Journal of Pharmaceutical Innovation (2019) 14:305–315
https://doi.org/10.1007/s12247-018-9357-6

ORIGINAL ARTICLE

The Assessment of pH-Induced Supersaturation and Impact of an Additional Drug on the Solution Phase Behavior of Saquinavir

Sujata D. Sakhare[1] · V. Sai Krishna Anand[1] · Aishwarya Karan[1] · K. Navya Sree[1] · S. G. Vasantharaju[1] · Girish Pai[2] · Swapnil J. Dengale[1]

Published online: 15 October 2018

Abstract

Purpose The goal of this study was to investigate the ability of saquinavir to generate the in vivo supersaturation and the impact of the presence of another solute, i.e., ritonavir, on the phase behavior of the former.

Method The phase behavior of saquinavir alone and in the presence of ritonavir was studied by pH shift supersaturation assay. The generation of drug-rich phase was confirmed by dynamic light scattering (DLS) and UV extinction method. The nature of precipitate generated after pH shift was investigated by employing DSC and XRPD. Further, the flux studies were performed by employing dialysis membrane using Franz diffusion cell.

Results Saquinavir precipitated in the amorphous form exhibiting type-II precipitation behavior generating the drug-rich phase and undergoing glass-liquid phase separation (GLPS) after the shift in pH towards higher side. The supersaturation advantage of saquinavir was marginally lowered in the presence of amorphous ritonavir. However, the free drug concentration of ritonavir was significantly reduced below the saturation solubility generating a subsaturated state. Both the drugs exhibited lowering in the chemical potential in the presence of each other, thereby reducing their flux/diffusion. The decrease in the free drug concentration and chemical potential were found dependent on the mole fraction of the solute present in the binary supersaturated solution.

Conclusion The findings of the phase behavior of weak bases in the presence of other solutes are of great value not only in fixed-dose combination and concomitantly administered drugs but also in formulating supersaturated systems like amorphous solid dispersions and co-amorphous systems.

Keywords Saquinavir · Glass-liquid phase separation · pH shift supersaturation · Amorphous

Introduction

In a quest to achieve higher pharmacological potency and specificity of targets like nuclear receptors, the new drug candidates are becoming increasingly lipophilic with suboptimal aqueous solubility [1]. Forty percent marketed drugs and 75% of developmental candidates belong to BCS class-II [2]. Pre-formulation scientists attempt to improve the solubility of such compounds to make them druggable. The aqueous crystalline solubility can be enhanced by employing the techniques like co-solvents, complexation, and size reduction [3–5]. Recently, there is a tremendous interest in generating a supersaturated state of a compound, which can be accomplished through amorphous solid dispersion, co-crystals, and salt forms [6–8]. Owing to their high chemical potential, supersaturated drug solutions exhibit a high rate of passive diffusion, thereby offering the unique advantage of the simultaneous improvement in solubility as well as permeability [9, 10].

Supersaturated solutions have a drug concentration in a solution exceeding its crystalline solubility. This maximum attainable supersaturation is given by the amorphous solubility. Amorphous solubility is not a true thermodynamic

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12247-018-9357-6) contains supplementary material, which is available to authorized users.

✉ Swapnil J. Dengale
swapnil.jd@manipal.edu

[1] Department of Pharmaceutical Quality Assurance, Manipal College of Pharmaceutical Sciences, Manipal Academy of Higher Education, Manipal, Karnataka 576104, India

[2] Department of Pharmaceutics, Manipal College of Pharmaceutical Sciences, Manipal Academy of Higher Education, Manipal 576104, India

solubility, since amorphous form is a metastable state, which tends to shift the equilibrium towards stable state through crystallization. Usually, amorphous solubility is 5–50× than its crystalline counterpart. At such a high concentration, the miscibility limit of the liquid form of the compound can be exceeded, leading to the phase separation, generating two phases in the solution, namely drug-rich phase and drug lean phase [10, 11]. The drug-rich phase is a colloidal system having particles whose particle size can be varied from 100 to 500 nm. If the drug-rich phase is present in the glass form, i.e., if the temperature of the medium is below glass transition temperature (Tg) of the water-saturated drug-rich phase, then the phenomenon is called as glass-liquid phase separation (GLPS) [12, 13]. For certain compounds like citric acid, the glass transition temperature of the water-saturated drug-rich phase is usually less than the temperature at which the experiments have been conducted. In such a situation, drug-rich phase exists in a super-cooled state; hence, this phenomenon is termed as liquid-liquid phase separation (LLPS) [10–12]. It has been proven in the literature that some compounds undergo rapid supersaturation generating LLPS/GLPS maintaining the solution concentration at highest supersaturation level, i.e., amorphous solubility [9, 13, 14]. From the physiological perspective, weak bases have high probability of generating supersaturated state in vivo as the pH of the gastrointestinal tract transitions significantly from the acidic (stomach pH 1 to 2) to alkaline environment (small intestine pH 5 to 7) [15–17]. The gradient pH of the gastrointestinal tract has implications for ionizable compounds which have pH-dependent solubility profile, in particular for ionizable weak bases having pKa in the range of 5 to 8. Such weak bases can be completely or partially solubilized in the stomach due to generation of predominant ionized species, while as the pH increases in the lower gastrointestinal tract, weak bases are amenable to precipitation due to predominance of unionized species. If the precipitated form does not crystallize immediately, then disordered drug-rich phase is formed while the solution concentration is maintained at amorphous solubility generating the supersaturated state with respect to crystal solubility. Such phenomenon which is called as in vivo supersaturation is desirable, as it can increase the overall exposure of a drug leading to the improvement in bioavailability. However, recent studies have demonstrated that the environment-sensitive fluorescence probe pyrene registers a more hydrophobic environment in the presence of the drug-rich phase as compared to single-phase aqueous solution [10]. It proves that the drug-rich phase has a potential to interact with additional species that is present in the solution. This interaction is akin to the partitioning of relatively lipophilic compounds (Logp > 1) into the C_{18} stationary phase of a reversed phase column. So, it is ingenuous to believe that the implications of this phenomenon are inconsequential, especially for techniques which have potential to generate in vivo supersaturation like solid dispersion, co-amorphous formulations. Furthermore, fixed-dose combinations and concomitantly administered drugs can suffer from such phenomenon, delivering a sub-optimal dose of either or both drugs depending upon the extent of supersaturation and physicochemical properties of the individual drugs.

Our laboratory is engaged in exploring the potential of co-amorphous systems to generate supersaturated solutions for improved bioavailability of BCS class-II drugs. During the solubility studies, in particular, pH shift assay experiments, we noticed that saquinavir maintains the concentration equivalent to the maximum achievable solution concentration, i.e., amorphous solubility after the pH shift towards the basic side regardless of its physical form, i.e., free base, mesylate salt, or developed co-amorphous forms. This observation prompted the systematic investigation of the phase behavior of saquinavir. Saquinavir is a weak base which has pH-dependent solubility and hence high potential to generate in vivo supersaturation [18]. Saquinavir is an anti-retroviral drug belonging to the class protease inhibitor. It has high potency but low bioavailability (4%) due to high clearance by the liver through CYP-3A4 metabolizing enzymes [19]. Saquinavir is always prescribed in combination with ritonavir, which boosts the performance by preferentially blocking CYP-3A4 enzyme system [20, 21]. The marketed formulation of ritonavir is amorphous solid dispersion [22]. Hence, in the light of the scientific evidence available in the literature that the amorphous solubility of a compound can be reduced by the concomitant presence of other solutes, it is not unreasonable to hypothesize that the free drug concentration of saquinavir and/or ritonavir is lowered in the presence of each other. This possibility seems very real as both the compounds are expected to be in their respective amorphous state in the alkaline environment of the small intestine and hence can mix ideally to form drug-rich phase undergoing LLPS/GLPS. In this context, we thought it worthy of an investigation to study the phase behavior of saquinavir in the presence of ritonavir.

Experimental Section

Materials

Saquinavir mesylate and free base were kindly gifted by Hetero Labs Limited (Hyderabad, India). Ritonavir was a kind gift from Aurobindo Pharma Limited (Hyderabad, India). The chemical structure of the compounds is represented in Fig. 1. Hydroxy propyl methyl cellulose (HPMC) was purchased from Loba Chemie limited (Mumbai, India). Semipermeable regenerated cellulose dialysis membrane was purchased from Spectrum Laboratories, Inc. (CA, USA) with a molecular weight cutoff of 3.5 kDa. HPLC grade solvents like acetonitrile and methanol were procured from Merck Life Sciences

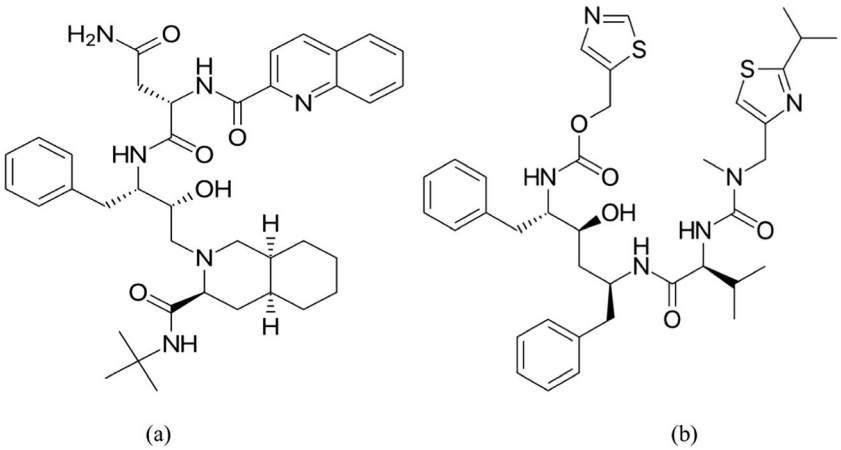

Fig. 1 Chemical structures of saquinavir (**a**) and ritonavir (**b**)

Private Limited (Mumbai, India). Ultrapure water was obtained from SIEMENS purification system (Munich, Germany) installed in the lab.

Methods

Equilibrium Crystalline Solubility by Shake Flask Method

The equilibrium crystal solubility of saquinavir mesylate salt, saquinavir free base, and ritonavir was determined in USP phosphate buffer (pH 6.8) at 37 °C for 12 h. The excess amounts of drugs were added to 2 mL USP phosphate buffer (pH 6.8) in separate vials. The resultant suspension was sonicated for 5 min, and subsequently, its pH was checked. The vials containing the excess drug in the buffer were kept in the orbital shaker for 12 h, maintaining the temperature 37 °C and rpm 150. The samples were retrieved after 12 h and centrifuged at 10000 rpm for 10 min using Remi C24 centrifuge (Mumbai, India). The supernatant of the resultant samples was diluted appropriately with the mobile phase. The samples were analyzed by HPLC method as described in "Analytical Techniques."

Amorphous Solubility Determination

Amorphous solubility of the drugs was determined by using ultracentrifugation method. Ten-microliter concentrated methanolic solution of the drug was spiked into 10 mL USP phosphate buffer (pH 6.8) containing 10 μg/mL HPMC and stirred at 37 °C. HPMC was added to the solution to inhibit crystal nucleation and growth throughout the duration of the experiment. The solution was then ultracentrifuged at 40,000 rpm in swinging bucket rotor (MLA-80) to palette the drug-rich phase using an Optima max-XP ultracentrifuge (Beckman Coulter Inc., Brea, CA, USA) for 20 min. The supernatant was then diluted appropriately and analyzed by HPLC method described in "Analytical Techniques."

Supersaturation Assay by pH Shift Method

The supersaturation potential of saquinavir (salt and free base) and ritonavir individually and in the presence of each other was evaluated by pH shift method. Phosphate buffer was used for the experiment due to its high buffer capacity at pH 2.0 and 7.0, which would mimic the stomach and intestinal environment respectively. The amount of drug equivalent to the human dose was equilibrated with the USP phosphate buffer (pH 2.0) for 10 min at 37 °C using magnetic stirrer. First sample was retrieved after 10 min (zeroth time point) followed by subsequent increase in the pH to a value 6.8 using potassium hydroxide solution. Stirring was continued for another 120 min while the temperature was maintained at 37 °C and samples were collected at intervals of 30 min. The collected samples were centrifuged for 20 min at 40,000 rpm at 37 °C, and supernatants were suitably diluted with mobile phase. The samples were analyzed using HPLC method as described in "Analytical Techniques."

Determination of Glass/Liquid-Liquid Phase Separation As mentioned in the above section, an excess amount of materials were equilibrated at 37 °C in the USP phosphate buffer pH 2.0; then, pH of the solution was shifted to 6.8 using potassium hydroxide to ensure that the concentration of both or either of the component was well above glass/liquid-liquid phase separation (GLPS/LLPS). The resulting solutions were cloudy. These solutions were characterized to confirm the formation of GLPS/LLPS by the following methods.

Dynamic Light Scattering Scattering intensity and particle size of the dispersed phase in the solution were monitored by dynamic light scattering (DLS) employing Nano zeta sizer (Nano ZS, Malvern Instruments, Westborough, MA, USA). The samples were introduced into the zeta sizer using a clear disposable zeta cell and analyzed for the particle size and dispersity.

UV Extinction Owing to the colloidal nature of drug-rich phase, it scatters UV light and the same can be detected at the non-absorbing (extinction) wavelength, which confirms the formation of GLPS/LLPS. The samples were analyzed at 400 nm using UV-visible spectrophotometer (UV-1800, Shimadzu Corporation, Kyoto, Japan).

Phase Behavior of Saquinavir in the Presence of Ritonavir

The drug-rich phase of saquinavir was generated by adding 1% *v/v* concentrated methanolic solution into the beaker containing 10 mL of USP phosphate buffer (pH 6.8). To these solutions, increasing concentrations of ritonavir, i.e., 10, 20, 30, and 40 μg/mL, were added. These solutions were then ultracentrifuged at 40000 rpm to remove the denser drug-rich phase, and the supernatant was analyzed by HPLC to determine the concentration of two drugs in the aqueous phase. The same set of experiment was done by generating the ritonavir drug-rich phase and adding increasing concentrations of saquinavir, i.e., 10, 20, 40, 60, and 100 μg/mL. The generation of drug-rich phase and subsequent LLPS/GLPS was confirmed by DLS and UV extinction method described in "Supersaturation Assay by pH Shift Method."

Flux/Diffusion Studies

Diffusion studies were carried out using the Franz diffusion cell apparatus through a semipermeable regenerated cellulose dialysis membrane (Spectrum Laboratories, Inc., CA, USA) with a molecular weight cutoff of 3.5 kDa. The effective surface area of dialysis membrane used for the study was 3.8 cm^2. Methanolic drug stock solutions were spiked to generate different concentrations in the donor compartment. Receiver compartment was filled with 15 mL of USP phosphate buffer (pH 6.8) and stirred at 60 rpm. All experiments were carried out at 37 °C using temperature-controlled magnetic stirrer. Samples were taken from the receiver compartment at regular time intervals and analyzed by HPLC method described in "Analytical Techniques."

Analytical Techniques

HPLC The sample analysis was carried on Waters HPLC (Alliance Waters 2695, Milford, MA, USA) separations module and UV dual lambda absorbance detector (Waters 2487). Grace smart C18 (250 × 4.6 mm ID × 5 μm) column was used for chromatographic separation with mobile phase constituting of a mixture of acetonitrile and 25 mM phosphate buffer (containing 3.042 g of potassium dihydrogen phosphate and pH adjusted to 3.2 with 85% of orthophosphoric acid) delivered isocratically at a flow 1.0 mL/min in the ratio of 50:50% *v/v*. The column temperature was set to 25 °C; column effluent was monitored at 238-nm wavelength.

X-ray Powder Diffraction X-ray powder diffractograms were recorded using Rigaku miniflex 600 X-ray diffractometer (Rigaku Co., Tokyo, Japan). The instrument was operated at 600 W (X-ray tube), with a fixed tube current of 15 mA and a voltage of 40 kV. The diffracted X-ray beam was monochromated by a graphite monochromator, and a standard scintillation counter was used as the detector. Diffraction intensities were measured by fixed time step scanning method in the range of 5–40° (2).

Differential Scanning Calorimetry Differential scanning calorimetry (DSC) measurements were carried out by a Shimadzu DT-60 apparatus (Shimadzu Corporation, Kyoto, Japan). Three to 5 mg of material was placed in a flat-bottomed aluminum pan of 0.1 mm thickness and crimped with an aluminum lid. The samples were placed into sample holder and heated from 25 to 200 °C at the heating rate of 10 °C (or as required in the experiment) per minute under a nitrogen flow (10 cc/min).

Results

Solubility

The amorphous solubility and equilibrium solubility of a crystalline form of saquinavir (both salt and free base form) and ritonavir in USP phosphate buffer pH 6.8 at 37 °C are summarized in Table 1. The equilibrium solubility of crystalline mesylate salt of saquinavir is approximately 15-fold higher than its free base counterpart. However, the amorphous solubility values of both mesylate salt and free base of saquinavir are comparable without any appreciable difference. Further, the amorphous solubility of ritonavir is significantly higher than the equilibrium solubility of a crystalline form, exhibiting the supersaturation ratio approximately 6. The amorphous solubility values determined in USP phosphate buffer pH 6.8 by UV extinction method were found to be in good agreement with those determined by ultracentrifugation followed by HPLC analysis. To confirm the effect of polymer concentration on the amorphous solubility, the experiments were carried out in the absence of the polymer. Both saquinavir and ritonavir did not show any significant difference in the amorphous

Table 1 The crystalline and amorphous solubility of saquinavir and ritonavir

Sample	Solubility (USP phosphate buffer, pH 6.8, ug/mL)		
	Ritonavir	Saquinavir base	Saquinavir salt
Crystalline	4.45 ± 0.33	2.97 ± 0.83	49.54 ± 6.42
Amorphous	36.76 ± 1.52	55.47 ± 2.59	62.32 ± 6.22

solubility values in the presence and absence of the polymer, ruling out the influence of polymer concentration on the amorphous solubility.

Precipitation Behavior of the Individual Drug by pH Shift Method

Owing to the weakly basic nature of ritonavir and saquinavir, they are expected to precipitate after an increase in the pH. The precipitation behavior of each drug was studied by pH shift assay wherein the drug was dissolved in the acidic medium mimicking the stomach conditions, and then, the pH was rapidly shifted to the neutral/alkaline side resembling the small intestine milieu. Both saquinavir and ritonavir demonstrated pH-dependent solubility. However, the ratio of the solubility of crystalline ritonavir in acidic to basic medium is less than twofold due to its weakly ionizable nature (Fig. 2). There is a difference in the solubility of the saquinavir free base and mesylate salt at the acidic side, the solubility of the former being significantly higher. Interestingly after the shift in the pH towards the alkaline side, both saquinavir free base and mesylate salt solubility values offset to a concentration corresponding to the amorphous solubility, and the concentration was maintained throughout the duration of the experiment (2 h). These findings demonstrate that there is a generation of supersaturated state of saquinavir with respect to its crystalline solubility after the shift in the pH. Further, the solution concentration of crystalline ritonavir fell to its equilibrium solubility after the pH shift. As ritonavir is manufactured in the form of an amorphous solid dispersion, we also investigated the precipitation behavior of neat amorphous ritonavir prepared by quench cooling method without employing any

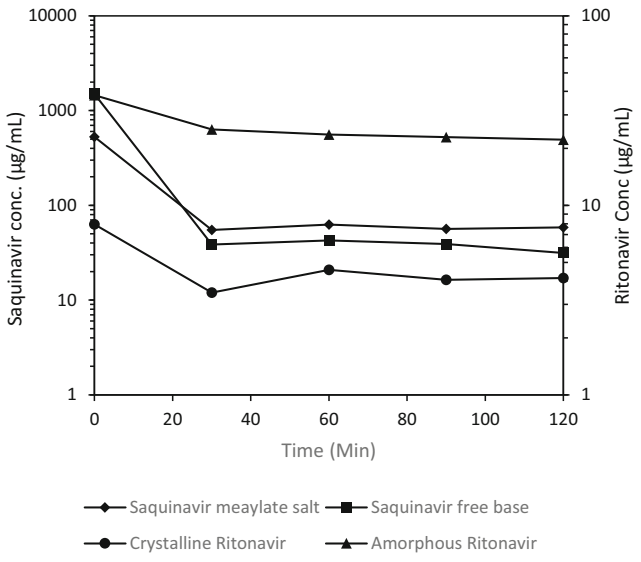

Fig. 2 Free drug concentration of saquinavir and ritonavir as a function of time obtained in pH shift supersaturation assay. (Zeroth time point indicates the free drug concentration before shift in the pH.) Error bars are not visible due to logarithmic scale

polymer. Interestingly, the solubility advantage of ritonavir was found to be endured after the transition of the pH towards higher value generating the supersaturated state with respect to crystalline solubility. However, ritonavir solution concentration was significantly less than its maximum achievable concentration, i.e., amorphous solubility in the medium.

As the solubility of saquinavir from both the forms, i.e., free base and mesylate salt, at low pH exceeded its amorphous solubility in the basic medium by factor 10–30 X, there exists a possibility of formation of a drug-rich phase of saquinavir following the pH shift leading to glass-liquid phase separation (GLPS). The generation of a colloidal phase after pH shift was confirmed by UV extinction and DLS method which showed a significant increase in the scattered light and particle count (KCPS) respectively. Nevertheless, if the colloidal phase generated following the pH shift is crystalline, then the phenomenon cannot be termed as GLPS. To confirm the glassy nature of the precipitated colloidal phase, a sample was taken after the pH shift, ultracentrifuged, and dried to investigate the nature of the colloidal phase. The dried sample was then analyzed by DSC and XRPD. Irrespective of the initial physical form (free base or mesylate salt) of the saquinavir, the precipitate showed no endothermic event confirming the DSC amorphicity of it. Further, the amorphous nature of precipitate was corroborated by XRPD where diffractogram demonstrated halo pattern (Supplementary material). These findings confirm that GLPS has occurred after the pH shift for saquinavir. The same phenomenon was investigated for ritonavir where crystalline ritonavir was found to be precipitated followed by pH shift. Nevertheless, amorphous ritonavir showed the presence of trace amounts of ritonavir polymorph V in the precipitate as evident by DSC thermogram which showed melting endotherm at 118 °C consistent with the literature value [23, 24] (Supplementary material).

Phase Behavior of Saquinavir in the Presence of Ritonavir

Phase behavior of saquinavir was studied in the presence of crystalline and amorphous ritonavir at the concentration equivalent to the human dose. As the solution phase behavior of free base and salt form of saquinavir was found analogous, and saquinavir mesylate is the marketed form, the phase behavior studies were performed employing the mesylate salt form of saquinavir. Neat amorphous ritonavir was prepared by quench cooling technique, and the amorphicity in the solid state was confirmed by DSC. The sample thermogram showed the glass transition temperature (Tg) at 52 °C which was consistent with the literature value [25]. Both saquinavir mesylate salt and ritonavir dissolved rapidly in the acidic medium. After pH shift, the solution concentration of saquinavir offset rapidly to a value corresponding to its amorphous solubility generating the supersaturated state with respect to crystalline

solubility which maintained throughout the duration of the experiment (2 h) (Fig. 3). Interestingly, the solution concentration of saquinavir in the presence of amorphous ritonavir was lower than that found in the presence of crystalline ritonavir. After pH shift, the saquinavir concentration was consistently found below the amorphous solubility in the presence of amorphous ritonavir, though the difference is not statistically significant.

Phase Behavior of Ritonavir in the Presence of Saquinavir

Phase behavior of amorphous ritonavir was studied in the presence of saquinavir mesylate salt form. The phase behavior of neat amorphous ritonavir is discussed in "Precipitation Behavior of the Individual Drug by pH Shift Method," where the solution was supersaturated with respect to the crystalline solubility of ritonavir after pH shift. The solution concentration of ritonavir in the presence of saquinavir was significantly less in the acidic medium before shifting the pH. Further, after pH shift, the solution concentration of ritonavir in the presence of saquinavir was dropped significantly to a value which is lower than its equilibrium solubility generating a subsaturated state (Fig. 4). This shows that the supersaturation advantage of amorphous ritonavir can be neutralized in the presence of another solute in the amorphous form. These findings are particularly interesting and may have adverse implications for fixed-dose combinations and concomitantly administered drugs.

Impact of an Additional Solute on the Supersaturation Ability of a Drug

Saquinavir demonstrated the ability to generate amorphous drug-rich phase after pH shift and subsequently underwent GLPS. Furthermore, it exhibited the potential to significantly

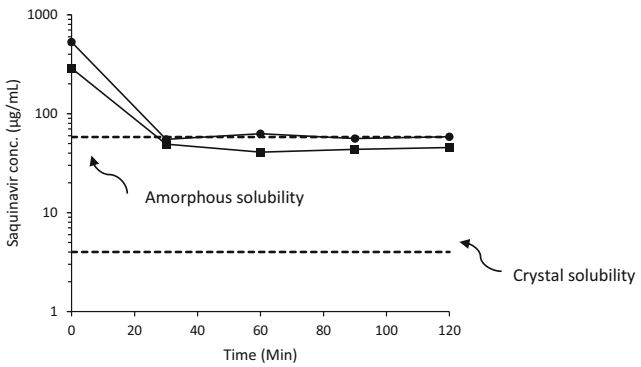

Fig. 3 Free drug concentration of saquinavir in the presence of crystalline (black circle) and amorphous (black square) ritonavir as a function of time in pH shift supersaturation assay. (Zeroth time point indicates the free drug concentration before shift in the pH.) Error bars are not visible due to logarithmic scale

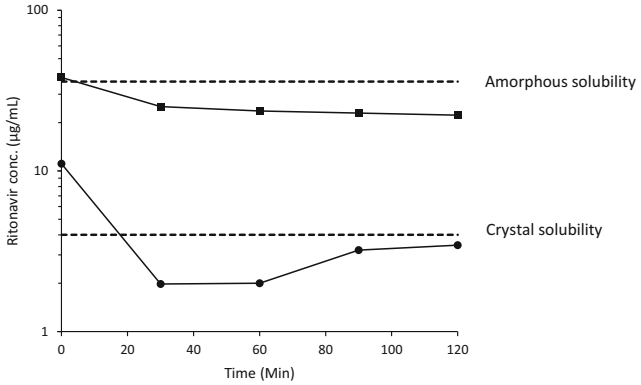

Fig. 4 Free drug concentration of ritonavir in the presence (black circle) and absence (black square) of saquinavir as a function of time in pH shift supersaturation assay. (Zeroth time point indicates the free drug concentration before shift in the pH.) Error bars are not visible due to logarithmic scale

decrease the bulk ritonavir concentration. It has been demonstrated previously that the amount of drug-rich phase generated has a profound impact on the free drug concentration available for absorption [10, 12]. It is expected that the unionized lipophilic drug added to the solution containing a drug-rich phase will preferentially distribute into the drug-rich phase lowering its bulk drug concentration. This hypothesis was tested by preparing fixed concentrations of saquinavir and adding increasing amounts of ritonavir into it. In the next set of experiments, the solution concentration of ritonavir was held well above its amorphous solubility and then increasing concentrations of saquinavir were added. The concentration of each drug achieved in the solution was estimated after removing denser drug-rich phase by ultracentrifugation. By employing such experimental design, the mutual influence of each solute on the tendency of the system to generate drug-rich phase and the ability to lower other solute concentration was systematically evaluated.

When the low amounts of drug(s) were added, the concentration of both the drugs in the supernatant was found the same as the amount spiked. However, at the higher drug concentration, the relationship between drug amount added and that recovered from the supernatant is no more linear (Figs. 5 and 6). As the total solute concentration increases, the concentration of each drug in the supernatant decreases. Interestingly, the simultaneous presence of both the solutes in the solution appears to lower the concentration of individual drug where drug-rich phase forms. Figure 5 shows the effect of increasing concentration of ritonavir on the phase behavior of saquinavir. Saquinavir concentration was maintained at the amorphous solubility by adding the excess amount, while increasing concentrations of ritonavir were spiked into the solution. At the low concentration of ritonavir, there is no significant decrease in the bulk drug concentration of saquinavir. However, the amount of ritonavir recovered in the supernatant was significantly less than that added. This may be due to preferential

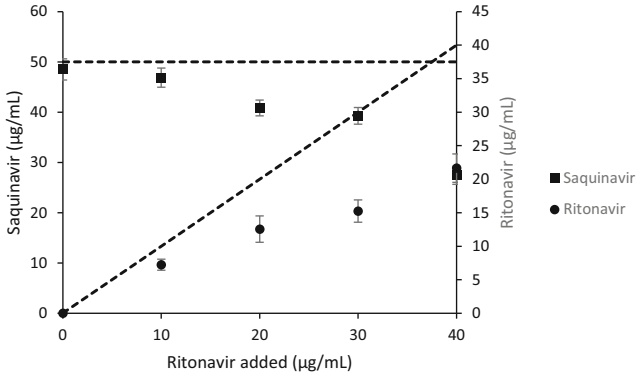

Fig. 5 Concentration of saquinavir in the supernatant when ritonavir was added in the increasing concentration to the solution. The horizontal dotted line represents the amorphous solubility of saquinavir, and the slanted dotted line represents the expected ritonavir concentration in the solution in absence of drug-rich phase

mixing of ritonavir in already formed drug-rich phase of saquinavir. Further, the bulk concentration of saquinavir begins to fall sharply after the spiked ritonavir amount exceeds 20 µg/mL. This may happen due to buildup of sizable amount of collective drug-rich phase of both the solutes in the solution. The other set of experiment where ritonavir concentration was held at its amorphous solubility and saquinavir was spiked in increasing concentration also showed the similar trend (Fig. 6).

Flux/Diffusion Studies

Permeability is a pre-requisite for efficacious bioavailability. The diffusion/flux experiments were conducted by employing Franz diffusion cell. Figures 7 and 8 shows the flux/diffusion of saquinavir and ritonavir respectively, as a function of time (min). The individual drug(s) in their amorphous forms exhibited the maximum flux/diffusion, while the flux/diffusion of

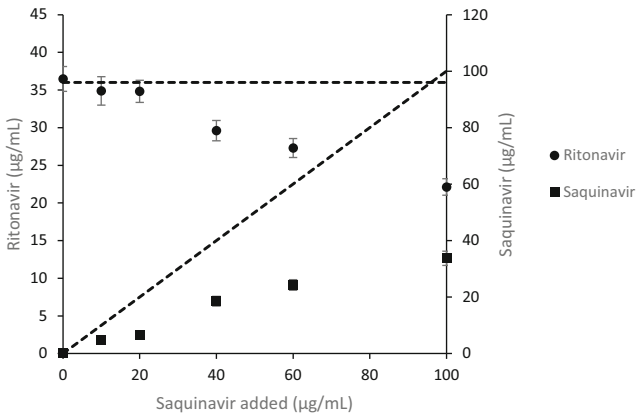

Fig. 6 Concentration of ritonavir in the supernatant when saquinavir was added in the increasing concentration to the solution. The horizontal dotted line represents the amorphous solubility of ritonavir, the slanted dotted line represents the expected saquinavir concentration in the solution in absence of drug-rich phase

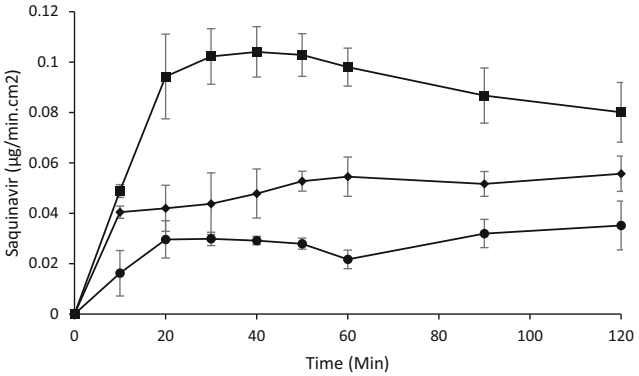

Fig. 7 Flux/diffusion of saquinavir (black square), saquinavir in the presence of 70 µg/mL (black diamond), and 100 µg/mL (black circle) ritonavir as a function of time

both the drugs was lowered in the presence of each other in the same solution. There is an evident influence of amount of drug-rich phase on the flux/diffusion. The flux/diffusion of the drug(s) was lowered by the presence of an increasing amount of other solutes present in the solution. The flux of both the drugs decreases as a function of increase in the amount of cumulative drug-rich phase in the solution.

Discussion

It is imperative to investigate the solution phase behavior of weakly basic compounds, as they have a high probability to generate supersaturated phase after transitioning into the alkaline intestinal milieu. If the compound is slow crystallizer exhibiting higher solubility in the acidic environment, then such a compound may form a drug-rich phase with subsequent GLPS/LLPS. These compounds may generate in vivo supersaturation in the intestine without employing any polymer or crystallization inhibitors. Such in vivo supersaturation is considered advantageous as it may increase the exposure of the drug. However, the presence of any additional drug in such in vivo supersaturated system due to fixed-dose formulation

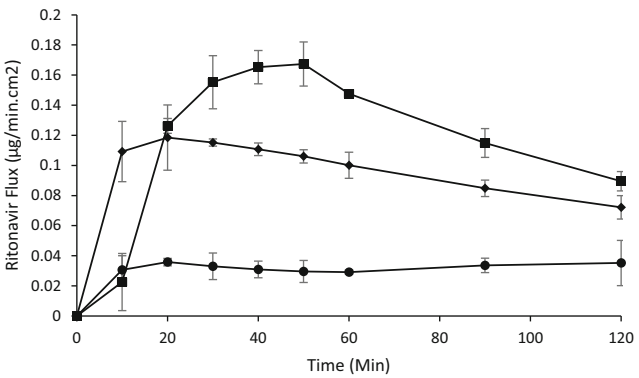

Fig. 8 Flux/diffusion of ritonavir (black square), ritonavir in the presence of 80 µg/mL (black diamond), and 100 µg/mL (black circle) saquinavir as a function of time

and concomitant administration cannot be inconsequential, especially if the other drug is weakly basic with slow crystallization behavior or formulated into ASD. The recent studies have shown that the free drug concentration equivalent to amorphous solubility and its chemical potential is reduced by the presence of a second additional solute in the same solution [10].

Depending upon the ability of a compound to retain supersaturation after the shift in the pH towards higher side, it can be classified into two types, i.e., type-I and type-II. Type-I compounds are ones which at the time of precipitation exhibit supersaturation with respect to its crystalline solubility. These compounds are also called as chasers as they generate metastable supersaturated state and chase the equilibrium solubility after the shift in the pH, while type-II compounds sustain the supersaturation and the solubility of their unionized form does not drop to the level of equilibrium solubility after pH shift. These compounds instantaneously respond to the change in the pH and called as non-chasers [26]. In this context, type-II compounds have high potential to generate in vivo supersaturation. From the pH shift supersaturation assay, it is evident that saquinavir and ritonavir showed type-II and type-I behavior respectively. In particular, type-II compounds like saquinavir can be misleading in the studies which are attempting to improve the solubility using various techniques. These compounds may show supersaturation advantage in both acidic and alkaline pH after formulating into efficacious form. However, once the pH shift experiments are conducted, the supersaturation advantage may endure regardless of any attempted solubility improvement technique. This behavior of type-II compounds has to be taken into consideration while attempting to improve their solubility and their real solubility advantage can only be claimed after carefully devising pH shift supersaturating assays.

Saquinavir is an ionizable compound formulated into the salt form. Salt forms are amenable to disproportionation across the pH gradient. It is imperative to determine pHmax for salt forms to fully understand their precipitation behavior. Saquinavir is a weak base and has pKa 7.1. Owing to its ionizable nature, saquinavir is expected to convert from salt to free base form after an increase in the pH. The pH where equilibrium is formed between ionized and unionized compounds with respect to both the crystalline salt and the crystalline base is called as pHmax [27]. It is important to estimate the pHmax to find out the so called disproportionation of weakly basic salts, i.e., conversion from salt to free base form. For a weak base, below pHmax, salt form predominates exhibiting high solubility, and above pHmax, the free base predominates [28]. The solubility of the free base depends upon in which form it precipitates, i.e., if the precipitated free base is crystalline, then the solubility of the compound may drop sharply; however, amorphous free base may exhibit higher solubility generating supersaturation. Based on the solid-state characterization of the saquinavir precipitate, we had demonstrated that it precipitated in the amorphous form. Nevertheless, we thought to corroborate the findings by estimating the pHmax of saquinavir to find out that which form predominated after shifting the pH to 6.8. The pHmax was estimated by the following formula:

$$\mathrm{pHmax} = \mathrm{pKa} + \log \frac{[B]s}{\sqrt{\mathrm{Ksp}}} \tag{1}$$

where Ksp is the solubility product of salt and $[B]s$ is the solubility of the crystalline free base. The pHmax of saquinavir was found to be 5.89 with respect to its pKa 7.1. This means that after the pH shift to the higher side, i.e., 6.8, the free base form of saquinavir predominated. These findings along with the solid-state characterization of saquinavir precipitate (refer to "Precipitation Behavior of the Individual Drug by pH Shift Method") prove that the supersaturation achieved by saquinavir after pH shift is indeed due to its precipitation in the amorphous form (see Supplementary material for diffractogram and thermogram of dried precipitate of saquinavir).

Trasi and Taylor (2015) reported that the presence of a second compound in the highly supersaturated system does not only decrease the chemical potential, but it also results in a decrease in solubility of each compound [10]. As both saquinavir and quench cool ritonavir retain in the amorphous form after a shift in the pH generating a highly supersaturated solution with respect to their respective crystalline solubility, it is expected that both these drugs may mix ideally and lower their free drug concentrations. The decrease in the solubility of saquinavir and ritonavir in the presence of each other can be explained by the following equation:

$$Sa\,(x1) = S_a^0(x1) \tag{2}$$

where S_a^0 is the solubility of the pure amorphous form of component 1 in the mixture and $x1$ is the mole fraction of the component 1 present in the binary mixture. $Sa\,(x1)$ is the decrease in the solubility of component 1 in the binary mixture. Equation 2 was used to predict the anticipated decrease in the solubility as a function of mole fraction of an individual drug present in the drug-rich phase. Figure 9 shows the free drug concentration in the solution as a function of drug-rich phase composition. The mole fraction of saquinavir was derived by collecting the drug-rich phase after ultracentrifugation. The collected drug-rich phase was re-suspended in the diluent and analyzed by HPLC method to establish the concentration of ritonavir and saquinavir; further, the relative concentration of two drugs was represented as a mole fraction of saquinavir. The free drug concentration of each drug was estimated by analyzing the supernatant of the ultracentrifuged solution using HPLC. The predicted and

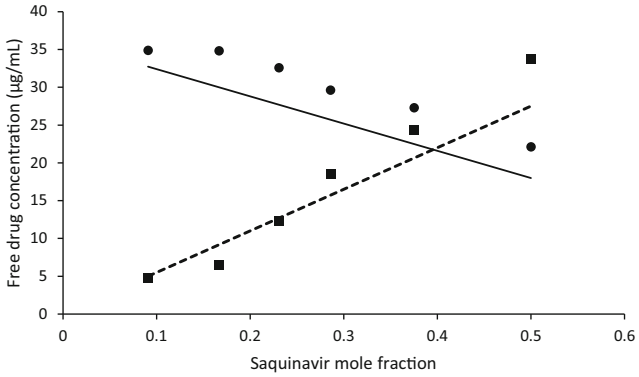

Fig. 9 Free drug concentration of saquinavir (black square) and ritonavir (black circle) as a function of drug-rich phase composition

experimental values are in good agreement indicating the ideal mixing of saquinavir and ritonavir. Thus, the decrease in the solution concentration of each drug is commensurate with the amount of that drug in the drug-rich phase. Perhaps, this explains the sharp drop of amorphous ritonavir concentration in the presence of saquinavir after pH shift. Owing to its highly ionizable nature, saquinavir attains relatively high solution concentration (almost ten times, Fig. 2) in the acidic medium (pH 2.0) before pH shift as compared to ritonavir solution concentration. After the pH shift, both the drugs remain in the amorphous phase and mix ideally with each other forming a drug-rich phase, which has a higher mole fraction of saquinavir and minimal mole fraction of ritonavir. As the system establishes the equilibrium between drug-rich phase and solution phase, the low mole fraction of ritonavir relative to saquinavir reflects in the significant lowering of free drug concentration of the former. Similarly, this also explains the marginal drop in the free drug concentration of saquinavir in the presence of amorphous ritonavir. However, it is not unreasonable to anticipate a significant drop in the saquinavir free drug concentration, if ritonavir was replaced by any other compound which has the potential to form large amount of drug-rich phase showing type-II behavior.

The rate of passive diffusion/flux of a solute is directly proportional to its chemical potential. As per the following equation, the chemical potential of an amorphous state is more than its crystalline counterpart.

$$\frac{\mu^0 - \mu_c^0}{RT} = In\,\frac{S_a}{S_c} \tag{3}$$

where the chemical potential of pure amorphous (μ^0) and crystalline phase $\left(\mu_c^0\right)$ is correlated with the solubility ratio of pure amorphous (Sa) and crystalline form (Sc). From Table 1, it is evident that Sa is greater than Sc, which demonstrates the higher chemical potential of the amorphous phase. However, for the compounds which mix ideally (in this case saquinavir and ritonavir), the chemical potential of a drug is lowered by the presence of a second solute in the amorphous mixture by a value proportional to its mole fraction [10, 12]. This can be explained by the simple thermodynamic equations.

$$\mu_1 = \mu_1^0 + RT.\mathrm{In}x_1 \tag{4}$$

where μ_1 is the chemical potential of the component 1 in the binary amorphous mixture and μ_1^0 is the chemical potential of the reference state (in this case the glassy phase). $x1$ is the mole fraction of the component 1 in the binary mixture.

The similar equation to represent the chemical potential of component 2 (μ_2) can be written as follows:

$$\mu_2 = \mu_2^0 + RT.\mathrm{In}(1{-}x_1) \tag{5}$$

Above equations demonstrate that the chemical potential of a given component is lowered by an amount which is directly related to the mole fraction of that component that is present in the binary amorphous mixture. As we found that the flux of both the drugs decreases as a function of the increase in the amount of cumulative drug-rich phase in the solution, the decrease in the flux/diffusion can be attributed to the overall decrease in the chemical potential of saquinavir and ritonavir in the binary mixture thereof.

The findings of this study are very important for weak bases, which have the potential to generate in vivo supersaturation due to their type-II precipitation behavior. Though in vivo supersaturation is advantageous to increase the exposure of a drug, such drugs would be expected to lower the in vivo supersaturation advantage in the presence of another solute. Such scenarios can be encountered when these drugs are co-formulated with other compounds or the concomitant administration of other drugs (like ritonavir to boost the performance of saquinavir). Flavonoids like naringin which is the part of the normal diet and has been shown to remain in the amorphous form may alter the exposure of type-II weak bases in vivo [29]. Further, the reduction in the free drug concentration may lead to decrease in the chemical potential of a drug lowering its permeability. This is the very alarming situation for the anti-infectives like saquinavir, because suboptimal exposure to the drug may mutate the surviving viruses leading to the drug resistance. Furthermore, we would want to point out that the in vitro pH shift supersaturation assay should be used as a tool in salt screening to assess the supersaturation advantage for compounds like saquinavir, as the equilibrium solubility studies may give false positives. In particular, the solubility advantage of a salt, polymorph, co-crystal, co-amorphous form may disappear after transitioning the pH towards the alkaline side, as the solubility may be higher due to precipitation of disordered phase regardless of the developed form.

Conclusion

In this study, the solution phase behavior of saquinavir was studied across the pH gradient and in the presence of ritonavir. Saquinavir is found to precipitate in the amorphous form in response to change in the pH, thus yielding supersaturated state with respect to its crystalline solubility. Further, the supersaturation advantage of saquinavir is marginally lowered in the presence of amorphous ritonavir. However, the solution concentration of amorphous ritonavir is found to decrease significantly in the presence of saquinavir after the shift in pH towards higher side. Both the drugs demonstrated the ability to lower the solution concentration and chemical potential of each other in the highly supersaturated system. The decrease in the free drug concentration and chemical potential is found dependent on the mole fraction of the solute present in the binary supersaturated solution. The decrease in the chemical potential showed to manifest in the lowering of flux/diffusion of both the drugs in the binary solutions. These findings may have implications for fixed-dose combination and concomitantly administered drugs. These implications may extend to supersaturation drug delivery strategies like amorphous solid dispersion and co-amorphous formulations.

References

1. Lipinski CA. Drug-like properties and the causes of poor solubility and poor permeability. J Pharmacol Toxicol Methods Elsevier. 2000;44:235–49.
2. Di L, Fish PV, Mano T. Bridging solubility between drug discovery and development. Drug Discov Today. Elsevier. 2012;17:486–95.
3. Khadka P, Ro J, Kim H, Kim I, Kim JT, Kim H, et al. Pharmaceutical particle technologies: an approach to improve drug solubility, dissolution and bioavailability. Asian J Pharm Sci. Elsevier. 2014;9:304–16.
4. Chaumeil JC. Micronization: a method of improving the bioavailability of poorly soluble drugs. Methods Find Exp Clin Pharmacol. Barcelona: JR Prous. 1998;20:211–6.
5. Loftsson T, Brewster ME. Pharmaceutical applications of cyclodextrins. 1. Drug solubilization and stabilization. J Pharm Sci. Wiley Online Library. 1996;85:1017–25.
6. Kawakami K. Modification of physicochemical characteristics of active pharmaceutical ingredients and application of supersaturatable dosage forms for improving bioavailability of poorly absorbed drugs. Adv Drug Deliv Rev. 2012;64:480–95.
7. Miller JM, Beig A, Carr RA, Spence JK, Dahan A. A win-win solution in oral delivery of lipophilic drugs: supersaturation via amorphous solid dispersions increases apparent solubility without sacrifice of intestinal membrane permeability. Mol Pharm. ACS Publications. 2012;9:2009–16.
8. Raina SA, Zhang GGZ, Alonzo DE, Wu J, Zhu D, Catron ND, et al. Enhancements and limits in drug membrane transport using supersaturated solutions of poorly water soluble drugs. J Pharm Sci. Elsevier. 2014;103:2736–48.
9. Indulkar AS, Gao Y, Raina SA, Zhang GGZ, Taylor LS. Exploiting the phenomenon of liquid-liquid phase separation for enhanced and sustained membrane transport of a poorly water-soluble drug. Mol Pharm ACS Publications. 2016;13:2059–69.
10. Trasi NS, Taylor LS. Thermodynamics of highly supersaturated aqueous solutions of poorly water-soluble drugs-impact of a second drug on the solution phase behavior and implications for combination products. J Pharm Sci Elsevier. 2015;104:2583–93.
11. Ilevbare GA, Taylor LS. Liquid-liquid phase separation in highly supersaturated aqueous solutions of poorly water-soluble drugs: implications for solubility enhancing formulations. Cryst Growth Des ACS Publications. 2013;13:1497–509.
12. Taylor LS, Zhang GGZ. Physical chemistry of supersaturated solutions and implications for oral absorption. Adv Drug Deliv Rev. 2016;101:122–42.
13. Sugihara H, Taylor LS. Evaluation of pazopanib phase behavior following pH-induced supersaturation. Mol Pharm ACS Publications. 2018;15(4):1690–9.
14. Mosquera-Giraldo LI, Taylor LS. Glass-liquid phase separation in highly supersaturated aqueous solutions of telaprevir. Mol Pharm. ACS Publications. 2015;12:496–503.
15. Sugawara M, Kadomura S, He X, Takekuma Y, Kohri N, Miyazaki K. The use of an in vitro dissolution and absorption system to evaluate oral absorption of two weak bases in pH-independent controlled-release formulations. Eur J Pharm Sci Elsevier. 2005;26:1–8.
16. Kostewicz ES, Brauns U, Becker R, Dressman JB. Forecasting the oral absorption behavior of poorly soluble weak bases using solubility and dissolution studies in biorelevant media. Pharm Res Springer. 2002;19:345–9.
17. Vertzoni M, Dressman J, Butler J, Hempenstall J, Reppas C. Simulation of fasting gastric conditions and its importance for the in vivo dissolution of lipophilic compounds. Eur J Pharm Biopharm Elsevier. 2005;60:413–7.
18. Pathak SM, Musmade P, Dengle S, Karthik A, Bhat K, Udupa N. Enhanced oral absorption of saquinavir with methyl-beta-cyclodextrin-preparation and in vitro and in vivo evaluation. Eur J Pharm Sci. 2010;41:440–51.
19. Noble S, Faulds D. Saquinavir. Drugs Springer. 1996;52:93–112.
20. Buss N, Snell P, Bock J, Hsu A, Jorga K. Saquinavir and ritonavir pharmacokinetics following combined ritonavir and saquinavir (soft gelatin capsules) administration. Br J Clin Pharmacol. Wiley Online Library. 2001;52:255–64.
21. Hill A, van der Lugt J, Sawyer W, Boffito M. How much ritonavir is needed to boost protease inhibitors? Systematic review of 17 dose-ranging pharmacokinetic trials. Aids LWW. 2009;23:2237–45.
22. Law D, Schmitt EA, Marsh KC, Everitt EA, Wang W, Fort JJ, et al. Ritonavir-PEG 8000 amorphous solid dispersions: in vitro and in vivo evaluations. J Pharm Sci Wiley Online Library. 2004;93:563–70.
23. Chemburkar SR, Bauer J, Deming K, Spiwek H, Patel K, Morris J, et al. Dealing with the impact of ritonavir polymorphs on the late stages of bulk drug process development. Org Process Res Dev. ACS Publications. 2000;4:413–7.
24. Bauer J, Spanton S, Henry R, Quick J, Dziki W, Porter W, et al. Ritonavir: an extraordinary example of conformational polymorphism. Pharm Res . Springer. 2001;18:859–66.
25. Dengale SJ, Ranjan OP, Hussen SS, Krishna BSM, Musmade PB, Gautham Shenoy G, et al. Preparation and characterization of co-amorphous ritonavir-indomethacin systems by solvent evaporation technique: improved dissolution behavior and physical stability without evidence of intermolecular interactions. Eur J Pharm Sci. 2014;62:57–64.
26. Hsieh Y-L, Ilevbare GA, Van Eerdenbrugh B, Box KJ, Sanchez-Felix MV, Taylor LS. pH-induced precipitation behavior of weakly basic compounds: determination of extent and duration of

supersaturation using potentiometric titration and correlation to solid state properties. Pharm Res . Springer. 2012;29:2738–53.
27. Bogardus JB, Blackwood RK. Solubility of doxycycline in aqueous solution. J Pharm Sci . Wiley Online Library. 1979;68:188–94.
28. Hsieh Y-L, Merritt JM, Yu W, Taylor LS. Salt stability-the effect of pHmax on salt to free base conversion. Pharm Res . Springer. 2015;32:3110–8.
29. Teja A, Musmade PB, Khade AB, Dengale SJ. Simultaneous improvement of solubility and permeability by fabricating binary glassy materials of talinolol with naringin: solid state characterization, in-vivo in-situ evaluation. Eur J Pharm Sci. 2015;78:234–44.