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DEVELOPMENT AND CHARACTERIZATION OF NOVEL CO-AMORPHOUS SYSTEM OF RITONAVIR WITH FUMARIC ACID: IMPROVED SOLUBILITY AND DISSOLUTION

Abhijit B Pawar[1], Punit B Parejiya[2*], Hetal K Patel[3], Prakruti Amin[4], Anuj A Patel[2], Het P Solanki[2]

[1]Ph.D Scholar (Pharmaceutical sciences), Kadi Sarva Vishwavidyalaya, Gandhinagar, Gujarat-382015, India

[2]Department of Pharmaceutics and Pharmaceutical Technology, KB Institute of Pharmaceutical Education and Research, Kadi Sarva Vishwavidyalaya, Gujarat-382023, India

[3]Department of Pharmaceutics and Pharmaceutical Technology, Arihant school of pharmacy and bio-research institute, Swarrnim Startup & Innovation University, Gandhinagar, Gujarat-382422, India

[4]Department of Pharmaceutics, Indus University, Shilaj, Gujarat-382115, India

***Authors for Correspondence:**

Dr. Punit B Parejiya

Associate Professor
Department of Pharmaceutics and Pharmaceutical Technology,
KB Institute of Pharmaceutical Education and Research,
Kadi Sarva Vishwavidyalaya Gandhinagar, Gujarat-382023
punit.parejiya@kbiper.ac.in
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Abstract

Ritonavir is highly lipophilic molecule that is practically insoluble in water, is widely used in low-dose fixed combinations, as a pharmacokinetic booster in antiretroviral regimens. Although it has favourable lipophilicity, ritonavir is reported to be a substrate of P-glycoprotein. Thus, the oral absorption of ritonavir could be limited by both dissolution and permeability, and is therefore considered a Class IV compound in the Biopharmaceutics Classification System. As the permeability is least impacted due to formulation strategies, hence the attempts were made to improve the solubility and dissolution rate. A novel coamorphous system of ritonavir was prepared with fumaric acid as low molecular weight coformer. Developed coamorphous system was meticulously characterized by advanced solid state characterization techniques such as DSC, modulated DSC, PXRD and PLM. Fit-to-purpose HPLC method was developed and validated with limited quantification parameters. Intermolecular interaction between ritonavir and fumaric acid was carefully studied by FTIR analysis. Developed coamorphous system showed ~6 fold improvement in the water solubility over crystalline ritonavir. Due to enhanced solubility, coamorphous system showed complete release in water as compared to crystalline ritonavir.

Introduction

Poor aqueous solubility remains one of the most persistent challenges in oral drug delivery, since a substantial proportion of new chemical entities and several marketed drugs fall under Biopharmaceutics Classification System (BCS) Class II and

IV, where dissolution rather than permeability is the rate-limiting step for absorption [1]. Among the strategies developed to overcome this limitation, conversion of a crystalline drug into its high-energy amorphous form has attracted considerable attention because the absence of long-range molecular order eliminates the lattice energy that must otherwise be overcome for dissolution, thereby improving apparent solubility and dissolution rate [2]. However, single-component amorpho=s systems are thermodynamically unstable and tend to recrystallize during storage, which has restricted their direct pharmaceutical application and necessitated stabilization strategies such as polymeric amorphous solid dispersion (ASD) [3].

As an alternative to polymer-based amorphous solid dispersions, the co-amorphous (CAM) approach has emerged as a promising formulation strategy over the last decade [4-6]. A co-amorphous system is defined as a single homogeneous amorphous phase comprising an active pharmaceutical ingredient (API) and a low molecular weight co-former, which may be a pharmacologically relevant API, an excipient, or an amino acid, held together through intermolecular interactions such as hydrogen bonding, ionic interaction, or heterodimer formation [7]. Compared with neat crystalline or amorphous material, co-amorphous systems have demonstrated improved physical stability, enhanced dissolution profiles, and, in several cases, superior pharmacokinetic performance [6-8]. Reported examples include indomethacin–arginine systems prepared by spray drying, which produced up to a 200-fold enhancement in intrinsic dissolution rate, and simvastatin–glipizide and simvastatin–amino acid combinations, which showed markedly improved amorphous-phase physical stability compared with the corresponding single-drug amorphous forms [9]. Curcumin–artemisinin and atorvastatin calcium–Fumaric acid co-amorphous systems have similarly shown improved solubility and pharmacokinetic profiles over their crystalline counterparts , while indomethacin–cimetidine and naproxen–cimetidine systems prepared by quench cooling, co-precipitation and ball milling illustrate that multiple preparation techniques can be employed to obtain the co-amorphous state [10-12]. Rational selection of a co-former, guided by molecular interaction potential, miscibility, and glass-forming ability, is considered central to a successful co-amorphous design [13, 14].

Ritonavir is a peptidomimetic HIV-1 protease inhibitor used, often in low-dose

fixed combinations, as a pharmacokinetic booster in antiretroviral regimens. It is a BCS Class IV compound with an aqueous solubility of approximately 0.00126 mg/mL, high lipophilicity (log P 3.9), and a reported melting point of 121–123°C. Its clinical relevance and notorious solubility-limited absorption, historically compounded by the emergence of a less soluble crystalline polymorph during commercial manufacture, have made it a benchmark molecule for solubility-enhancement research. Several approaches have previously been explored to enhance ritonavir bioavailability, including polyethylene glycol- and Gelucire-based amorphous solid dispersions, which improved in vitro dissolution and, in rodent pharmacokinetic studies, produced substantially higher plasma concentrations and area under the curve relative to the crystalline drug [15]. More recent work has examined polymer screening approaches such as hydroxypropyl methylcellulose acetate succinate and polyvinylpyrrolidone-vinyl acetate based amorphous solid dispersions for ritonavir, prepared through film casting and hot-melt techniques, further confirming the amorphous route as the dominant solubility-enhancement strategy for this molecule [16].

Despite the extensive work on polymer-based amorphous solid dispersions of ritonavir, its behaviour in low molecular weight co-amorphous systems, which avoid the processing and physical-stability limitations associated with high-viscosity polymeric carriers, remains largely unexplored [17]. The present investigation was therefore aimed at developing the novel coamorphous system of Ritonavir with fumaric acid by solvent evaporation. Developed coamorphous system was evaluated by advanced solid state characterization tools such as differential scanning calorimetry (DSC), modulated-DSC, Thermgravimetric analysis, Powder X-ray diffractometry and Polarized light microscopy. Intermolecular interaction between ritonavir and fumaric acid was confirmed by FT-IR. Aqueous solubility and powder dissolution of developed coamorphous system was evaluated against plain drug. Finally, the physical stability of developed coamorphous system was evaluated at high temperature and humidity conditions.

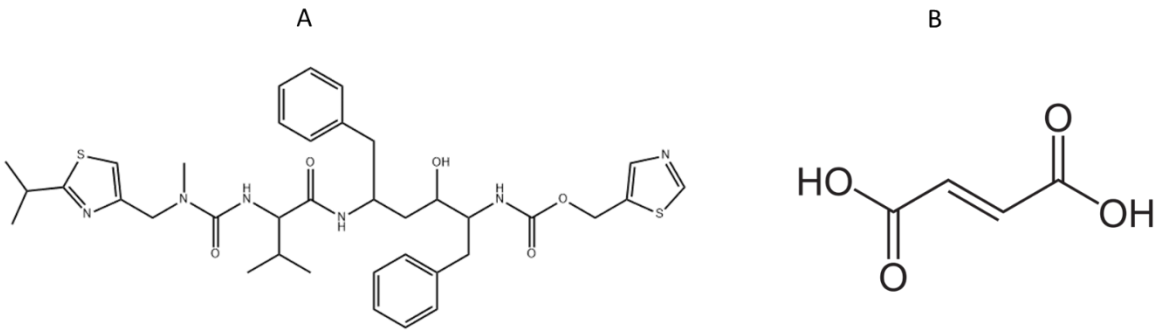

Fig.1. Chemical structure of (A) Ritonavir and (B) Fumaric acid

2. Materials and Methods

2.1 Materials

Ritonavir was procured as a gift sample from Jigs Chemical Limited, Ahmedabad, India. Fumaric acid was purchased from Sigma-Aldrich (USA). Trifluoroacetic acid was procured from Sisco Research Laboratories Pvt. Ltd. (India). Methanol and acetonitrile were procured from Honeywell International India Pvt. Ltd. Deionized water was obtained from a Milli-Q ultrapure water system (Millipore, USA).

2.2 Preparation of co-amorphous system of ritonavir and Fumaric acid by solvent-assisted grinding

Solvent evaporation was employed to prepare the co-amorphous system of ritonavir with the selected co-former, Fumaric acid, at a 1:1 molar ratio. Fumaric acid was selected as the co-former based on its established hydrogen-bond-accepting pyridine nitrogen and amide carbonyl groups, its favourable interaction potential with ritonavir's carbamate, thiazole and hydroxyl hydrogen-bond donor/acceptor groups, its Generally Recognized As Safe (GRAS)/pharmacopoeial status, and its documented success in stabilizing the co-amorphous form of the structurally comparable BCS Class II statin atorvastatin calcium [1].

An accurately weighed 1:1 molar mixture of ritonavir and Fumaric acid was dispensed and dissolved in methanol under stirring. After 30 minutes of stirring content was transferred to round bottom flask and subjected to solvent evaporation under vaccum conditions. After complete solvent evaporation, the solid was scratched from round bottom flask and analysed by powder X-ray diffractometry (PXRD) for residual crystalline impurities. The obtained solid mass was passed through a 40 µm sieve to produce powder of consistent particle size, which was then dried under vacuum.

Samples were packed in an air-tight container and stored under refrigeration (2–8°C) to avoid temperature-induced recrystallization until further characterization.

2.3 Development of Reverse phase-High performance liquid chromatography (RP-HPLC) method

Chromatographic separation was carried out on a Waters Separation Module 2695 equipped with a Waters 2996 photodiode array detector and Empower 3 chromatography data software. Separation was achieved on an X-Bridge C18 column (150 × 4.6 mm, 5 µm particle size) maintained at 40°C using a gradient mobile phase comprising 0.01% trifluoroacetic acid in water (mobile phase A) and acetonitrile:methanol (50:50 v/v; mobile phase B), with the gradient programme 0 min/5% B, 1 min/5% B, 4 min/100% B, 6 min/100% B, 10 min/5% B and 12 min/5% B. The flow rate was maintained at 1.0 mL/min, injection volume was 10 µL, sample temperature was maintained at 25°C, and the total run time was 12 min. Acetonitrile: water (50:50 v/v) was used as the diluent. Under these conditions ritonavir eluted at a retention time of approximately 6.2 min.

A stock solution of ritonavir was prepared in diluent and serially diluted to obtain working solutions of 10, 25, 50, 100, 200 and 500 µg/mL. Each concentration was injected in triplicate (n = 3) and the mean peak area was plotted against nominal concentration to construct the calibration curve. Linear regression analysis was performed to determine the slope, intercept and coefficient of determination (R^2). The limit of detection (LOD) and limit of quantification (LOQ) were estimated from the residual standard deviation of the regression line and the slope of the calibration curve, using the standard relationships LOD = 3.3σ/S and LOQ = 10σ/S, in accordance with ICH Q2(R1) guidance, where σ is the residual standard deviation of the regression and S is the slope of the calibration curve.

2.4 Powder X-Ray Diffraction (PXRD)

PXRD was employed to understand the solid-state characteristics of as such Ritonavir and coamorphous system. Nearly 15-20 mg of solid sample was spread uniformly on to the sample holder and the measurements were carried out using Bruker D8 Advance diffractometer (BrukerAXS, Karlsruhe, Germany), employing Cu-Kα X-radiation with a wavelength (λ) of 1.5406 Å. The instrument operated at 40 kV and 40 mA power. Samples were scanned from 3-40Θ, with a precise step size of 0.02° and a rapid scan time of 0.3 seconds. Subsequently, we

meticulously analyzed the recorded diffractograms using DIFFRAC EVA (version V5.2) diffraction software.

2.5 Thermal analysis (DSC, and m-DSC)

We recorded the DSC curves for all samples using a DSC250 instrument from TA Instruments, equipped with TRIOS software. Approximately 5-10 mg of powdered samples were carefully placed in hermetically sealed aluminum pans. Our investigation covered Ritonavir, physical mixture, and its novel forms (co-amorphous system). The temperature range spanned from 25°C to 300°C, with a heating rate of 10°C/min, all under a continuous nitrogen flow with a purging rate of 50 mL/min.

We employed Modulated Differential Scanning Calorimetry (MDSC) to assess the glass transition temperature (Tg) of developed co-amorphous system. The experiments were conducted using a DSC250 instrument from TA Instruments, equipped with TRIOS software. This instrument featured a refrigerated cooling system. Prior to analysis, the sample cell was thoroughly purged with dry nitrogen at a flow rate of 50 mL/min. we weighed 4-5 mg of co-amorphous samples and heated them up to 300°C. The analysis was performed at a heating rate of 2°C/min, with a modulation amplitude of ±1°C and a modulation period of 60 seconds.

2.6 Polarized light microscopy (PLM)

PLM was used to check crystallinity of as such ritonavir and developed coamorphous formulation. Small amount of sample was placed on a clean glass slide and examined under Olympus BX51 polarized light microscope which contains crossed polarizers and a red compensator (U-TP530). Images were taken at 10× magnification using transmitted light. If crystalline impurities are present, it appears as bright birefringent regions against the dark background, which makes it very easy to distinguish between crystalline and amorphous parts in samples [18].

2.7 Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of as such ritonavir, physical mixture and coamorphous formulations were recorded using an FTIR multiscope spectrophotometer (PerkinElmer, Buckinghamshire, UK) equipped with Spectrum v3.02 software to identify functional groups and evaluate possible intermolecular interactions between the drug and co-former. Absorption spectra were recorded in the wavenumber range of 450–4000 cm^{-1}.

2.8 Percentage assay (% assay)

An accurately weighed quantity of the co-amorphous system, equivalent to 50 mg of

ritonavir, was prepared in triplicate and transferred to a 100 mL volumetric flask. The volume was adjusted to 100 mL with diluent, and the contents were sonicated until complete dissolution was achieved. The resulting solution was further diluted appropriately with the same diluent and subjected to HPLC analysis for quantification of ritonavir.

2.9 Saturation solubility of optimized Co-amorphous system in water

The saturation solubility of ritonavir, physical mixture and coamorphous formulation was evaluated by dispersing an excess quantity of each sample in purified water. For solubility determination, nearly 50 mg equivalent of as such ritonavir, physical mixture and coamorphous formulation was dispersed in 2 mL of respective buffer. The mixtures were transferred into sealed containers and placed in a shaking water bath (LAUDA, H20 SW), where they were maintained at 37 ± 0.5 °C with continuous agitation at 200 RPM to reach equilibrium. At pre-specific time intervals (Initial, 2 h, and 6 h), samples of about 500 µL were withdrawn and subjected to centrifugation to separate any undissolved material. From the resulting supernatant, 100 µL of the clear solution was carefully collected, appropriately diluted with the respective medium/diluent, and then quantified for drug content using a HPLC method. All the measurements were done in triplicate to ensure the reproducibility.

2.10 Powder dissolution

Accurately weighed quantities of as such ritonavir, physical mixture and coamorphous formulation equivalent to 400 mg of ritonavir were individually filled into size 2 hard gelatin capsules and subjected to dissolution testing. The dissolution study was carried out using a Electrolab EDT-08LX (Electrolab India Pvt. Ltd., India) dissolution apparatus (USP Type II) dissolution apparatus operated at a paddle rotation speed of 75 RPM. The dissolution medium consisted of 900 mL of pH 4.0 acetate buffer maintained at 37 ± 2°C throughout the study. All the measurements were done in triplicate to ensure the reproducibility.

2.11 Physical stability

Physical stability of the CAMs was systematically evaluated under varied storage conditions. Samples were kept in open glass vials in a humidity chamber (Newtronic Lifecare Equipment Pvt. Ltd., India) maintained at 40 ± 2 °C / 75 ± 5% RH, in a Stericell SC55ECO hot air oven (BMT Medical Technology s.r.o., Czech Republic) for 60 °C. At defined time intervals (2 and 4 weeks), samples were withdrawn and characterized using PXRD

and DSC to investigate potential alterations in solid-state properties.

2.12 Statistical analysis

All calibration measurements are expressed as mean values of triplicate injections (n = 3). Linear regression, residual analysis and derived analytical figures of merit (LOD, LOQ) were computed using standard regression statistics. A coefficient of determination (R^2) ≥ 0.99 was considered acceptable evidence of assay linearity across the tested concentration range.

3 Results and Discussion

3.1 Physicochemical suitability of ritonavir

The compiled physicochemical profile presented (Table 1) for ritonavir supports its selection as a challenging but appropriate candidate for formulation intervention. Its very low aqueous solubility of 0.00126 mg/mL, BCS class II classification, and lipophilic character reflected by log P of 3.9 together indicate that dissolution rather than permeability is likely to be the major barrier to oral performance. In practical formulation terms, these attributes justify investigating solid-state modification strategies intended to improve the apparent solubility and dissolution behavior of the drug [16].

Table 1: Physicochemical properties of Ritonavir

Properties	Ritonavir
Molecular formula	$C_{37}H_{48}N_6O_5S_2$
Molecular weight (g/mol)	720.944
Melting point (°C)	121-123 °C
Appearance	white colored powder
Log P	3.9
pKa	2.8
Aqueous solubility (mg/mL)	0.00126 mg/mL
Hydrogen Bond donor count	4
Hydrogen Bond acceptor count	9
Use	Anti-Viral
Dose (mg)	25 mg and 50 mg (FDC)
BCS class	Class II
Problem associated with drug	Low solubility

3.2 Development and validation of the RP-HPLC assay

The chromatographic conditions used for quantification of ritonavir are summarized in Table 2. Under the described gradient, ritonavir produced a well-resolved, symmetrical peak at a retention time of 6.2 min with a total run time of 12 min, indicating suitability of the method for routine and stability-related quantification.

The calibration curve was linear over the range of 10–500 µg/mL (Fig. 2). Linear regression of the mean peak area against nominal concentration yielded the equation Area = 6733.7 × Concentration + 60194.2, with a coefficient of determination (R^2) of 0.9994, confirming excellent linearity across the tested range. The residuals were randomly distributed with no systematic trend, and the percentage deviation of the back-calculated concentrations from nominal values remained within ±10% for all calibration levels (Table 3). Based on the residual standard deviation of the regression, the LOD and LOQ were calculated as 15.12 µg/mL and 45.82 µg/mL, respectively.

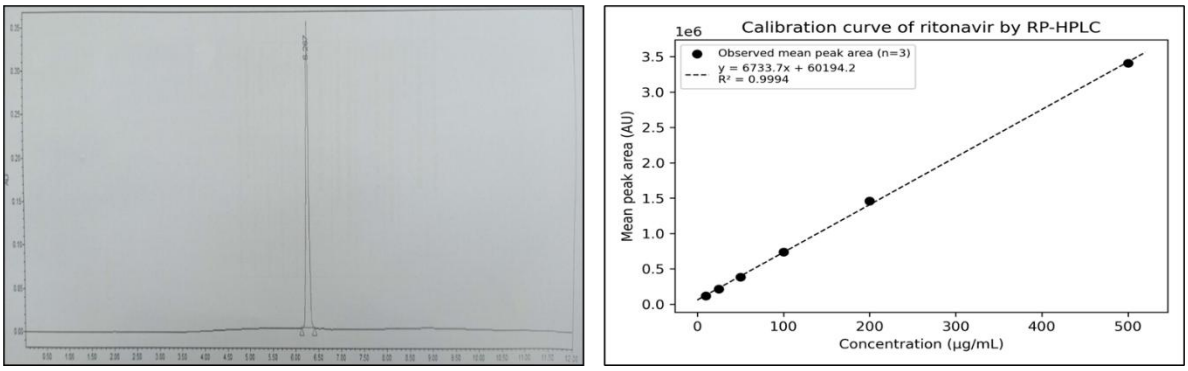

Fig. 2. RP-HPLC chromatogram and calibration curve of ritonavir (10–500 µg/mL).

3.3 PXRD

As depicted in fig 3-B, as such ritonavir showed characteristics Bragg's peaks at 2θ values like 6.8, 8.4, 10.6, 14.6, 14.9, 16.3, 18.1, 19.0, 21.5, 22.2, 23.5° in its diffraction pattern, which was in line with the literature report [19]. As confirmed in Fig 3-D, the developed coamorphous system showed the hollow pattern in PXRD, which confirms the complete amorphization, without any crystalline impurities.

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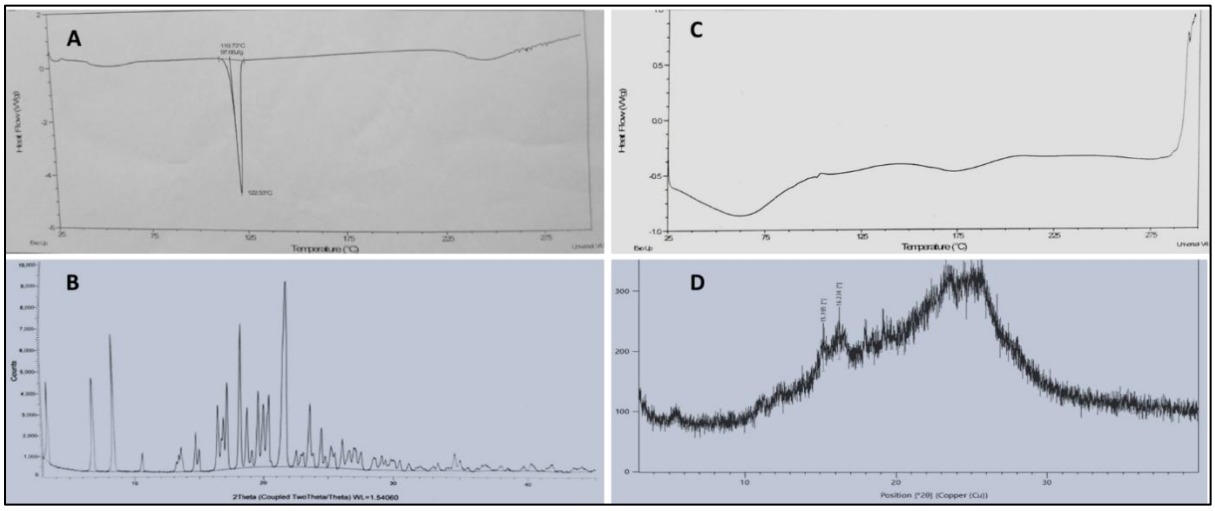

Fig.3: Characterization of as such ritonavir (A: DSC; B: PXRD) and coamorphous system (C: DSC; D: PXRD)

3.4 DSC and m-DSC

DSC demonstrated a sharp melting endotherm at 122.53 °C, closely matching the reported melting point range of 122-123 °C therefore confirming the crystalline identity of the received sample [20, 21]. The corresponding PXRD interpretation, namely the presence of sharp peaks matching pure ritonavir, independently supports the same conclusion. Agreement between thermal and diffraction evidence strengthens confidence that the starting material existed predominantly in crystalline form prior to any amorphization attempt. Developed coamorphous system did not show any melting endotherm, which further confirms he amorphous transition of crystalline ritonavir during processing.

As confirmed in fig.4, developed coamorphous system showed a single glass transition event at around 37.17°C which confirms homogenous amorphous transition of ritonavir with fumaric acid. Modulated DSC showed no melting endotherm which further supports the DSC and PXRD of amorphous transition.

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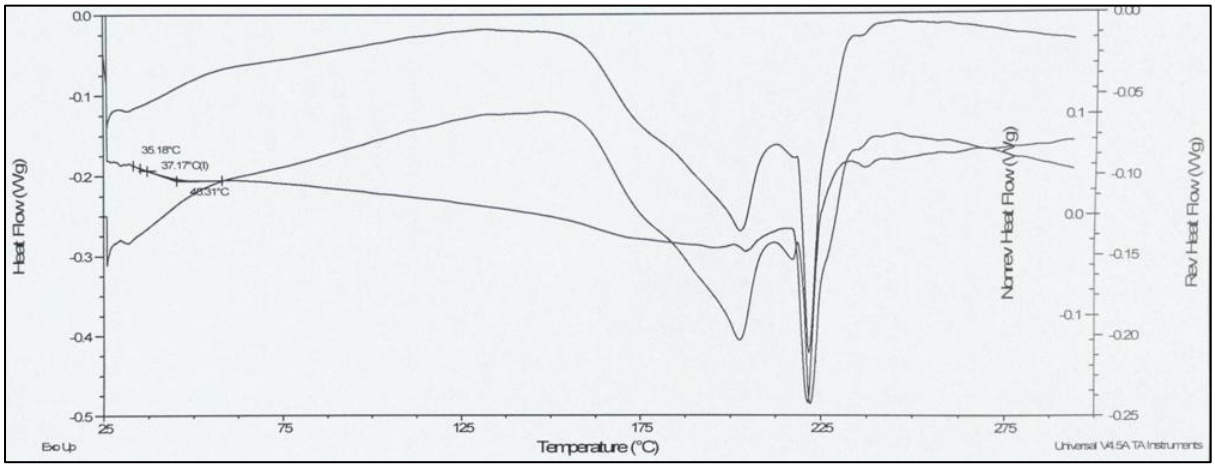

Fig.4.: modulated DSC of developed coamorphous system

3.5 Polarised light microscopy

Polarized light microscopy (PLM) experiment was intuitively performed to evaluate presence of crystalline impurities in coamorphous systems. In Fig.5-A birefringence phenomenon was obviously observed in as such ritonavir, due to crystalline nature of ritonavir. Whereas the absence of birefringence for coamorphous system (Fig.5-B) confirms the complete amorphization of ritonavir. The PLM observations were found to be in good agreement with the PXRD and DSC data of coamorphous system.

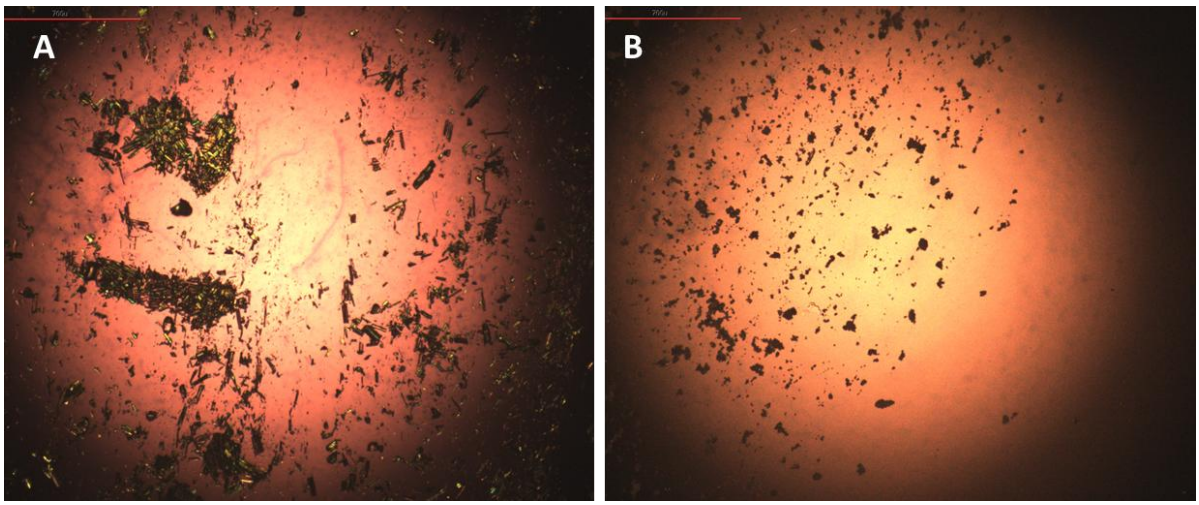

Fig. 5: Polarized light microscope images of (A) crystalline ritonavir and (B) coamorphous system

3.6 FTIR

The FTIR spectrum of ritonavir matches with the literature reported characteristic peaks at 3357.73 cm-1, 3025.35cm-1, 2964.47 cm-1 and 1714.67 cm-1, which correspond to N-H stretching of secondary

amine, C=C stretching, C-H stretching and C=O stretching, respectively [22, 23]. The FTIR spectra confirms the identity of pure form of ritonavir. The FTIR spectrum of developed coamorphous formulation showed broadening and reduced intensity of the N-H stretching and carbonyl stretching, indicating involvement of these groups in intermolecular interactions. Physical mixture retained all the characteristic vibrational frequencies of ritonavir with slightly reduced intensities.

These spectral changes hints at the formation of strong ionic hydrogen bonding interactions between the carboxylate group of fumaric acid and the secondary amine (N–H) of ritonavir in the developed co-amorphous system.

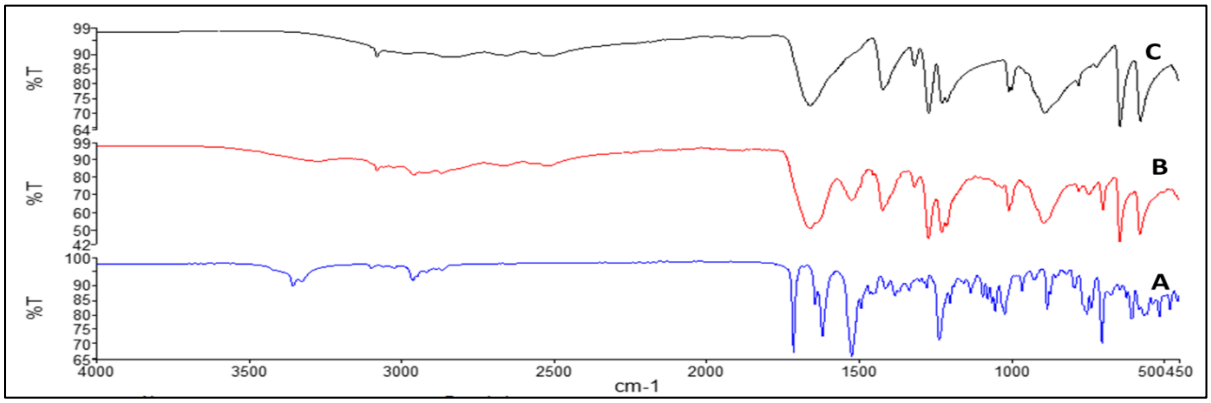

Fig.6: FTIR spectra of (A) ritonavir, (B) coamorphous system and (C) physical mixture

3.7 % assay

Assay of the developed coamorphous system was done with the validated HPCL method to estimate the potency of developed coamorphous system. Assay of the developed coamorphous system was observed to be 98.65% by HPLC, which confirms homogeneity of drug content in developed coamorphous system.

3.8 Saturation solubility in water

Solubility is the key prerequisite for bioavailability, especially for BCS class-II drug candidates. Ritonavir is a weakly basic molecule, whose solubility is reported to be practically insoluble in water (~5µg/mL) [24]. The saturation solubility of the ritonavir was tested in water as it can have significant impact on the *in-vivo* behaviour. Hence saturation solubility of ritonavir, physical mixture and coamorphous system was evaluated in water up to 24 Hr at 37°C. As shown in Figure 7, coamorphous system consistently exhibited the highest solubility as compared to crystalline ritonavir across all time points. The physical mixtures demonstrated minimal improvement over

as such ritonavir due to acidic microenvironment created by fumaric acid. Co-amorphous system showed nearly 6-folds improvement in the aqueous solubility as compared to as such ritonavir, which can be correlated with the complete amorphization. These findings highlight the enhanced solubility achieved through co-amorphization, with fumaric acid, relative to both the physical mixtures and the as such drug.

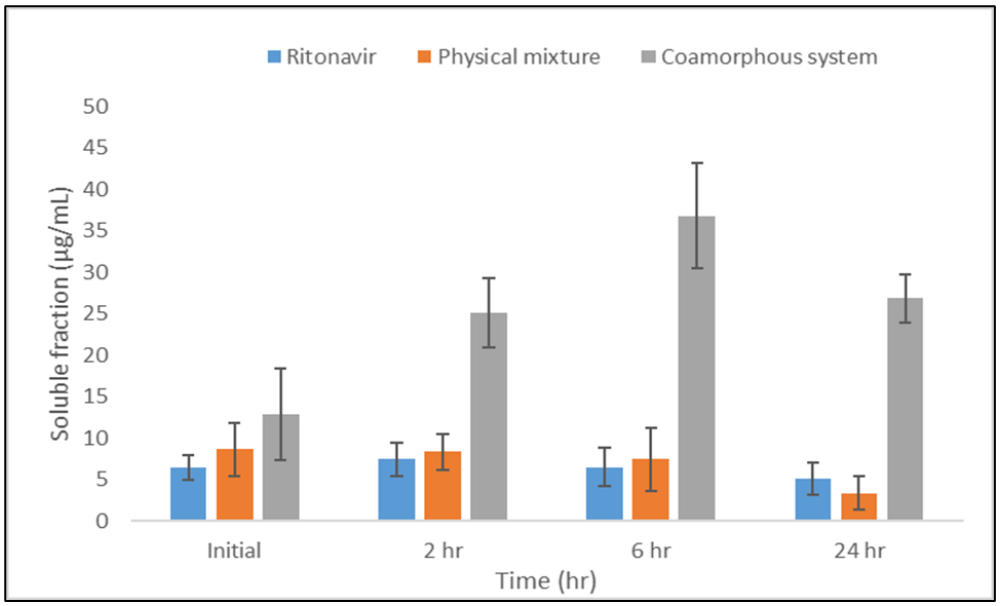

Fig.7: Saturation solubility of ritonavir, physical mixture and coamorphous system

3.9 Powder dissolution

A powder dissolution test was performed under sink conditions in purified water along with the crystalline drug and the physical mixture to investigate the dissolution behaviour of the co-amorphous formulations. As depicted in Fig.8, the coamorphous system showed complete release of nearly 99.5% at 45 min, compared to 11.5% and 24.8%, respectively for crystalline counterpart and physical mixture. This enhancement can be attributed to the amorphization due to molecular-level mixing of ritonavir with fumaric acid, a highly water-soluble co-former, which reduces the solvation energy required for ritonavir molecules to enter the aqueous phase. Furthermore, the absence of lattice energy in the disordered amorphous state removes the energy barrier required for disrupting the crystalline structure. Collectively, these factors might have contributed to faster dissolution and improved solubility of coamorphous system over the as such ritonavir. The significantly improved solubility of coamorphous system can be correlated to improved solubility in water.

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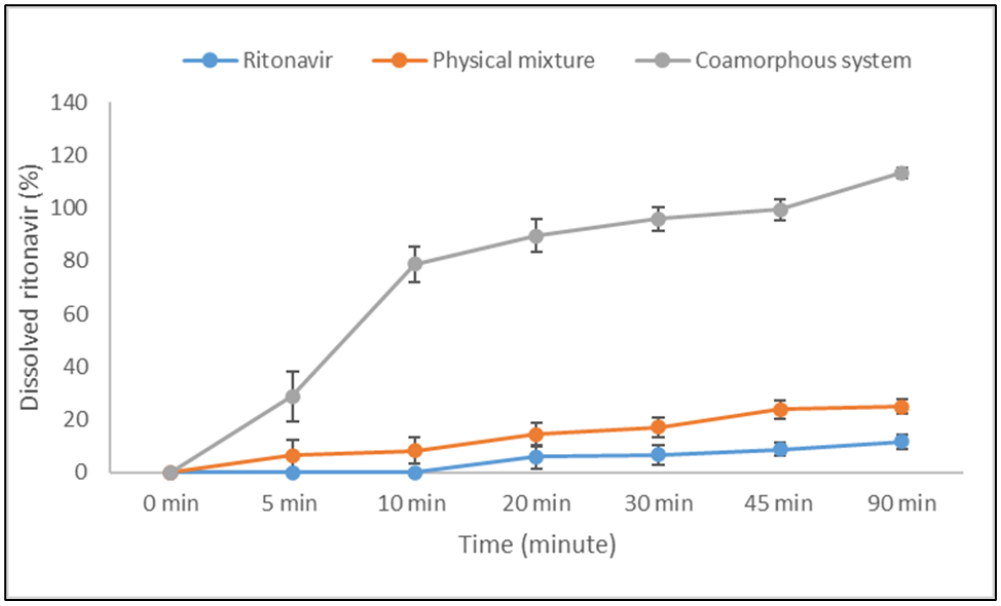

Fig.8: Powder dissolution of ritonavir, physical mixture and coamorphous system

3.10 Physical stability

The physical stability of the coamorphous formulation was evaluated by PXRD after storage in an open container under accelerated (40 °C/75% RH) and stress conditions (60 °C) for up to 30 days. The initial CAM exhibited a broad diffuse halo pattern, confirming its amorphous nature. This halo pattern was retained 3, 7, 15 and 30 days under both storage conditions without the appearance of any distinct sharp diffraction peaks, indicating that the CAM remained physically stable and did not show any signs of recrystallization throughout the study period. The diffractograms of stress exposed samples were largely similar to that of the initial coamorphous formulation.

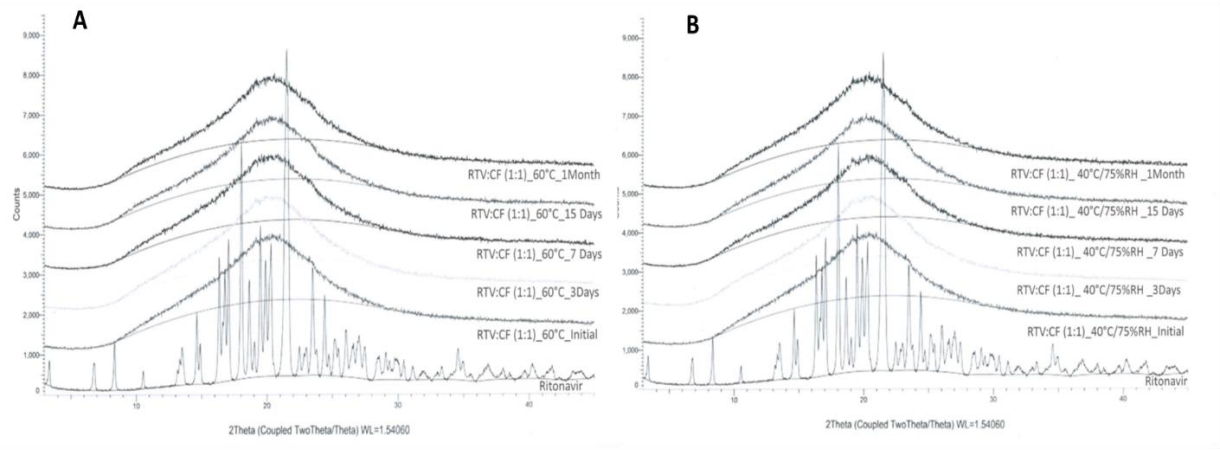

Fig.9: Physical stability of coamorphous system at (A) 60°C and (B) 40°C/75% RH

Conclusion

This study covers an crystallographic identity of the drug through DSC and PXRD, with an observed melting point (122.53°C) closely matching literature values. The novel coamorphous system of ritonavir with fumaric acid at 1:1 molar ratio was developed with rotary evaporation. Methanol was selected as a solvent of choice by performing the qualitative solubility of ritonavir in different organic solvents. The developed coamorphous system resulted in single Tg at nearly 37.17°C, which confirms the homogeneity of drug and coformer at selected molar ratio. FTIR data confirm the molecular interaction between drug and coformer, however the complete amorphization might have happened due to intimate mixing and efficient solvent evaporation with rotary evaporation alongside molecular interaction. Complete amorphous transition of crystalline ritonavir in presence of fumaric acid was confirmed by advance solid state characterization techniques such as DSC, m-DSC, PXRD and PLM. Aqueous solubility of developed coamorphous system showed more than 6-fold improvement as compared to crystalline ritonavir. Also, the powder dissolution of coamorphous system was observed to be significantly better as compared to crystalline ritonavir. Coamorphous system showed no signs of recrystallization at elevated temperature and humidity conditions up to 30 days.

The outcome of this extensive study suggests the significance of development of stable co-amorphous system and role of small molecules for improvement of physicochemical properties of BCS class II and IV drugs. The relatively unexplored area of co-amorphization, has huge potential to overcome the solubility related challenges of poorly soluble molecules.

Authors contributions

All authors have contributed to the design of the study, carrying out of the experiments, data analysis, and preparation of the manuscripts.

Conflict of interest

The authors declare no conflict of interest.

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