Pharm Res (2018) 35: 158
https://doi.org/10.1007/s11095-018-2441-2

RESEARCH PAPER

Crystallization from Supersaturated Solutions: Role of Lecithin and Composite Simulated Intestinal Fluid

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Received: 18 April 2018 / Accepted: 5 June 2018 / Published online: 18 June 2018

ABSTRACT

Purpose The overall purpose of this study was to understand the impact of different biorelevant media types on solubility and crystallization from supersaturated solutions of model compounds (atazanavir, ritonavir, tacrolimus and cilnidipine). The first aim was to understand the influence of the lecithin content in FaSSIF. As the human intestinal fluids (HIFs) contain a variety of bile salts in addition to sodium taurocholate (STC), the second aim was to understand the role of these bile salts (in the presence of lecithin) on solubility and crystallization from supersaturated solutions,

Methods To study the impact of lecithin, media with 3 mM STC concentration but varying lecithin concentration were prepared. To test the impact of different bile salts, a new biorelevant medium (Composite-SIF) with a composition simulating that found in the fasted HIF was prepared. The crystalline and amorphous solubility was determined in these media. Diffusive flux measurements were performed to determine the true supersaturation ratio at the amorphous solubility of the compounds in various media. Nucleation induction times from supersaturated solutions were measured at an initial concentration equal to the amorphous solubility (equivalent supersaturation) of the compound in the given medium.

Results It was observed that, with an increase in lecithin content at constant STC concentration (3 mM), the amorphous solubility of atazanavir increased and crystallization was accelerated. However, the crystalline solubility remained fairly constant. Solubility values were higher in FaSSIF compared to Composite-SIF. Longer nucleation induction times were observed for atazanavir, ritonavir and tacrolimus in Composite-SIF compared to FaSSIF at equivalent supersaturation ratios.

Conclusions This study shows that variations in the composition of SIF can lead to differences in the solubility and crystallization tendency of drug molecules, both of which are critical when evaluating supersaturating systems.

KEY WORDS biorelevant media · crystallization · nucleation · simulated intestinal fluids · supersaturation

ABBREVIATIONS

FaSSIF	Fasted state simulated intestinal fluid
HIF	Human intestinal fluid
J_{amorph}	Flux at amorphous solubility
NIT	Nucleation induction time
SGC	Sodium glycocholate
SGCDC	Sodium glycochenodeoxycholate
SGDC	Sodium glycodeoxycholate
SGUDC	Sodium glycoursodeoxycholate
SIF	Simulated intestinal fluid
SR	Supersaturation ratio
SR_{amorph}	Supersaturation ratio at amorphous solubility
STC	Sodiun taurocholate
STCDC	Sodium taurochenodeoxycholate
STDC	Sodium taurodeoxycholate
STUDC	Sodium tauroursodeoxycholate

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INTRODUCTION

Supersaturating dosage forms are gaining increasing interest as a strategy to overcome the problem of poor aqueous solubility common to many emerging drugs. Supersaturation can potentially be attained *in vivo* via several pathways such as by employing amorphous solid dispersions (1,2), lipidic/emulsifying formulations (3,4), salts and cocrystals (5), prodrug conversion to an active moiety (6), and also upon gastrointestinal transit for weakly basic compounds (7–9). The success of supersaturating systems as a formulation strategy can be attributed to their ability to increase solution concentration in excess of the crystalline solubility. In contrast to solubilization strategies (such as micellar surfactants, cyclodextrins) which increase the crystalline solubility and decrease the solute thermodynamic activity, a supersaturating system with the same total drug concentration will have higher free drug concentration (10,11). Because the rate of transport across a biological membrane is dictated by activity (i.e. free drug concentration, not the total drug concentration) at the same total drug concentration, supersaturating systems exhibit superior *in vivo* performance in comparison to a solubilizing system (12–14), when crystallization is avoided.

The advantages of a supersaturating formulation can be offset by their inherent metastability. A supersaturated solution has a concentration higher than that produced by dissolving the thermodynamically stable crystalline form of a compound. Thus, in a supersaturated solution a driving force for crystallization exists which can ultimately result in a decrease in solution concentration (15,16). This in turn can negatively impact the bioperformance of a supersaturating formulation. Therefore, it is of utmost importance to understand the crystallization tendency of supersaturated solutions produced from enabling formulations. It is well known that crystallization can be influenced by additives, present either in the formulation or in the media such as polymers or surfactants. Polymers tend to inhibit crystallization (17,18) whereby their efficiency as inhibitors is a function of polymer hydrophobicity and structure. Ilevbare *et al.* showed that an effective polymer had a balance between hydrophilic and hydrophobic moieties and possessed bulky side groups (19). This enables drug-polymer interaction in an aqueous environment thereby disrupting the nucleation process. Commonly employed surfactants, such as sodium dodecyl sulfate and polysorbate 80 have been shown to induce crystallization (20). Surfactants can induce crystallization via heterogeneous nucleation or by decreasing the interfacial energy between the solute in the solution and the emerging crystal (21). In addition to formulation components, the medium composition can also impact the crystallization process and outcome (22–24).

As maintaining the maximum supersaturation for a time duration corresponding to the absorptive timeframe in the intestinal tract is critical to maximize *in vivo* performance, *a priori* knowledge of the physical stability of supersaturating solutions *in vivo* can be advantageous for formulation design and *in silico* oral absorption modelling. It is well known that the pH of human gastro-intestinal fluids increases from the stomach to the intestine (25). Additionally, the intestinal fluids contain bile salts and phospholipids (such as lecithin) which can form micelles, mixed micelles and vesicles which can then impact formulation performance (26–28). Thus, in order to closely mimic the *in vivo* performance, biorelevant media, such as simulated human fluids containing bile salts and phospholipids, are commonly employed to evaluate formulations. Simulated intestinal fluids such as the fasted/fed state simulated intestinal fluid (Fa/FeSSIF) are commercially available as convenient, ready-to-use powders. Two versions of FaSSIF are currently available, FaSSIF-V1 (version 1) and FaSSIF-V2 (version 2). These media have the same bile salt content but differ in the lecithin content (Table I), and also employ different buffers. Sodium taurocholate (STC) is used in Fa/FeSSIF as a representative bile salt because of its low *pKa* of 1.8 (29) which renders it readily soluble at the varying pH conditions of the gastro-intestinal environment, and reduces any propensity to precipitate due to pH change (30).

Riethorst *et al.* carried out a detailed characterization of human intestinal fluids (HIFs) and showed that the HIF, in addition to STC, contains a complex mixture of several different bile salts including sodium glycocholate (SGC), sodium glycodeoxycholate (SGDC), sodium glycochenodeoxycholate (SGCDC), sodium glycoursodeoxycholate (SGUDC), sodium taurodeoxycholate (STDC), sodium taurochenodeoxycholate (STCDC), sodium tauroursodeoxycholate (STUDC) (31). The structures of these bile salts is shown in Fig. 1. These can be broadly classified as glyco-conjugated (glycine as the conjugated amino acid side) or tauro-conjugated (taurine as the conjugated amino acid side). Within each class, the bile salts vary with respect to the presence/absence and location/orientation of hydroxyl groups. Among these eight bile salts, SGUDC and STUDC contribute minimally to the total bile salt composition (1.3 and 0.6% respectively). The median value of the total bile salt concentration in HIF was found to be 3.3 mM which is close to the STC concentration in FaSSIF.

Table I Composition of different biorelevant media employed in this study

	FaSSIF-V1	FaSSIF-V2	Composite-SIF
STC	100%	100%	12%
STCDC	–	–	12%
STDC	–	–	6%
SGC	–	–	28%
SGCGC	–	–	27%
SGDC	–	–	15%
Total bile salt	3 mM	3 mM	3 mM
Lecithin	0.75 mM	0.2 mM	0.75 mM

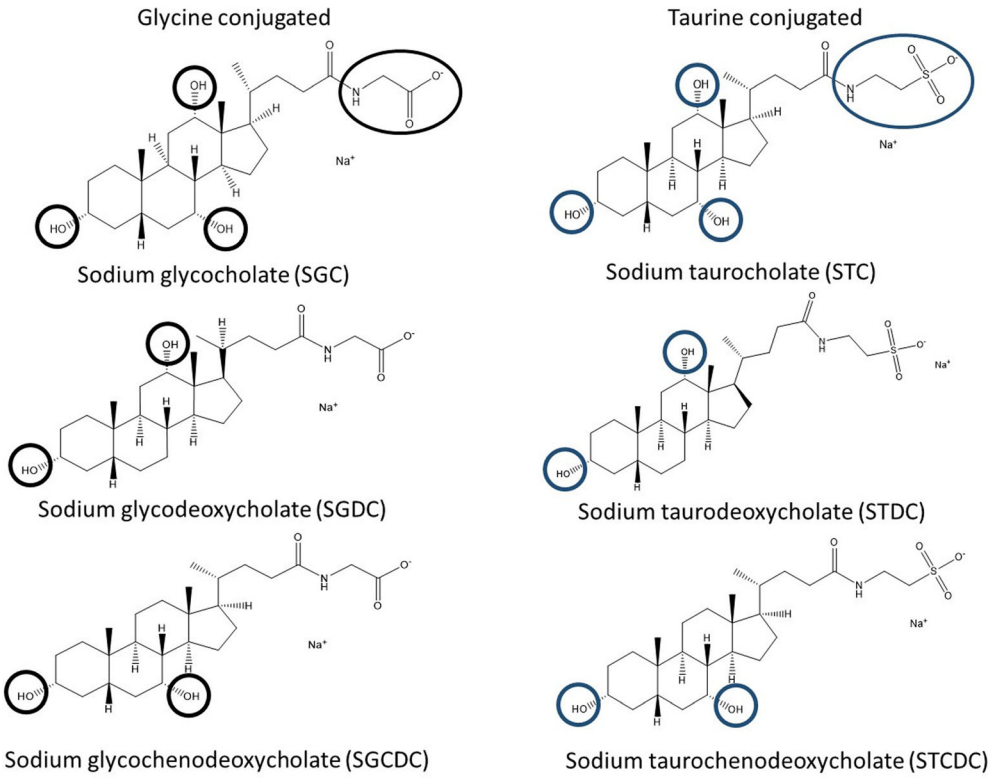

Fig. 1 Molecular structure of bile salts abundant in HIF.

The two major constituents of intestinal fluids, namely lecithin and bile salts are surface active and thus, can potentially impact solubility and crystallization kinetics of compounds. Chen *et al.* noted that STC can delay or inhibit crystallization of several compounds (32). Li *et al.* carried out an exhaustive study using thirteen bile salts and demonstrated that individual bile salts differ in their efficiency as crystallization inhibitors (33). The inhibitory effect was found to be somewhat related to the hydrophobicity of the bile salts. This suggests that a SIF which contains a variety of bile salts may exhibit a different impact on crystallization from commercial SIF. The impact of lecithin as a component of SIF on drug crystallization kinetics is still an unknown. Therefore, the goals of this study were twofold: to understand the impact of 1) lecithin content and 2) bile salt composition on solubility and solution crystallization kinetics.

In an elaborate review on solubility of drug compounds in SIF and HIF, Augustijns *et al.* found a strong correlation (R^2 = 0.85) between the crystalline solubility determined in SIF and that determined in HIF (34). This suggests that different bile salts present in a medium in conjunction with lecithin may solubilize the crystalline form of the drug to the same extent. In other words, a minimal change in crystalline solubility can be expected due to a change in biorelevant media composition. This can in turn result in similar dissolution profiles when carrying out dissolution studies of non-supersaturating formulations in biorelevant media with different bile salt compositions. However, another important physicochemical parameter for supersaturating drug compounds is the amorphous solubility. Amorphous solubility is the solution concentration at which the solute in solution is in a metastable equilibrium with the amorphous drug. Currently, there is a lack of understanding as to how the amorphous solubility is impacted by the bile salt composition in SIF. Hence, an additional aim of the study was to determine the amorphous solubility in Composite-SIF and compare this value to that achieved in FaSSIF.

To address the aims outlined above, the impact of Composite-SIF, and FaSSIF containing different amounts of lecithin, on the solubility and crystallization of four structurally different, poorly water soluble model compounds was assessed. Composite-SIF is a new SIF that we have developed, composed of the six most prevalent bile salts (Table I) present in HIF, using the mean values determined by Riethorst *et al.* (Table I). It contains the same lecithin amount used in FaSSIF version 1 (V1). Both crystalline and amorphous solubility values were determined, and nucleation induction time measurements were performed at the same supersaturation ratio in the various media.

Materials

Atazanavir, ritonavir, and tacrolimus were purchased from Chemshuttle, Inc. (Hayward, CA). Cilnidipine was obtained from Euroasia Chemicals Pvt. Ltd. (Mumbai, India). The molecular structures of these compounds are shown in Fig. 2.

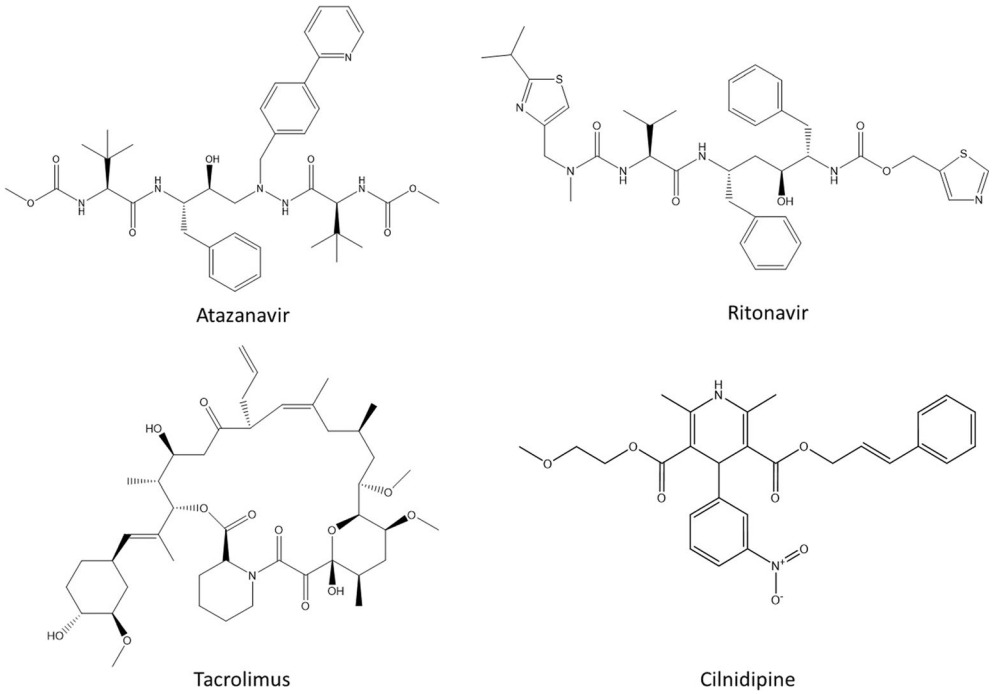

Fig. 2 Molecular structures of the drugs used in this study.

FaSSIF/FeSSIF/FaSSGF powder was procured from Biorelevant (London, UK). STC was purchased from Biosynth International, Inc. (Itasca, IL). STDC was procured from Ark Pharm, Inc. (Libertyville, IL). STCDC and SGCDC were obtained from Matrix Scientific (Columbia, SC). SGC was purchased from Chem-Impex International, Inc. (Wood Dale, IL) and SGDC was obtained from Calbiochem (San Diego, CA). Lecithin was acquired from Crescent Chemical Co. (Islandia, NY). The structures of the bile salts are given in Fig. 1. Methanol and dichloromethane were purchased from Sigma-Aldrich (St. Louis, MO). Aqueous buffer (pH 6.5, ~270 mOsmol) was prepared using sodium hydroxide, sodium chloride and monobasic sodium phosphate monohydrate, obtained from Fisher Chemical-Fisher Scientific (Hampton, NH).

METHODS

Preparation of Biorelevant Media

FaSSIF-V1 was prepared directly by dissolving the FaSSIF/FeSSIF/FaSSGF powder into pH 6.5 buffer according to the manufacturer directions. The composition of FaSSIF-V1 is given in Table I. To investigate the impact of lecithin on nucleation induction time, media with varying lecithin concentrations (0.01 to 0.75 mM), but the same STC concentration (3 mM), were prepared by diluting the FaSSIF-V1 with 3 mM STC buffered solution. The medium with 3 mM STC and 0.2 mM lecithin has the same amount of these components as the commercially available version 2 of FaSSIF (FaSSIF-V2). In the commercially available FaSSIF-V2, the buffer system and ionic strength are also different from that used for FaSSIF-V1; maleate instead of phosphate. Herein, the same phosphate buffer system as described above was used for all systems in order to systematically study the impact of lecithin concentration and/or bile salt composition on induction times. Composite-SIF with a composition as given in Table I was prepared by dissolving the bile salts in buffer such that the total bile salt concentration was 3 mM (similar to commercial FaSSIF). Lecithin, at a concentration of 0.75 mM (similar to FaSSIF-V1), was introduced into the Composite-SIF by dissolving it in dichloromethane and adding this organic solution to the aqueous bile salt mixture. This resulted in a turbid emulsion which was stirred constantly at 500 rpm at 50°C for 30 min to evaporate dichloromethane from the aqueous solution. This procedure produced a clear micellar solution with no perceptible odor of dichloromethane. Composite-SIF containing a lower lecithin content (0.2 mM) was also prepared by diluting the Composite-SIF with a 3 mM solution of bile salt mixture in order to compare the impact of FaSSIF-V2 and the new SIF on nucleation induction time from supersaturated systems. To deconvolute the impact of the different bile salts present in Composite-SIF, solutions of individual bile salts at a concentration of 3 mM and containing 0.75 mM lecithin were prepared. Lecithin was introduced in a similar manner as described above.

Micelle Size Determination of FaSSIF-V1 and Composite-SIF

The size of micelles formed in FaSSIF-V1 and Composite-SIF was determined by dynamic light scattering (DLS) using a

Malvern Zetasizer Nano ZS system (Malvern Instruments Inc., Westborough, MA) equipped with a backscatter detector. The scattering from the particles was collected at 173° angle.

Solubility Studies

Crystalline solubility of atazanavir, ritonavir, tacrolimus and cilnidipine was determined in pH 6.5 buffer, FaSSIF-V1 and Composite-SIF. To determine the crystalline solubility, excess crystalline drug was added to the desired medium and equilibrated at 37°C for 24 h. The undissolved crystalline drug was then separated by filtration using 1 μm syringe filters. Glass fiber filters were used for atazanavir, whereas PTFE filters were used for ritonavir, tacrolimus and cilnidipine. The concentration of the drug in the filtrate was determined by high performance liquid chromatography (HPLC) with an Agilent 1260 Infinity system (Agilent Technologies, Santa Clara, CA). A 15 cm × 4.6 mm Ascentis® C18 HPLC column (Sigma-Aldrich St. Louis, MO) with 5 μm particle size was used for atazanavir, ritonavir and cilnidipine. The mobile phase consisting of 0.1% trifluoroacetic acid in water (aqueous phase) and acetonitrile (organic phase) was pumped at a flow rate of 1 mL/min. An aqueous:organic phase ratio of 60:40, 55:45 and 50:50 was used for atazanavir, ritonavir and cilnidipine respectively. 80 μL was used as the injection volume. A retention time of less than 5 min was obtained for atazanavir and ritonavir whereas, cilnidipine was eluted in 15 min. An ultraviolet (UV) detector was used to detect atazanavir and ritonavir at a wavelength of 210 nm while 240 nm was used for cilnidipine. For tacrolimus, a 15 cm × 4.6 mm Hypersil GOLD C8 HPLC column with 3 μm particle size (Thermo Fisher Scientific, Waltham, MA) was used. The mobile phase was composed of acetonitrile, methanol, water, and 0.6% phosphoric acid (46:18:36:0.1). The column was maintained at 50°C. A flow rate of 1 mL/min and injection volume of 80 μL was used. The retention time was 10 min. Detection was carried out by a UV detector at 210 nm. For all the compounds, a calibration plot with $R^2 = 0.999$ was constructed over the range 0.05 to 10 μg/mL which was then used for determining drug concentrations. If required, the supernatant solutions were diluted with the mobile phase to obtain concentrations within the limits of the calibration plot.

The amorphous solubility of the four compounds was determined at 37°C in pH 6.5 buffer, FaSSIF-V1 and Composite-SIF using the solvent-shift method (35). A concentrated stock solution (~25 mg/mL) of the drug was prepared in organic solvent. This solution was then introduced into the desired aqueous medium using a Harvard PHD 22/2000 syringe pump (Harvard Apparatus, Holliston, MA) at a particular flow rate. The drug solutions thus obtained were constantly stirred at 300 rpm and monitored for a change in scattering with drug concentration by measuring extinction at a non-absorbing wavelength using a UV/vis spectrophotometer (SI Photonics, Tuscon, Arizona), coupled with a fiber optic dip probe. An interval of 10 s was used between each acquisition. The drug concentration at which an increase in scattering was observed was taken as the amorphous solubility. The desired aqueous medium was blanked for UV absorption before the introduction of drug solution and no interference in scattering was seen due to the medium during data acquisition. The concentration of the stock solution and the flow rate of the syringe pump was chosen such that the experiment time was less than 10 min.

The crystalline and amorphous solubility of atazanavir was also determined in 3 mM solutions of the six individual bile salts both in the absence and presence of 0.75 mM lecithin as well as in 3 mM STC solutions containing varying amounts of lecithin (0.01 to 0.75 mM).

Determination of Supersaturation Ratio (SR)

The supersaturation ratio (*SR*), given by Eq. 1, is defined as the ratio of activity of the solute in the solution (a) to the activity of the solute at a standard state (a^*) (36). The standard state is taken as the crystalline state, and thus a^* is the activity of the solute at the crystalline solubility.

$$SR = \frac{a}{a^*} \tag{1}$$

Diffusive flux (J) across a membrane, assuming sink conditions on the receiver side, also depends directly on the activity of the donor solution and this relationship can be given by Eq. 2 (12).

$$J = \frac{Da}{h\gamma_m} \tag{2}$$

where, D is the diffusion coefficient of the solute, h is the thickness of the membrane and γ_m is the activity coefficient of the solute in the membrane, which are all constants for a particular system and drug. Equations 1 and 2 can be combined to obtain relationships between *SR* and J as given in Eq. 3.

$$SR = \frac{J}{J^*} \tag{3}$$

where, J^* is the flux obtained at the crystalline solubility assuming a crystalline standard state. As the nucleation-induction time experiments were carried out at the amorphous solubility, *SR* at amorphous solubility (SR_{amorph}) was determined. Here, SR_{amorph} is equal to $\frac{J_{amorph}}{J^*}$. SR_{amorph} was determined experimentally by measuring J_{amorph} and J^* using a side-by-side diffusion cell (PermeGear Inc., Hellertown, PA). The donor compartment was separated from the receiver compartment using a Spectra/Por® 1 regenerated cellulose membrane, molecular

weight cut off value of 6–8 kD (Spectrum Laboratories Inc., Rancho Dominguez, CA). 34 mL of the desired aqueous medium, stirred and maintained at 37°C, was added to the donor and receiver chambers. SR_{amorph} for the model compounds was determined in the different media used to evaluate the nucleation induction times. A concentration of drug corresponding to the amorphous or crystalline solubility was added to the donor chamber by aliquoting a concentrated drug solution prepared in methanol to determine J_{amorph} or J^{*} respectively. Crystallization was not observed over the experimental time frame (~40–60 min) when a concentration equal to the amorphous solubility was used. A surface area of 7.07 cm^2 was available for mass transport across the membrane. A 200 µL aliquot was withdrawn from the receiver chamber at the desired time points and the concentration was determined by the HPLC method described in the previous section. J can also be defined by Eq. 4.

$$\mathcal{J} = \frac{dm}{Adt} \tag{4}$$

where, $\frac{dm}{dt}$ is the rate of mass transfer of the solute across a membrane with a cross sectional area, A. J was determined by making plots of concentration achieved in the receiver compartment as a function of time. The slope of such plots gave the value of J by factoring in the volume of the receiver medium and A.

Determination of Nucleation-Induction Time (NIT)

Nucleation-induction time (NIT) or the time required for detectable nuclei to form from a supersaturated system was determined in several media. A concentrated drug solution was prepared by dissolving the drug in methanol. An aliquot of this solution was introduced into 20 mL aqueous medium such that the drug concentration was equal to the amorphous solubility. Using this approach, an equivalent SR, SR_{amorph} was maintained across the different NIT experiments. The single phase supersaturated solution thus obtained was constantly stirred at 300 rpm and maintained at 37°C. The solution was then monitored with time using a UV/vis spectrophotometer (SI Photonics, Tuscon, Arizona), coupled with a fiber optic dip probe to measure changes in solution light scattering by measuring the extinction at a non-absorbing wavelength. A 1 min time interval was used between each acquisition. A scattering event in this experiment was attributed to crystallization or formation of detectable nuclei from the supersaturated solution. The time point at which an increase in scattering above the noise level was observed was taken as the NIT. The NIT ($t_{induction}$) determined in this work can be given by Eq. 5.

$$t_{induction} = t_{nucleation} + t_{growth} \tag{5}$$

Here, $t_{nucleation}$ is the true nucleation time or time required for the first nuclei clusters to form and t_{growth} is the time required for the clusters to grow to a size detectable by the UV/vis spectrophotometer. Similar to amorphous solubility measurements, the desired aqueous medium was blanked for UV absorption prior to introduction of drug solution and no interference in scattering was seen due to the medium during data acquisition. The impact of Composite-SIF and FaSSIF-V1 on NIT was studied for all four model compounds. Atazanavir alone was used to study the impact of lecithin amount in FaSSIF, individual bile salts in the presence of lecithin and to compare the impact of lower lecithin containing FaSSIF (FaSSIF-V2) and Composite-SIF on NIT.

RESULTS

Micelle Size Determination in FaSSIF-V1 and Composite-SIF

A clear solution was obtained for Composite-SIF while FaSSIF-V1 was slightly translucent. A unimodal size distribution of micelles with a z-average of 49 ± 4 nm was obtained for FaSSIF-V1 which is consistent with literature reports (37,38). Composite-SIF showed a bimodal size distribution. 56% of the micelles had a mean size of 63 ± 4 nm while 44% of the micelles were 3.9 ± 0.4 nm in size. Due to the complexity of the composition of Composite-SIF, it can be expected that structures of varying sizes can form resulting in a bimodal size distribution.

Solubility of Crystalline and Amorphous Forms

Figure 3 shows the crystalline and amorphous solubility values of atazanavir in pH 6.5 buffer, 3 mM STC solution and different media prepared with 3 mM STC and varying lecithin concentrations (0.01 to 0.75 mM). The crystalline solubility of atazanavir in different media is ~ 1 µg/mL. Thus, STC both in presence/absence of lecithin does not seem to solubilize crystalline atazanavir. The amorphous solubility in pH 6.5 buffer is 65 µg/mL. In 3 mM STC solution, the amorphous solubility decreases to 38 µg/mL. Upon addition of lecithin to 3 mM STC solution, the amorphous solubility increases with an increase in lecithin concentration. It should be noted that the amorphous solubility in the highest concentration lecithin-containing solution (FaSSIF-V1) increases only by a factor of 1.3 in comparison with the solubility value in neat pH 6.5 buffer.

Figure 4 shows the crystalline and amorphous solubility values of atazanavir in 3 mM individual bile salt solutions with a constant lecithin concentration of 0.75 mM. The crystalline solubility does not change significantly in the presence of bile salts and lecithin. The amorphous solubility for the various

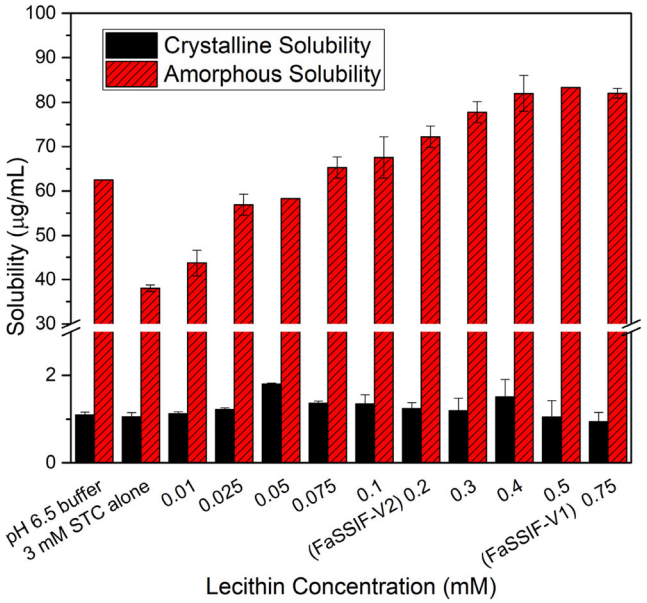

Fig. 3 Impact of lecithin concentration in a 3 mM STC solution on crystalline and amorphous solubility of atazanavir at 37°C. The value obtained in pH 6.5 buffer is given for reference.

systems ranges from 82 to 93 µg/mL, which translates to a solubility enhancement of 1.3 to 1.5 times compared to pH 6.5 buffer in the absence of additives.

Table II gives the crystal and amorphous solubility values of the four model compounds in pH 6.5 buffer, Composite-SIF and FaSSIF-V1. Compared to buffer solubility, about a 3 to 4 fold increase in crystalline and amorphous solubility is observed for tacrolimus. In the case of cilnidipine, a 40 and 80 fold increase in crystalline solubility was seen in Composite-SIF and FaSSIF-V1 respectively, whereas the amorphous solubility increased by 16 to 30 fold. The

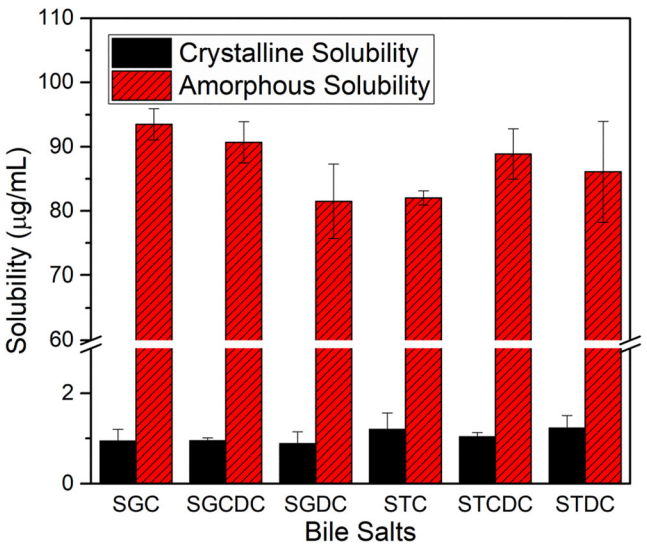

Fig. 4 Crystalline and amorphous solubility of atazanavir in a 3 mM solution of individual bile salts with 0.75 mM lecithin at 37°C.

crystalline solubility of ritonavir increases by a factor of 2 in Composite-SIF and 3 in FaSSIF-V1, whereas the amorphous solubility enhancement is from 1.3 and 1.9 in Composite-SIF and FaSSIF-V1 respectively.

Determination of SR_{amorph} (Supersaturation Ratio at the Amorphous Solubility)

Figure 5 shows SR_{amorph} for atazanavir determined in media containing 3 mM STC with varying lecithin concentrations. SR_{amorph} decreases in the 3 mM STC solution compared to neat buffer. Upon addition of lecithin, SR_{amorph} increases. This increase is seen for lecithin concentrations up to 0.075 mM lecithin. Above this, SR_{amorph} remains fairly constant. Thus, atazanavir solutions containing 3 mM STC and a lecithin content equal to or higher than 0.075 mM have an equivalent SR_{amorph}. SR_{amorph} was also determined for 3 mM solutions of six individual bile salts in presence of 0.75 mM lecithin. For all systems, SR_{amorph} was found to be ~65. Thus, all these solutions have a thermodynamically equivalent *S*.

Table III gives SR_{amorph} values determined for atazanavir, ritonavir and tacrolimus in pH 6.5 buffer, Composite-SIF, and FaSSIF-V1. Due to the limitations of the analytical method used in this study combined with slow diffusion, determination of J^* was not possible for cilnidipine and tacrolimus. The table gives the value of J_{amorph} for these compounds instead of SR_{amorph}. It is apparent that SR_{amorph} values are similar for atazanavir and ritonavir in the various media. As J_{amorph} values for tacrolimus and cilnidipine are similar in the buffer and solubilizing media, it can be supposed that these drugs do not mix with the bile salts and lecithin constituting the Composite-SIF. The enhancement in solubility is thus purely due to entrapment in the micellar structure. Hence, it can be assumed that J^* values for tacrolimus and cilnidipine will be similar in the different media resulting in equivalent values of SR_{amorph}. Similar values of SR_{amorph} for atazanavir to those in FaSSIF-V1 and Composite-SIF were obtained for FaSSIF-V2 and the corresponding Composite-SIF.

Determination of Nucleation-Induction Time (NIT)

Figure 6 shows the impact of lecithin amount on the NIT of atazanavir. In the absence of any additive, atazanavir crystallizes in 170 min at a supersaturation of SR_{amorph}. In the presence of STC alone, the NIT is prolonged whereby crystallization is inhibited for up to 600 min. Upon incorporation of lecithin into the medium, the NIT decreases, i.e. crystallization is induced. The NIT was found to decrease with an increase in lecithin content. Since the SR_{amorph} is not equivalent for the different systems compared here, experiments were also carried out at select lecithin concentrations where an equivalent *SR* was maintained in order to confirm that the observed differences in NIT were not caused by the differences

Table II Crystalline and amorphous solubility values of the model compounds in different media

		Buffer	FaSSIF-V1	Composite-SIF
Atazanavir	Crystal	1.1 (0.2)	1.2 (0.3)	1.3 (0.3)
	Amorphous	65 (2)	82 (1)	82 (2)
	Amorphous/Crystalline solubility ratio	59.1	68.3	63.1
Ritonavir	Crystal	2.5 (0.2)	6.7 (0.2)	4.9 (0.1)
	Amorphous	30 (1)	56 (2)	38 (2)
	Amorphous/Crystalline solubility ratio	12	8.4	7.8
Tacrolimus	Crystal	1.5 (0.2)	6.5 (0.3)	4.8 (0.1)
	Amorphous	47 (2)	210 (4)	160 (3)
	Amorphous/Crystalline solubility ratio	31.3	32.3	33.3
Cilnidipine	Crystal	0.063 (0.0)	5.1 (0.1)	2.7 (0.1)
	Amorphous	2.3 (0.2)	66 (2)	37 (1)
	Amorphous/Crystalline solubility ratio	36.5	12.9	13.7

Values in parentheses give standard deviation (n = 3)

in SR_{amorph}. It was observed that the NIT values did not change with a change in *SR* (data not shown).

Figure 7 shows the impact of individual bile salts (all containing 0.75 mM lecithin) on the NIT of atazanavir. It is evident that the individual bile salts differ in their impact on nucleation. The chenodehydroxy bile salts inhibit crystallization for longer time periods (longer NIT) followed by dehydroxy bile salts, whereas, the trihydroxy bile salts show shorter values of NIT.

Figure 8 shows a comparison of NITs for different drugs in Composite-SIF and FaSSIF-V1. In the absence of bile salts or lecithin, the NIT of ritonavir, cilnidipine and tacrolimus was found be 320 ± 150, 210 ± 90 and 340 ± 100 min respectively. It is readily apparent that the NITs of atazanavir, ritonavir and tacrolimus are longer in composite-SIF than those observed in FaSSIF-V1. In other words, supersaturation is maintained for a longer duration in Composite-SIF than FaSSIF-V1. No significant difference was observed in the case of cilnidipine. This may be due to a similar impact of different bile salts on the crystallization of cilnidipine or in this case, crystallization may be governed completely by lecithin and not by the bile salts. Figure 9 compares the impact of the two different versions of FaSSIF and Composite-SIF on the NIT of atazanavir. For a particular medium type, it can be seen that the lecithin amount can impact crystallization. Between the two groups, it is evident that the NIT is longer in Composite-SIF compared to FaSSIF, consistent with the results observed above.

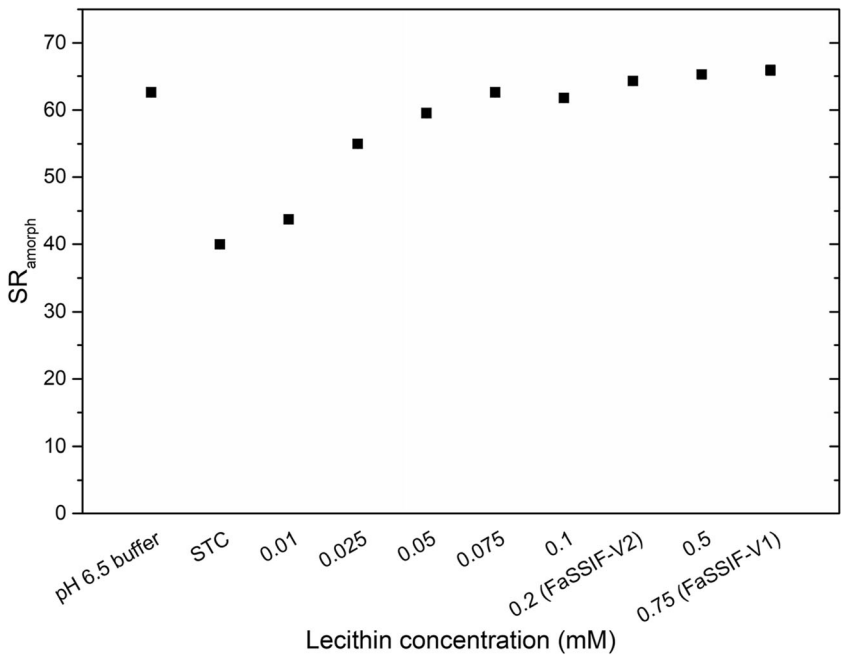

Fig. 5 SR_{amorph} values for atazanavir in different media containing 3 mM STC and varying lecithin content determined at 37°C. Value obtained in pH 6.5 buffer is given for reference. Values were determined from flux measurements.

Table III SR_{amorph} values for the model compounds in different media

	Buffer	FaSSIF-V1	Composite-SIF
Atazanvir	63 (3)	65 (3)	64 (2)
Ritonavir	9.6 (0.5)	9.2 (0.3)	10 (0.4)
Tacrolimus*	0.027 (0.001)	0.028 (0.003)	0.031 (0.002)
Cilnidipine*	0.0014 (0.0001)	0.0018 (0.0001)	0.0017 (0.0002)

*J_{amorph} values in $\mu g/min.cm^2$ are reported instead of SR_{amorph}

Values in parentheses give standard deviation ($n = 3$)

DISCUSSION

Dissolution Media: Evolution and Gaps

Dissolution testing of solid oral dosage forms is routinely carried out to evaluate and compare the performance of different formulations, as well as to predict the *in vivo* exposure and demonstrate bioequivalence or inequivalence between the formulations (39). The dissolution rate depends directly on the solubility of the compound, which in turn is impacted by the type of the medium used (40). Thus, to closely predict the *in vivo* performance of the drug by a dissolution method, it is important that the dissolution medium chosen can simulate the gastro-intestinal (GI) environment. It is known that the human GI environment is complex with variation in pH along the GI tract and the presence of solubilizing bile salts and phospholipids (25–28). The pH and concentration of bile salts and phospholipids is also impacted by food intake. The HIF contains a multitude of bile salts; STC, STDC, STCDC, SGC, SGDC and SGCDC are the most abundant bile salts (31). Thus, to simulate such a highly complex environment it becomes obvious that simple aqueous buffer is not adequate. Hence, in 1998, Dressman *et al.* first proposed biorelevant dissolution media to simulate the fasted and fed states of the gastric and intestinal environments (30). Galia *et al.* demonstrated that, compared to Biopharmaceutics Classification System (BCS) I drugs, the dissolution rate of poorly aqueous soluble BCS II drugs was highly impacted by simulated intestinal fluids (41). The composition proposed by Galia *et al.* was used for commercially available Fa/FeSSIF-V1. STC is used as a representative bile salt in Fa/FeSSIF because of its low *pKa* which results in good solubility at different pH values (30). Since their introduction, biorelevant media have gained popularity in the pharmaceutical community as a surrogate for human fluids. Indeed, better correlations between *in vitro* dissolution data using biorelevant media and *in vivo* plasma profiles have been achieved (42–46). Jantratid *et al.* updated the composition of the media and this has been used to produce

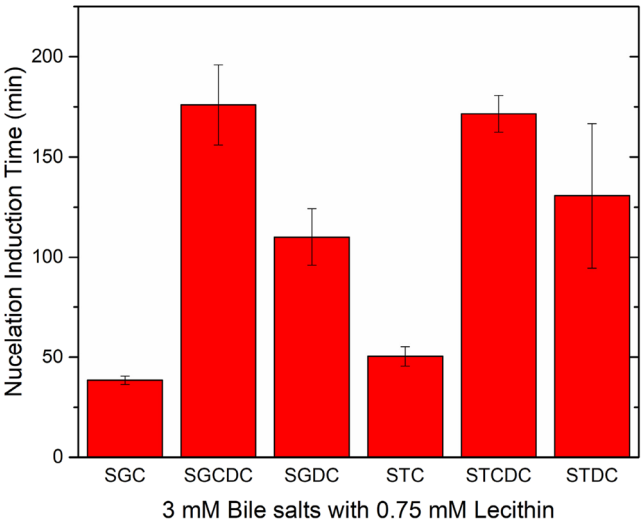

Fig. 7 Impact of individual bile salts in the presence of 0.75 mM lecithin on the nucleation induction time of atazanavir determined at 37°C. In the absence of any additives, the NIT of atazanavir was found to be ~ 170 min.

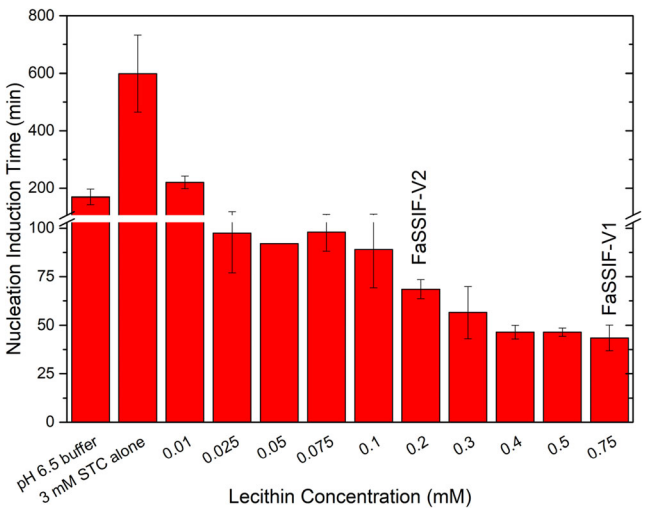

Fig. 6 Nucleation Induction Time (min) of atazanavir in different media containing 3 mM STC and varying lecithin content determined at 37°C. Value obtained in pH 6.5 buffer is given for reference.

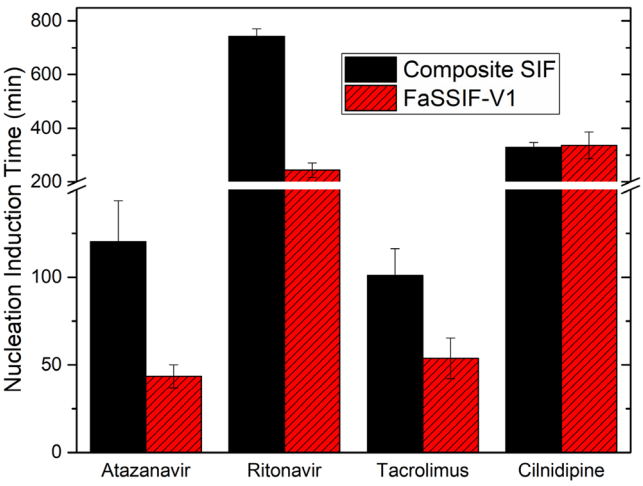

Fig. 8 Comparison of the impact of Composite-SIF and FaSSIF-V1 on the nucleation induction time of different model compounds at 37°C. In the absence of bile salts or lecithin, the NIT of atazanvir, ritonavir, cilnidipine and tacrolimus was found be ~170, 320, 210 and 340 min respectively.

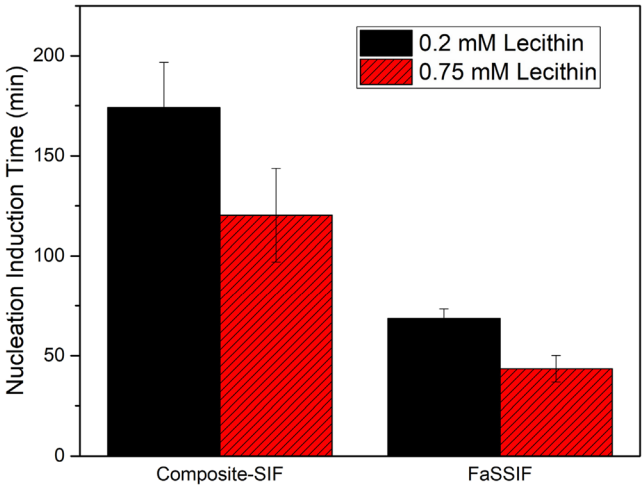

Fig. 9 Comparison of the impact of Composite-SIF and FaSSIF prepared using different lecithin concentrations on the nucleation induction time of atazanvir determined at 37°C. The 0.2 mM lecithin-containing medium corresponds to FaSSIF-V2. In neat buffer, the NIT of atazanavir was found to be ~170 min.

commercial FaSSIF-V2 (47). Recently, a version 3 of FaSSIF (FaSSIF-V3) was introduced by Fuchs *et al.* (48). This version contains SGC in addition to STC in 1:1 ratio along with lecithin, lysolecithin, sodium oleate and cholesterol. Of the several bile salts present in the HIF, the composition of FaSSIF-V3 was chosen based on the correlation of the solubility of the model compounds with HIF and the surface tension of the medium. The choice of the bile salts in biorelevant medium has historically been governed by consideration of their impact on crystalline solubility and dissolution rate.

While dissolution testing has been the primary focus when developing a suitable biorelevant media, less attention has been directed towards studying the impact of biorelevant media composition on the extent and the duration of supersaturation. Using HIF aspirated from healthy volunteers, Bevernage *et al.* first demonstrated that supersaturation can be attained and maintained in HIF by adding an inhibitory excipient (49,50). As the success of a supersaturating system is a direct function of the extent and longevity of the supersaturation, it is important to understand the impact of biorelevant media choice on the amorphous solubility (highest extent of supersaturation) and the crystallization tendency of the drug. Using STC alone as a representative bile salt, a good estimation of the crystal solubility of a variety of compounds in HIF can be achieved (34,51). However, the corresponding impact of bile salt choice on the amorphous solubility and supersaturation is still underexplored.

Impact of Biorelevant Media on Solubility

In order to successfully evaluate supersaturating formulations, it is important to understand the impact of the composition of biorelevant media chosen on both the crystalline and the amorphous solubility values. In this work, the impact of lecithin content and the bile salt composition of the SIF employed on the amorphous solubility was elucidated. For atazanavir, although the crystalline solubility was found to be largely unaffected by medium composition, the amorphous solubility was highly impacted by the presence of STC and lecithin. STC alone lowered the amorphous solubility compared to the value observed in pH 6.5 buffer. This decrease can be attributed to mixing of the bile salt with the drug resulting in a decrease in the thermodynamic activity of the drug in the amorphous phase and consequently the amorphous solubility (52). Consecutive additions of lecithin to the STC-containing medium, increased the amorphous solubility of atazanavir. This is because STC can now interact with lecithin molecules to form micellar structures which can incorporate drug molecules, instead of mixing with the amorphous drug aggregates. In turn, this leads to an increase in atazanavir amorphous solubility compared to that achieved in solutions that only contain STC. This observation suggests that the differences in lecithin content in human fluids will impact the amorphous solubility which in turn may affect the bioperformance of an enabling formulation. Thus, it can be speculated that the differences in the composition of intestinal fluids in human subjects can potentially result in inter-subject variability. Mixing between STC and atazanavir also explains the observed decrease in SR_{amorph} in the presence of 3 mM STC solution relative to buffer alone (Fig. 5). The free drug concentration available for mass transport across the membrane decreases due to mixing, resulting in a lower value of SR_{amorph}. With an increase in lecithin concentration, SR_{amorph} increases but reaches a maximum value at a lecithin concentration of 0.075 mM. This result can be explained based on the concentration of free drug. The free drug concentration in 3 mM STC solutions increases from 38 μg/mL in the absence of lecithin to 65 μg/mL (Fig. 5) in the presence of 0.075 mM lecithin. This increase is due to reduced mixing of atazanavir molecules with STC molecules as a result of lecithin incorporation in the system. STC can form micellar structures with lecithin instead of interacting and mixing with atazanavir and this results in an increase in the number of free atazanavir molecules in the system. Above 0.075 mM lecithin, atazanavir can undergo solubilization, albeit to a minor extent, in the micellar structures. Although, the total solution concentration can be higher than 65 μg/mL due to solubilization, the free drug concentration that dictates S is equal to 65 μg/mL, resulting in a similar maximum value of SR_{amorph}.

The impact of bile salt composition in the presence of lecithin on solubility was studied for the four compounds using Composite SIF and FaSSIF-V1 (Table II). Except for atazanavir, both crystalline and amorphous solubility is enhanced significantly by the SIFs. Solubility values in FaSSIF are nearly 1.5 to 2 fold higher than in Composite-SIF. The amorphous-to-crystalline solubility ratios are also presented in

Table II. The lower extent of enhancement in the amorphous solubility than that observed for the crystalline form in comparison to buffer alone, in the case of ritonavir and cilnidipine, is consistent with previous observations where supersaturation and solubilization occurred simultaneously (10,11). A difference in micellar solubilization mechanism for concentrations corresponding to the crystalline and amorphous forms has been shown to result in a difference in the extent of solubility enhancement for the two forms (11). It must be noted that the thermodynamic supersaturation at the amorphous solubility is still equivalent across various media as shown in Table III, reiterating that solubility or concentration values are not accurate measures of supersaturation and thus, cannot be used as a surrogate to determine supersaturation ratio. A marginal enhancement in solubility compared to neat buffer was seen for atazanavir, both in higher and lower lecithin containing media (Table II). The difference in solubility in FaSSIF compared to Composite-SIF could be attributed to the varying extent of solubilization by the different bile salts/lecithin micelles. Thus, the choice of biorelevant medium can impact the solubility of the drug and these effects should be considered.

At the crystalline solubility, the solute in the solution phase exists in equilibrium with the crystalline solid phase whereas, at the amorphous solubility, an equilibrium exists between the solute in the solution phase and the amorphous drug. A maximum in the solute thermodynamic activity or supersaturation ratio is attained at the amorphous solubility (53). Thus, at the amorphous solubility, the rate of membrane transport or flux, which bears a direct relationship with solute activity, also reaches the highest value. Upon exceeding the amorphous solubility, the highly supersaturated system undergoes liquid-liquid phase separation (LLPS) resulting in a continuous solution phase with a concentration equal to the amorphous solubility and a dispersed phase composed of nano-sized amorphous drug-rich droplets (35). As the solution phase free drug concentration cannot exceed the amorphous solubility, no further enhancement in flux is observed upon LLPS. However, the nanodroplets have been shown to maintain the maximum flux as long as they are present, providing a reservoir of drug (54). This can be advantageous during *in vivo* absorption. Here, absorption across the biological membrane takes place from the continuous solution phase, whereas the amorphous nanodroplets can redissolve rapidly into the continuous solution phase and replenish any of the absorbed drug to maintain the solution concentration at the amorphous solubility and the flux across the membrane at the maximum value. Thus, a formulation undergoing LLPS can exhibit enhanced absorption and therefore, superior oral bioavailability in comparison to formulations that do not undergo LLPS (55). Due to the impact of media composition on the amorphous solubility, as observed in this study, the same drug formulation may or may not undergo LLPS based on the choice of biorelevant medium used. This in turn can lead to differences in performance and subsequently impact the design, evaluation, comparison, screening and selection of formulations. Differences in the amorphous solubility values due to media composition can also impact *in silico* or pharmacokinetic (PK) modeling of oral absorption process as knowledge of the amorphous solubility is critical to determine the maximum absorptive flux or the rate of absorption across a biological membrane. Additionally, the amorphous solubility can also be critical to estimate the dissolution rate of a formulation, in particular where the dissolution kinetics are governed by the amorphous drug (56,57).

Impact of Biorelevant Media on Supersaturation

A supersaturated solution is thermodynamically metastable compared to a solution saturated at the crystalline solubility. As a result, there is driving force for crystallization. The rate of crystal nucleation (J_N) from a supersaturated system is directly related to the extent of supersaturation (Eq. 6) (58).

$$J_N = v^* z n \exp\left(\frac{-16\pi\gamma^3 v^2}{3k_B{}^3 T^3 (\ln S)^2}\right) \tag{6}$$

Here v^* represents rate of attachment of a monomer to the nucleus, z is the Zeldovich factor which is the probability of formation or dissolution of the crystal nucleus, n is the number density of molecules in solution per unit time, γ is the interfacial tension between the crystal nucleus and the solution, v is the molecular volume, k_B is the Boltzmann constant and S is the supersaturation ratio. Crystallization depends on the molecular structure of the crystallizing solute and the medium in which supersaturation is generated (22,24,59). Intuitively, when supersaturation is generated *in vivo*, crystallization kinetics will be impacted by the HIF composition. In a comprehensive study, Li *et al.* showed that the different bile salts found in HIF inhibit crystallization to varying extents whereby the degree of nucleation inhibition was related to the hydrophobicity of the bile salt (33). This observation suggests that studying supersaturating systems in a biorelevant medium prepared with only STC is likely to yield different results from a medium containing the other bile salts present in HIF; STC constitutes only 12% of the total bile salt concentration in HIF. However, HIF contains lecithin in addition to bile salts. Thus, it is important to understand the impact of bile salts on crystallization in the presence of lecithin. The systematic study carried out on atazanavir to evaluate the impact of lecithin in presence of 3 mM STC highlights that STC alone is an effective crystallization inhibitor of atazanavir (Fig. 6). However, the presence of lecithin as low as at 0.01 mM diminishes the crystallization inhibitory effect of STC. The inhibition effect was further reduced with increasing lecithin to a point worse than in buffer alone. This can be due to 1) the incorporation of STC

molecules into the mixed micelles formed with lecithin rendering STC molecules unavailable for crystallization inhibition and/or 2) crystallization induction by lecithin molecules possibly by adsorption on the growing crystal nucleus with consequent reduction of the interfacial energy between the solute in the solution and the emerging crystal. Thus, the amount of lecithin added to the medium can influence the crystallization outcome and therefore, the use of FaSSIF-V1 *versus* V2 to evaluate the crystallization propensity of a supersaturated solution can lead to different outcomes.

Comparison of Composite-SIF, which closely mimics HIF in terms of bile salt composition, with FaSSIF-V1 showed that nucleation induction times for atazanavir, ritonavir and tacrolimus were longer in Composite-SIF relative to in FaSSIF-V1, i.e. crystallization was delayed in the former medium (Fig. 8). This can be readily explained based on the results displayed in Fig. 7, which show the impact of individual bile salts (in the presence of lecithin) on the crystallization of atazanavir. It is evident that the induction time varies quite considerably with the type of bile salt present in the medium. The longest induction times were observed for SGCDC and STCDC followed by SGDC and STDC while shortest times were observed for SGC and STC. The chenodehydroxy and the dehydroxy bile salts constitute 60% of the Composite-SIF. As these bile salts are totally absent in commercial FaSSIF which employs only STC, crystallization is faster in FaSSIF as compared to Composite-SIF. This shows that bile salts have different properties and hence, a single bile salt may not be representative of HIF when preparing biorelevant media for the evaluation of supersaturating systems. Bevernage *et al.* observed a good correlation between the impact of FaSSIF-V1 and HIF collected from healthy volunteers on the crystallization onset in supersaturated drug solutions (49). However, inferences from this study need to be drawn cautiously, as the supersaturation was inferred based on solution concentrations rather than solute thermodynamic activity. It has been shown that when supersaturation occurs simultaneously with micellar solubilization, employing total solution concentration to determine the extent of supersaturation can lead to errors in estimation of the thermodynamic supersaturation, which is the actual driving force for nucleation and crystal growth (11). These errors arise due to changes in the extent or mechanism of micellar solubilization as a function of concentration. Given the recent introduction of FaSSIF-V3, it is worth discussing the possible impact of this biorelevant medium on crystallization. FaSSIF-V3 contains SGC and STC in 1:1 ratio along with lecithin, lysolecithin, sodium oleate and cholesterol. Herein, the induction time was found to be shortest in a medium containing either SGC or STC in the presence of lecithin. Hence, it can be expected that crystallization kinetics may be similar in FaSSIF-V1 and FaSSIF-V3. Thus, currently available biorelevant medium containing STC alone or with SGC may not be representative of the other bile salts present in the HIF in the context of evaluating crystallization from supersaturating systems. In this study, both lecithin content and bile salt composition were found to impact the extent and duration of supersaturation. Clearly the next step is to perform a careful evaluation of crystallization kinetics and amorphous solubility in HIF and identify similarities and differences with various simulated media in order to identify the most appropriate composition.

CONCLUSIONS

Biorelevant media are commonly employed to evaluate the dissolution performance of pharmaceutical formulations and to develop *in vitro-in vivo* correlations. However, less consideration is given to how the biorelevant media composition impacts crystallization from supersaturated systems. Herein, differences in supersaturation duration for poorly water soluble model drugs could be attributed to the specific composition of the biorelevant medium employed. Both lecithin content as well as bile salt composition was found to impact nucleation induction time. This suggests that depending on the type of biorelevant media used to evaluate the supersaturating formulation, variations in crystallization tendency can be observed and these, may or may not correlate well with *in vivo* performance. Therefore, close attention should be paid to media composition when evaluating the performance of supersaturating dosage forms and their relevance to the *in vivo* conditions likely to be encountered should be considered.

ACKNOWLEDGEMENTS AND DISCLOSURES

The authors would like to acknowledge AbbVie Inc. for providing research funding for this project. Purdue University and AbbVie jointly participated in study design, research, data collection, analysis and interpretation of data, writing, reviewing, and approving the publication. Anura S. Indulkar was a graduate student at Purdue University. Lynne S. Taylor is a professor at Purdue University. Lynne S. Taylor has no additional conflicts of interest to report. Anura S. Indulkar, Shweta A. Raina, Yi Gao, and Geoff G. Z. Zhang are employees of AbbVie and may own AbbVie stock.

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