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OPTIMIZATION AND EVALUATION OF SIROLIMUS NANODISPERSION DRUG-LAYERED PREDNISOLONE TABLETS FOR THE MANAGEMENT OF THROMBOCYTOPENIA

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ABSTRACT

Background: Thrombocytopenia is a hematological disorder characterized by a reduced platelet count, which increases the risk of bleeding and impairs hemostasis. The present study aimed to develop and optimize a fixed-dose combination tablet comprising a sirolimus nanodispersion layered onto a prednisolone core tablet using a Box–Behnken experimental design as a potential therapeutic strategy for thrombocytopenia. **Methodology:** Sirolimus nanodispersion drug-layered tablets were prepared and optimized using a three-factor, three-level Box–Behnken design. The optimized formulation was evaluated for critical quality attributes, including hardness and friability, and subsequently experimentally validated. Therapeutic efficacy was investigated in cyclophosphamide-induced thrombocytopenic Wistar rats by assessing platelet count, bleeding time, clotting time, and hematological parameters, including red blood cell count, hemoglobin, and hematocrit. **Results and Discussion:** The optimized formulation exhibited acceptable mechanical properties, with hardness ranging from 5.3 to 5.5 kg/cm² and friability below 1%, confirming the robustness of the optimization process. In vivo evaluation demonstrated a marked improvement in platelet recovery following treatment. The platelet count increased from 39.87 ± 8.82 × 10^3/mm³ in the disease control group to 132.62 ± 21.76 × 10^3/mm³ and 154.57 ± 7.40 × 10^3/mm³ after once-daily and twice-daily administration, respectively. Furthermore, treatment significantly improved bleeding time, clotting time, and hematological parameters, indicating enhanced therapeutic efficacy. **Conclusion:** The optimized sirolimus nanodispersion drug-layered prednisolone tablet demonstrated favorable pharmaceutical characteristics and significant therapeutic efficacy in the cyclophosphamide-induced thrombocytopenia model. The developed fixed-dose combination represents a promising formulation strategy for improving the management of thrombocytopenia and warrants further preclinical and clinical investigation.

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INTRODUCTION

Immune thrombocytopenia (ITP) is an acquired autoimmune disorder marked by the autoantibody-induced death of platelets and diminished platelet generation. Recent evidence suggests that dysfunction of Treg cells may contribute to the pathogenesis of ITP. They are essential for maintaining peripheral tolerance by inhibiting self-reactive lymphocytes. When these regulatory cells are compromised, patients exhibit activated autoreactive T lymphocytes that target platelets and dysregulated cytokine production, thereby hastening platelet destruction. The impaired function or reduced quantity of Tregs in individuals with ITP makes the growth of functional Treg cells a promising treatment strategy. Moreover, several clinical trials have shown that successful therapies for ITP can enhance Treg cell levels following the restoration of platelet counts. While the precise mechanism remains unclear, these findings indicate a strong potential for Treg cells as future biomarkers for therapeutics [1-3]. Sirolimus is a Biopharmaceutics Classification System (BCS) class II drug with poor water solubility and dissolution-rate-limited oral bioavailability, particularly resulting in varying systemic exposure. Nanodispersion technology has been studied to overcome these drawbacks by reducing particle size and increasing surface area, thereby enhancing dissolution rates and improving the oral bioavailability of poorly soluble drug substances, including mTOR inhibitors. Under these circumstances, nanodispersion drug layering technology has been considered to achieve uniform deposition of sirolimus on the prednisolone core tablet formulation in a fixed-dose combination, thereby improving bioavailability while retaining the stability of the drug formulation. Sirolimus is an immunosuppressive agent categorized as a mammalian target of rapamycin (mTOR) inhibitor. It has been used to manage several immune-mediated illnesses, including autoimmune lymphoproliferative syndrome (ALPS), autoimmune hemolytic anemia (AIHA), Evans syndrome, and immune thrombocytopenic purpura (ITP).

Presently, there is less evidence concerning the efficacy of sirolimus in these circumstances, which may restrict its generalizability. Prednisone (PDN) is often utilized as the principal therapy for ITP. Administered daily for a maximum of 4 weeks at a dose of 0.5 to 2 mg/kg of body weight, followed by a tapering period. A small proportion of patients attain persistent remission, whereas around two-thirds are expected to have an initial response. Furthermore, extended corticosteroid therapy often results in a greater incidence of unfavorable consequences than positive outcomes [4-6]. Prednisolone is a synthetic glucocorticoid structurally related to the endogenous corticosteroid cortisol produced by the adrenal cortex. It is widely used for its potent anti-inflammatory and immunosuppressive effects. It is used to treat several illnesses, including arthritis, hematological and immunological disorders, ophthalmic and dermatological conditions, respiratory ailments, malignancies, and severe allergies. A fixed-dose combination (FDC) is a formulation that delivers several active pharmacological substances as a single entity by combining two separate drugs into one pill. These fixed-dose combination (FDC) formulations constitute a substantial segment of the global pharmaceutical market and have gained increasing clinical and commercial acceptance. The worldwide pharmaceutical sector had a significant increase in fixed-dose combination (FDC) approvals throughout the 2000s. Unlike a single-drug maximum dose, the FDC multidrug therapy formulation may have a synergistic, extended therapeutic effect, thereby improving safety and efficacy while reducing adverse effects. A reduction in the frequency of missing prescriptions led to improved patient care. The decrease in expenses and dose requirements led to improved patient compliance [7, 8].

However, these advantages are accompanied by specific drawbacks associated with FDC formulations, including drug solubility, compatibility, disintegration characteristics, dosing, potential drug interactions, and the properties of the specific pharmaceuticals. Subsequent issues may emerge with the utilization of FDC, including exclusion from treatment regimens, less dose flexibility for certain drugs, and complications in ascertaining the origin of side effects. In the context of immune thrombocytopenia (ITP), fixed-dose combination (FDC) therapy is a rational strategy to simultaneously target immune-mediated platelet destruction and impaired platelet recovery.

Prednisolone remains the first-line treatment because of its rapid anti-inflammatory and immunosuppressive effects; however, prolonged corticosteroid therapy is frequently associated with relapse and systemic adverse effects. Sirolimus, an inhibitor of the mammalian target of rapamycin (mTOR), enhances regulatory T-cell function and suppresses autoreactive lymphocytes, thereby restoring immune tolerance. Consequently, combining prednisolone with sirolimus is expected to provide complementary therapeutic effects by promoting rapid platelet recovery, reducing corticosteroid

dependence, and improving long-term disease control. To evaluate this therapeutic approach, cyclophosphamide (50 mg/kg, i.p.) was used to induce thrombocytopenia in rats, as this model has been established as a simple, reliable & reproducible method for experimental thrombocytopenia [9–13]. The primary objective of the study was to develop and optimize a sirolimus nanodispersion drug-layered tablet on a prednisolone core using a Box–Behnken experimental design. The optimized formulation was characterized for particle size, physical properties (e.g., strength & elasticity), and drug content. Subsequently, the optimized formulation was evaluated in a rat model of cyclophosphamide-induced thrombocytopenia to assess its effects on platelet recovery, bleeding time, clotting time, and hematological parameters.

MATERIAL AND METHOD

Materials

Sirolimus and prednisolone were received as gift samples from Concord Biotech Limited, India, and Murli Krishna Pharma Pvt. Ltd., India, respectively. Lactose monohydrate, polyvinyl pyrrolidone (PVP-K30), and hydroxypropyl methylcellulose were purchased from HI Media in Mumbai, India. Talc, magnesium stearate, and polyethylene glycol 6000 were procured from Nice Chemie Pvt. Ltd., in Mumbai, India. Sucrose, anhydrous calcium sulphate, and povidone-30 were procured from Reidel India Chemicals in Mumbai. Titanium dioxide, polyethylene glycol 400, alpha-tocopherol, and poloxamer 188 were procured from Himedia Laboratories Pvt. Ltd.

PREPARATION OF FIXED-DOSE COMBINATION TABLET

Preparation of Prednisolone Core Tablet

Polyvinylpyrrolidone (PVP), hydroxypropyl methylcellulose (HPMC), and prednisolone were each passed through a #40-mesh sieve to break up agglomerates and obtain a uniform particle size before blending. After sieving the powder through a #40-mesh screen, lactose monohydrate was added in equal amounts. After that, the mixture was mixed well for the required period. Mix the prior powder combination with the remaining lactose monohydrate until thoroughly blended, then co-sift through a #40-mesh sieve. After sieving talc through #40 mesh, mix it with the prior mixture for 5 minutes. Sifted magnesium stearate should be blended for 5 minutes using a #60-grit filter in a suitable blender. A tablet with a target weight of 100.0 ± 3.0 mg (97.0 to 104.0 mg) is produced by crushing the lubricated mix using a 5.5 mm round punch in the final phase [14].

Seal Coating

The #40 mesh screen sieved Hydropropyl methyl cellulose. In a stainless-steel vessel, dissolve hydroxypropyl methyl cellulose in deionized water. Keep stirring with a pneumatic stirrer until a homogeneous solution is reached. Add polyethylene glycol and mix to make a clear, uniform solution. After integrating the sifted talc into the previous solution, mix until a uniform dispersion is achieved. Use a Neo coater to coat prednisolone core pills with the seal coating dispersion from the previous stage until the required weight is reached [15].

Preparation of Sirolimus Nanodispersion

Sirolimus nanodispersion was prepared by wet media milling using a Willy A. Bachofen (WAB) Dyno-Mill. An aqueous dispersion containing 5% w/v sirolimus was stabilized with 2% w/v Poloxamer 188 and 1% w/v PVP K-30. The milling chamber was loaded to 75% capacity with 0.3 mm yttrium-stabilized zirconia beads. Particle-size reduction was achieved through sequential milling for 30, 60, 90, and 120 min at agitator tip speeds of 10, 12, 14, and 15 m/s, respectively. Product throughput was maintained at 30–40 kg/h using a pump speed of 30–40 rpm. Temperature was controlled using a cooling-water jacket (3.2–4.0 L/h), maintaining the product outlet temperature between 12 and 14 °C. A target median particle size (D0.5) of approximately 100 nm was selected to enhance dissolution and bioavailability. Post-milling characterization confirmed particle-size values of D0.1 = 0.08 µm, D0.5 = 0.10 µm, and D0.9 = 0.38 µm. The nanodispersion was further evaluated for particle size and zeta potential to confirm nanoscale characteristics and physical stability. Sirolimus nanodispersion was prepared using WAB-Dynomill technology to reduce particle size and improve dissolution characteristics. The process parameters used for particle size reduction are given in Table 1.

Barrier Coating-1

Mix filtered water and sifted polyvinyl pyrrolidone in a stainless-steel vessel. Use a continuous pneumatic agitator to mix the components until a homogeneous solution is reached. While stirring, slowly add sugar to the prior solution until it is homogeneous. The mixture was stirred for a sufficient duration before the gradual addition of anhydrous calcium sulphate. The tablet was coated with a barrier coating solution using a Neo coater until the desired weight was reached. The coating composition and processing conditions were optimized during formulation development [16].

Table 1: WAB- Dynomill process parameters for particle size reduction

S. No.	Time (min)	Tip Speed (m/s)	Product Throughput (Kg/hr.)	Product pump RPM	Product Outlet Temperature (°C)	Cooling Water		Particle Size in (µn)		
						Flow rate (Lit./hr.)	Outlet Temperature (°C)	D (0.1)	D (0.5)	D (0.9)
1	30	10	30	30	12	3.2	10	0.15	0.20	1.2
2	60	12		30	13	3.5	12	0.12	0.15	0.10
3	90	14	40	40	14	3.8	12	0.10	0.12	0.72
4	120	15		40	12	4.0	13	0.08	0.10	0.38

[D(0.1): diameter below which 10% of particles fall, D(0.5): median particle diameter, D(0.9): diameter below which 90% of particles fall]

Drug Layering of Sirolimus Nanodispersion

The prepared sirolimus nanodispersion was layered onto the barrier-coated prednisolone core tablets using a Neo coater until the desired weight gain was achieved.

Optimization of Sirolimus Drug Layering formulation using a computational method in Design Expert v13.

The Box-Behnken design and Design-Expert software were used to optimize three independent variables in the formulation of a sirolimus-layered nanodispersion. The independent variables are HPMC E5 LV, sucrose, and calcium sulfate anhydrous, each defined at three levels based on the composition range observed in the formulation batches. The design produced 17 batches (Table 2) of experimental runs to analyze the effects of varying formulation variables on the tablets' physical properties. Response variables were determined for mechanical properties; specifically, the measured means of the resulting tablets' hardness and friability were recorded. Data generated from the test formulations were analyzed using response surface methodology (RSM) to relate the independent variables to the dependent (response) variables and select the optimal formulation with the desired mechanical properties.

The formulation factors are HPMC E5 LV, sucrose, and calcium sulphate anhydrous, which effectively control coating characteristics, tablet mechanical strength, and the coating's processing performance for drug layering. Hardness and friability of tablets were response variables because they are critical quality attributes (CQAs) that directly determine a tablet's structural integrity, mechanical strength, and resistance to damage during packaging and transport. Evaluating these responses allows the design to precisely optimize and predict how variations in formulation factors influence the final physical durability of the compressed tablet. Some additional quality attributes, such as particle size distribution, drug content, and post-compression characteristics, were evaluated separately to support the overall quality of the optimized formulation [17].

Table 2: Box–Behnken design matrix for optimization of sirolimus nanodispersion drug-layered tablets.

		Factor 1	Factor 2	Factor 3	Response 1	Response 2
Std	Run	A: HPMC E5 LV	B: Sucrose	C: Calcium sulphate anhydrous	Hardness	Friability
		mg	mg	mg	kg/cm²	%
6	1	10	21.659	5.5	5.48	0.56
5	2	3.127	21.659	5.5	5.32	0.81
10	3	6.5635	25	5.5	5.44	0.56
1	4	3.127	18.318	5.505	5.3	0.87
14	5	6.5635	21.659	5.505	5.46	0.48
4	6	10	25	5.505	5.49	0.56
2	7	10	18.318	5.505	5.47	0.56
8	8	10	21.659	5.51	5.5	0.56
11	9	6.5635	18.318	5.51	5.35	0.81
3	10	3.127	25	5.505	5.33	0.87
17	11	6.5635	21.659	5.505	5.45	0.48
15	12	6.5635	21.659	5.505	5.46	0.48
7	13	3.127	21.659	5.51	5.34	0.81
13	14	6.5635	21.659	5.505	5.47	0.48
9	15	6.5635	18.318	5.5	5.41	0.56
16	16	6.5635	21.659	5.505	5.45	0.48
12	17	6.5635	25	5.51	5.43	0.56

Coating Uniformity Evaluation

The uniformity of sirolimus nanodispersion on the barrier-coated prednisolone core tablets is determined by measuring the drug content of each coated tablet. A total of 10 tablets were randomly taken from the optimized formulation batch after performing the drug layering process. Each tablet was weighed individually and ground into a fine powder using a mortar and pestle. An accurate amount equivalent to 2 mg of sirolimus was transferred to a 100 mL volumetric flask. About 70 mL of methanol was added to the flask, and the mixture was sonicated for 15 minutes to ensure complete extraction of sirolimus from the surface coating. The total volume was brought to 100 mL with methanol, and the mixture was then mixed thoroughly. The solution was filtered through a 0.45 μm nylon membrane filter, and proper dilutions were prepared using methanol.

The absorbance of individual samples was recorded at 296 nm using a UV–Visible spectrophotometer (UV-1800, Shimadzu Corporation, Kyoto, Japan). The sirolimus content was calculated using the established calibration curve. The coating uniformity was indicated by % drug content, mean ± standard deviation, and % relative standard deviation. Low %RSD indicated uniform deposition of sirolimus nanodispersion on the tablet surface. The percentage drug content was calculated using the following equation:

$$\textbf{\textit{Drug Content}}\ \% = \frac{\textbf{\textit{Measured Drug Content}}}{\textbf{\textit{Theoretical Drug}}} \times \mathbf{100}$$

The percentage relative standard deviation was calculated as:

$$\textbf{\textit{RSD}} = \frac{\textbf{\textit{Standard Deviation}}}{\textbf{\textit{Mean}}} \times \mathbf{100}$$

Coating Weight Gain Uniformity

To evaluate coating uniformity, twenty tablets were weighed individually before and after the drug-layering process using an analytical balance (AUW220D, Shimadzu Corporation, Kyoto, Japan; accuracy ±0.1 mg). The coating weight gain was calculated using the following equation:

$$\textbf{\textit{Coating Weight Gain}}\ (\%) = \frac{\textbf{\textit{Wf}} - \textbf{\textit{Wi}}}{\textbf{\textit{Wi}}} \times \mathbf{100}$$

Where $\textbf{\textit{Wi}}$ *= initial tablet weight before coating, and* $\textbf{\textit{Wf}}$ *= final tablet weight after coating*

The mean coating weight gain, standard deviation, and %RSD were calculated. Uniform coating was considered acceptable when the variation in coating weight gain among tablets was minimal (RSD < 2%).

***In Vitro* Dissolution Study**

The dissolution study for the pure sirolimus API, the marketed sirolimus tablet, and the optimized sirolimus nanodispersion layered tablet was conducted using the USP dissolution apparatus II (paddle method) instructions. Under the same temperature condition of 37.0 ± 0.5 °C, the dissolution study was conducted in 900 mL of phosphate buffer (pH 6.8) at a paddle speed of 50 rpm. Samples were collected at designated time intervals (5, 10, 15, 20, 30, 45, 60, 70, 80, and 90 min) in 5 mL volumes from the dissolution chamber, with each sample replaced by the same volume of fresh solution to maintain sink conditions. Sirolimus concentration in solution was quantified using a UV-Visible spectrophotometer (UV-1800, Shimadzu Corporation, Kyoto, Japan) at the established λmax value. Cumulative percentage drug release (%CDR) was calculated using a pre-established calibration curve, and dissolution tests were repeated 3 times (n = 3). Dissolution profiles obtained from optimized nanodispersion layered tablets were compared with those of the pure sirolimus API and a marketed sirolimus tablet. The drug-release process and the dissolution data obtained from the optimized nanodispersion layered tablets were modeled using different kinetic models, including Zero-order, First-order, Higuchi, Korsmeyer–Peppas, and Hixson–Crowell. The linear regression analysis was performed using OriginPro 2024 (OriginLab Corporation, Northampton, MA, USA), and the coefficient of determination (R^2) was used to evaluate the fits. For the Korsmeyer-Peppas model, only the first ≤60% cumulative drug-release data were used, in accordance with standard kinetic analysis.

***IN-VIVO* STUDY**

Thrombocytopenia in rats

Thrombocytopenia is characterized by a decreased platelet count. Many medicinal plants treat and prevent thrombocytopenia caused by illnesses or medicines. The usual range for thrombocytes in healthy individuals is 150-400×10^9/L of blood. Platelet counts of 50-100×10^9/L indicate moderate-to-severe thrombocytopenia, with few symptoms. Thrombocytopenia is linked to dengue, ITP, malignancy, chikungunya, aplastic anemia, drug-induced ITP, and hemolytic uremic syndrome. Treatment of thrombocytopenia depends on its etiology and severity. Corticosteroids, immunoglobulins, and splenectomy are the first-line therapy for chronic idiopathic thrombocytopenic purpura, although 25–30% of patients fail. Because it causes stable thrombocytopenia, cyclophosphamide, an antineoplastic and immunosuppressive drug, will be used as

a toxicant in this investigation. Thrombocytopenia was induced using cyclophosphamide (50 mg/kg, i.p.) administered on Day 1; the animals are simple, viable, and stable [18-20].

Biological Evaluation

Male Wistar rats (200–250 g) of either sex were purchased from AT & T. All animal handling procedures in the experiment were carried out following the strict guidelines set down by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. The experimental design was also approved by the Institutional Animal Ethics Committee (IAEC) of our Institute (Approval No.): RIPS/IAEC/2025-26/01/33. After one week of acclimatization, rats were randomized into four groups (n=6 per group) based on body weight and basal whole-blood counts. Cyclophosphamide (Endoxan N 200mg) was purchased from AT & T, Hyderabad. The white crystalline powder of Cyclophosphamide was dissolved and applied.

Rats in Group-I served as vehicle control and were administered with distilled water throughout the study. **Rats in Group-II to Group-IV** (CYP treated) were intraperitoneally administered with cyclophosphamide at a dose of 50mg/kg. i.p., with a dose volume 5ml/kg based on body weight on day 1 of the experiment. Reference & test compounds for the treatment will be administered to the respective treatment groups based on body weight, as per the study design (Table 3). Each group consisted of six animals, which is consistent with commonly employed sample sizes in preliminary pharmacological studies evaluating thrombocytopenia models [21].

Table 3: Procedure to assess platelet enhancement capability of sirolimus nanodispersion drug stacking technology over prednisolone core tablets in Wistar rats with cyclophosphamide-induced thrombocytopenia.

SN	No. of animals	Group	Day 1-3	Day 4-14	Purpose
I	6	Normal control (Naïve Control)	0.5% CMC (1 ml/kg, p.o.)	0.5% CMC (1 ml/kg, p.o.)	To study the normal physiological parameters
II	6	Disease control	Cyclophosphamide (50 mg/kg, i.p)	0.5% CMC (1 ml/ kg, p.o.)	To serve as disease control
III	6	Developed Formulation of Single dose [Prednisolone + Sirolimus (QD)]	Cyclophosphamide (50 mg/kg, i.p)	10mg Prednisolone and 2mg Sirolimus /kg, p.o. (Once a day)	To assess the effect of the developed formulation in a single dose
IV	6	Developed Formulation of Double dose [Prednisolone + Sirolimus (BID)]	Cyclophosphamide (50 mg/kg, i.p)	10 mg Prednisolone and 2 mg Sirolimus /kg, p.o. (Twice a day)	To assess the effect of the developed formulation in double dose

The dosage ratios of 10 mg per kg body weight for prednisolone and 2 mg per kg body weight for Sirolimus were based on previous pharmacological studies [22], which demonstrated that both drugs are effective immunosuppressants and immunomodulators in rodent models. The primary therapeutic agent in these studies was prednisolone, owing to its rapid suppression of immune-mediated platelet destruction. The concentration of Sirolimus was lower to provide long-term mTOR-mediated immunity while avoiding complications associated with excessive immunosuppression. The main purpose of incorporating the ratio of 10:2, the acute therapeutic response versus long-term immunity, was to validate the formulation for a combination strategy [23].

Study Protocol: All test formulations were delivered as suspensions in 0.5% CMC. The animal equivalent dose of the formulation was derived from the human therapeutic dosage indicated on the product label, which specifies a daily dose of 60 mg of Prednisolone and 40 mg of Sirolimus. The animal equivalent dose was calculated using the FDA-recommended body-surface-area conversion method (Km factor) [24]. The selected dose range was consistent with previously reported preclinical studies [22, 23] and was intended to achieve sustained therapeutic exposure while minimizing the risk of treatment-related toxicity.

Oral administration was selected to mimic the intended clinical route of delivery and to evaluate the performance of the developed formulation under physiologically relevant conditions. The 14-day dosing schedule was designed to provide sustained drug exposure to assess platelet recovery and hematological responses in the cyclophosphamide-induced thrombocytopenia model, while allowing routine monitoring of animal health and tolerability [25, 26]. Approximately 0.5–1.0 mL of blood was collected from the retro-orbital plexus of rats under mild anesthesia using glass capillaries.

The collected blood samples were immediately transferred into tubes containing disodium ethylenediaminetetraacetic acid (EDTA) as an anticoagulant for hematological analysis. Blood samples were collected one hour before the commencement of the experiment and thereafter on days 7 and 14 following the administration of the formulation.

Blood was collected from the retro-orbital plexus of rats under mild anesthesia using glass capillaries and preserved with disodium ethylenediaminetetraacetic acid for the assessment of biochemical markers. The total platelet count (PC), total leucocyte count (TLC), and differential leucocyte count (DLC), encompassing neutrophils, lymphocytes, monocytes, eosinophils, and basophils, were assessed [27-29]. The typical behaviors, fur luster, dietary intake, hydration, weight fluctuations, and death rate of the rats were meticulously monitored. The animals were weighed daily for 14 days [30-32]. All animals completed the study protocol, and data from all animals were included in the final analysis. The study was conducted without blinding, and the investigators were aware of the treatment groups throughout the experiment and data analysis.

EVALUATED PARAMETERS

Physical parameters

The measurements taken included fur luster, diet and drinking amounts, and changes in weight and mortality rate.

Key parameters

A comprehensive blood count (CBC) was performed, including measurements of Total Platelet Count (PC) and Total Leucocyte Count (TLC). The Differential Leucocyte Count (DLC) was also determined, specifying the counts for neutrophils, lymphocytes, monocytes, eosinophils, and basophils.

Determination of clotting time

Clotting time was determined using blood collected from the retro-orbital venous plexus under mild anesthesia at the designated sampling time points during the 14-day study. Blood was collected into a glass capillary tube (internal diameter 0.9–1.1 mm; length 10 cm).

The capillary tube was kept horizontal and gently broken at 30-second intervals until a fibrin thread appeared. Clotting time was recorded as the interval between blood collection and the first appearance of a fibrin thread [26].

Determination of bleeding time

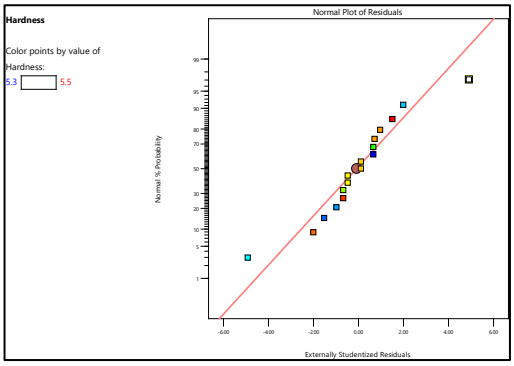
The bleeding duration was measured using a modified Duke technique, in which the animal was restrained in a rat holder with its tail exposed. The animal's tail was first washed with hot water and rectified alcohol, then punctured with a sterile needle. The tail was blotted dry onto Whatman® filter paper until bleeding ceased. Bleeding duration was measured in seconds [26].

RESULTS AND DISCUSSIONS

Optimization of Sirolimus Drug Layering formulation

ANOVA was used to analyze the effects of formulation variables on tablet hardness and friability, based on a quadratic model, using the Box−Behnken experimental design. The model was statistically significant for hardness, as evidenced by an F value of 23.12 and a corresponding p-value of 0.0002. HPMC E5 LV had the largest impact on hardness ($F = 163.94$, $p < 0.0001$) of all the factors examined, followed by sucrose ($F = 9.93$, $p = 0.0161$). Additionally, the quadratic terms A^2 ($F = 10.99$, $p = 0.0128$) and B^2 ($F = 12.97$, $p = 0.0087$) were significant, indicating a quadratic relationship between the factors and hardness. The factors: calcium sulfate (C) and interaction terms produced p-values greater than 0.05, suggesting that these factors may possess relatively less influence on the hardness of the tablets. The data showed that the same model used for friability also had an F-value of 15.13 and a p-value on the order of 0.00084, which suggested it was significantly different from 0, as both values confirmed through statistical testing that the resulting HPMC E5 LV (A) significantly contributed to friable tablets with an F-value of 62.99 and p-value ≤ 0.0001. The interaction of the sugar (sucrose) and the interaction between sucrose (B) and calcium sulfate anhydrous (C) produced moderate differences between each other within the friable tablet population with an F-value of 6.27 and p-value of 0.007. The quadratic terms A^2 ($F=37.43$, $p=0.00049$) and B^2 ($F=12.58$, $p=0.0094$) indicated curved surface effects were also present. Statistically significant terms are considered to be p-values < 0.05. Therefore, all p-values greater than this were shown to be less significant. This data shows that HPMC E5 LV concentration has the major effect on hardness and friability in the sirolimus nanodispersion tablet. Model adequacy was confirmed using Design-Expert software. The selected quadratic model showed satisfactory adjusted and predicted R^2 values with a non-significant lack-of-fit, indicating that it adequately described the experimental data and was suitable for formulation optimization. The optimized formulation was subsequently validated experimentally,

confirming the predictive capability of the developed model. Therefore, the selected models were used for desirability-based optimization of the sirolimus nanodispersion drug-layered tablets. The quadratic polynomial equations show the model relationships between the formulation variables (HPMC E5 LV (A), sucrose (B), and calcium sulfate anhydrous (C)) and response variables (tablet hardness and friability). The polynomial equations specify how HPMC E5 LV (A), sucrose (B), and calcium sulfate anhydrous (C) affect tablet hardness and friability through their linear, interaction, and quadratic effects.



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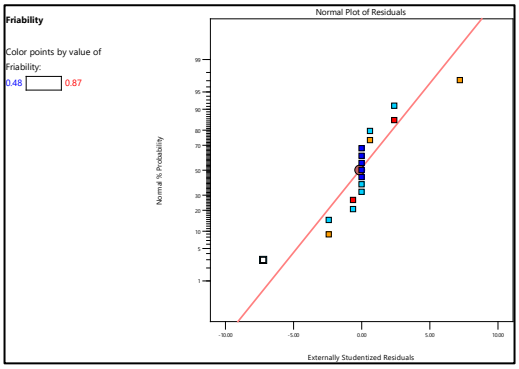

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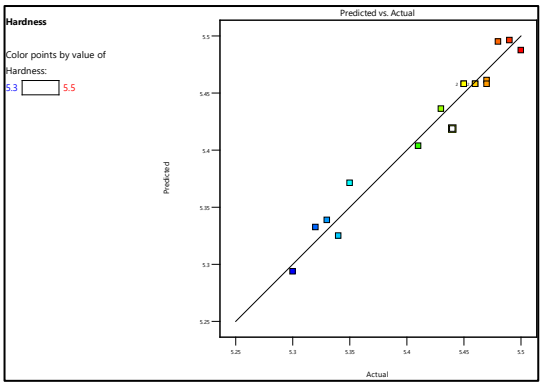

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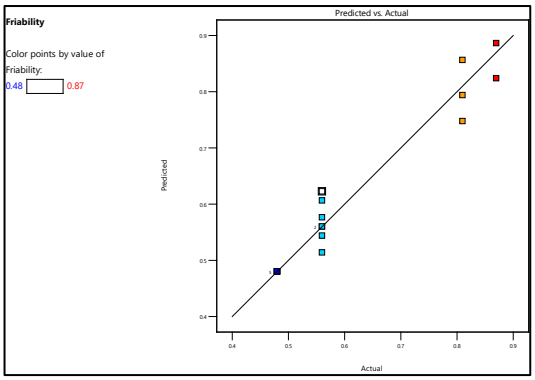

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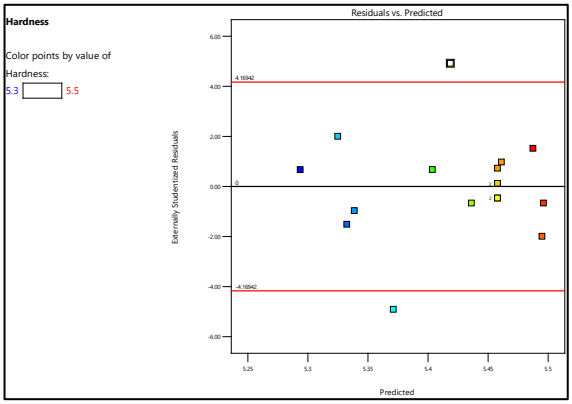
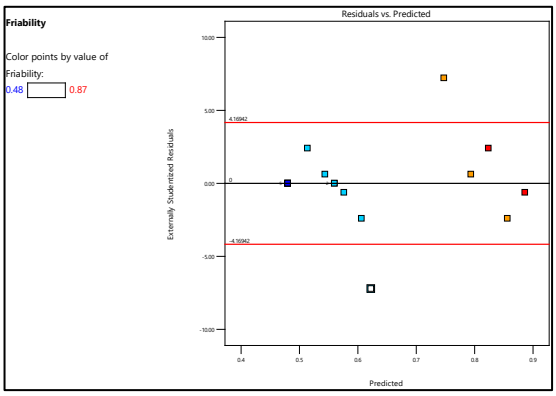
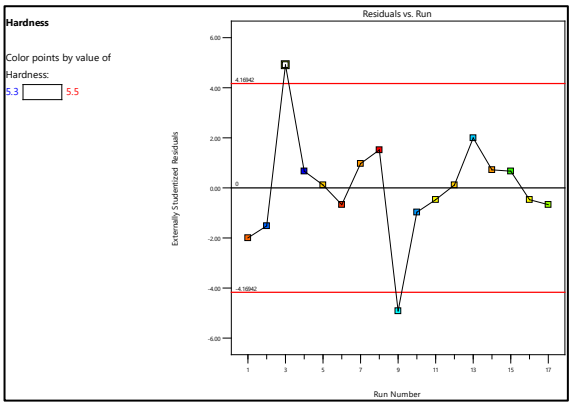


(e)



(f)



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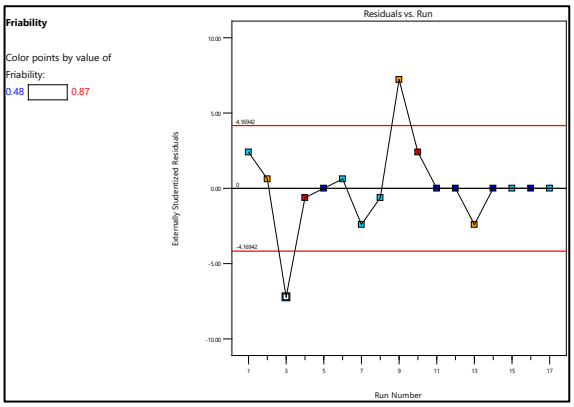

(h)

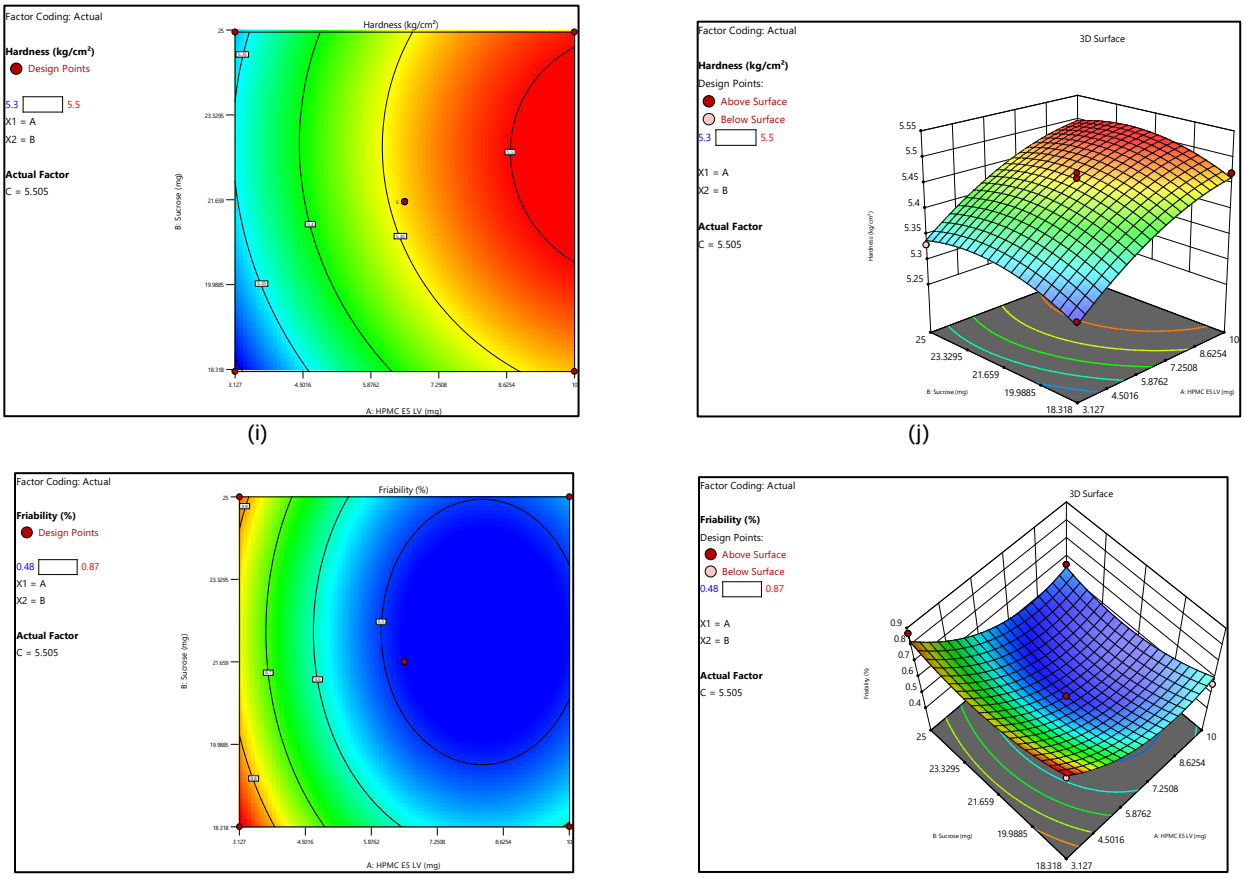

Figure 1: Diagnostic plots for evaluation of the quadratic models generated by Box–Behnken design. (a) Predicted versus actual plot for hardness, showing good agreement between experimental and predicted values. (b) Predicted versus actual plot for friability. (c) Normal probability plot of residuals for hardness. (d) Normal probability plot of residuals for friability. (e) Residuals versus predicted response for hardness. (f) Residuals versus predicted response for friability. (g) Residuals versus run order for hardness. (h) Residuals versus run order for friability. The random distribution of residuals and close agreement between predicted and observed responses indicate the adequacy and predictive reliability of the selected quadratic models. (i) 2D contour plot and (j) 3D response surface plot showing the effect of HPMC E5 LV and sucrose on tablet hardness; (k) 2D contour plot and (l) 3D response surface plot showing the effect of HPMC E5 LV and sucrose on tablet friability.

The 2D contour plot and 3D response surface plot in Figure 1(i, j) illustrate the combined influence of HPMC E5 LV (A) and sucrose (B) on tablet hardness while keeping calcium sulfate constant. The increase in polymer concentration positively affects hardness, indicating that higher HPMC levels contribute to stronger tablet mechanical properties.

In Figure 1(k, l), 2D contour and 3D response surface plots illustrate the combined influence of HPMC E5 LV (A) and sucrose (B) on tablet friability at a constant level of calcium sulfate (C). The plots indicate that increasing HPMC conc. reduces friability, suggesting improved tablet mechanical strength and stability.

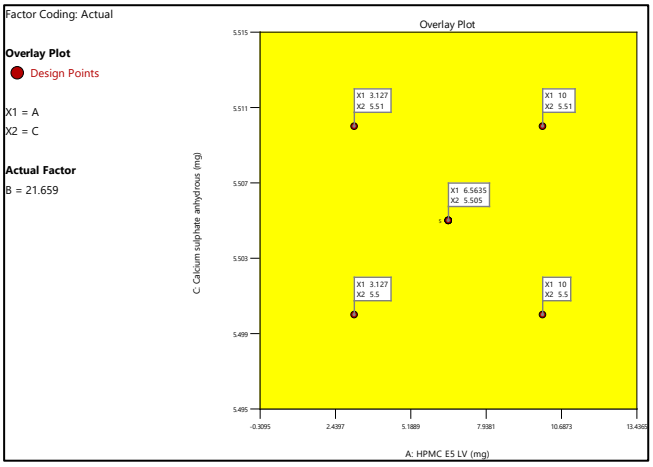

Figure 2: overlay plot of optimized formulations

Table 4: Coefficient table for the Box–Behnken design models of hardness and friability.

	Intercept	A	B	C	AB	AC	BC	A^2	B^2	C^2
Hardness	5.458	**0.08125**	**0.02**	-0.0037	-0.0025	1.61E-18	0.0125	**-0.029**	**-0.0315**	**-0.019**
p-values		**< 0.0001**	**0.0161**	0.5731	0.7886	1	0.2063	**0.0128**	**0.0087**	**0.0664**
Friability	0.48	**-0.14**	-0.0312	0.03125	-8.05E-18	1.26E-18	**-0.0625**	**0.14875**	**0.08625**	**0.05625**
p-values		**< 0.0001**	0.1198	0.1198	1	1	**0.0407**	**0.0005**	**0.0094**	**0.0539**
Response	**Model**	**Sequential p-value**	**Lack-of-Fit p-value**		**Adjusted R^2**		**Predicted R^2**		**Model Selection**	
Hardness	Linear	5.70×10^{-5}	0.0053		0.7661		0.7035		–	
	2FI	0.9122	0.0029		0.7109		0.4928		–	
	Quadratic	**0.0054**	**0.64**		**0.9256**		**0.5377**		**Selected**	
	Cubic	0.0277	Aliased		0.9838		–		Aliased	
Friability	Linear	0.0306	–		0.3648		0.1738		–	
	2FI	0.8182	–		0.2443		−0.3699		–	
	Quadratic	**0.0008**	**0.53**		**0.8882**		**0.2176**		**Selected**	
	Cubic	Aliased	Aliased		1		–		Aliased	

A = HPMC E5 LV, B = Sucrose, C = Calcium sulphate anhydrous. The selected quadratic models showed Adjusted R^2 values of 0.9256 (hardness) and 0.8882 (friability). Predicted R^2 values were 0.5377 and 0.2176, respectively. The lack-of-fit was non-significant for both models (Hardness: p = 0.64; Friability: p = 0.53. ***p-value shading: p < 0.1*** *(highly significant),* ***0.1 ≤ p < 0.2*** *(moderately significant), and* ***p ≥ 0.2*** *(not significant).*

Design-Expert software was used for Numerical optimization based on the desirability function to create optimized formulations with the desired tablet characteristics (hardness and friability). The design space (reflected in the overlay plot in fig. **2**) included the region where both hardness and friability were satisfied, as indicated by simulating separate response surfaces for each of them. Based on the maximum desirability value, the model generated five optimized formulations, which were then prepared experimentally and evaluated to validate the Box–Behnken design's predictive ability and the appropriateness of the optimized composition for developing sirolimus nanodispersion drug-layered tablets.

Experimental validation of the optimized formulation

The optimized formulation predicted by the Box–Behnken design was experimentally validated to assess the predictive accuracy of the developed quadratic model. As summarized in Table **5**, the experimentally observed responses were in close agreement with the model-predicted values. The prediction errors for hardness and friability were 1.92% and 1.22%, respectively, indicating excellent predictive performance. The low prediction errors, together with the narrow model-predicted standard deviations, demonstrate the robustness, adequacy, and reliability of the optimization model for developing the sirolimus nanodispersion drug-layered tablet. Prediction error (%) was calculated as:

$$\textbf{\textit{Prediction error}}\ (\%) = \frac{\textbf{\textit{Observed} − \textbf{\textit{Predicted}}}}{\textbf{\textit{Predicted}}} \times 100$$

Calculation:

- **Hardness** = |5.581 − 5.47573| / 5.47573 ×100 = **1.92%**
- **Friability** = |0.536 − 0.542628| / 0.542628 ×100 = **1.22%**

Table 5: Experimental validation of the optimized Box–Behnken model

Response	Predicted value	Observed value	Prediction error (%)
Hardness (kg/cm2)	5.476	5.581	1.92
Friability (%)	0.543	0.536	1.22

PREPARATION AND EVALUATION OF FIXED-DOSE COMBINATION TABLET

Evaluation of powder blends (Pre-compression study)

The compressible composites of the Sirolimus immediate-release layer demonstrate a Carr's index ranging from 2.38 to 4.76%, a Hausner's Ratio ranging from 1.02 to 1.05, and an angle of repose ranging from 26.31±0.5 to 28.80±0.8. The Hausner's Ratio was consistently 1.03 across all batches, and the Carr's index was uniformly 3.63%. The angle of repose for Prednisolone's instantaneously compressible composites ranged from 26.76±0.7 to 29.51±0.5. Pre-compression experiments conducted in accordance with USP criteria have demonstrated that all immediate-release and sustained-release formulations exhibit acceptable flow and compression properties. Tables 6 and 7 demonstrate that magnesium stearate functions as a lubricant in these quick-release, instantaneously compressible powder blends, while talc serves as a diluent, thereby improving

their flow and compressibility. The nanodispersion drug-layering technology provides a substantial pharmaceutical benefit over conventional fixed-dose combination tablets. Sirolimus, a BCS Class II drug with dissolution-limited absorption, was nanosized to <400 nm by Dynomill processing. This significantly increases the surface area, the rate of dissolution, and gastrointestinal absorption. In contrast to conventional combined tablets, where the two drugs are uniformly mixed, nanodispersion layering technology enables the spatial separation of prednisolone (core) and sirolimus (layered coat), thereby reducing drug-drug interactions during compression and improving chemical stability. Moreover, the uniform deposition of nanosized sirolimus on the barrier-coated core enables dose uniformity and controlled release kinetics.

The layered system may also reduce inter-individual pharmacokinetic differences and increase systemic bioavailability compared with conventional FDC tablets. Therefore, the observed improvement in platelet recovery may be associated with the formulation design and nanosized drug dispersion; however, pharmacokinetic studies are required to confirm any enhancement in bioavailability.

Table 6: Pre-compression parameters (D1- D5)

Optimized Formulation Code	Angle of Repose (θ)	Bulk density (g/ml)	Tapped density (g/ml)	Carr's Index (%)	Hausner's Ratio
D1	28.22±0.5	0.41±0.005	0.42±0.005	2.38	1.02
D2	27.29±0.1	0.40±0.005	0.42±0.005	4.76	1.05
D3	26.31±0.5	0.41±0.005	0.42±0.005	2.38	1.02
D4	28.80±0.8	0.41±0.005	0.42±0.005	2.38	1.02
D5	28.51±0.7	0.40±0.005	0.41±0.005	2.54	1.05

±S.D. n=3

Table 7: Pre-compression parameters of (A1-A5).

Optimized Formulation Code	Angle of Repose (θ)	Bulk density (g/ml)	Tapped density (g/ml)	Carr's Index (%)	Hausner's Ratio
F1	26.76±0.7	0.53±0.006	0.55±0.005	3.63	1.03
F2	28.71±0.4	0.53±0.006	0.55±0.005	3.63	1.03
F3	28.61±0.4	0.53±0.006	0.55±0.005	3.63	1.03
F4	29.51±0.5	0.53±0.006	0.55±0.005	3.63	1.03
F5	27.54±0.3	0.67±0.004	0.56±0.003	3.53	1.05

Particle size distribution (PSD) of optimized sirolimus nano-dispersion by Malvern Mastersizer

The distribution of particle size for optimized sirolimus nano-dispersions formulation F1, F2, F3, and F4 was assessed using a Malvern particle size analyzer, as shown in Figures 3, 4, 5, and 6, respectively. All formulations showed a uniform distribution, indicating no significant aggregates and demonstrating that the average size of particles created by each formulation was similar, with each having the same mean particle size value ranging from 268.4 to 273.2 nm, with PDI values of 0.316 to 0.342, which indicates that they have a good degree of homogeneity and stability.

The optimized formulation (F-4) had an average particle size of 268.4 nm. It produced a distribution profile so narrow that it provided a larger area of contact, allowing greater absorption of poorly soluble sirolimus during dissolution than would normally occur without the nano-dispersion process, owing to its smaller size. The stable sirolimus nano-dispersion formulations were prepared with particle sizes in the nanometer range.

EVALUATION OF TABLET (POST COMPRESSION STUDY)

The overall appearance of the manufactured tablets (F1–F4) was assessed. The white, spherical tablets were pleasing to the touch.

Weight Variation

The percentage weight variation of the tablets from samples F1–F4 was determined to be between 0.18 and 4% of the average tablet weight, which is in accordance with the standard (approximately ±5.0% deviation for tablets of over 324mg).

Thickness

The thickness of tablets from each batch was measured at random, yielding a mean of 0.50±0.005 cm. The standard deviation values were confirmed to fall within the prescribed range after calculation.

Hardness

The pulverized tablets from all batches F1–F4 were determined to be within the range of 5.3 ± 0.057 to 5.5 ± 0.115 kg/cm2 (n = 3), as indicated by the standard record.

Friability Test

Batches F1–F4 exhibited friability values between 0.48 and 0.87, which are within the standard range (1%, n=3).

Drug content

UV spectroscopy was employed to evaluate the drug content of the prepared tablets (batches F1–F4). The absorbance of sirolimus in methanol was measured at 296 nm (λmax). The assay was determined to be within the standard record limit (92.5%-107.5%), with a variance of 98.0% to 99.70%. The prepared tablets (batches A1–A4) were subjected to UV spectroscopic analysis. The absorbance of prednisolone in methanol was measured (λmax) at 263 nm. The assay results were within the 95%–105% standard record limit, ranging from 99.30% to 102.07% (Table 8).

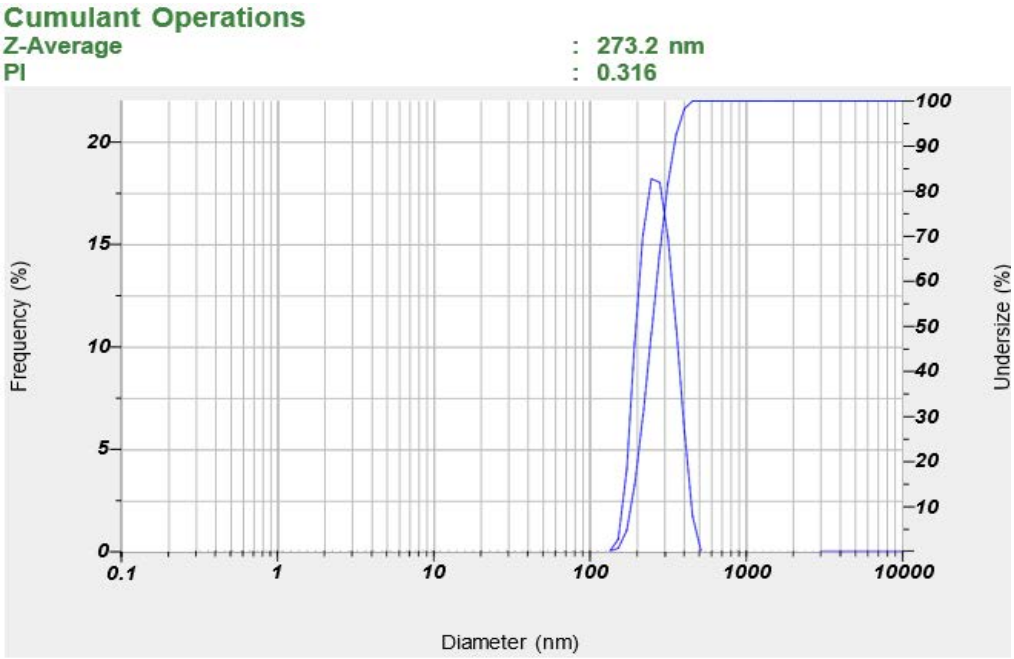

Figure 3: Particle size of F1

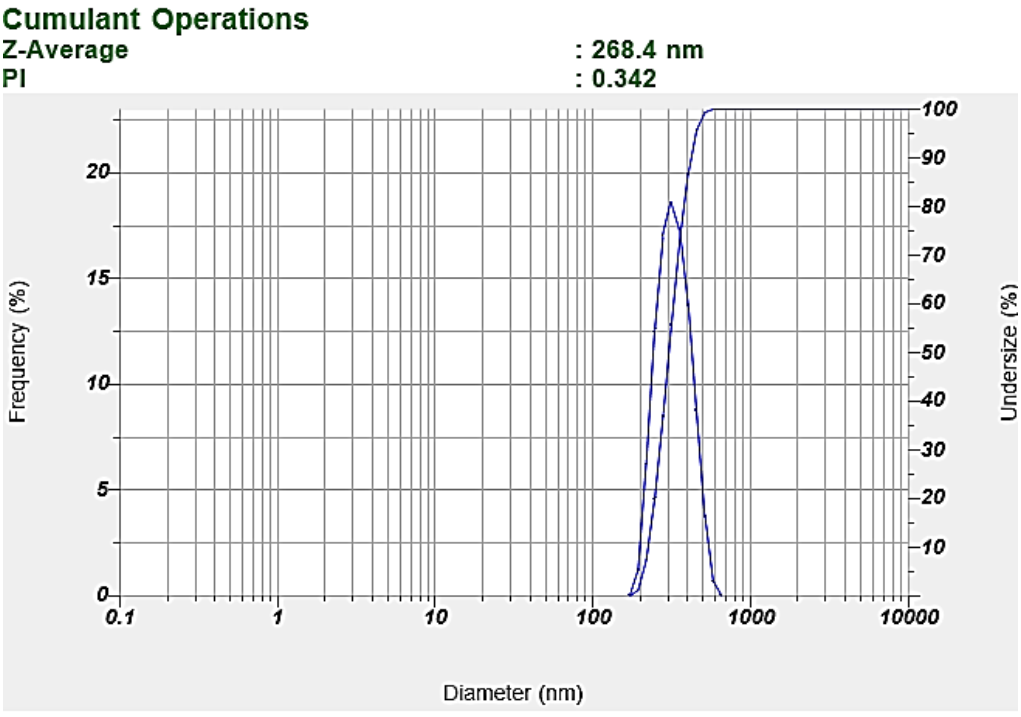

Figure 4: Particle size of F2

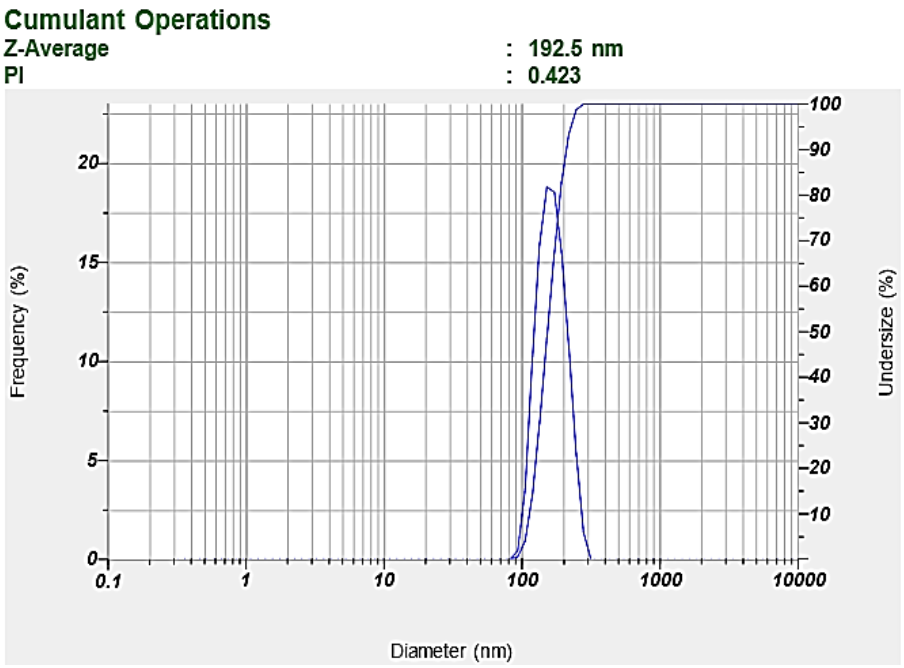

Figure 5: Particle size of F3.

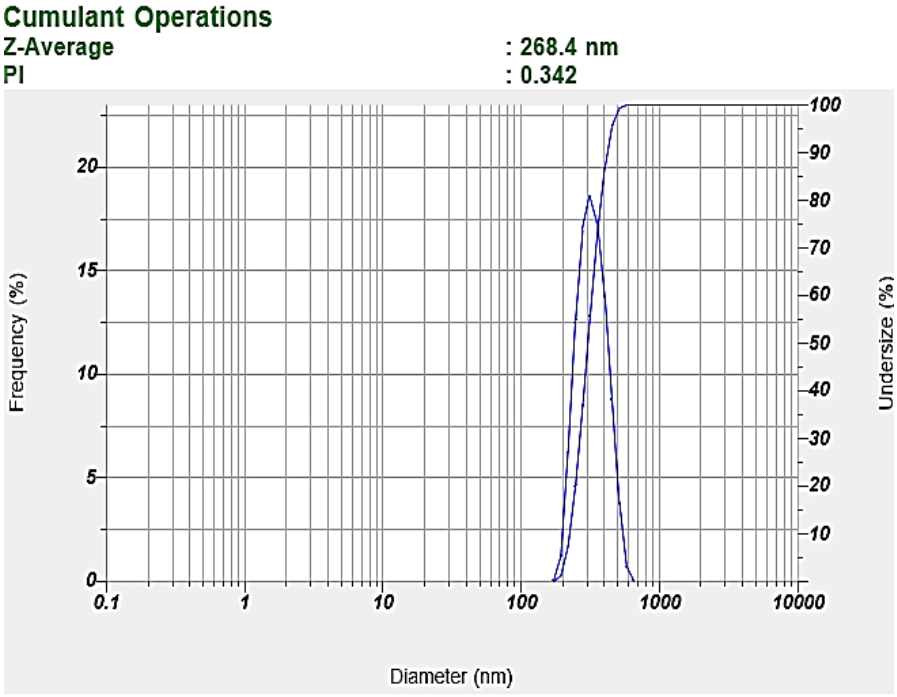

Figure 6: Particle size of F4.

Table 8: post-compression evaluation parameters

Optimized Formulation Code	Average Weight (gm)	Thickness (cm)	Hardness (Kg/cm^2)	Friability (%)	Assay (%)	
					Sirolimus	Prednisolone
F1	0.448	0.55±0.00 5	5.5 (≈ 540 N)	0.87	99.70	100.60
F2	0.527	0.55±0.005	5.5 (≈ 540 N)	0.56	98.00	99.30
F3	0.533	0.55±0.005	5.5 (≈ 540 N)	0.56	99.65	102.07
F4	0.531	0.55±0.005	5.5 (≈ 540 N)	0.48	99.33	101.69
F5	0.508	0.55±0.00 5	5.4 (≈ 530 N)	0.81	100.81	99.83”

value is represented as mean ± SD (n = 3), Friability ≤ 1.0% (USP guideline), Hardness and weight variation acceptance ranges. [* 1 kgf ≈ 9.81 N]

The selection of a layered tablet design was beneficial over a traditional fixed-dose matrix tablet because sirolimus has very poor solubility in water and dissolution-limited oral absorption. Therefore, using a nanosized dispersion layer to disperse sirolimus into nanodroplet form improves rapid wetting and enhances dissolution.

In addition, having sirolimus physically separated from prednisolone minimizes drug-drug interactions and excipient-drug interactions during storage and manufacturing of these products, thus improving stability. Additionally, the prednisolone barrier coating ensures that equal amounts of nanosized sirolimus are uniformly deposited, thereby providing greater dosing accuracy for these two drugs than if they were mixed or administered separately. Therefore, the developed layered nanodispersion system showed promising formulation characteristics, although additional studies are needed to confirm its impact on *in vivo* performance and therapeutic outcomes.

Coating Uniformity

To evaluate the coating uniformity of the optimized sirolimus nanodispersion layered tablet (F4), the drug content of ten individual coated tablets was measured. The drug content of each tablet is shown in Table 9, where the drug content ranges from 98.39% to 99.97%. The mean drug content was 99.39 ± 0.56%, and the %RSD was 0.57%. The low variation in drug content shows the reproducibility of the optimized drug-layering procedure.

Uniformity of the coating is an important quality attribute for drug-layered tablet formulations because non-uniform drug deposition may lead to variability in drug loading and dissolution behavior. The good coating uniformity of the optimized formulation results from optimized coating process parameters. Because Poloxamer 188 and PVP K30 were incorporated into the nanodispersion, the suspension's stability increased, and particle agglomeration was significantly reduced during spraying, thereby achieving uniform sirolimus coating across the tablet. A low %RSD for the coating (0.57%) indicates that the coating procedure was completed and that tablets with uniform drug distribution were obtained.

Coating Weight Gain Uniformity

The uniformity of coating weight gain of the optimized sirolimus nanodispersion layered tablets (F4) was determined by weighing each of the resultant tablets before and after they were subjected to the drug-layering process, as presented in Table 10. The optimized formulation had an average initial weight of 200.03±0.10 mg, which increased to 204.98±0.11 mg after drug-layering, indicating a coating weight gain of 4.95±0.02 mg. The calculated coating weight gain was 2.48±0.01%, with %RSD of 0.40, indicating good tablet-to-tablet uniformity during the coating process.

The minimal variability in the coating weight gain indicates that the developed drug-layering process resulted in continuous deposition of the sirolimus nanodispersion onto the barrier-coated prednisolone core tablets. The level of coating uniformity achieved in the study is an important quality parameter of a drug-layered tablet formulation, as it enables accurate loading of the active substance, reduces inter-tablet differences in drug dosage, and consequently provides uniform dissolution behavior.

The high coating uniformity is achieved through optimized coating parameters. Moreover, the use of Poloxamer 188 and PVP K30 in the nanodispersion ensured stable suspension and prevented particle aggregation, thereby resulting in uniform deposition of sirolimus on the tablet surface. The %RSD value of 0.40% further confirms that the process was reproducible and enabled the production of tablets with uniform coating thickness and coating deposition.

Table 9: Drug content-based coating uniformity of the optimized sirolimus nanodispersion layered tablet (F4).

Tablet No.	Drug Content (mg)	Drug Content (%)
1	1.98	99.89
2	1.99	99.56
3	1.99	99.61
4	1.99	99.82
5	1.98	98.78
6	1.99	99.91
7	1.98	99.01
8	1.99	98.39
9	1.99	99.97
10	1.97	98.94
Mean ± SD		**99.39 ± 0.56**
%RSD		**0.56**

***In vitro* dissolution study**

The *in vitro* dissolution profiles of the pure sirolimus API and the commercial sirolimus tablet differed from those of the newly formulated nanodispersion layered tablet. Of the three formulations, the nanodispersion layered tablet achieved the

highest cumulative drug release over the dissolution period. At 30 minutes, the optimized formulation released approximately 68.61% ± 0.25 sirolimus, compared with 56.37% ± 0.21 and 38.36% ± 0.39 for the commercial sirolimus tablet and pure sirolimus API, respectively.

At 60 minutes, the formulation achieved a cumulative drug release of 89.88% ± 1.04, whereas the commercial tablet had only 67.18% ± 1.11 and the pure sirolimus API only 51.29% ± 1.20. After 90 minutes of dissolution, the optimized formulation had the highest cumulative drug release of 97.39% ± 0.13, compared with the commercial sirolimus tablet at 79.33% ± 0.41 and the pure sirolimus API at 59.35% ± 1.26 (Figure 6).

Table 10: Coating weight gain uniformity of the optimized sirolimus nanodispersion layered tablet (F4).

Tablet No.	Weight (mg)			Coating Weight Gain (%)
	Initial	Final	Gain	
1	200.12	205.09	4.97	2.48
2	199.95	204.9	4.95	2.48
3	200.08	205.04	4.96	2.48
4	200.15	205.11	4.96	2.48
5	199.88	204.82	4.94	2.47
6	200.1	205.07	4.97	2.48
7	199.97	204.91	4.94	2.47
8	200.06	205.01	4.95	2.47
9	200.04	205	4.96	2.48
10	199.91	204.84	4.93	2.47
Mean ± SD	**200.03 ± 0.10**	**204.98 ± 0.11**	**4.95 ± 0.02**	**2.48 ± 0.01**
%RSD	**0.05**	**0.05**	**0.4**	**0.4**

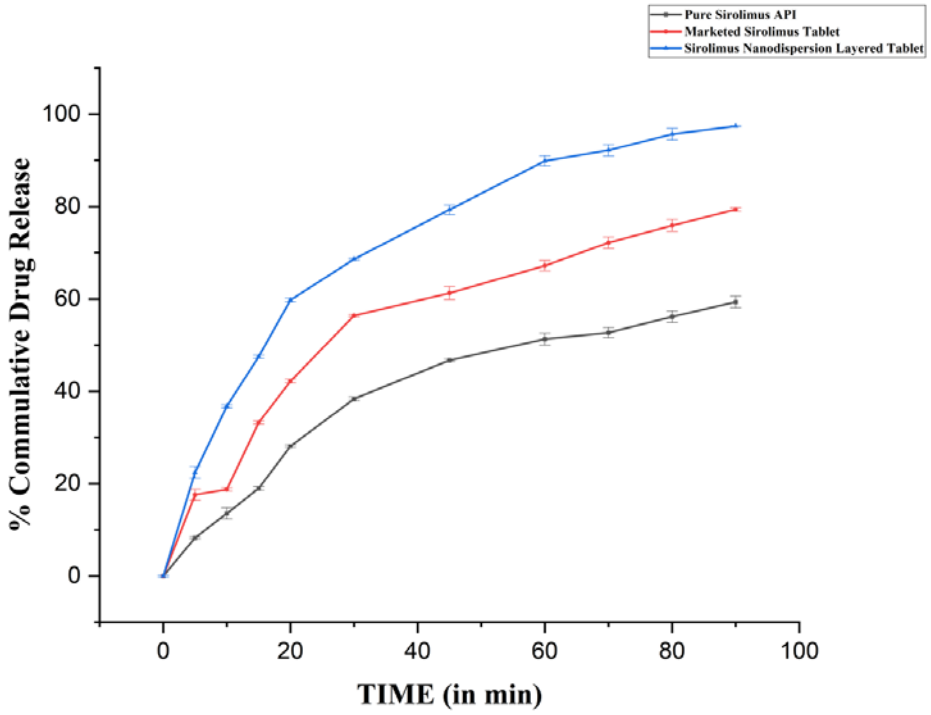

Figure 6: *In vitro* dissolution profiles of pure sirolimus API, marketed sirolimus tablet, and the developed sirolimus nanodispersion layered tablet in phosphate buffer (pH 6.8) at 37 ± 0.5 °C using the USP Apparatus II (paddle method). Data are presented as mean ± SD (n = 3).

The improvement in dissolution observed with the formulation can be explained by reducing sirolimus particle size to the nanometer range, thereby increasing the effective surface area available for dissolution per the Noyes-Whitney equation. Additionally, the use of Poloxamer 188 and PVP K30 to stabilize the nano-dispersion improved particle wettability and reduced aggregation, thereby increasing the dissolution rate. On the other hand, the uniform coating of the prednisolone core using the nano-dispersion increased the drug's surface area, thereby leading to greater dissolution than with the pure drug or the marketed formulation. Moreover, the nano-dispersion drug layering technology can effectively address the dissolution issues associated with poorly soluble sirolimus, thereby improving its bioavailability. Sirolimus is released; various

models, such as the Zero-order, First-order, Higuchi, Korsmeyer-Peppas, and Hixson-Crowell kinetic models, were evaluated using the obtained dissolution data. The correlation coefficients are shown in Table 11. Pure sirolimus API was best fitted to the Higuchi model, with a correlation coefficient (R^2) of 0.9750±0.07.

This indicates that the drug release process is predominantly diffusion of the drug from the drug particles into the dissolution fluid. Likewise, the marketed sirolimus tablets were found to be well-fit by the Higuchi model (R^2 = 0.9740±0.24). The sirolimus nanodispersion layered tablets produced for this study exhibited the highest degree of conformity with the First-order kinetic model (R^2 = 0.9927±0.18), with the Hixson–Crowell model (R^2 = 0.9823±0.12) and the Higuchi model (R^2 = 0.9762±0.29) ranking second and third, respectively. This perfect compatibility with the First-order kinetic model indicates that drug release from the formulation is concentration-dependent, with the rate of release decreasing as the amount of drug in the formulation decreases. This behavior is characteristic of immediate-release formulations containing highly dispersed drug particles, in which rapid wetting and dissolution occur due to the increased surface area resulting from nanosizing. The shift from diffusion-controlled release, as observed in the pure drug and marketed formulations, to concentration-dependent release, as observed in the optimized formulation, underscores the importance of nanodispersion drug layering technology in altering the dissolution properties of sirolimus.

The kinetic studies provide evidence that the developed nanodispersion layered tablet improved the dissolution profile of sirolimus and altered the drug-release mechanism relative to the pure drug and the marketed formulation. The results demonstrate that the developed formulation is a promising approach for processes that improve the drug's oral bioavailability while retaining rapid and reproducible drug release.

Table 11: Drug release kinetic analysis of pure sirolimus API, marketed sirolimus tablet, and developed sirolimus nanodispersion layered tablet.

Formulation	Zero-order	First-order	Higuchi	Korsmeyer–Peppas	Hixson–Crowell
Pure Sirolimus API	0.9089±0.12	0.9569±0.09	**0.975**±0.07	0.9168±0.49	0.9428±0.24
Marketed Sirolimus Tablet	0.8808±0.37	0.9717±0.19	**0.974**±0.24	0.9414±0.31	0.9485±0.57
Developed Nanodispersion Layered Tablet	0.849±0.16	**0.9927**±0.18	0.9762±0.29	0.9767±0.71	0.9823±0.12

IN-VIVO STUDY

Effects of sirolimus nanodispersion drug layering technology over prednisolone core tablets on platelet count

In the present study, thrombocytopenia was induced by intraperitoneal administration of cyclophosphamide (50 mg/kg) on Day 1. Platelet counts were significantly decreased (39.875 ± 8.825 103/mm3; $p<0.001$) in the disease control animals, as shown in Figure 7, compared with the Normal control (288.25 ± 24.5 103/mm3), which was treated with 0.5% CMC only. Administration of 10 mg Prednisolone and 2 mg Sirolimus /kg, p.o., twice a day, increased platelet production, i.e., 90.57 ± 7.40 $10^3/mm^3$ as shown in Table 12.

The increases in platelets seen after using both prednisolone and sirolimus are due to both medications (corticosteroid) working together [33]. The observed response may also have been influenced by the known immunosuppressive effects of prednisolone [34] and the inherent biological variability of the experimental animals. Sirolimus is a selective inhibitor of the mammalian target of rapamycin (mTOR) [35], which plays a pivotal role in T cell proliferation and differentiation. In the context of immune thrombocytopenia, dysfunctional regulatory T cells (Tregs) fail to inhibit autoreactive cytotoxic T lymphocytes that mediate platelet destruction. mTOR inhibition selectively enhances Treg proliferation and functional recovery while inhibiting effector T-cell responses. This leads to the restoration of immune tolerance and a decrease in antibody-mediated platelet destruction. Prednisolone, on the other hand, rapidly inhibits inflammation and immune responses by suppressing cytokine expression, macrophage activation, and autoantibody production.

The combination of prednisolone and sirolimus offers immediate immune inhibition of platelet destruction and subsequent immune reconstitution by Tregs, respectively. These results suggest that combination therapy may improve platelet recovery in the cyclophosphamide-induced thrombocytopenia model.

Table 12: Effect of sirolimus nanodispersion drug layering technology over prednisolone core tablets on platelet counts

Group-no	Groups	Dose/Route	Platelet count (10^3/mm^3)
1	Naïve control (Animal=6)	0.5% CMC (1 ml/kg, p.o.)	288.25 ± 24.5
2	Disease control (Animal=6)	0.5% CMC (1 ml/ kg, p.o.)	39.87 ± 8.82***
3	Prednisolone	10 mg /kg, p.o, BID	46.32 ± 10.62***
4	Prednisolone + Sirolimus (QD)	10 mg/kg, p.o.; QD+ 2 mg/kg, p.o.; QD	132.62 ± 21.76
5	Prednisolone + Sirolimus (BID)	10 mg/kg, p.o.; BID+ 2 mg/kg, p.o.; BID	154.57 ± 7.40

**** Data are expressed as Mean±SEM (n = 6), using one-way ANOVA followed by post hoc comparison test. *p<0.05, *p<0.01, ***p<0.001***

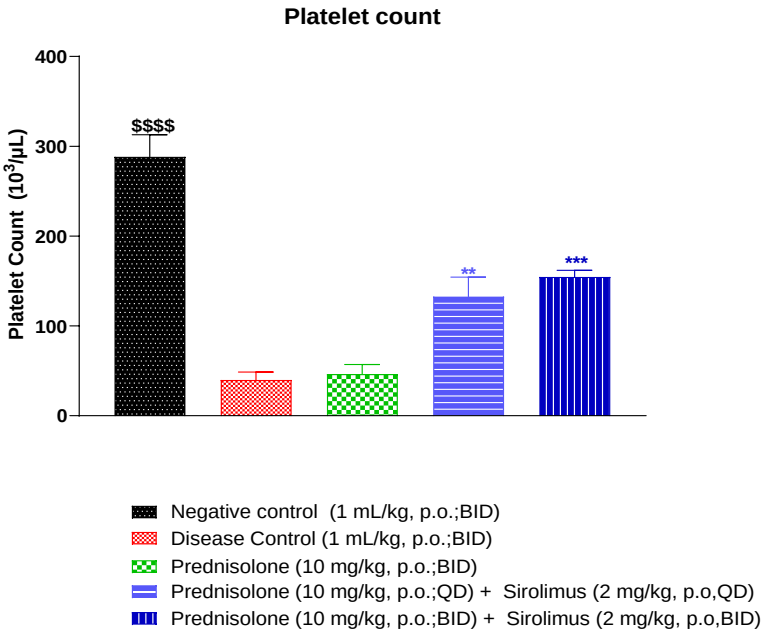

Figure 7: Effect of sirolimus nanodispersion drug layering technology over prednisolone core tablets on platelet count in cyclophosphamide-induced thrombocytopenic rats. Data are expressed as mean ± SEM (n=6). Statistical significance indicated as *p < 0.05, **p < 0.01, ***p < 0.001 compared with Disease Control group.

Effects of sirolimus nanodispersion drug layering technology over prednisolone core tablets on Bleeding time

Blood loss time in negative control animals was 44 ± 2.19 seconds. After inducing thrombocytopenia with cyclophosphamide, blood loss time was significantly higher than in the negative control group, at 62.85 ± 9.6 seconds (P < 0.001). Animals that were treated in groups A & B with Once daily oral doses of either 10mg of Prednisone or 2mg /kg of Sirolimus showed a significant reduction in blood loss time compared with positive control animals, at 45.5 ± 2.47 seconds (P < 0.001) and 51.85 ± 2.71 seconds (P < 0.05), respectively.

Effects of sirolimus nanodispersion drug layering technology over prednisolone core tablets on Clotting time

Animals in the negative control group in Table 13, who received no treatment except 0.5% CMC, exhibited a mean clotting time of 116 ± 4.75 seconds, while those in the positive control group treated with cyclophosphamide had a significantly increased mean clotting time (p < .05), at 157.7 ± 37.5 seconds, compared to the positive controls (Figure 8). Administration of Prednisolone (10 mg/kg) significantly reduced clotting time compared with the disease control group, decreasing to 100.87 ± 8.41 seconds (p < .01). Administration of Prednisolone (10 mg/kg) and Sirolimus (2 mg/kg) in the developed formulation significantly reduced clotting time compared with the disease control group via p.o.; the animals had a significantly (p < .001) shorter clotting time of 86.71 ± 3.29 seconds than the positive controls treated with only cyclophosphamide (Figure 9).

Effects of sirolimus nanodispersion drug layering technology over prednisolone core tablets on WBC counts

The mean WBC count for the negative control, as presented in Table 14, was 6.73 ± 2.16 (103/mm^3). In comparison, the mean WBC count for the positive control (cyclophosphamide treated

for only 3 days) was 3.28 ± 0.93 (103/mm^3). The positive controls have significantly lower WBC counts than the negative control, indicating thrombocytopenia in rats, but this difference is not statistically significant (p>0.05). When the rats were given either 10 mg prednisolone and 2 mg sirolimus per kg by mouth two times daily there was no significant increase (p>0.05) in their WBC count versus that of the control (4.65 ± 1.02 103/mm^3), however, WBC counts were significantly elevated in the Prednisolone + Sirolimus (BID) treatment group compared with the disease control group (8.17 ± 1.33 103/mm^3) as shown in figure 10.

Table 13: Effect of sirolimus nanodispersion drug layering technology over prednisolone core tablets on clotting time and bleeding time

Grp. No.	Groups	Dose/Route	Clotting time in seconds(Mean±SEM)	Bleeding Time
1	Naïve control (Animal=6)	0.5% CMC (1 ml/kg, p.o.)	112.9 ± 8.95	61.6 ± 4.75
2	Disease control (Animal=6)	0.5% CMC (1 ml/ kg, p.o.)	209.7 ± 22.31* a	157.7 ± 37.5* a
3	Prednisolone	10 mg /kg, p.o, BID	146.8 ± 12.52** b	100.87 ± 8.41** b
4	Prednisolone+Sirolimus (QD)	10 mg/kg, p.o.; QD+2 mg/kg, p.o.; QD	123.3 ± 6.79*** b	86.71 ± 3.29*** b
5	Prednisolone+Sirolimus (BID)	10 mg/kg, p.o.; BID+2 mg/kg, p.o.; BID	112.4 ± 9.89	67.57 ± 7.40

Data are expressed as mean±SEM (n = 6), using one-way ANOVA followed by post hoc comparison test *p<0.05, *p<0.01, ***p<0.001 [a = compared with Naïve Control group; b = compared with Disease Control group]

Table 14: Effect of sirolimus nanodispersion drug layering technology over prednisolone core tablets on WBC, RBC, and Hemoglobin counts

Grp. No.	Groups	Dose/Route	WBC (103mm3)	RBC (106mm3)	Hb (g/dl)
1	Naïve control (Animal=6)	0.5% CMC (1 ml/kg, p.o.)	6.73±2.16	3.02±0.14	13.06±0.6
2	Disease control (Animal=6)	0.5% CMC (1 ml/ kg, p.o.)	3.28±0.93	1.04±0.17***	11.08±1.4
3	Prednisolone	10 mg /kg, p.o, BID	4.65±1.02	0.76±0.04	12.23±0.4
4	Prednisolone+Sirolimus (QD)	10 mg/kg, p.o.; QD+ 2 mg/kg, p.o.; QD	6.17±1.33	1.84±0.48	12.97±0.7
5	Prednisolone+Sirolimus (BID)	10 mg/kg, p.o.; BID + 2 mg/kg, p.o.; BID	6.57 ± 1.4	2. 10 ± 0.78	13.6±0.6

Data are expressed as mean±SEM (n = 6), using one-way ANOVA followed by post hoc comparison test. *p<0.05, *p<0.01, ***p<0.001.

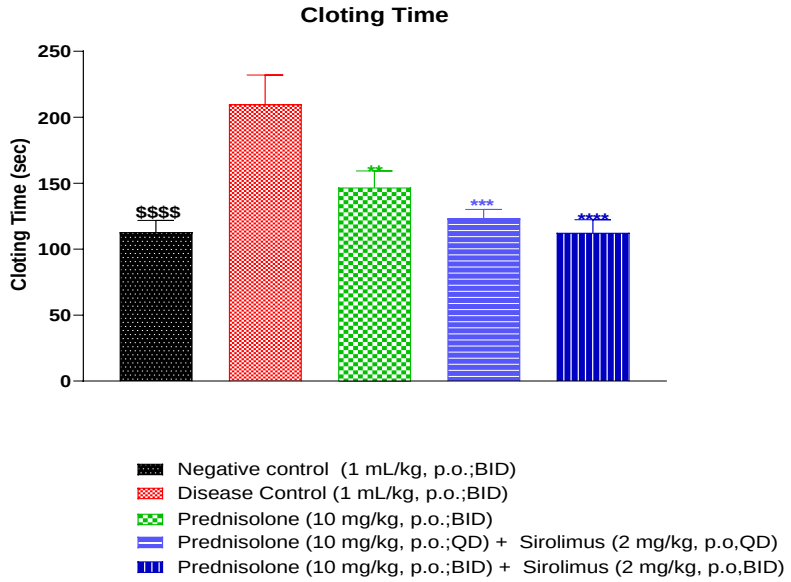

Figure 8: Effect of developed formulation on clotting time in cyclophosphamide-induced thrombocytopenia model. Data are expressed as mean ± SEM (n=6). Statistical significance indicated as *p < 0.05, **p < 0.01, ***p < 0.001 compared with Disease Control group.

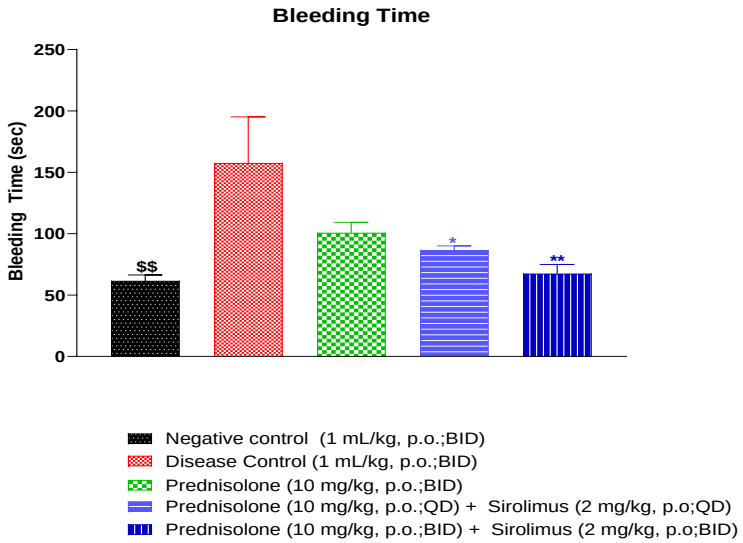

Figure 9: Effect of sirolimus nanodispersion layered tablets on bleeding time following cyclophosphamide-induced thrombocytopenia. Data expressed as mean ± SEM (n=6). Significance levels indicated by asterisks (*p < 0.05, **p < 0.01, ***p < 0.001).

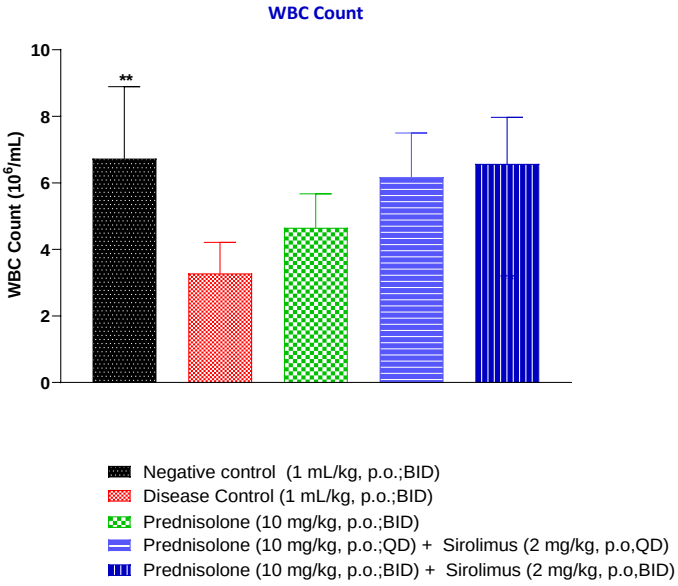

Figure 10: Changes in total WBC count following treatment with prednisolone and sirolimus nanodispersion layered tablets in thrombocytopenic rats, mean ± SEM (n=6)

Effects of sirolimus nanodispersion drug layering technology over prednisolone core tablets on RBC Counts

The average number of red blood cells in the negative control group was 3.02 ± 0.14 x 106/mm3, while in the positive control group, the number of red blood cells after 3 days of treatment with cyclophosphamide was determined to be 1.04 ± 0.17 x 106/mm3 which is significantly lower ($p < 0.001$) than that of the negative control indicating the presence of thrombocytopenia in the rats (Figure 11). Treatment with 10 mg of prednisolone and 2 mg of sirolimus (p.o.) twice daily increased the average number of red blood cells to 1.84 ± 0.48 x 106/mm3 in the rats receiving this treatment.

Effects of sirolimus nanodispersion drug layering technology over prednisolone core tablets on Hemoglobin Counts

The rat doses were normalized to the human equivalent dose (HED) using the standard body-surface-area normalization technique based on Km factors. According to FDA guidelines for dose conversion, the Km factor for rats is 6, whereas that for adult humans is 37. Based on this technique, the dose of

prednisolone in rats (10 mg/kg) would be approximately 1.62 mg/kg in humans, whereas the dose of sirolimus in rats (2 mg/kg) would be approximately 0.32 mg/kg in humans. These values are within the clinically relevant therapeutic ranges as reported for immunosuppressive and anti-inflammatory therapies, indicating the potential translational efficacy of the developed fixed-dose combination. However, further clinical studies are warranted to validate the safety and efficacy of the developed fixed-dose combination in humans. The average amount of hemoglobin found in the negative control group was recorded at 13.06 ± 0.68 g/dl, while the average amount of hemoglobin found in the positive control group (the rats treated with cyclophosphamide for three days) was only recorded as 11.08 ± 1.47 g/dl. The decrease in hemoglobin observed in the disease control group compared with the naïve control group suggests hematological alterations following cyclophosphamide administration. However, the differences among the experimental groups were not statistically significant ($p > 0.05$). When comparing the hemoglobin level from the positive control to that of the test groups with 10 mg/ kg of Prednisone and 2 mg/kg of Sirolimus administered orally twice daily, the hemoglobin level was reported as 12.23 ± 0.46 g/dl, which was also statistically insignificant when compared to the positive control, as depicted in Figure 12.

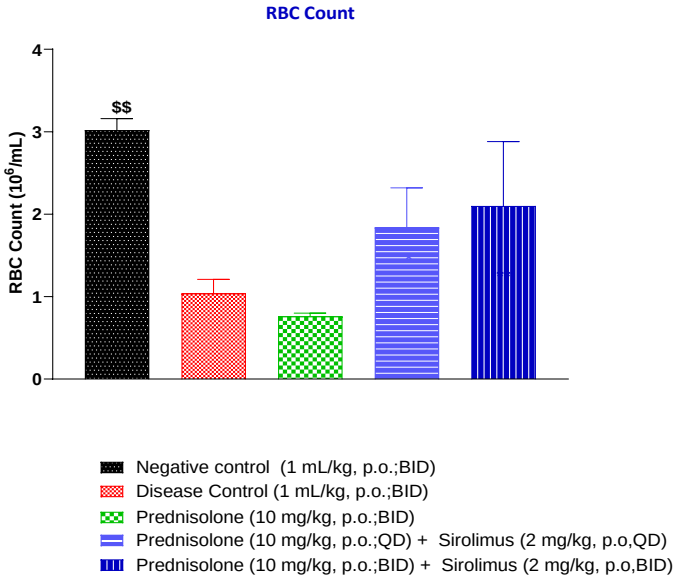

Figure 11: Effect of developed formulation on RBC count in cyclophosphamide-induced thrombocytopenia model, mean ± SEM (n=6).

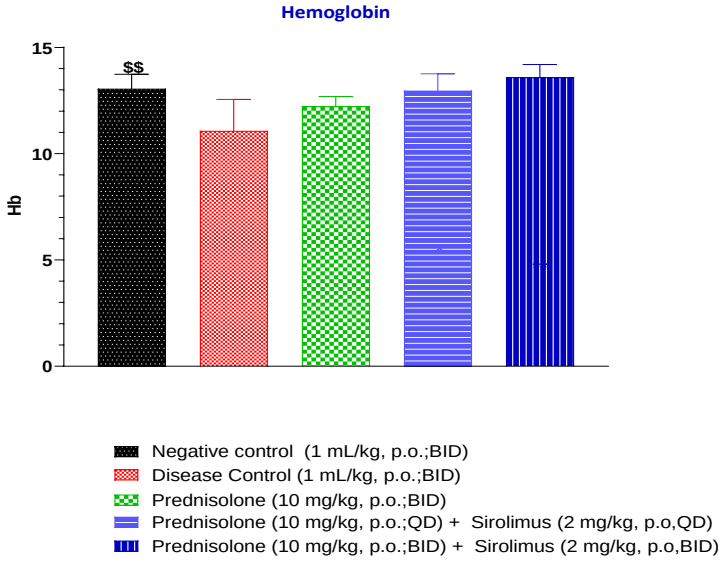

Figure 12: Hemoglobin levels following treatment with sirolimus nanodispersion layered prednisolone tablets in thrombocytopenic rats. Data shown as mean±SEM (n=6). Statistical significance indicated by *p<0.05, **p<0.01, ***p<0.001.

Some limitations need to be addressed. First, the pharmacokinetic analysis was not performed; thus, although the improved therapeutic effect is indicated, the increased absorption and bioavailability of sirolimus nanodispersion could not be directly ascertained. Future studies focusing on pharmacokinetic and bioavailability analyses are needed to provide a clear indication of the role of nanoscale formulations in improving drug pharmacokinetics. Second, histopathological analysis of hematopoietic organs such as the bone marrow and spleen was not performed; thus, the current study is limited in its understanding of the mechanisms underlying platelet count recovery and hematological reconstitution. Comprehensive histopathological and immunological studies would help elucidate the underlying biological mechanisms, particularly those related to megakaryocyte function and immune modulation. Furthermore, molecular mechanism studies focusing on the mTOR pathway and Treg modulation were not addressed in the current study but are of prime importance in future research.

CONCLUSION

The present study successfully developed a nanodispersion drug-layered tablet, in which sirolimus was coated onto prednisolone core tablets, using a Box–Behnken experimental design. Optimization studies demonstrated that HPMC E5 LV concentration significantly influenced tablet hardness and friability, while the optimized formulation exhibited satisfactory physicochemical properties and mechanical strength. *In vivo* evaluation in cyclophosphamide-induced thrombocytopenic rats showed that the developed formulation enhanced platelet count and hematological parameters compared with the disease control group. The observed therapeutic effects may be attributable to the combined action of sirolimus and prednisolone; however, the contributions of improved bioavailability and underlying mechanisms could not be directly confirmed without pharmacokinetic studies.

The experimental results demonstrate promising therapeutic effects in a cyclophosphamide-induced thrombocytopenia model. However, additional pharmacokinetic, histopathological, molecular, and long-term studies are required to confirm the underlying mechanisms and establish the clinical relevance of these findings. While the formulated product exhibited desirable physical and chemical properties and showed promising therapeutic effects in treating thrombocytopenia, the study did not include any pharmacokinetic, bioavailability, histopathological, or molecular studies to evaluate the mechanisms underlying improved drug absorption and the pharmacological response. Since pharmacokinetics, histopathology, and molecular biomarkers were not evaluated, the mechanisms underlying enhanced drug absorption and immunomodulation are best inferred from the observed pharmacological response and the available literature. Future work will need to evaluate the pharmacokinetics and histopathology of organs and examine biomarkers to elucidate the mechanism of action and potential translational benefit of this formulation. In addition, cyclophosphamide-induced thrombocytopenia may not fully recapitulate the complex pathophysiology of human immune thrombocytopenia (ITP); therefore, the findings should be interpreted with caution given the limitations of the experimental model. Therefore, the clinical relevance and translational applicability of the developed formulation remain preliminary and require confirmation through pharmacokinetic, toxicity, and mechanistic studies.

FINANCIAL ASSISTANCE

NIL

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

The study was conceptualized and designed by Shekhar Verma and Achal Mishra. Pradeep Jena and Mayank Garhewal performed the experiments and collected the results. Madhuri Vaghel analyzed the results and wrote the initial draft of the manuscript. Shekhar Verma and Pradeep Jena edited and reviewed the first draft. All authors have reviewed and approved the final manuscript.

DECLARATION OF USE OF AI TOOLS

The authors used ChatGPT to assist with language editing and improving readability. All content, analysis, and conclusions were reviewed and verified by the authors. The authors take full responsibility for the originality, accuracy, and integrity of the work.

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