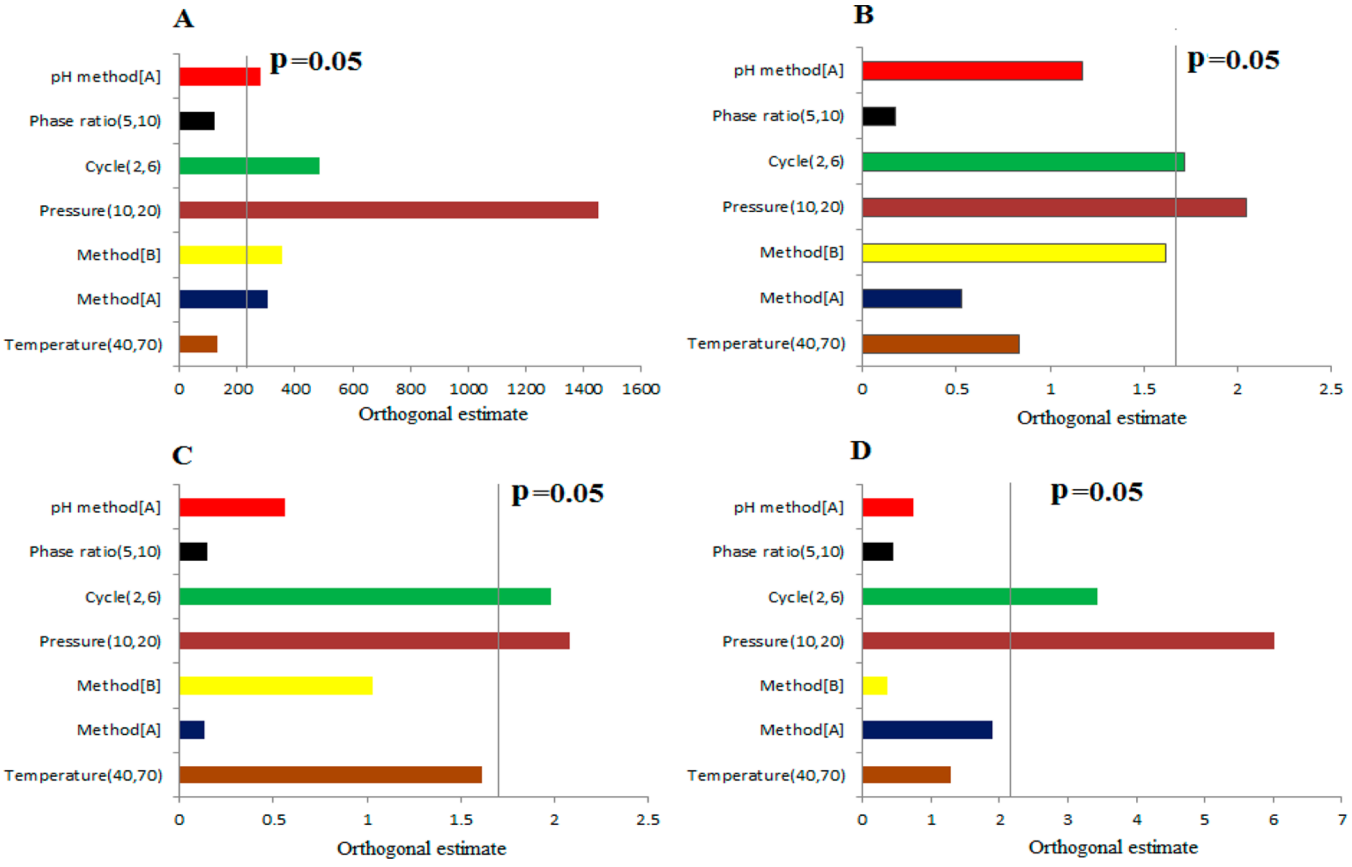

Figure 5. Surface profilers showing the effect of microfluidizer pressure and number of pressure cycles on the microemulsion: (A) Z-average, (B) turbidity, (C) zeta potential, and (D) viscosity.

Width of the droplets size distribution is defined by polydispersity index (PDI, a dimensionless number), and its value varies between 0 (ideal single distribution) and 1 (complete random distribution). Generally, distribution with PDI value smaller than 0.2 was considered as monodistributed, while any PDI value of greater than 0.7 would indicate a high polydispersity (broad distribution). Samples with high PDI value (e.g., > 0.7) are not usually appropriate for using cumulated analysis (i.e., Z-average) and may require distribution analysis on a peak-by-peak basis. In this study, PDI values varied from 0.241 ± 0.005 (F19) to 0.455 ± 0.029 (F16) (Figure 1E).

Turbidity (Y_4) is one of the qualitative parameters of dispersed systems since it is related to droplet size; it may be clinically relevant. The dispersed particles/droplets cause scattering and reduction of transmitted light intensity. The turbidity of the prepared formulation ranged from 702.33 ± 0.58 (F21) to 6299.33 ± 3.21 NTU (F15) (Figure 3A). There is a direct relationship between turbidity of dispersed systems and their particle size.[35] Mixing order of ingredients showed either positive or negative effect on the turbidity depending upon the method employed to produce microemulsion. Mixing order B (X_{1B}) produced smaller droplets compared to mixing order A (X_{1A}) and consequently yielded smaller and larger values of turbidity, respectively. The variables X_{3I}, X_5, and X_6 (Figures 4A and 5B) showed negative effect on the turbidity. This was again related to droplet size. These factors produced smaller droplets and hence decreased the value of turbidity. Furthermore, a correlation coefficient of 0.9760 was obtained between turbidity and Z-average, which illustrated the direct relationship between turbidity and droplet size.

3.3.2. Zeta Potential. Zeta potential is one of the physical properties of the dispersed system describing its stability and might also serve as a good QTPP. The dispersed particles or droplets with large zeta potential (either positive or negative) would repel each other and prevent the occurrence of droplet fusion (coalescence). The optimum value of zeta potential should be greater than 25 mV (positive or negative) to confer stability to a dispersed system.[34] In this study, the negative charge is imparted by carboxylate group of CCA polymer. The zeta potential of the prepared cyclosporine microemulsion varied from −40.63 (F14) to −58.47 mV (F9), while the zeta potential width varied from −4.28 (F18) to −9.64 mV (F15) (Figure 3B). The zeta potential response best fitted to the following linear model equation taking its width into consideration:

$$Y_5 = -42.06 - 1.57X_{1A} + 2.17X_{1B} - 0.09X_2 + 1.20X_{3I} - 1.22X_4 + 2.15X_5 + 1.99X_6$$

None of the studied independent factors significantly (p > 0.05) affected the zeta potential except the microfluidizer pressure (X_5) and number of pressure cycles (X_6) (Figure 4B). Higher microfluidizer pressure and a greater number of pressure cycles generate smaller droplets due to intense and repetitive effect of shear forces (Figure 5C). Since zeta potential is a surface phenomenon, decreasing the particle size yields a greater number of smaller droplets, and thereby increases their

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Table 3. Results of ANOVA and lack of fit tests

			ANOVA		lack of fit		
responses	R^2	R^2-adjusted	F ratio	prob > F	R^2 max	F ratio	prob > F
$D_{90,chord}$ (Y_1, primary emulsion)	0.739	0.652	8.51	0.0005	0.997	29.71	0.0025
$D_{90,chord}$ (Y_2, raw emulsion)	0.737	0.649	8.39	0.0006	0.998	43.24	0.0012
Z-average (Y_3)	0.946	0.917	32.59	0.0010	0.999	14.08	0.0681
turbidity (Y_4)	0.979	0.968	87.47	0.0001	0.999	6.49	0.1409
zeta potential (Y_5)	0.646	0.456	3.39	0.0273	0.986	4.48	0.1963
viscosity (Y_6)	0.696	0.532	4.26	0.0118	0.989	4.97	0.1792
osmolality (Y_7)	0.823	0.728	8.64	0.0005	0.989	2.69	0.3013
surface tension (Y_8)	0.371	0.032	1.09	0.4211	0.875	0.74	0.7032
contact angle (Y_9)	0.387	0.057	1.17	0.3803	0.887	0.81	0.6716
pH (Y_{10})	0.180	−0.262	0.40	0.9897	0.986	10.14	0.0931
Q_{3h} (Y_{11})	0.241	−0.168	0.59	0.7544	0.942	2.18	0.3558

effective surface area when compared to a microemulsion containing larger droplets.[35]

3.3.3. Viscosity. Viscosity is one of the CQA that determines clinical performance and physical stability of the dispersed systems. It has been reported that increasing the viscosity of an ophthalmic preparation increases the contact time between the formulation and eye tissue, which results in increased ocular availability of drug and hence improved clinical outcome.[36] The high viscous ophthalmic dispersion systems, to some extent, also inhibit the droplet fusion by reducing their movement.[37] The viscosity to the cyclosporine microemulsion was imparted by CCA polymer, and it varied from 25.13 ± 0.12 (F21) to 41.17 ± 0.67 cps (F15) (Figure 3C). The effect of investigated formulation and process variables on the viscosity of microemulsion best fitted to the following linear equation:

$$Y_6 = 30.35 + 0.76X_{1A} - 1.41X_{1B} - 0.21X_2 - 0.57X_{3I} - 1.31X_4 - 2.07X_5 - 2.20X_6$$

Microfluidizer pressure (X_5) and number of pressure cycles (X_6) showed significant ($p < 0.05$) negative impact on the viscosity of cyclosporine microemulsion (Y_6) (Figure 4C). The viscosity of CCA dispersion was about 480 cps. This high viscosity was even maintained during the stage of raw emulsion formation. Emulsion viscosity decreased with an increase in microfluidizer pressure and number of pressure cycles. There was a dramatic decrease in the viscosity of microemulsion after the first pressure cycle and such a decrease continued until six cycles but to a lower degree than that of the first cycle (Figure 5D). It is possible that high microfluidizer pressure caused disentanglement and fragmentation of CCA polymer and thereby decreased the viscosity of microemulsion. Similar effects have been observed with high pressure processing of guar gum, gum arabic, sodium alginate, sodium carboxymethyl cellulose, hydroxyethyl cellulose, and sulfated galactan.[38,39] This was attributed to disruption of covalent bond that produced smaller polymer fragments, reduced molecular weight, and, consequently, caused a decrease in the polymer viscosity.

3.3.4. Osmolality. The osmolality of the human eye is 303.7 ± 22.9 mOsm/kg.[40,41] Ophthalmic drug products should be isotonic in order to prevent irritation in the eye. Hypertonic or hypotonic solution/dispersion damages the epithelia of the eye. Hypertonic tears have been implicated in KCC disease and thus widely used for its diagnosis.[42] Glycerin is the primary ingredient of the microemulsion formulation responsible for its osmolality, and without glycerin, the osmolality was 12−15 mOsm/kg. With the investigated formulation and process variables, the osmolality values ranged from 249 (F5) to 287 mOsm/kg (F13) (Figure 3D), and the data best fitted to the following linear equation:

$$Y_7 = 271.71 - 2.59X_{1A} + 0.58X_{1B} - 0.56X_2 - 0.75X_{3I} + 0.27X_4 + 6.14X_5 + 3.73X_6$$

The R^2 was modest between experimental and model predicted values but agreement was good between R^2 and adjusted-R^2. The factors significantly ($p < 0.05$) affecting the osmolality of the microemulsion were microfluidizer pressure (X_5) and number of the pressure cycles (X_6) (Figure 4D). Higher processing pressure and increased number of the pressure cycles showed positive influence on the osmolality of the microemulsion (Y_7). This was indirectly related to water evaporation during microemulsion preparation as it was subjected to increased pressure and successive pressure cycles. As the preparation passed through the microfluidizer it was subjected to intense impact and shear forces in the interaction chamber resulting in elevated temperature of the preparation. The degrees of rise in preparation temperature depend upon the processing pressure and number of pressure cycles. Although attempts were made to cool the preparation by passing it through ice slurry, there was significant increase in the formulation temperature. The raised temperature caused an increase in water evaporation and thereby increased the solute concentration. Since osmolality is a colligative property and dependent upon the solute content, the osmolality of the microemulsion increased with solvent evaporation.

3.3.5. Lack of Fit and ANOVA. Model correctness was assessed by lack of fit test, with replicate experiments (pass if statistically insignificant). In this study, lack-of-fit is insignificant ($p > 0.05$) for all the responses except $D_{chord,90}$ of primary and raw emulsion (Table 3). The maximum R^2 values were greater than 0.942 in all the responses except surface and contact angle. These values measured the proportion of variation in the responses that could be attributed to model rather than to random error. The ANOVA on the observed means of the responses showed that investigated formulation and processing variables significantly ($p < 0.05$) affected droplet size, zeta potential, viscosity, turbidity, and osmolality (Table 3). However, the surface tension, contact angle, pH, and drug diffusion were not significantly ($p > 0.05$) affected by the processing variables. On the basis of the values of F ratio, responses could be rank ordered as pH < Q_{3h} < surface tension < contact angle < zeta potential < viscosity < $D_{chord,90}$ (raw emulsion) < $D_{chord,90}$ (primary emulsion) < osmolality < Z-

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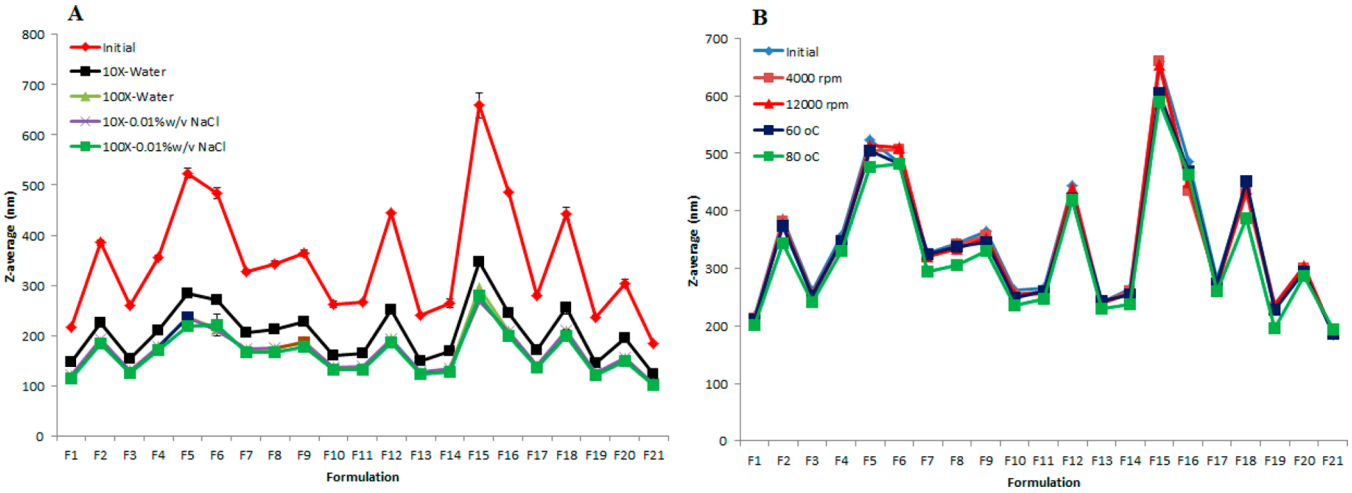

Figure 6. Accelerated stability results showing the effect of (A) dilution with deionized water and 0.1% w/v NaCl and (B) temperature and centrifugal force on Z-average of the microemulsion.

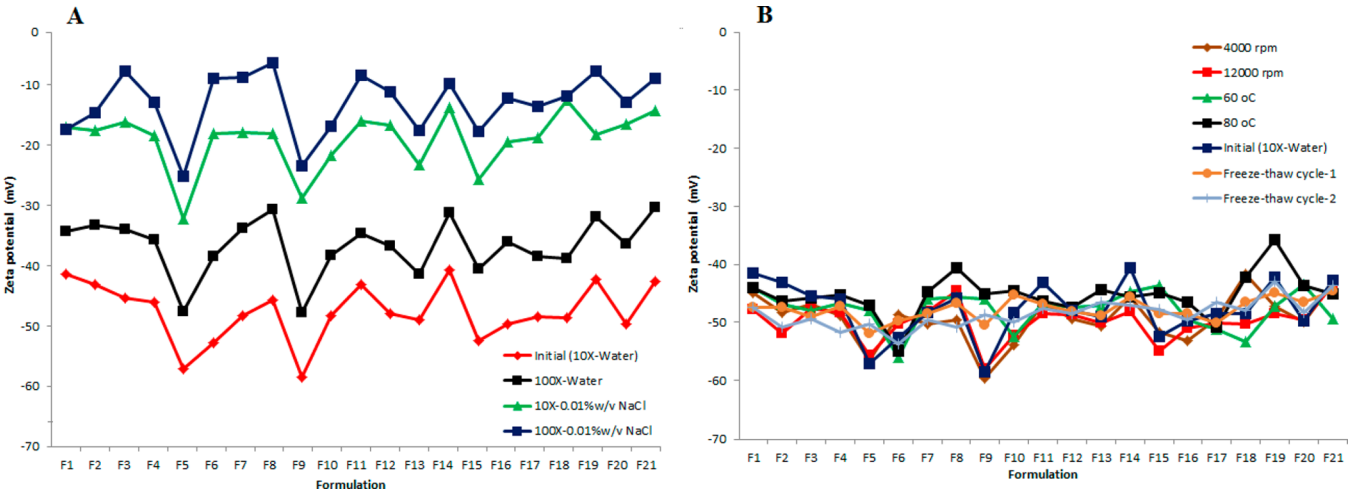

Figure 7. Accelerated stability results showing the effect of (A) dilution with deionized water and 0.1% w/v NaCl and (B) temperature and centrifugal force on zeta potential of the microemulsion.

average < turbidity (Table 3). In other words, least errors were observed in Z-average and turbidity, while highest errors were found in pH, Q_{3h}, surface tension, and contact angle.

3.3.6. Accelerated Stability Testing. Progressive dilution with deionized and sodium chloride solution caused a decrease in droplet size and zeta potential (Figures 6A and 7A). The decrease in droplet size on subjecting to dilution was about 50%. This may be explained by various/concentric layers of CCA polymer molecules surrounding the droplets. Upon dilution, polymer molecules may move into the bulk media and correspondingly cause shrinkage of the outer layers of the droplet leading to decrease in size. Similarly, dilution with deionized water caused about 20% reduction in the zeta potential. This further supports the hypothesis of CCA molecules departing from the droplets since droplet surface charges were induced by the polymer. It was noticed that the presence of electrolytes significantly affected the zeta potential. Electrolytes are known to decrease zeta potential of the dispersed system and destabilize it. Sodium chloride solution (0.1% w/v) caused about 60% decrease in the zeta potential value. This drastic decrease in zeta potential is possibly due to two reasons: neutralization of the carboxylate ions by electrolytes and dilution effect. Higher concentration of electrolyte was not used in the stability testing studies as large increase in conductivity of dispersed system prevented the accurate measurement of the zeta potential.

Freezing induces the formation of ice crystals, and this may cause droplets to elongate and flatten. Furthermore, freezing affects the lipophilic and hydrophilic properties of the emulsifier. The lipophilic portion of the emulsifier loses its mobility and hydrophilic portion becomes dehydrated as water molecules freeze.[42] As the microemulsion thaw, water molecules release and travel through the microemulsion. The cyclosporine microemulsion formulations passed this test as it healed before coalescence occurred. This was indicated by an insignificant change in the droplet size after subjecting the formulation to two freeze−thaw cycles (data not shown). Temperature is known to cause destabilization of emulsions resulting in phase inversion and emulsion breaking.[43] Such a destabilization was not observed with the cyclosporine microemulsion formulations when they were stored at 60 °C as shown by insignificant change in the Z-average droplet size and zeta potential (Figures 6B and 7B). About 10% decrease in Z-average droplet size was observed when heated at 80 °C. This might be related to change in surfactant solubility as well as change in droplet shape and morphology. The hydrophobic

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part of the surfactant gets exposed to heat, enabling more effective interactions with the drug−oil droplet and result in reduction of droplet size.[24,25]

Accelerated stability testing under centrifugal force is commonly used to induce and test instability in dispersed systems. It tests the influence of gravitational force and could be used to predict shelf life of the product under normal storage conditions.[44] Cyclosporine microemulsion formulations were subjected to centrifugation for 30 min at 4000 and 12 000 rpm, which are equivalent to 1306*g* and 11 752*g*. This mimics the effect under normal *g*-force for 0.9 and 8.2 months at room temperature, respectively.[45,46] At the end of the centrifugal test, all formulations exhibited sign of phase separation under 11 752*g* conditions, but all of them redispersed to form a homogeneous dispersion upon gentle shaking. No change in size or zeta potential was observed (Figures 6B and 7B) indicating the cyclosporine microemulsion stability for at least 8 months at room temperature.

The selection of the in vitro testing methods was based on the relevance of the product qualities (i.e., emulsion droplet size, viscosity, zeta potential etc.) to the clinical outcome as each quality response can impact the in vivo performance of the cyclosporine microemulsion formulation. Experimental data indicated that out of the ten investigated qualities, three (emulsion droplet size, viscosity, and zeta potential) are sensitive to the manufacturing processes, despite being Q1/Q2 to the RLD. For all three qualities, two manufacturing processes (homogenization pressure and pressure cycles) were identified as having the greatest impact. Accordingly, these two processing variables may be considered as the most critical manufacturing variable potentially affecting the clinical outcome.

The complex manufacturing processes, less understood drug delivery technologies, and emerging sources of materials provide significant variation in product quality and performance of drug products posing a threat to public health. Evidence of the public health risk is evident from the filings contained in the FDA Drug Quality Reporting System (DQRS), FDA Adverse Event Reporting System (FAERS), and Field Alert Reports (FARs). The reported defects are primarily due to product- and process-related variables. To address such fundamental mechanistic concerns across the pharmaceutical industry, ICH issued a guidance for industry Q8(R2) Pharmaceutical Development that describes an enhanced design and process approach called "Quality by Design (QbD)".[1] It calls for identification and evaluation of raw materials variability, relationship to process, and their impact on product quality and performance. Hence, in this study, too, QbD approach has been used to understand the product and process variables that are likely to show impact on clinical outcome of cyclosporine ophthalmic microemulsion products. Better understanding of the relationship of these variables to product quality will assist in risk management, improving problem detection, and promote timely risk control measures (ICH Q9) and maintenance of a state of control (ICH Q10) throughout the lifecycle.[2]

The modern science-based pharmaceutical quality assessment system used in this report is expected to add significant knowledge to the pharmaceutical development, control, and regulation of generic drug products across the globe. Furthermore, the study results are expected to guide pharmaceutical scientists in understanding the significance of molecular pharmaceutics in product quality and performance.

4. CONCLUSIONS

The formulation and process variables affecting the CQA of cyclosporine microemulsion were studied. The investigated formulation (mixing order of ingredients, phase volume ratio, and pH adjustment method) and process (temperature of primary and raw emulsion formation, microfluidizer pressure, and number of pressure cycles) variables did not affect surface tension, contact angle, pH, and drug diffusion. However, the formulation and process variables significantly ($p < 0.05$) affected the droplet size, zeta potential, viscosity, osmolality, and turbidity of cyclosporine microemulsion formulations. Cyclosporine microemulsion formulation with a smaller droplet size resulted in higher percent of drug diffusion when compared to a formulation with a larger droplet size. Drug diffusion study from a dialysis tube may be used to discriminate a poorly performing product from a good performing product having a possible difference in droplet size of cyclosporine emulsion. There was a decrease in emulsion droplet size on dilution with water, electrolyte, or surfactant solution. Undiluted microemulsion should be used for accurate droplet size measurements. Diluted microemulsion droplet size measurement could lead to misleading results and misinterpretation of the data. Similarly, ten times diluted microemulsion could be used for zeta potential measurement, and further dilution would decrease its value. Cyclosporine microemulsion formulations were stable when subjected to low and high temperature and centrifugal force. The key CQA of cyclosporine microemulsion to be measured and controlled were droplet size, turbidity, zeta potential, viscosity, and quantity of drug diffused from a dialysis membrane as they might impact the clinical outcome of the products. Any significant change in the values of these parameters might indicate an effect of processing variables on product performance. Two products of cyclosporine ophthalmic microemulsion may be considered bioequivalent if they have similar Q1 and Q2 as well as in vitro quality metrics (droplet size, zeta potential, viscosity, turbidity, and quantity of drug diffused from a dialysis membrane).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Surface tension, contact angle, pH, and in vitro drug diffusion. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

This scientific contribution is intended to support regulatory policy development. The views presented in this article have not been adopted as regulatory policies by the Food and Drug Administration at this time.

The authors declare no competing financial interest.

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