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FORMULATION AND EVALUATION OF STABLE NANOSUSPENSION OF SELECTIVE POOR LY SOLUBLEDRUG RITONAVIR

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ABSTRACT

Ritonavir is a commonly used antiretroviral agent, which is used in the treatment of HIV. The solubility of this compound in biological fluids limits its bioavailability to less than 5%. In light of its physicochemical and biopharmaceutical properties, Ritonavir was selected as a drug candidate for evaluating Nanosuspension-based formulations to enhance solubility and bioavailability. **Materials and methods:** An analytical method was developed and the particle size distribution was reported. Initial preformulation studies were done. Nanomill (Netzsch) media milling was used for the size reduction of drug particles. The physicochemical properties of the nanosuspension were evaluated using standard procedures. **Results:** The study was found to exhibit the highest solubility in 1.2pH HCl (for Ritonavir). In the preformulation studies data, it was inferred that Ritonavir has passable flow properties. From the stability studies by DSC analysis, it was reasonable to believe that there was no interaction between drug and excipients used in the Nanonisation process used in Ritonavir Nanosuspension preparation. The results of drug excipients compatibility studies suggest that there was no significant change in the physical appearance of premixture blends when stored at 40^0C/75% RH for a period of 4 weeks when compared to the initial sample. The average particle size of the Ritonavir optimized Nanosuspension thus produced was found to be about 400nm with PDI of 0.268 indicating good physical stability of Nanosuspension. The zeta potential of -25.4mV for optimized Ritonavir Nanosuspension indicates the good physical stability of Nanosuspension produced. The Dissolution of

Ritonavir Nanosuspension found to be 87% in selected dissolution media when compared to the dissolution of 41.6 and 56% corresponding to Ritonavir plain drug and Ritonavir suspension. **Conclusion:** According to the present research, preparation of Nanosuspensions using the Media Milling method (top-down approach) significantly enhanced the solubility and dissolution rate of Ritonavir, a poorly soluble drug.

INTRODUCTION

In recent decades, nanoparticle engineering has been developed and investigated for pharmaceutical applications.[1] The use of nanotechnology can solve the problems associated with the various approaches described previously. Nanotechnology is the scientific and engineering practice at the nanoscale, which is 10-9 m. Nanosuspensions are colloidal dispersions of nano-sized drug particles stabilised by surfactants.[2] Nanosuspensions are dispersed nanoparticles of a poorly water-soluble drug in absence of a matrix.[3] These are useful for increasing the solubility of drugs that are difficult to dissolve in either water or lipids. By increasing the solubility of the active compound, the rate of flooding increases, and the maximum plasma level is reached more rapidly. The production of Nanosuspensions has been commercialized through methods such as media milling and high-pressure homogenization.[4] Ritonavir is a commonly used antiretroviral agent, which is used in the treatment of HIV- viral proteinase enzyme. Ritonavir is a BCS class II and IV drug. It prevents the cleavage of the gag-pol polyprotein, resulting in noninfectious, immature viral particles. It has very low bioavailability of not more than 5% due to its limited solubility in biological fluids. Due to its dissolution as well as permeability, Ritonavir is classified as a class II compound under Biopharmaceutical Classification (BCS). It is possible to improve therapeutic efficacy by improving aqueous solubility in such cases. The in vitro and in vivo performance of PEG-amorphous Ritonavir solid dispersions was evaluated with different levels of drug loading. Invitro dissolution was done in 0.1N HCl. Intrinsic dissolution of solid dispersions indicated a 10-fold improvement when compared with crystalline counterparts. In vivo study results indicate significant increase in AUC and Cmax over crystalline drug. 10% amorphous dispersion exhibited increases of 22 and 13.7-fold in AUC and Cmax respectively.[5-7]

Based on their physicochemical and biopharmaceutical properties, Ritonavir was selectedas a drug candidate for developing Nanosuspension based formulations for improving the solubility and bioavailability by enhancing the rate and extent of dissolution. The aim of the

work is to enhance the solubility, dissolution rate and oral bio availability of poorly soluble drug Ritonavir by formulating it into Nanosuspension with poloxamer as a stabilizing agent and evaluation of physical and chemical parameters.

MATERIALS AND METHODS

Analytical method development: Calibration curve of Ritonavir in 1.2pH Hydrochloric acid buffer (0.1N HCl): 10mg of drug was transferred into 100ml volumetric flask and then made up to 100ml with 10ml methanol and 90ml 0.1N HCl. From this 2, 4, 6, 8 and 10ml was taken andmade up to 10ml with 0.1N HCl and their absorbance was measured at 246nm.

Preformulations studies: Preformulation involves the application of biopharmaceutical principles to the physicochemical parameters of drug substances with the goal of designing an optimum drug delivery system. It provides information on the nature of the drug substance and framework for drug combination with pharmaceutical excipients in the dosage form.[8]

(**Lachman L, Liberman H.A (1998).** Hence, the following preformulation studies are carried out.

i) Organoleptic evaluation
ii) Flow Properties
iii) Particle size distribution
iv) Solubility/Saturation
v) Compatibility studies

i) Organoleptic evaluation: The color, odor and taste of the drug were evaluated and recorded using descriptive terminology.

ii) Flow Properties.

- **Bulk density:** It was determined by measuring the volume of a known mass of powder sample that has been passed through an appropriate screen into a graduated cylinder (Method I). Approximately 10gms of the test sample, M was introduced into a 25 mL dry measuring cylinder without compacting. The powder was levelled carefully and the unsettled apparent volume V0, read to the nearest graduated unit. Bulk density was calculated, in g per mL, by the formula, (*M*) / (*V*o). Generally, replicate determinations are desirable for the determination of this property.
- **Tapped density:** Cylinder containing the sample was tapped mechanically by raising the cylinderand allowing it to drop under its own weight using a suitable mechanical tapped

density tester that provides a fixed drop of 14 ± 2 mm at a nominal rate of 300 drops per minute. The tapped density was calculated, in g per mL, by the formula, $(M) / (V_f)$.

- **Measures of Powder Compressibility:** The compressibility index and Hauser ratio are measures of the property of a powder to be compressed. These differences are reflected in the compressibilityindex and the Hauser's ratio.

$$\text{Compressibility Index} = \frac{\text{(Tapped Density - Bulk Density)}}{\text{Tapped Density}} \times 100$$

Hauser's Ratio = Tapped density/Bulk density.

iii) Particle size distribution: The particle size distribution was measured by Malvern Mastersizer 2000S. The system uses laser diffraction analysis consists of an optical instrument, the sample dispersion units Hydro S, Hydro P and Scirocco. The sample particles dispersed in suitable medium pass through the focused beam of light and scatter the light at spatial angles. [9] (**Dhaval J. P, 2010**). The reported particle-size distribution typically includes Dv10,Dv50 and Dv90, which are the percentages of particles below the given size.

iv) Saturation solubility: Solubility of Ritonavir in different buffers was determined by shake flask method. The filtrateswere then analyzed by UV spectrophotometer at 246nm and 248nm respectively to evaluate the amount of Ritonavir dissolved.

v) Drug Excipients compatibility: Physical mixtures of Drug and excipients were prepared by grinding specific ratios (mentioned below in table 4.7) for drug and excipients in a mortar. Sample of 3-4grams was taken and loaded in a glass vial, covered with rubber stopper, sealed with aluminum cap and labeled properly. Samples were observed and the physical description was recorded forinitial evaluation and loaded into stability chamber of 40^0C and 75% RH for 4 weeks to study compatibility of the drug with the selected excipients. At the end of 4^{th} week samples were removed, again observations were recorded to have a comparison with the Initial recorded observation.

Preparation of Ritonavir Nanosuspension: Nanosuspension is a suspension containing the drug particles in nanosized range. For the present study, media milling by nano mill (Netzsch) was chosen for size reduction ofRitonavir drug particles as simple beaker method was not able to reduce the particle size of the molecule in the nonorange. The compositions of various trials undertaken are given in Table 1.

Table 1: Composition of Ritonavir Nanosuspensions.

S.No	Ingredients (mg/ml)	Formulation codes			
		F1	F2	F3	F4
1	Ritonavir	100	100	100	100
2	Poloxamer F 407	-	5	10	20
3	Water	1ml			

Manufacturing procedure for the preparation of Ritonavir Nanosuspension: The poloxamer was dissolved in required quantity of water and stirred to get clear solution. Then Ritonavir was added to the poloxamer solution and stirring was continued for 30min. The resultant dispersion was passed through colloidal mill for about 15mins to get the homogenous smooth dispersion. The resultant dispersion was passed through Nano mill till particles of desired size range were obtained.[10, 11]

Media milling: Freshly prepared dispersion of the Ritonavir drug was placed in nano mill container. The milling chamber was charged with milling media (Zirconium Oxide Beads) then rotated at a very high shear rate (60 rpm, 0.2-2 bar) under controlled temperatures for several hours (1-4 hours). The high energy shear forces are generated as a result of the impaction of the milling media with the dispersed drug resulting into breaking of microparticulate drug into Nanoparticulate drug. In-between samples were collected for the physical characterization of the sample. Various process parameters like volume of beads, stirring time and stirring speed were carefully monitored and optimized parameters were maintained for the formulation of Ritonavir Nanosuspension. The optimized milling parameters maintained during manufacturing of Ritonavir Nanosuspensions are given in table 4.9.[12, 13]

Particle size analysis: The particle size distribution of the Nanosuspension was determined by photon correlation spectroscopy, using a Zeta seizer nano series instrument (Malvern Instruments, Worcestershire, UK). This technique yields the mean particle diameter and the range of the particle size distribution and polydispersity index (PDI). All the data presented are the mean values of the results on three independent samples produced under identical conditions. Atomic force microscopy is used for visualization of particle shape. To compare the size and the size distribution of the Ritonavir drug, in Nanosuspensions samples were dispersed in water, using an ultrasonic bath for 60min. The sonication was used to disperse the particles into individual particles so that the accurate measurement will be done properly.[13]

Particle charge (zeta potential): The zeta potential of a Nanosuspension is governed by both the stabilizer and the drug itself. In order to obtain a Nanosuspension exhibiting good stability, for an electrostatically stabilized Nanosuspension a minimum zeta potential of (+or) 15mV is required. Electrophoretic mobility measurements were performed with Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) at 25°C. Approx. 1mL of wet-milled Nanosuspension diluted by filtered water to obtain count rate in the 200-400. The zeta potentials were calculated by the instrument according to the Helmholts–Smoluchowski's equation. The measurements were performed in triplicate.

X-ray powder diffraction analysis: The physical states of API in the different samples were evaluated by X-ray powder diffraction (XRPD). Diffraction patterns were analyzed with a Miniflex II X-ray diffractometer (Rigaku Co. Tokyo, Japan), where the tube anode was Cu with K = 15,405 A.The pattern was collected with a tube voltage of 30 kV and a tube current of 15mA of in stepscan mode (4◦/min). The instrument was calibrated by using Si. A chemo metric method was used to evaluate the X-ray results. The self-modeling curve resolution (SMCR) method is a chemo metric procedure used for two and three component systems to deconvolve raw spectroscopic data and to obtain an analytical solution in band form, which provides a clearer interpretation. A computer program involving the use of SMCR and multivariate curve resolution (MCR) methods was employed to analyze the XRPD data and to study the interactions between API and the stabilizer in the formulated Nanosuspensions of Ritonavir.

Differential scanning calorimetry: Thermal properties of drug, polymer and Nanosuspension were investigated using a METTLER differential scanning calorimeter thermal analysis controller with an intracooler- 2 cooling. About 3 to 5 mg of product was placed in perforated aluminum sealed 50µl pans and the heat runs for each sample was set from 40°C to 150°C at 10°C/min, under an inert environment using nitrogen. The apparatus was calibrated using pure metals like indiumwith known melting points and heat of fusion (ΔH fusion).

Saturation solubility studies: Solubility studies were done by shake flask method. An excess amount of dried Nanosuspension of drug Ritonavir was taken along with the desired solvent. The volumetric flasks were then fixed onto a water bath shaker and shaken for 24 hours at 37˚C ± 1ºC. Samples were removed after the specified time and filtered through 0.45µ m nylon 66 syringe driven membrane filter unit. The filtrates were then analyzed by

UV spectrophotometer at 246nm to evaluate the amount of Ritonavir dissolved. This study was done to understand the effect of Nano milling on solubility of the drug Ritonavir in particular media.[12]

Microscopy test: The samples (before and after Nanonization) were visualized by using optical microscope (LEICA, DFC 395) at 40X zoom. Samples of Nanosuspensions were mounted on the aluminum stubs with the help of carbon double-sided tape (Nisshin EM Co. Ltd., Tokyo) and sputter coated with Platinum by using Auto fine coater (JEOL, JFC - 1600) for 90sec under vacuum (3Pa) and observed under the Scanning Electron Microscope (JEOL, JSM-6380) at a magnification of 500X.

Freeze thaw study: Freeze-thaw testing was conducted by exposing the optimized Nanosuspensions of Ritonavir to freezing temperature (2-8˚C) for 24hrs then allowing to thaw at room temperature for 24hrs. The above said cycle repeated for 7 times. After this the freeze thawed sample was analyzed for any particle size changes and agglomeration and drug release study in finalized release media for Ritonavir drug molecule.

Fourier Transform Infrared Spectroscopy (FTIR): The FTIR spectra were recorded for pure drug Ritonavir, polymer (poloxamer) and optimized dried Nanosuspension formulation using KBr pellet technique. The pellets were prepared using KBr hydraulic press under hydraulic pressure of 150kg/cm^2. The spectrums were scanned over 3600-400 cm-1 at ambient temperature with a resolution of 4cm^{-1}, using FTIR 2500 apparatus and spectra were recorded.

A. Flow Properties

- **Sedimentation volume:** The rate of separation of the suspensions were determined by keeping 50ml portion of each suspension in stoppered measuring cylinder and stored undisturbed at roomtemperature. The separation of clear liquid was noted at an interval of 2W and 4W. The sedimentation volume, F (%), was then calculated using the following equation: F = 100Vu/Vo.

Where Vu is the ultimate volume of the sediment, Vo is the original volume of the suspension.

- **Pourability:** This test is carried out on optimized suspension, after mixing thoroughly to ensure that the final preparation is pourable and will not cause any problem during filling and during handling of the dosage form.

- **Redispersion:** Fixed volume of each suspension (50 ml) was kept in calibrated tubes which were stored at room temperature for various time intervals (2W, 4W). At regular interval of 2W, one tube was removed and shaken vigorously to redistribute the sediment and the presence of deposit if any was recorded. The time taken to redisperse the sedimented suspension was recorded. The method essentially consisted of holding the sample tube straight in upright position between two fingers with thumb at the bottom and the middle finger at the top followed by the almost uniform rotation through 180 degree and brought back through the same path. The pair of successive upward and downward movement each of approximately equal force, constituted one complete shake. The number of shakes required for complete elimination of sediment from the bottom of the tube was recorded. At this juncture the sample was observed for homogeneity of the suspension and the total time (sec) recorded to redisperse the sedimented suspension. This was based on the empirical understanding that not more than that force should be required and the same that is routinely applied by the consumer in the event of "shake well before use" maximum care was taken to exert approximately the same amount of force every time and the same time interval. (**Anwar K,2014)**

B. Chemical evaluation

- **Assay of Ritonavir in Nanosuspensions:** 10ml of Ritonavir Nanosuspension was taken and dissolved in about 200ml methanol and sonicated for 30min, the volume was adjusted to 500ml using 0.1N HCl continuous sonication for 5min. Further 3ml of this solution was diluted to 100ml with 0.1N HCl. Filtered through a 0.45µm membrane filter and analyzed by measuring the absorbance at 246nm against blank using UV spectrophotometer. The readings were taken in triplicate (Shimandzu UV-1700).
- ***In-vitro*** **dissolution of Nanosuspension:** The release rate of Ritonavir from Nanosuspension was determined using USP dissolution testing apparatus II (Paddle type). The dissolution test was performed using 900 ml 0.1 N HCl, at 37 ± 0.5˚C and 50 rpm/min. At the predetermined time interval, 10mL samples were withdrawn filtered through a 0.45µm membrane filter and replaced with fresh medium to keep the volume constant. 5ml of this solution was diluted to 10ml with medium and absorbance was taken at 246 nm using a Shimadzu UV/vis double-beam spectrophotometer. Cumulative percentage drug released was calculated using an equation obtained from a calibration curve.[14]

RESULTS AND DISCUSSION

The present research work has been carried out with an aim to increase the solubility and dissolution rate of Ritonavir by formulating into a Nanosuspension to make it stable and patient friendly. Ritonavir Nanosuspension was prepared by pearl milling technique using Zirconium beads as milling media and Poloxamer 407 as stabilizer.

Analytical method development: The calibration data of Ritonavir was subjected to liner regression. The calibration range was found to be 20 to 100 **µg/ml** with R^2 value of 0.9998. Slope of the regression was found to be 0.0124 with intercept of regression line was found to be 0.0008.(Table 2, Fig 1).

Table 2: Calibration data for the estimation of Ritonavir (N=6) in 0.1N HCl.

Concentration µg/ml	Absorbance ± SD
20	0.258 ± 0.012
40	0.489 ± 0.014
60	0.745 ± 0.015
80	0.991 ± 0.021
100	1.25 ± 0.018

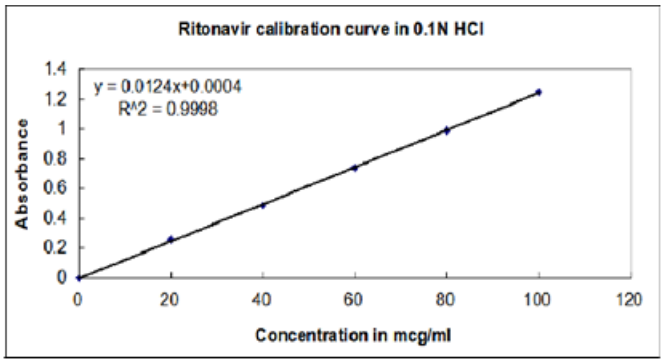

Fig 1: Calibration plot for the estimation of Ritonavir in 0.1 N HCl.

Preformulations study: The organoleptic and flow properties of both Ritonavir API are given in Table 3. The Carr's index and Hausner's ratios categorize the investigational drug, Ritonavir having very poor flow properties. The particle size distribution (D90) of the plain API before Nanonisation was found to be 14micron for Ritonavir.

Table 3: Organoleptic and flow properties of Ritonavir.

Properties	Observations of Ritonavir
Description	White to off white powder
Color	White
Taste	Bitter

Odor	Odorless
Test	**Ritonavir**
Bulk density	0.191 gm/ml
Tapped density	0.464gm/ml
Carr's index	58.8%
Hausner's ratio	2.43
PSD of API (micron)	
D10	0.7
D50	4.6
D90	13.9

Saturation solubility: The solubility data from Table 4 reveals Ritonavir belongs to poorly soluble class. For Ritonavir, solubility is comparatively more at pH 1.2 buffers - hence further all evaluations were aimed at this particular buffer media.[15]

Table 4: Saturation solubility.

S. No.	Medium	Solubility (mg/ml) of Ritonavir
1	Water	0.1568
2	pH 1.2	0.2637
3	pH 4.5	0.0568
4	pH 6.8	0.0284
5	pH 7.4	0.0426

Drug-Excipient's compatibility: The physical observations of drug excipients compatibility studies suggest that there is no change in the physical appearance of mixtures (Drug: Excipient) when stored at 40°C ± 2°C / 75% ± 5% RH for 1 month for Ritonavir. Hence the said excipient was deemed to be compatible and selected for further formulation development and optimization studies. (Table 5)

Table 5: Drug – Excipient's compatibility study (For Ritonavir).

S.No.	Binary mixture	Physical observations	
		Initial	40°C/75%RH for 1 month
1	Ritonavir	White powder	White powder
2	Ritonavir: Poloxamer 407	White powder to off-white powder	White powder to off-white Powder

Preparation of Nanosuspensions: Formulation F1 was not suitable for milling as the drug could not be dispersed properly for the purpose of milling. In case of F2 the poloxamer concentration was insufficient to effectively disperse the drug and in F4 too high concentration of poloxamer caused much foam. F3 was selected for further studies as there was less foaming and greater stability of the Ritonavir Nanosuspension which is indicated by least PDI. (Table 6) The prepared Ritonavir Nanosuspension exhibited tense bluish

opalescence, indicating successful formation of the Nanosuspension of Ritonavir. The Ritonavir Nanosuspension was transparent whereas the micro suspension was turbid which is shown in Fig 2.

Table 6: Formulation parameters of Ritonavir Nanosuspension.

Formulation code	Time	Agitation speed	Particle size(nm)	Polydispersity Index (PDI)	Remarks
F1	4hrs	3000	-	-	Drug could not be dispersed properly.
F2	4hrs	3000	456	0.424	Stabilizer concentration was insufficient
F3	4hrs	3000	384	0.266	Dispersion of drug in the polymer is good
F4	4hrs	3000	356	0.289	High quantity of foam Generation. Dispersionof drug in the polymer is good

Comparison of Ritonavir Nano and Micro suspension

Figure 2: Pictures showing Ritonavir Nanosuspension and Micro suspension.

Optimization of Process Parameters for Ritonavir Nanosuspension: By keeping the time and agitation speed constant, volume of milling media was optimized. 60% volume of milling media was found to be appropriate as it gave minimum particle size distribution. PDI was found to be better with 60ml of milling media compared to that of 50ml and 70ml of media. At 60ml milling media the particle size of the Nanosuspension produced was also found to be very less. Hence 60ml milling media was finalized for further optimization trials. The minimum PDI signifies the uniform distribution of the particles and stability of the Nanosuspension produced. By varying the speed of the mill, there was no much impact was found on particle size of the formulation. Hence milling speed of 3000rpm was finalized for further optimization trials. All the corresponding valuesare given in Table 7 and 8.

Table 7: Optimization of milling media volume for Ritonavir.

S. No.	Volume of millingmedia (ml)	PDI	PSD (nm)	Agitation speed
1	50	0.562	652	3000
2	60	0.266	384	3000
3	70	0.326	435	3000

Table 8: Optimization of milling speed for Ritonavir.

S. No.	Time	Milling Speed	PSD (nm)
1	4	2500	512
2	4	3000	384
3	4	3500	370

Evaluation of nanosuspension

Particle Size Distribution Analysis by Malvern particle size analyzer: The mean particle size and particle size distribution are two important characteristic parameters because they affect the saturation solubility, dissolution rate, physical stability even *in-vivo* behavior of Nanosuspensions. The polydispersity index (PDI) is an important parameter that governs the physical stability of Nanosuspensions and should be as low as possible for long term stability of Nanosuspensions. A PDI value of 0.1 to 0.25 indicates a narrow size distribution.(Table 9)

Table 9: Particle size analysis of Ritonavir Nano suspensions.

S. No.	Time (min)	Mill speed rpm	Pump rpm	Average Particle size (nm)			
				Before drying			After drying and redispersion
Batch no of the formulations				F2	F3	F4	F3
1	60	3000	70	950.4	864.8	870.6	850.3
2	120	3000	70	790.4	633.7	694.4	646.8
3	180	3000	70	610.3	425.4	4990.5	420.1
4	240	3000	70	517.4	384.6	418.0	408.2

Table 10: Effect of dilution on particle size of Ritonavir Nanosuspension.

S.No.	Dilution	Z- avg (nm)	PDI
1	1:1	392.3	0.281
2	1:10	383.7	0.248
3	1:100	395.9	0.259
4	1:500	373.3	0.235
5	1:1000	389.2	0.268

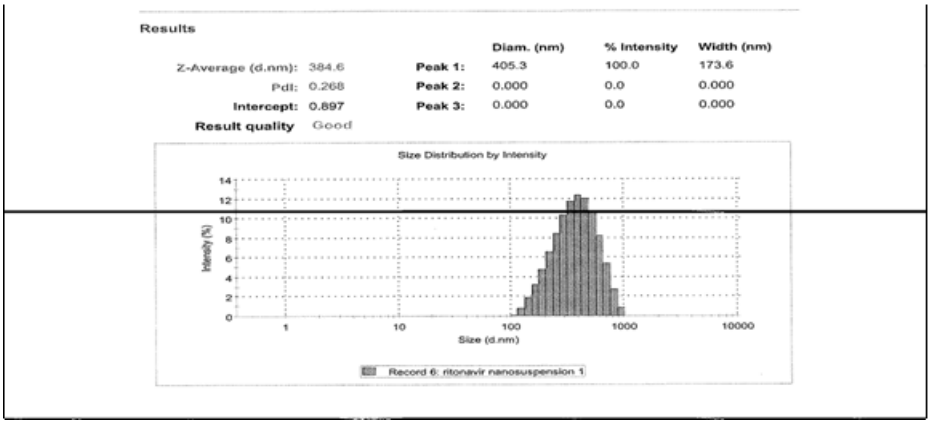

Fig 3: Particle size distribution of Ritonavir Nanosuspension F3.

Effect of milling time on particle size was optimized by keeping the speed and milling media volume constant, using 0.4mm of zirconium oxide beads. The particle size of the Nanosuspension formulations were evaluated by Malvern particle size analyzer (Zetasizer) and the results showed that the particle size of formulations F2 (Drug: Poloxamer – 1:0.05), F3(Drug: Poloxamer – 1:0.1) and F4 (Drug: Poloxamer – 1:0.2) were reduced to nanometric range. The particle size distribution of formulation F3 was found to be around 400nm with polydispersity index of 0.268**.** Particle size determination was also performed after drying the Nanosuspension and then redispersion of same dried Nanosuspension in water and Z-avg was found to be slightly higher when compared to the Nanosuspension prepared freshly. However, the difference in particle size results obtained found to be negligible when compared with eachother (Fresh Nanosuspension and Dried Nanosuspension). There was no significant change in particle size distribution on dilution of the Nanosuspension. PDI values were not altered after dilution which shows that the particles are within narrow size range. After 4 weeks all the above Nanosuspension formulations (F2, F3, F4) were again tested for particle size distribution to check whether any cluster/growth/ agglomeration and found to be 530, 377, 429nm respectively. The physical stability study results of F2 (Drug: Poloxamer – 1:0.05), F3 (Drug: Poloxamer – 1:0.1) and F4 (Drug: Poloxamer – 1:0.2) at room temperature for 1 month was found to be satisfactory. Sedimentation of suspension was observed after 2weeks for F2 (Drug: Poloxamer- 1:0.05) and no sedimentation was observed after 2 weeks and a slight sedimentation was observed after 4 weeks for formulations F3 (Drug: Poloxamer – 1:0.1) and F4 (Drug: Poloxamer – 1:0.2), which were readily redispersible. (Table 10, Fig 3).

Zeta potential: The determination of the zeta potential of a Nanosuspension is essential as it gives an idea about the physical stability of Nanosuspension. The zeta potential of a

Nanosuspension is governed by both stabilizer and the drug itself. Zeta potential also effect on circulation of Nanosized particles in blood stream and absorption into body membrane. After 4 hours of milling the zeta potential was found to be -24.3mV, -25.4mV and -19.5mV for F2, F3 and F4 respectively. As the zeta potential of the Ritonavir Nanosuspension is greater than ±15 mV, it can be concluded that the Nanosuspensions are deemed to be stable. All the results are given in Table 11. Zeta potential of final formulation was found to be increasing with the time of milling.

Table 11: Zeta potential of Ritonavir Nanosuspension F2, F3 & F4.

S. No.	Time (min)	Mill Speed (rpm)	Pump rpm	F2	F3	F4
1	0	3000	70	-4.9	-6.8	-5.9
2	60	3000	70	-8.9	-9.1	-8.8
3	120	3000	70	-14.1	-18.7	-14.9
4	180	3000	70	-18.8	-20.6	-16.8
5	240	3000	70	-24.3	-25.4	-19.5

X-Ray Diffraction studies (XRD): The assessment of the crystalline state and particle morphology together helps in understanding the polymorphic or morphological changes that a drug might undergo when subjected to Nanonising. X-Ray diffraction has been used to analyze potential changes in the inner structure of API nanocrystal during the formulation development. The extent of such changes depends on the chemical nature and physical hardness of the active ingredient. The change in the solid state of the drug particles can be identified by X- ray diffraction analysis and supplemented by differential scanning colorimetry. In order to get an actual understanding of particle morphology, the techniques such as scanning electron microscopy (SEM), atomic force microscope or transmission electron microscopy (TEM) are preferred.

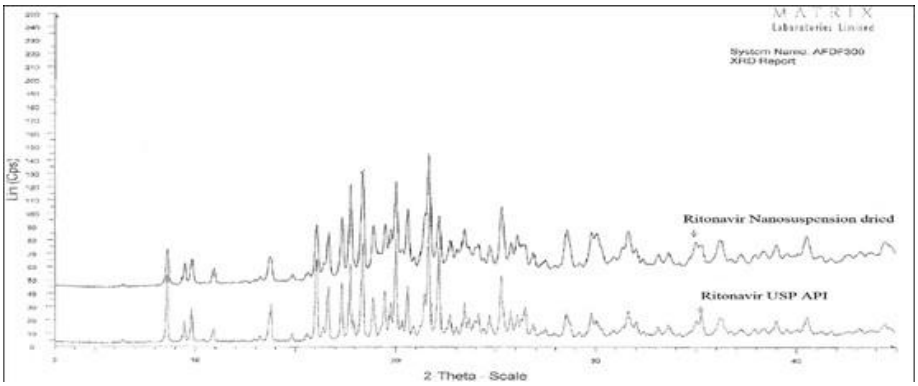

Figure 4: XRD data of Ritonavir and Ritonavir formulation.

The X-ray diffraction analysis was performed for Ritonavir pure drug and dried Ritonavir Nanosuspension. The obtained patterns reveal that the crystallinity of the drug in Nanosuspension formulation was not affected. The characteristic peaks of the Ritonavir drug molecule were found to be present in Nanosuspension. The comparative diffractograms are been shown in Fig 4.

Differential scanning calorimetry

Calorimetry is a primary technique for measuring the thermal properties of materials to establish a connection between temperature and specific physical properties of substances. DSC is a thermal analysis apparatus measuring how physical properties of a sample change, along with temperature against time. During a change in temperature.

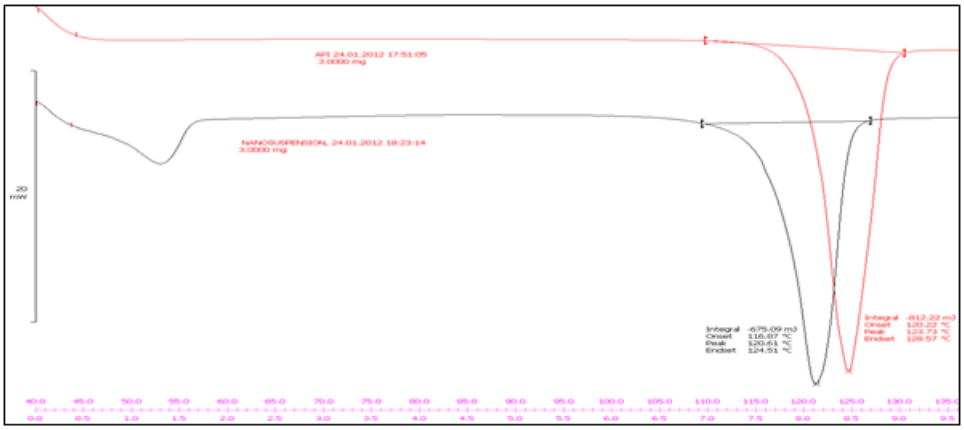

Fig 5: DSC graph of Ritonavir API and Ritonavir Nanosuspension.

DSC curves obtained for Ritonavir API and Ritonavir Nanosuspension were shown in Fig 5. Pure Ritonavir API had a sharp endothermic peak at 124°C that corresponded to the melting point of API. In Ritonavir Nanosuspension, a small but shifted API endothermic peak was observed which was found at 120.6°C. The DSC graph shows that there is no interaction between Ritonavir and Poloxamer.

Saturation Solubility: Tremendous fold of increase in saturation solubility was observed when the investigational drug was formulated into Nanosuspension. This may be due to Increase in surface area of the drugs because of Nanonisation. The fineness of the dispersed particles causes them to dissolve more quickly owing to their higher dissolution pressure and leads to an increased saturation solubility. This may enhance bioavailability of drugs compared to other micro particular systems. The results are given in Table 1 2 .

Table 12: Saturation solubility of Ritonavir API in different media before and after Nanosuspension formulation.

Solvent	Ritonavir	
	Pure drug	Nano suspension
Water	0.1568	1.621
pH 1.2	0.2637	3.521
pH 4.5 Acetate buffer	0.0568	0.924
pH 6.8 Phosphate buffer	0.0284	0.834
pH 7.4 buffer	0.0426	0.921

Morphology by microscopy and SEM imaging: The optical microscopic images showed great differences between suspension and Nanosuspension. In suspension the particles of the drug were found to be large irregular. However, after Nanonisation the particles disappeared and the drug became small and uniform which is been shown in Fig 6 for Ritonavir Nanosuspension.

A scanning electron microscope (SEM) is a type of Electron microscope that produces images of a sample by scanning it with a focused beam of electrons. The electrons interact with atoms in the sample, producing various signals that can be detected and that contain information about the sample's surface topography and composition. SEM has been used to determine PSD, surface topography, texture and examine the morphology of fractured or sectioned surface. The electron beam is generally scanned in a raster scan pattern and the beam's position is combined with the detected signal to produce an image. The Nanonised dried optimized formulation were screened through SEM to show the better particulate nature of the drug. The SEM images of both the dried optimized Nanosuspensions are shown in Fig 7 for Ritonavir Nanosuspension.[16]

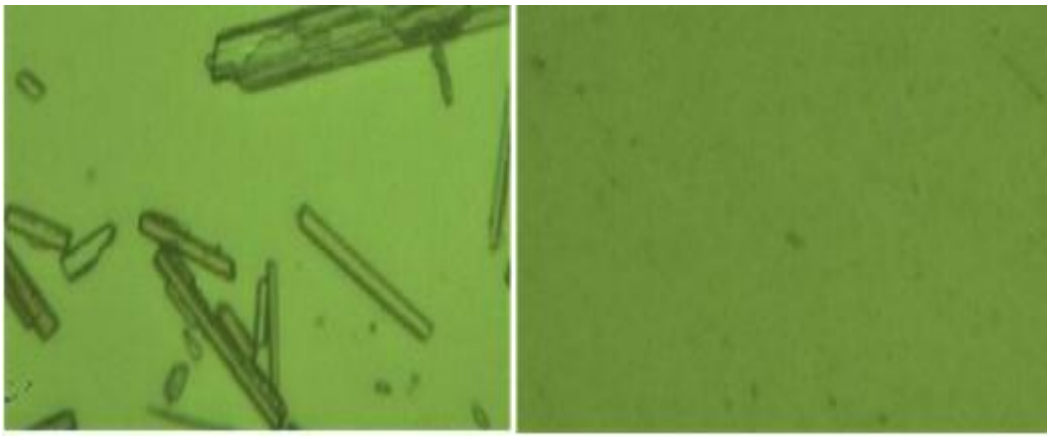

Fig 6: Optical microscopic image of Ritonavir suspension & Nanosuspension at 40X.

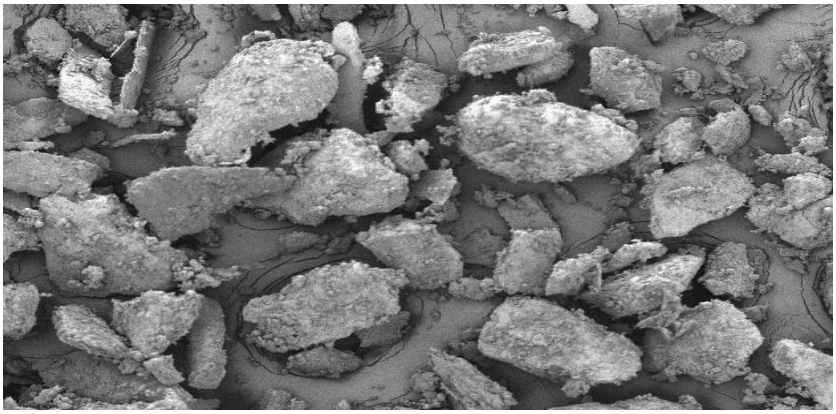

Figure 7: SEM image of Ritonavir Nanosuspension at 500X.

Freeze thaw study on Nanosuspension: Freeze thaw cycle testing is a part of stability testing that allows determining if the Nanosuspension will remain stable under various conditions. This type of test puts the sample through a series of extremely rapid temperature changes that it may encounter during normal shipping and handling processes. There was no agglomeration found in Ritonavir Nanosuspension. Particle size by zeta sizer was found to be 397nm for Ritonavir Nanosuspension after freeze thaw cycle study. The particle size of the Nanosuspension was found to be comparable to the Initial one. Release pattern was found to be satisfactory and comparable to the initial one. Hence it can be concluded that the Nanosuspension thus prepared was deemed to be stable. The data is given in Table 13.

Table 13: Effect of Freeze thaw cycle.

Tests	**Ritonavir Nanosuspension**		
	Initial		**After F/T cycle**
Particle size	**384.6**		**378.2**
Dissolution	**Time (min)**	**In 0.1N HCl***	
	15	73 ± 2.1	68 ± 2.4
	30	81 ± 1.7	77 ± 2.1
	45	88 ± 2.9	86 ± 2.2
	60	91 ± 2.6	92 ± 1.6
	90	94 ± 1.2	92 ± 1.2
	120	95 ± 1.7	93 ± 1.2

* (N=6, Mean ± S.D)

FT-IR studies

The FTIR spectrum of Ritonavir drug substance, poloxamer and dried Ritonavir Nanosuspension was recorded on Perkin - Elmer spectrum one FTIR spectrophotometer by using KBR pellet method and the spectrum is shown in Fig 8. Peaks at wavenumbers 3326, 2957, 1455, 1030, 873, 751, 704cm$^{-1.}$ in Ritonavir drug substance are considered to be

characteristic peaks. All these characteristic peaks are also observed in dried Nanosuspension indicating that there is no interaction between drug substance and excipients due to the process of Nanonisation.

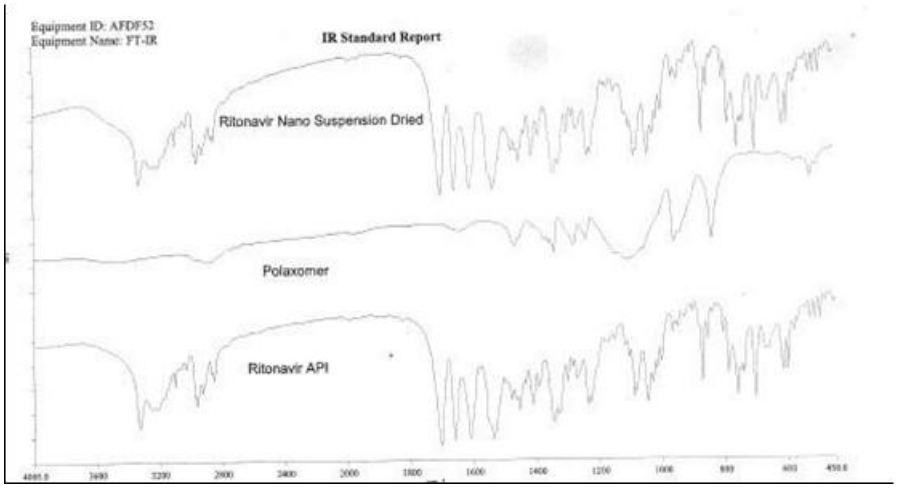

Fig 8: Comparative FTIR spectra of Ritonavir, poloxamer and Ritonavir Nano suspension.

Flow properties of Nano suspensions: The sedimentation volume was measured for the suspensions and was found that the suspensions showed the F values from 0.75 to 0.97 for Ritonavir Nanosuspensions. Greater the value of F, the more stable the product. When F=1, no sediment is apparent and caking is absent and suspension is esthetically pleasing and found to be stable. Tingstad indicated that a flocculated suspension that settles to a level that is 90% of the initial suspension height (F=0.9) and no further is probably deemed to be satisfactory. The suspensions, formulated were checked for their pourability from the bottle, it was found that Ritonavir Nanosuspensions were easily pourable from the bottle. (Table 14).

Table 14: Redispersibility values of Ritonavir Nanosuspensions.

Flow properties	After 2 weeks			After 4 weeks		
	F2	F3	F4	F2	F3	F4
Redispersibility (%)	100	100	100	100	100	100
Time taken to redisperse (Sec)	12	5	6	20	6	7
Sedimentation volume	0.75	0.96	0.97	0.75	0.92	0.94

Chemical evaluation of Nanosuspension: The assay values of Ritonavir Nanosuspensions were found to be 99.7%, 99.9% and 99.5% for F2, F3 and F4 respectively.

Dissolution studies: Nanosuspension thus produced for Ritonavir is able to increase both the dissolution velocity and saturation solubility. Size reduction indicates the enhancement of effective surface area which intern increase dissolution pressure as well as dissolution velocity. Becauseof reduction in particle size due to Nanonisation, solubility increases which change the surface tension leading to increase saturation solubility.

Percent drug released in 0.1N HCl was found to be 95% and 84% from F3 and F4 Ritonavir Nano suspension respectively. The dissolution study was also carried out on dried sample of Ritonavir Nano suspension and found to be 87%. To compare the dissolution of Ritonavir Nano suspension, a separate dissolution study on Plain Ritonavir and Ritonavir Nanosuspension before Nanonisation process was subjected for dissolution studies and the results were found to be 41.6% and 56% respectively. All these results are given in Table 15 and shown in Fig 9. The increase in accessible surface area to the dissolution medium and hydrophilic surfactant coating on the particle surfaces may be the reason for the increase in dissolution rate and extent.

Table 15: *Invitro* release from Ritonavir drug, Ritonavir suspension, Ritonavir Nanosuspension and Reference product in 0.1N HCl.

Time (min)	Cumulative percentage of Ritonavir released*				
	Ritonavir Plain API	Ritonavir Suspension	F3	F3 (Nanosuspension -Dry)	F4
0	0	0	0	0	0
15	9 ± 0.6	15 ± 1.6	73 ± 2.1	69 ± 0.9	68 ± 1.7
30	16 ± 1.6	23 ± 1.9	81 ± 1.7	76 ± 1.9	74 ± 1.2
45	23 ± 2.1	31 ± 2.1	88 ± 2.9	79 ± 2.1	78 ± 1.9
60	28 ± 2.4	39 ± 2.3	91 ± 2.6	81 ± 2.2	80 ± 2.1
90	36 ± 1.9	48 ± 2.5	94 ± 1.2	83 ± 1.1	82 ± 1.5
120	41.6 ± 1.5	56 ± 1.2	95 ± 1.7	87 ± 0.9	84 ± 1.1

* (N=6, Mean ± S.D)

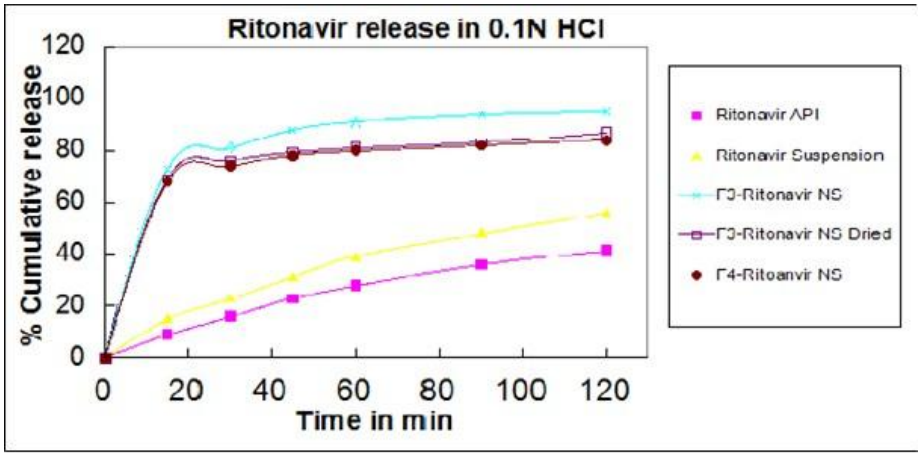

Figure 9: *Invitro* dissolution profile plot of Ritonavir drug, Ritonavir suspension, and Ritonavir Nanosuspension in 0.1N HCl.

The Nanonisation technology is particularly promising for improving the oral absorption and bioavailability of BCS class II drugs. It has been estimated that 40% of new chemical entities currently being discovered are poorly water-soluble nature. Many of the potential drugs are abandoned in the early stages of development due to solubility problems. Therefore, it is more important that methods for overcoming solubility limitations should be identified and applied commercially such that potential therapeutic benefits of these agents can be realized.[17-19]

The present work to enhance the solubility, dissolution rate and bio availability of poorly soluble drug Ritonavir by formulating it into Nanosuspension with Poloxamer as a stabilizing agent. The optimized Nanosuspension of Ritonavir was evaluated for physical and chemical properties.

1. Saturated solubility studies performed on Ritonavir API in micronized form. The study found to exhibit highest solubility in 1.2pH HCl (for Ritonavir). Hence same media was selected as a dissolution media for further studies.
2. In the present investigation, an attempt was made to formulate Ritonavir Nanosuspension using poloxamer as dispersing/stabilizing agent with the use of media milling technique.
3. The preformulation studies were done initially, which include determination of flow properties (bulk density, Tapped density, Carr's index, Hauser's ratio and angle of repose), particle size distribution, saturation solubility studies, drug-excipient compatibility study. From the data it was inferred that Ritonavir has passable flow properties. From the stability studies by DSC analysis, it was reasonable to believe that there was no interaction between drug and excipients used in Nanonisation process used

inRitonavir Nanosuspension preparation.

4. The results of drug excipients compatibility studies suggest that there was no significant change in the physical appearance of premixture blends, when stored at 40^0C/75% RH for a period of 4 weeks when compared to initial sample.
5. Different batches were taken to reduce the particle size of drug by changing the milling speed and volume of milling beads volume by keeping size of the bead and time duration for Nanonisation cycle as constant. Bead size of 0.4microns and milling time of 4hrs produced particles of 400nm size range when operated at a milling speed of 3000rpm with60% volume of milling media. The Ritonavir Nanosuspension thus produced did not showany stability related problems when stored for 1 month at 40^0C/75%RH.
6. Saturated solubility studies were again performed on optimized Ritonavir Nanosuspension. Tremendous increase in solubility in all the medias which were tried on plain micronized drug. This may be due to increase in surface area of the drug due to Nanonisation process.
7. The Dissolution study profiles of micronized drug in suspension and Nanonised drug in suspension was compared and remarkable improvement was found for Nanonised drug in selected media for Ritonavir.
8. The average particle size of the Ritonavir optimized Nanosuspension thus produced was found to be about 400nm with PDI of 0.268 indicating good physical stability of Nanosuspension.
9. The zeta potential of -25.4mV for optimized Ritonavir Nanosuspension indicates the good physical stability of Nanosuspension produced.
10. The availability of characteristic peaks of Ritonavir in Ritonavir Nanosuspension reveals that the original crystal habitat of the Ritonavir drug before Nanonisation is not been changed.
11. In DSC the two melting transitions in the system made up of drug (Ritonavir) and poloxamer signifies there is no interaction between drug (Ritonavir) and poloxamer.
12. The optical microscopic images and SEM images reveals of great difference between Ritonavir suspension and Nanosuspension. The small uniform regular shaped particles are the outcome of nanotechnology approach.
13. The FTIR study also indicating that there is no interaction between Ritonavir drug substance and poloxamer due to Nanonisation process.
14. The Dissolution of Ritonavir Nanosuspension found to be 87% in selected dissolution media when compared to the dissolution of 41.6 and 56% corresponding to Ritonavir

plaindrug and Ritonavir suspension. Based on this *in-vitro* dissolution study it is evident that Ritonavir Nanosuspension exhibit high dissolution rate and extent probably due to increase in surface area because of Nanonisation.

The Nanosuspension technology proves to be a promising approach in drug delivery system to enhance the solubility of drugs belongs to BCS class II and ultimately the bioavailability and can be formulated into oral dosage forms for efficient drug delivery with better patient compliance.

CONCLUSION

The results of the present investigation clearly indicated that the preparation of Nanosuspensions by top-down approach (Media milling) method greatly improved the solubility and dissolution rate of poorly soluble drug, Ritonavir. Poloxamer used as an inert stabilizer/surfactant to stabilize the Nanosuspension.

CONFLICTS OF INTEREST: Nil.

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