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Development, Optimization and Evaluation of Ritonavir Nanosuspension by Pearl Milling Technology

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Background: Current days HIV-1 infection in CNS increases. Current antiretroviral treatment does not give cure from HIV-1 completely. Bioavailability is poor by using conventional approaches.

Objective: To prepare and optimize the nanosuspension for nasal route, containing antiretroviral drug ritonavir by pearl milling technology. To develop an effective novel nose to brain formulation which will reaches to brain.

Method: Ritonavir dispersion was fabricated using zirconium oxide beads and stabilizer.

Result & Conclusion: Optimized batch shows average size 90.4 nm PDI 0.253 respectively. Zeta potential -15.6 mV. The FTIR shows no drug excipient interactions and they are compatible with each other. DSC peaks are evident that the drug had changed its crystalline structure to amorphous which is clearly evident that the solubility of both the drugs is enhanced due to the reduction in size. Pearl milling is seen promising to reduce the particle size with suitable stabilizers.

Keywords: Neurological disorder, Antiretroviral Therapies, nanosuspension, media milling.

1. Introduction

According to WHO, HIV contribute globally as a major health concern, there are around 36.3 million lives in the world infected with HIV. UNAIDS report 2020 , shows that nearly 37.7 million people worldwide are affected by Human immunodeficiency infection (HIV) at

the end of 2020, nearly 1.5 million newly contaminated with HIV and 6.8 lack lives died from AIDS-associated conditions.[1]

Contribute as a major health concern, HIV-related neurological disorders, such as HIV-1 virus infection, are on the rise these days. The treatments that are currently available merely lower the plasma viral level; certainly fail to eliminate the virus entirely.The central nervous system (CNS) is not completely reached by the traditional dose form administration method . Its tardy onset of action, hepatic metabolism, and gastrointestinal discomfort are all caused by oral administration. Additionally, some techniques are intrusive, which has led to patient noncompliance. An efficient innovative formulation that will directly reach the brain or central nervous system must be developed in order to solve these issues.[2–6]

A drug's solubility is crucial to its efficacy. Particularly for medications that are poorly soluble, nanosuspensions have proven to be a more economical and technically efficient choice [7].

Sub-micron colloidal dispersions of pure medication particles in an external liquid are known as nanosuspensions. Because of their higher dissolution pressure and increased saturation solubility, the smaller particles dissolve more quickly.[8]

Ritonavir is a novel medicine class that works differently than the majority of the antiretroviral therapies for AIDS that were before on the market. By limiting the HIV viral proteinase enzyme, the medication stops the gag-pol polyprotein from being cleaved, producing immature, noninfectious viral particles. Ritonavir is currently available in tablet, pill, and oral liquid dose forms. Ritonavir's strong permeability and limited solubility in water enable it to be classified as a class-II medication by the BCS.

Media milling technique is considered as modernised version of ball mill in which Grinding balls, also known as milling media, cause impaction of scattered drug particles and mechanical attrition. They are made of a range of materials, including ceramics, yttrium-stabilized glass, highly cross-linked polystyrene resins, and zirconium oxide. It can produces very fine particle size and suitable for toxic chemicals as it is performed in closed vessels. It also shows the continuous operation.[9] In the present research, nanosuspension technology was used to enhance the solubility of ritonavirutilizing zirconium oxide beads, glycerin, and poloxamer 407 used as a stabilizer. Zeta potential, DSC study, drug content and particle size, polydispersity index, and *in-vitro* diffusion were analyzed in comparison to the pure drug.

2. MATERIAL AND METHOD:

2.1 Material:

Ritonavir and poloxamer were obtained from Glenmark Reseach Centre, Sinnar. All other materials used were of analytical grade and were produced from commercial sources.

2.2 Method:

The preparation was done by pearl milling. In this method, glycerin was dissolved in water further added poloxamer. A magnetic stirrer was used to agitate a vial containing a specified

amount of zirconium beads. The medication was then gradually added to the solution above. For six hours, the earlier mentioned mixture was agitated at 1200–1500 RPM using a magnetic stirrer.

2.3 Characterization of drug and excipient:

2.3.1 Preformulation study:

Melting point checked with the capillary tube sealed on one side placed in Thieles tube.

2.3.2 UV method for determination of Ritonavir:

The standard solution was prepared by taking 10 mg of Ritonavir was first dissolved in few ml of methanol in volumetric flask of capacity 100 ml. The volume of solution was made up using water and phosphate buffer pH 6.5 respectively to get solution of concentration 100 ppm.Dilutions were prepared between10-60 ppm. The absorbance was measured at 238 nm using UV- Visible spectrophotometer (Shimadzu Co, Japan).

2.3.3 Saturation solubility studies:

An orbital shaker was used to evaluate the saturation solubility. The extra drug was incorporated to 25 mL of solvent stored at room temperature for 48 hours at 100 RPM in an orbital shaker. For fifteen minutes, the equilibrated sample was centrifuged at 3000 RPM. The supernatant was filtered through a 0.45 μm Whatman filter paper. A UV spectrophotometer was used to quantify the drug content in the supernatant at 238 nm. [10]

2.3.4 Compatibility study of drug with Polymers by FTIR:

The FTIR spectrophotometer (Shimadzu co., Japan) used for analysis. Drug and the KBr taken in the proportion of 1:9 after blending that properly, mixture were placed in FTIR spectrophotometer and scanned between wave number 500-4000 cm^{-1}.The mixture of medication, polymers, and surfactant was sealed in vials and stored at room temperature for 21 days. The samples were then examined for any changes in their infrared spectrum. [11]

2.3.5. Trial Batches using Media milling method:

Using varying formulation and process settings, trial batches are considered to achieve a particle size of less than 250 nm and a polydispersity index of approximately 0.9.

Formulation parameter: 1)Polymer concentration 2)Surfactant concentration

Process parameter: 1) Speed 2) Bead volume

Table 1 : Trial batches

Batch No.	Drug(%)	Poloxamer407(%)	PVA(%)	HPMC 5 CP (%)	Glycerol(%)	Bead (gm)	RPM
T1	0.1	0.1	-	0.05	0.1	10	1500
T2	0.1	0.05	-	-	-	8	1300
T3	**0.1**	**0.1**	**-**	**-**	**0.1**	**10**	**1500**

T4	0.1	0.05	-	0.1	-	8	1300
T5	0.1	0.1	0.1	-	0.1	10	1500

2.4 Experimental design (optimization of formulation 2^3 factorial design): While designing the experiments particle size is considered as the dependant variable. For the optimization factorial design having 3 factors with 2 level used in the study. Design with leves is mentioned in the table 2.

Table 2 : Independent factor & their coded value

Actual parameter	Coded	Actual value	Coded value
Polymer concentration (%)	A	0.05% 0.1%	-1 +1
Bead volume (gm)	B	4 6	-1 +1
Speed (RPM)	C	1300 1500	-1 +1

Table 3 : Different Optimized batches by design expert

Formulation code	**Drug Ritonavir(%)**	**Poloxamer 407(%)**	**Glycerol (%)**	**Bead (gm)**	**Speed (RPM)**
F1	1	0.05	0.1	8	1300
F2	1	0.1	0.1	8	1500
F3	1	0.05	0.1	8	1500
F4	1	0.1	0.1	10	1300
F5	1	0.05	0.1	10	1500
F6	**1**	**0.1**	**0.1**	**10**	**1500**
F7	1	0.05	0.1	10	1300
F8	1	0.1	0.1	8	1300

3. EVALUATION of NANOSUSPENSION:

3.1 Determination of pH :

4 A digital pH meter (Systronics, India) was used to measure the pH.

4. Determination of particle size and polydispersity index (PDI):
Photon correlation spectroscopy (Zeta-sizer Ver. 7.01, Malvern Instruments) was used to measure the mean size of the particles and the polydispersity index of the particles. A drop of nanosuspension was mixed with pure water using a syringe, put in a test tube, agitated vigorously, and then put in a tiny, disposable polystyrene cuvette. Light scattering was seen at 25 °C. [12]

4.1.1 Determination of zeta potential:
Photon correlation spectroscopy measures particle size, was utilized to determine the zeta potential (Zeta-sizer Ver. 7.01, Malvern Instruments). Zeta potential was used to determine surface charges.[13]

4.1.2 Characterization by Differential Scanning Calorimetry (DSC):
Differential Scanning Calorimetry (DSC) is a technique used to evaluate the drug's crystallinity, final formulation, sample heat absorption or release, and Potential polymorphic changes of Ritonavir in nanosuspension [14]. DSC measurements were made for both the optimized formulation and the pure medication. Following drying and precise weighing of 2–5 mg, the samples were put in an aluminium pan and hermetically sealed with an aluminium lid. A nitrogen gas flow rate of 50 mL per minute was used to purify the system after it had been heated to 10°C per minute.

5. Determination of drug content
Drug content for nanosuspension
Stock solution of nanosuspension was made in methanol filtered using Whatman paper (0.45 µm) analyzed at λ max 238 nm by UV-spectrophotometer. Total ritonavir content (TDC) was calculated by equation -1 [15]

$$TDC = \frac{\text{Vol. Total}}{\text{Vol. aliquot}} \times \text{Drug amount in aliquot} \times 100 \quad \ldots\ldots\ldots (1)$$

3.2.4 *In- vitro* diffusion study:
The in-vitro drug release study was conducted using a 16 ml Franz diffusion cell. A nanosuspension drug containing 5 mg was applied to a cellulose dialysis membrane (MWCO 12,000 g/mole; Himedia Laboratories Pvt. Ltd.). The donor and receptor compartments were maintained at 37º C and continuously stirred at 100 rpm, with the dialysis membrane placed in between.After adding phosphate buffer (pH 6.5) to the receiver compartment, samples were removed on a regular basis. The same volume of new medium was then added. The UV spectrophotometer was used to assess drug release at λ max 238 nm.[16,17]

3.2.5 Comparative study of pure drug and nanosuspension by diffusion method:
A USP dissolution testing instrument (type II) with rotating paddles at 50 rpm was used to compare pure ritonavir and ritonavir nano suspension using 900ml of 0.1N HCl as the dissolution medium. During the experiment, the temperature was kept at 37 ± 0.50 °C. To maintain a constant volume of dissolution medium, 10 mLof samples were taken out at different times and refilled with the same volume of dissolution media. After passing the samples through a 0.45µ filter, the absorbance at 238 nm was measured for analysis.

4. Results and Discussion:

4.1 Melting point:

Ritonavir (API) was observed for organoleptic properties such as melting point, color and odor. Physical changes (from solid to liquid) in the drug's melting point were noted and compared with the normal range.The melting range of ritonavir sample was observed between 124-126℃ results matches with reported Ritonavir melting range.

4.2 UV- Spectroscopy:

In UV- Spectroscopy study, the maximum wavelengths (λmax) i.e. maximum absorption of Ritonavir in water, methanol, phosphate buffer (pH 6.5) was to be found 239 and 238nm respectively. The reported λmax of Ritonavir in methanol and phosphate buffer (pH 6.5) is 240 and 238 nm (Indian Pharmacopoeia 2014).[18]

4.3 Determination of solubility:

Table 1 : Solubility of drug and formulation

Sr. No	Solvents	Ritonavir (mg/mL)	Formulation (mg/mL)
1.	Distilled water	0.0025 ±0.18	0.068 ±0.25
2.	Phosphate buffer (pH 6.5)	0.0350±0.22	0.359 ±0.27

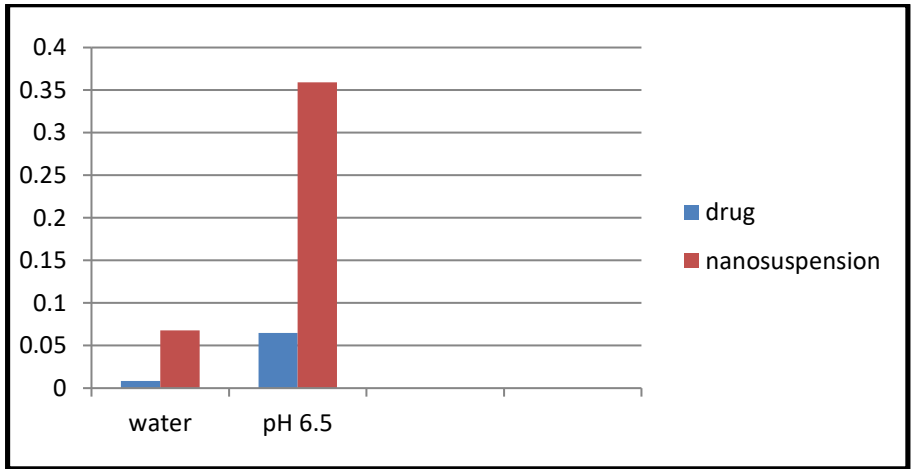

Figure 1 : Solubility profile of drug & formulation in water & phosphate buffer

By observing the above results, the solubility of the drug formulation get increased due to nanosizing. There are 10-12 fold increase in solubility of Ritonavir in distilled water and PBS pH 6.5. Solubility increases due to reduction in particle size by pearl milling. Amphiphiles helps to minimize the hydrophobicity of the drug [19]. Particles with a larger surface area are more exposed to the medium.

4.4 FTIR analysis of drug and excipients:

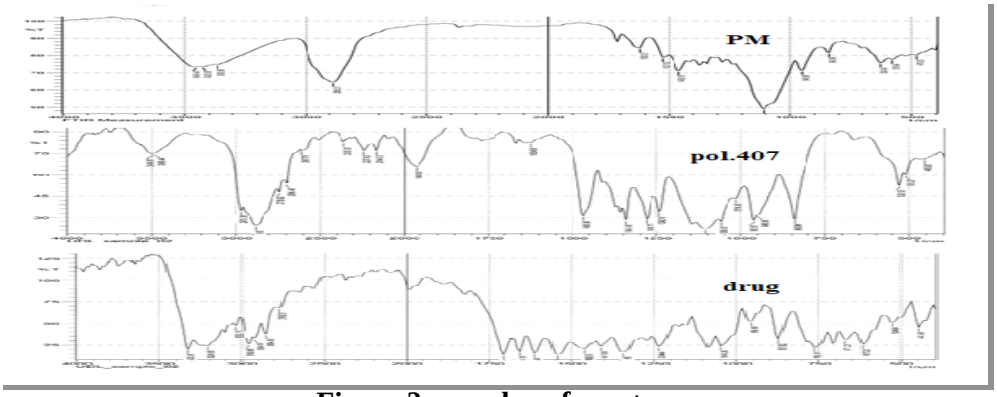

Figure 2 : overlay of spectra

The principle peaks of 3325, 2962, 1658, 1543, 1458 and 1049 cm^{-1} aliphatic secondary amine, CH stretch alkane, Amide, Aromatic Nitro group, methyl C-H, 1° amine and C-H respectively. Since the principle peaks for the drug and excipients are observed, we may conclude that there is no interaction and that the excipients are compatible.

4.5 Optimization by 3 factor and 2 level factorial design:

Three factors and a two-level factorial design were used in this study. All eight possible combinations were used in the experimental experiments. Three factors were chosen: X1-Stabilizer Concentration, X2-Bead and X3-Speed were selected as factors.

Table 3: Design Summary

Code	Factors	Unit	Low	High	Low Coded	High Coded
A	Conc. of stabilizer	%	0.05	0.1	-1	+1
B	Bead	gm	4	6	-1	+1
C	Speed	RPM	1300	1500	-1	+1

Table 4 : Different Batches Given By Factorial Design and Their Evaluation

Run	A: Pol.407 %	B: bead Gm	C: speed RPM	Particle size m
1	0.05	6	1300	111.4
2	0.1	6	1500	90.4
3	0.1	4	1500	123.8
4	0.05	4	1500	98.3
5	0.05	6	1500	95.2
6	0.1	4	1300	150.6
7	0.1	6	1300	105.5
8	0.05	4	1300	130.9

Table 5: ANOVA to check significance of value

Source	Sum of Squares	df	Mean Square	F value	p-value prob> F	
Model	2944.05	5	588.81	135.16	0.0074	**Significant**
A-pol.407	148.78	1	148.78	34.15	0.0281	
B-bead	1277.65	1	1277.65	293.29	0.0034	
C-speed	1028.31	1	1028.31	236.05	0.0042	
AB	390.60	1	390.60	89.66	0.0110	
BC	98.70	1	98.70	22.66	0.0414	

Residual	8.71	2	4.36			
Cor total	2952.76	7				

The significance of the model is indicated by its F-value of 135.16. The likelihood that a "Model F-value" this large might be caused by noise is merely 0.74%. Model terms that have "Prob> F" values below 0.0500 are considered significant. A, B, C, AB, and BC are crucial terms in the model.

Table 6: Fit Statistics

Standard deviation	2.09	**R-Square**	0.9970
Mean	113.26	**Adjusted R-Square**	0.9897
Coefficient of variation %	1.84	**Predicted R- Square**	0.9528
PRESS	139.40	**Adequate Precision**	34.259

The "Pred R-Squared" 0.9528 is in reasonable acceptance with the "Adj R-Squared" of 0.9897. "Adeq Precision" estimates the ratio of signal to noise. More over four is a desirable ratio. An adequate signal is indicated by ratio of 34.259.

Final Equation In Terms Of Coded Factor is equation-2,

Particle Size = +113.26+4.31*A-12.64*B-11.34*C-6.99*AB +3.51*BC---------(2)

The largest influence on size is caused by factor A speed and the interaction between B and C. As we increase the speed it shows negative impact on particle size that is decreases size. Additionally, a decrease in particle size is noted when the quantity of Poloxamer 407 decreases.

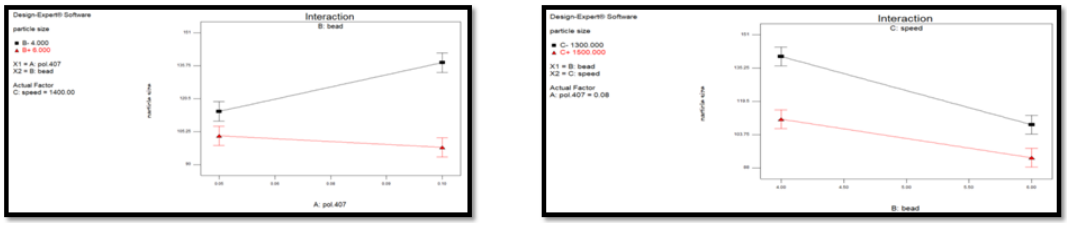

Figure 3 : Interactions plots of AB & BC

When the response varies according to the settings of two variables, interaction takes place. It suggests that one factor's impact depends on the other's level. The interaction between factor BC is shown in the above picture as two planes that are very parallel to each other, indicating that there is no interaction between the variables.On the other hand, factor BC indicates that factors B and C interact, and the change is positive.The impact of particle size changes depends on the speed and bead level.

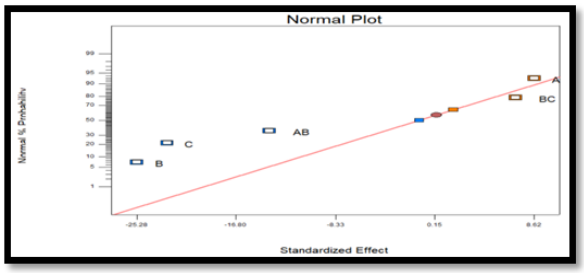

Figure 4 : Normal plot

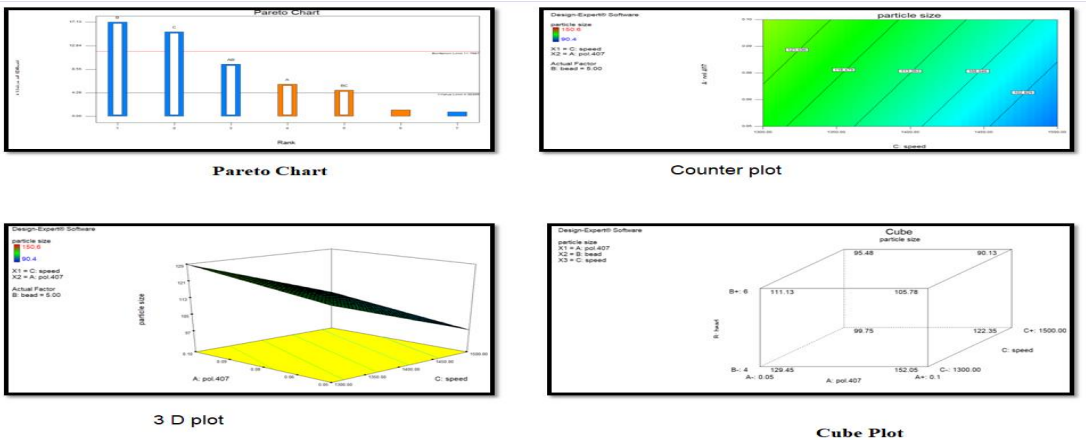

Figure 5 : Various plots by DOE

As seen in the 3D plot and counter plot, the impact of RPM, bead, and particle size was investigated. We could see from the counter plot and 3D plot that higher particle sizes were found at low RPM and bead levels, whereas smaller particle sizes were shown at higher levels. As a result, RPM and bead have the opposite influence on particle size.Formulation F2 watch selected as an optimized batch because of lesser particle size and optimum Zeta potential. Another optimised formulation was prepared according to the predicted model and evaluated for the response. Result of predicted batches and experimental batches with residual error.

Table 7: **Predicted and experimental results**

Response	Target	Predicted value	Experimental value	Residual error (%)
Particle size (nm)	<250	113.26	116.44	2.08

4.6 pH of batches:

In order to prevent irritation and pain during formulation delivery, it is crucial to construct a dosage form with a pH range comparable to the intranasal formulation, which is between 4.5 and 6.5. Additionally, this pH raises the formulation's absorption at that location [20]. According to the data, the pH of the optimized product was 6.0.

Table 8: pH

Batch	pH
F1	6.2 ± 0.1
F2	5.4 ± 0.07
F3	6.6± 0.2
F4	6.4 ± 0.1
F5	4.8 ± 0.2
F6	**5.4 ± 0.5**
F7	5.6 ± 0.4
F8	5.2 ± 0.3

4.7 Size and polydispersity index:

Table 9: Results of trial batches

Batch No	Particle size (nm)	Polydispersity index (PDI)
T1	123	0.399
T2	142.3	0.453
T3	**102.4**	**0.391**
T4	187.5	0.386
T5	172.2	0.409

Trial batches were obtained using the pearl milling technique in order to optimize the formulation. According to the above table, the largest particle size and PDI (T4 batch) occurred at low surfactant concentrations, minimal speeds, and bead volumes. However, particle size and PDI decreased when surfactant content and speed increased (T3 batch).

Table 10 : Results for F1-F9 batches

Batch no.	Size (nm)	PDI
F1	111.4	0.326
F2	**90.4**	**0.253**
F3	123.8	0.302
F4	98.3	0.420
F5	95.2	0.361
F6	150.6	0.478
F7	105.5	0.542
F8	130.9	0.265

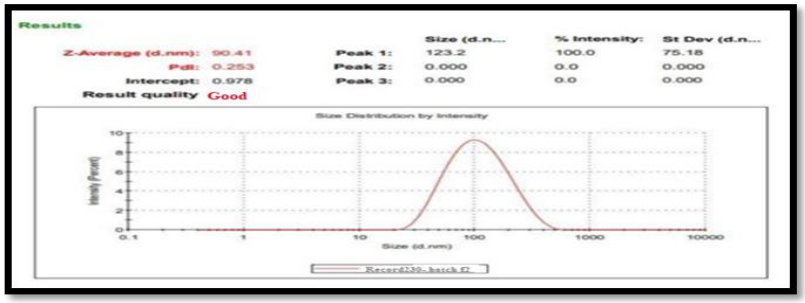

Figure 6 : Size and PDI of F2 batch

Particle size is decreased by changing the formulation and process parameters. Less than 300 nm is the ideal range for medication delivery that targets the brain [21]. Batch F2 exhibits a PDI of 0.253 and a minimum particle size of 90.4 nm. The formulation's particle size homogeneity increases with decreasing polydispersity. A limited particle size distribution is indicated by a PDI of 0.253, which falls within the desired range. One crucial factor that can be utilized to evaluate stability is the nanosuspension's particle size distribution.A large interfacial area for drug absorption or penetration across biological membranes is provided by the small particle sizes of nanosuspension, which increases the integrated medication's relative bioavailability. [12]

4.8 Zeta potential analysis:

Figure 7 :Zeta potential of optimized batch (F3) of nanosuspension

For the dispersed system standard zeta potential range is ±30 mV. The ideal value for nanosuspension is ±20 mV [10]. The optimized batch (F2) zeta potential value was determined to be -15.6 mV, falling within a range.Combinations of polymers and stabilizers were used to stabilize dispersions. Zeta potential could control by steric and electrostatic stabilization in dispersions.

5.9 DSC Thermogram:

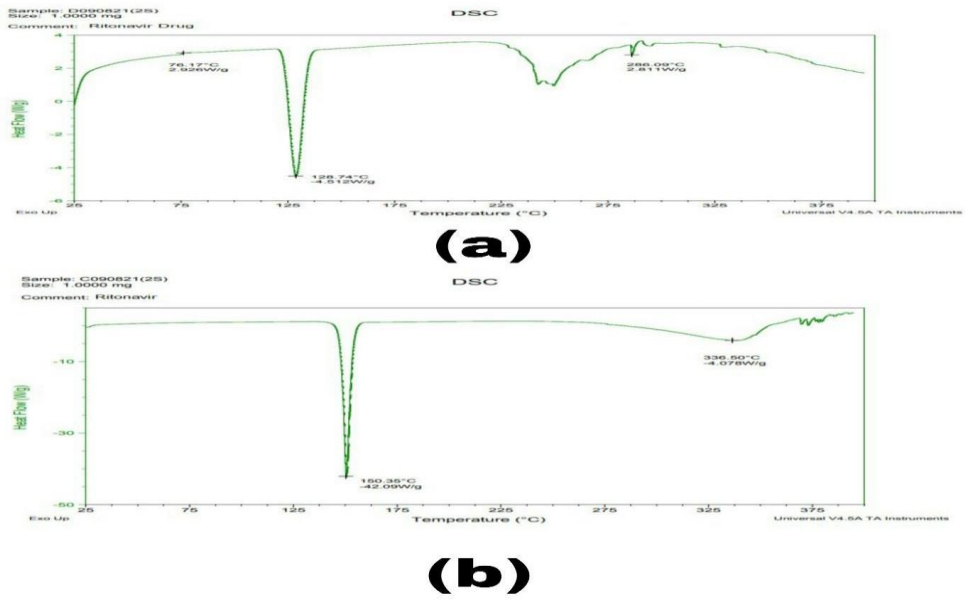

Figure 8 : DSC of (a) drug and (b) formulation

The spectra shows a distinct transition for the medication at 128.74°C and for the formulation at 150.35°C. The melting point of ritonavir is actually between 121 and 123°C. Ritonavir's physical state changes as a result of the pearl milling process, which causes its peak to move to the exothermic peak. We may draw the conclusion that the medication transforms from a crystalline to a amorphous state when thermal energy is applied.[22]

5.10 Drug content

Table 11 : Drug content of nanosuspension

Sr. No.	Batch no.	Drug content (%)	% Drug release after 1 hour
1.	F1	90.15 ± 3.80	73.65
2.	**F2**	**92.63 ± 1.92**	**84.67**
3.	F3	86.19 ± 2.34	80.07
4.	F4	84.76 ± 2.05	86.23
5.	F5	93.31 ± 1.95	79.20
6.	F6	95.29 ± 1.95	77.79
7.	F7	91.56 ± 2.15	75.10
8.	F8	89.21 ± 1.29	65.64

Drug content for nanosuspension is given in above table 11. Batches show drug content between 84-95%. F2 give drug content 92.63 %. The drug content was found to be less due to the processing in milling while recovery of product

5.11 *In- vitro* diffusion study

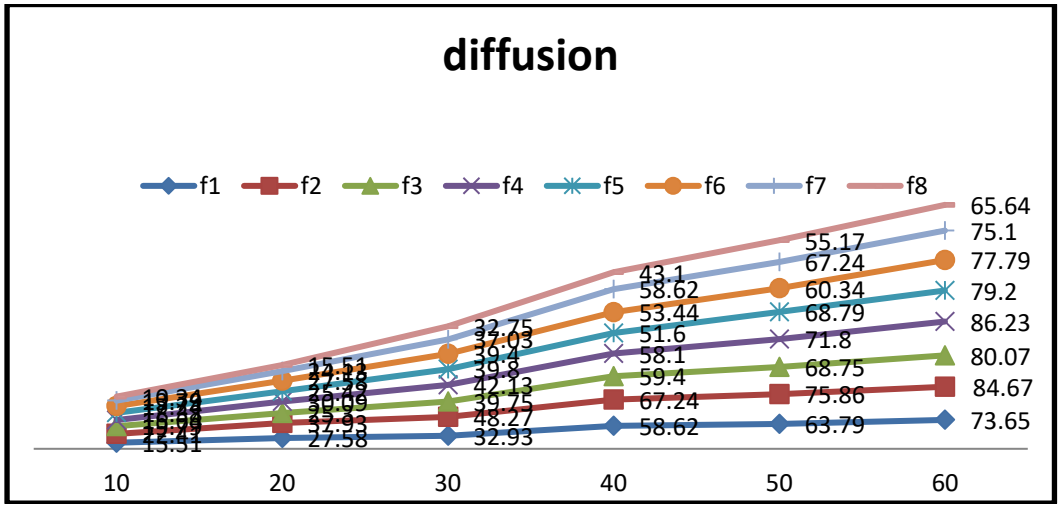

Figure 9 : Diffusion profile of optimized batches

From table 11 diffusion results F2 shows higher diffusion of 84.67% of drug release in one hour. By pearl milling amorphous drug increases in surface area with decrease in the particle size. Thus, by increasing the surface area increases diffusion via the membrane and that improves bioavailability.

Table 12: Dissolution comparison

Time	Pure drug (%)	Optimize batch (%)
10	3.35	77.53
20	10.58	80.03
30	27.18	84.69
40	35.02	87.24
50	43.79	90.76
60	51.32	92.27

When compared to a pure medication, the produced nanosuspension's in-vitro diffusion profile indicates an enhanced rate of dissolution. Comparing the 92.27 percent drug release to the 51.37% release of pure drug, the difference is significant. It will be verified that the

above formulation effectively boosts the drug Ritonavir's solubility and dissolution.

6. Conclusion

A promising method for enhancing the saturation solubility, rate of dissolution, and bioavailability of medications with hydrophobic and lipophobic properties is nanosuspension.The nanosuspension technique has many advantages, such as simple method of preparation, less requirement of excipients, increased dissolution rate and solubility, increases the bioavailability leading to decrease in the dose and ease of large-scale manufacturing.

Ritonavir's solubility and dissolution were significantly improved. The significant increase in ritonavir bioavailability may be explained by the drug's improved solubility at pH. By choosing the right formulation and process parameters, we may determine that the media milling technique is a useful tool for stabilizing particles and reducing their size.

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