Compromised In vitro Dissolution and Membrane Transport of Multidrug Amorphous Formulations

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Abstract

Herein, the thermodynamic properties of solutions evolving from the non-sink dissolution of amorphous solid dispersions (ASDs) containing two or more drugs have been evaluated, focusing on the maximum achievable supersaturation and tendency of the system to undergo liquid-liquid phase separation (LLPS). Ritonavir (RTV) and atazanavir (ATV) were co-formulated with polyvinylpyrrolidone to produce ASDs with different molar ratios of each drug, and the dissolution profile of each drug was studied under non-sink conditions. The phase behavior of the supersaturated solutions generated by ASD dissolution was compared to that of supersaturated solutions generated by antisolvent addition. Dissolution of an ASD containing RTV, ATV and lopinavir (LPV) was also investigated. A thermodynamic model was used to predict the maximum achievable supersaturation for ASDs containing two and three drugs. In addition, a transport study with Caco-2 cells was conducted to evaluate the impact of co-addition of drugs on membrane transport. It was found that the formulation containing a 1:1 molar ratio of RTV and ATV achieved only 50% of the supersaturation attained by dissolution of the single drug systems. The maximum achievable concentration of ATV decreased linearly as the molar fraction of ATV in the formulation decreased and a similar trend was observed for RTV. For the dispersion containing a 1:1:1 molar ratio of RTV, ATV and LPV, the maximum concentration of each drug was only one third of that achieved for the single drug formulations. The decrease in the achievable supersaturation was well-predicted by the thermodynamic model for both the binary and ternary drug combinations. These observations can be explained by a decrease in the concentration at which the drugs undergo LLPS in the presence of other miscible drugs, thereby reducing the maximum achievable supersaturation of each drug. The reduced free drug concentration was reflected by a decreased flux across Caco-2 cells for the drug combinations compared to drug alone. This study sheds light on the complex dissolution and solution phase behavior of

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multicomponent amorphous dosage forms, in particular those containing poorly water soluble drugs, which may undergo supersaturation *in vivo*.

Keywords: Dissolution; Solubility; Supersaturation; Multicomponent; Amorphous; Coamorphous

1. Introduction

Recently, interest in developing formulations containing more than one active pharmaceutical ingredient (API) in fixed dose combinations is growing and the number of products reaching the market is increasing [1-3]. Fixed dose combinations are finding utility in treating diseases that require multiple drugs, including cancer, infectious diseases, diabetes and cardiovascular disorders. They may also be employed as an approach in life cycle management, maximizing revenue and profits [4].

Various new strategies to formulate multicomponent drugs are being pursued. For example, several cocrystals of drug combinations, where two or more drugs are present in the crystal lattice, have been reported recently [5-8]. These studies have focused on crystal engineering and the formation mechanism of these solids rather than their solubility or dissolution advantages.[4-6] More generally, the aqueous solubility of cocrystals has been mathematically modeled and this form has been predicted to dramatically increase the solubility of poorly soluble drugs, when the coformer is highly soluble [9, 10]. However, the cocrystal often does not result in the expected increase in drug concentration due to rapid precipitation of the native drug crystal in the dissolution medium [11]. Another recent approach is to make a miscible amorphous blend of the two drugs and these systems has been termed coamorphous formulations [12-14]. The coamorphous material has been suggested as a potential approach to overcome solubility problems of poorly water soluble drugs [13-15]. Single phase coamorphous materials were reported to have better solid-state stability against crystallization than single compounds alone, and enhanced dissolution for the model compounds investigated [13-15].

Both of these strategies have been introduced recently as a means to enhance drug solubility and hence bioavailability, as well as to combine drugs in the formulation. However, the dissolution characteristics, supersaturation potential, and phase behaviour of the solutions evolving from these new formulations have not been widely studied [16]. These characteristics are vital for achieving successful products and avoiding unpredictable performance [17].

Amorphous solubility and liquid-liquid phase separation

Amorphous formulations have been widely investigated for their potential to overcome solubility-limited drug delivery [12]. The maximum amount of drug that can dissolve at a molecular level in aqueous media (free drug) is related to the amorphous solubility of the drug. If the amorphous solubility is exceeded (scheme 1), and in the absence of crystallization, liquid-liquid phase separation (LLPS) occurs whereby a metastable equilibrium exists between free drug in the bulk aqueous solution (drug-poor phase) and a non-crystalline, water saturated, drug-rich phase, which is typically colloidal.

Scheme 1. Schematic presentation of drug (1) distribution between the drug-rich phase and the drug-poor phase. The blue circles are the drug-rich phase.

LLPS has been reported for several materials [18-20]. The relationship between the amorphous solubility and the concentration where LLPS is typically observed has provided an approach to experimentally measure the amorphous solubility without the need to dissolve amorphous material; rather the amorphous phase is created *in situ* in the experiment [19].

Scheme 2. Schematic drawing of energy and solubility relationships of crystalline, amorphous and highly supersaturated solutions generated by dissolution of an amorphous solid dispersion. LLPS: liquid-liquid phase separation

Scheme 2 explains the relationship between solubility and the free energy of the drug in different forms. When, for example, an amorphous solid dispersion (ASD) is introduced to aqueous media, the dissolution process starts and a highly supersaturated solution can be achieved. If this supersaturated solution is unstable (concentration exceeds spinodal decomposition point) or metastable (concentration exceeds binodal point) with respect to both the amorphous and crystalline forms of the drug, then LLPS occurs until a metastable equilibrium is established between drug-poor and drug-rich phases. This two phase system

will persist for a certain period of time prior to crystallizing, with the duration depending on several factors such as formulation components and the propensity of the drug to crystallize [21].

Recently, our group has introduced experimental methods to determine the maximum supersaturation level that can be approached by measuring the concentrations at which LLPS or glass-liquid phase separation occur [19, 22]. These experimental measurements have been compared to theoretical predictions with good agreement being observed [23]. Awareness of the maximum achievable supersaturation for a given compound, as well as how to maintain this supersaturation during the course of absorption, are key for successful formulation of poorly water soluble components [24]. These aspects have been not widely studied for multicomponent systems where it is important to understand the interplay of multiple drugs in solution and its impact on the supersaturation of each component. Therefore, fundamental understanding of the molecular and thermodynamic processes involved in the dissolution of drug combinations and the effect on the subsequent supersaturation is of great interest.

In this study, a model system of two drugs used to treat human immunodeficiency virus (HIV) infections, atazanavir (ATV) and ritonavir (RTV), was investigated. The recommended dosing regimen is once daily at a dose of 100 mg RTV and 300 mg ATV. RTV inhibits intestinal and hepatic cytochrome P450 isoform 3A (CYP3A), thereby decreasing the metabolism of ATV which is a CYP3A substrate. The chemical structures and selected physiochemical properties of these drugs are presented in Fig. 1. and Table 1 respectively.

Table 1. Some chemical and physical properties of RTV and ATV including molecular weight (MW), pKa, log P, melting onset (T_m) and to melting enthalpy (ΔH (kJ/mol)).[25]

Drug	MW (g/mol)	pKa	log P	Ionization behavior	T _m (K)	ΔH (kJ/mol)	T _g (K)
RTV	720.9	2.8	5.2	Weak base	399	69	323
ATV	704.9	4.3	4.5	Weak base	483	75	377
LPV	628.8	NA	4.7	Neutral	368	ND	350

ND: Not determined

Fig. 1. Molecular structures of RTV ATV and LPV.

The maximum achievable supersaturation level of each drug following dissolution of a combination amorphous formulation under non-sink conditions was studied and rationalized based on our understanding of the LLPS phenomenon. Combining these drugs negatively influenced the maximum achievable supersaturation level for each drug, which in turn was shown to reduce the membrane transport rate across Caco-2 cells. Similar effects were seen for a ternary system in which lopinavir (LPV) was added to ATV and RTV. Using a simple thermodynamic model based on the reduced chemical potential of each component in the mixtures [26, 27], it was possible to predict the reduced supersaturation.

2. Material and methods

2.1 Materials

Ritonavir (RTV), atazanavir (ATV) sulfate and lopinavir (LPV) were obtained from Attix (Toronto, Ontario, Canada). Polyvinylpyrrolidone (PVP) K29/32 was purchased from Sigma Aldrich Co. (St. Louis MO, USA). Acetonitrile (HPLC grade) and methanol were obtained from Macron Chemicals Phillipsburg, NJ). Milli Q water was used for all aqueous solutions. The medium used in the experiments dissolution and flux experiments was 50 mM pH 6.8 sodium phosphate buffer. The buffer components were supplied from VWR, USA. HBSS pH 6.5 was used for the cell-based assays where all ingredients were purchased from Sigma Aldrich Co. (Stockholm, Sweden). Molecular structures of the model compounds are shown in Fig. 1.

2.2 High Performance Liquid Chromatography

The concentrations of RTV, ATV and LPV solution were analyzed using an Agilent 1260 high performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA) equipped with a UV/Vis spectrometer detector. RTV and ATV were separated using a Zorbax Column Eclipse XDB-C18 4.6 mm x 25 cm at ambient temperature with a total run time of 17 min. The mobile phase was 55% v/v acetonitrile and 45% v/v water and the flow rate was 0.75 mL/min using an isocratic method. Absorbance of RTV, ATV and LPV was monitored at 240 nm. The injection sample volume was 50 μ L. Standard solutions were prepared by dissolving mixtures of RTV, ATV and LPV in 50 mL of mobile phase and analyzed with HPLC to prepare a calibration curve. The calibration curves for the compounds had a $R^2 \geq 0.999$.

The concentrations of drugs in cell study (except for solutions corresponds of crystalline solubility) were analyzed using an Agilent 1290 high performance liquid chromatography

(HPLC) system (Agilent Technologies, Santa Clara, CA) equipped with a diode array detector. The column and procedures were as described above with an injection volume of 20 μL . For solutions containing a concentration corresponding to the crystalline solubility, a Waters Xevo TQ MS with electrospray ionization was used to measure compound concentration. The mass spectrometer was coupled to an Acquity UPLC system (Waters, Milford, MA) equipped with a Waters BEH C18 $2.1 \times 50 \text{ mm}$ ($1.7 \mu\text{m}$) column at 60°C and an autosampler at 10°C . The injection volume was $10 \mu\text{L}$. The mobile phase was composed of two solvents: solvent A, 5% acetonitrile and 0.1% formic acid in water; and solvent B, 0.1% formic acid in acetonitrile. The chromatographic run consisted of a linear gradient at a flow rate of 0.5 mL/min . The gradient comprised an increase from 5% to 90% of solvent B from 0.5 to 1.2 min, followed by a hold from 1.2 to 1.6 min and a return to the initial conditions at 1.7 min until the end of the run (2 min).

2.3 Differential Scanning Calorimetry

Thermal analyses of the samples were carried out on a DSC Q2000 (TA instrument, USA) which was calibrated for temperature and enthalpy using indium and for heat capacity calibrations using sapphire. Samples were crimped in non-hermetic Tzero aluminum pans and scanned under a continuously purged dry nitrogen atmosphere (flow rate: 50 mL/min). The instrument was equipped with a refrigerated cooling system. The thermodynamic parameters were calculated using the TA Universal Analysis 2000 software (TA Instruments New Castle, DE, USA). The onset of melting and heat of fusion were determined using a heating rate of 10°C/min . The glass transition temperature (T_g) was determined using a heating rate of 20°C/min .

The solid dispersions were analyzed by performing a heat-cool-heat cycle to remove any residual water. The samples were heated to 110 °C which is above the T_g of ATV, held for 1 min, then cooled to -20 °C at a heating rate of 20 °C/min before heating again at the same rate to 220 °C.

2.4 Powder X-ray diffraction

Powders were analyzed using a Shimadzu XRD-6000 (Shimadzu Corporation, Kyoto, Japan) equipped with a Cu-K α source and set in Bragg–Brentano geometry between 5 and 35° 2 θ at 8 or 4°/min with a 0.04° step size.

2.5 Preparation of amorphous and crystalline forms of ATV

ATV sulfate (1 g) was converted to the free base form by dissolving the salt form in 50 mL of methanol. The solution was then titrated with 0.1 M aqueous sodium hydroxide solution until a precipitate was observed. The suspension was vacuum filtered and the precipitate was washed with water to remove any soluble residue. The solid white precipitate was vacuum dried in an oven at room temperature for 24 hours. The resultant solid was analyzed by powder x-ray diffraction and DSC and was confirmed to be amorphous. The crystalline form of ATV was thereafter produced by suspending 250 mg of the amorphous form in 1:1 volume mixture of water and methanol and stirred at room temperature for 96 hours. DSC and XRPD measurements confirmed that the crystalline form had been isolated (Fig. S1, supporting info) [28].

2.6 Crystalline Solubility Measurements

The equilibrium solubility values of crystalline RTV and ATV and their 1:1 w/w physical mixtures were measured by adding 10 mg (20 mg in case of the physical mixture) to 15 ml of

50 mM sodium phosphate buffer pH 6.8. Samples were placed in a water bath for 48 hours at 37 °C and shaken continuously. The supernatant was separated from excess solid by ultracentrifugation at 35 000 rpm (210053 g) in an Optima L-100 XP ultracentrifuge equipped with Swinging-Bucket Rotor SW 41 Ti (Beckman Coulter, Inc., Brea, CA) for 30 min. The concentration of the supernatant was thereafter determined by HPLC. The solid forms at the end of the experiment were found to be unchanged relative to the starting material based on DSC.

2.7 Melting Point Depression

All materials used for melting point (T_m) depression experiments were passed through a 250 μ m mesh and were vacuum dried for at least 72 hours prior to mixing. Binary mixtures of crystalline RTV and amorphous ATV were prepared by geometric mixing at concentrations of 0, 5, 10, 20, 30, 40, 50, and 60 mole % of ATV. The T_m onset was measured using DSC at a scan rate of 1 °C /min.

2.8 Miscibility Investigation

Binary mixtures of amorphous ATV and crystalline RTV were evaluated using a heat-cool-heat cycle in the DSC. The mixture was heated to 134 °C which is above the T_m of RTV and held isothermally for 2 min to assure melting of RTV. Then the sample was cooled to -20 (well below the T_g of both drugs) at 20 °C/min and heated to 210 °C above the T_m of ATV at the same heating rate and the T_g of the mixture was noted.

2.9 Determination of Amorphous Solubility by UV/Vis Spectrophotometric Method

The same methodology was used to determine the amorphous solubility of both RTV and for ATV.[19, 23] Each drug was dissolved in methanol at a concentration of around 10 mg/mL.

The methanol solutions were gradually added to 15 ml of a 50 mM sodium phosphate buffer pH 6.8, at 37 °C. The titration was performed using a syringe pump to continuously add the drug solution at an infusion speed of 10 μ L/min, with continuous stirring at a speed of 300 rpm (Corning NY, USA). At the end of the experiment, less than 1% of methanol was added to the aqueous medium and this amount was subsequently confirmed not to affect the solubility of either compound. At least four experiments were performed for each drug. The intensity of light scattered from the phosphate buffer solution as a function of added drug concentration was measured with a SI Photonics UV/vis spectrometer (Tucson, AZ, USA) fiber optically coupled with a dip probe of path length 10 mm. Light scattering was detected by monitoring the extinction at a non-absorbing wavelength of 450 nm. When LLPS occurred, the baseline signal increased sharply due to the formation of scattering species. The concentration where the extinction increased for each system was estimated by fitting the data in the regions of low and high extinction using linear regression analysis and determining the intersection of the two curves.

2.10 Preparation of Solid Dispersions

ASDs of RTV and ATV were prepared by using solvent evaporation. Solutions were made by dissolving a total of 1g mixture containing 90% w/w PVP and 10% w/w of a RTV and ATV mixture at various molar ratios, in 5 mL methanol. The drug mixtures ranged from 0 - 100% ATV in 10 mol% intervals. The solvent was removed using a rotary evaporator (Brinkman Instruments, Westbury, NY, USA) with heating by a water bath maintained at 55°C. The samples were then placed under vacuum for 24 h to remove any residual solvent. A formulation containing 90% w/w PVP and a 10% w/w mixture of RTV, ATV and LPV at a 1:1:1 molar ratio was prepared in similar manner as described above. Dispersions were

prepared in duplicate and evaluated by DSC and PXRD to confirm the amorphous nature of the dried material.

2.11 Dissolution of Solid Dispersions

Dissolution testing was performed on several solid dispersions: those containing 90% w/w PVP with 10 % w/w RTV alone, ATV alone, those containing a 1:1 molar ratio of RTV and ATV, and a 1:1 physical mixture of the RTV alone and ATV alone dispersions. Around 150 mg of powder was added to 15 mL of the buffer and stirred at 300 rpm in a jacketed beaker (37°C). The dissolved concentrations were evaluated at different time points (5, 15, 30, 60 and 120 min) and three independent experiments were performed at each time point. Samples were subjected to ultracentrifugation at 35 000 rpm (210053 g) in an Optima L-100 XP ultracentrifuge equipped with Swinging-Bucket Rotor SW 41 Ti (Beckman Coulter, Inc., Brea, CA) for 45 min. The supernatant was collected, diluted with mobile phase, and concentration was determined by HPLC as described above. A control experiment using 300 mg of powder containing 90%w/w PVP and 5%w/w of each drug was performed at the same conditions and samples were taken after 60 min. Similar experiments were also conducted to measure the dissolution behavior of 90% w/w PVP with 10 % w/w of 1:1:1 molar ratio of RTV, ATV and LPV.

2.12 Determination of Maximum Supersaturation Levels Achieved

The maximum achievable free drug concentration for each ASD, and for supersaturated solutions created by antisolvent addition, was tested in the same medium as used for the dissolution study. For ASDs, around 150 mg of powder was added to 15 mL of the buffer and stirred at 300 rpm, 37°C for 30 min. For antisolvent addition, sufficient amount of RTV dissolved in MeOH to generate the amorphous solubility was added to 15 ml of 50 mM

sodium phosphate buffer pH 6.8, 37°C containing different amounts of ATV. Similar experiments were done by adding ATV to solutions containing different amounts of RTV. The supernatant was separated by ultracentrifugation at 35 000 rpm (210053 g) in an Optima L-100 XP ultracentrifuge equipped with Swinging-Bucket Rotor SW 41 Ti (Beckman Coulter, Inc., Brea, CA) for 45 min. The samples were then diluted and analyzed by HPLC.

2.13 Nanoparticle Tracking Analysis (NTA)

NTA measurements were performed with a NanoSight LM20 system (NanoSight, Amesbury, United Kingdom), equipped with a 532 nm laser. Antisolvent addition was used to generate supersaturated solutions in buffer in order to investigate the drug-rich phase formed following LLPS for ATV, RTV or a mixture of both drugs at various concentrations. All measurements were performed in triplicate at 37°C and analysis was performed by NanoSight using a beta version of NTA 3.0 software. Three videos, each 60 s long, were recorded and analyzed in batch mode. Solutions for antisolvent measurements were made by dissolving drugs in methanol. The methanol and buffer used in the NTA experiments were filtered through a 0.22µm nylon membrane filter.

2.14 Cell Culture

Caco-2 cells obtained from American Tissue Collection, Rockville, MD were maintained in an atmosphere of 90% air and 10% CO₂ as described previously (Artursson, 1990). For transport experiments, 3.3×10^5 cells were seeded on polycarbonate filter inserts (12 mm diameter; pore size 0.4 µm; Costar) and allowed to grow and differentiate for 21-30 days before the cell culture monolayers were used for transport experiments.

2.15 Transport Protocol

The intestinal membrane transport was determined from transport rates across Caco-2 cell monolayers. Donor solutions of ATV alone and RTV alone, as well as a 1:1 molar ratio of ATV-RTV were prepared. Methanol stock solutions of 10 mg/mL were used to produce solutions above the amorphous solubility of each drug by adding 30 μ L of each stock solution to 3 mL (or 20 μ L to 2 mL in the second run) of Hank's balanced salt solution, containing HEPES at pH 6.5 (HBSS pH 6.5). The final concentration of methanol was ~1%. The amount of each drug added was held constant for the single component and binary drug solutions, and always above the amorphous solubility. The applied concentration was proven non-toxic to the monolayers, as assessed by integrity measurement using the paracellular marker [14 C]-mannitol (57.3 mCi/mmol purchased from Perkin-Elmer Life Sciences, Boston, MA). In addition, solutions equivalent to the crystalline solubility of ATV and RTV were generated to compare the flux at the crystalline vs amorphous solubility across Caco-2 cell monolayers.

The transport experiments were run for 60 minutes, and were initiated by the application of the drug solution to the (apical) donor side (n=3, repeated twice except for solutions at crystalline solubility). The commonly used pH gradient of pH 6.5 in the apical chamber and pH 7.4 in the basolateral chamber was used to mimic the absorption in the small intestine. The experiment was performed at 37°C and the filter inserts were stirred at 500 rpm on IKA-Schüttler MTS4 to obtain data that were unbiased by the aqueous boundary layer. The receiver chambers were sampled continuously, with 600 μ L removed for analysis at time 15, 30 and 60 minutes. These samples were replaced with equal volumes of pre-heated receiver solution. The samples were analyzed with HPLC-UV immediately after the experiment was terminated. As different supersaturated systems are evaluated by the same procedures, the only difference is the free drug available for membrane transport. The flux equation can be given by [29]

$$J = \frac{dM}{dt.A} = \frac{Da}{h\gamma_m} \text{ Eq. 1}$$

J is the flux of drug molecules diffusing across the cell membrane. (dM/dt) is the change in mass by unit time. A , D , a , γ_m and h are the membrane cross-sectional area, the solute diffusion coefficient, the solute thermodynamic activity, the activity coefficient of the solute in the membrane and the thickness of the membrane respectively. If A , D , γ_m , and h remain constants, then the flux is directly proportional to the thermodynamic activity [24], whereby activity is given as:

$$a = \gamma C \quad \text{Eq. 2}$$

and C is the drug concentration.

3. Results

3.1 Amorphous solubility of single component systems

In order to gauge the amorphous solubility advantage for RTV and ATV, the crystalline and amorphous solubilities were determined, whereby the latter values were estimated from UV extinction measurements[19, 23] (supporting information, Fig. S2). These values are summarized in Table 2.

Table 2. Molar solubilities at 37 °C in pH 6.8 buffer for crystalline ATV and RTV ($S_{\text{Crystalline}}$), as a mixture of both crystalline forms (S_{Mixtures}), and in amorphous form ($S_{\text{Amorphous}}$). The ratio of the amorphous solubility to the crystalline solubility, $S_{\text{Amorphous}}/S_{\text{Crystalline}}$, is also shown. Results are the average and standard deviations of at least three measurements.

Drug	$S_{\text{Crystalline}}$ (M)	S_{Mixtures} (M)	$S_{\text{Amorphous}}$ (M)	$S_{\text{Amorphous}}/S_{\text{Crystalline}}$
RTV	$(3.2 \pm 0.9) \times 10^{-6}$	$(4.9 \pm 0.4) \times 10^{-6}$	$(45.2 \pm 2.0) \times 10^{-6}$	~14
ATV	$(1.6 \pm 0.2) \times 10^{-6}$	$(1.6 \pm 0.2) \times 10^{-6}$	$(132.1 \pm 2.0) \times 10^{-6}$	~83
LPV*	$(4.29 \pm 0.2) \times 10^{-6}$ *	ND	$(27.2 \pm 2.0) \times 10^{-6}$	~6

*Results from reference [27]

The amorphous solubility of RTV is approximately a factor of 14 higher than that of the crystalline reference, while amorphous ATV is ~80 fold more soluble as compared to its crystalline counterpart. To understand the origin of this difference, factors contributing to the

observed solubility should be considered. Although RTV and ATV have some chemical similarity, their crystal properties are quite different whereby RTV has a considerably lower melting point and enthalpy of fusion (Table 1). The solubility barriers for crystalline drugs in water are the need to disrupt the crystal packing, and the hydrophobicity of the molecules. The following equation describes the crystal and solvation contributions to the free energy of solution:

$$\Delta G_{\text{solution}} = \Delta G_{\text{fusion}} + \Delta G_{\text{solvation}} \quad \text{Eq. 3}$$

Therefore, disrupting the crystal structure to form an amorphous solid reduces the barrier to only the solvation contribution. The solvation contribution (γ) was evaluated from the following equation:[30]

$$\log \chi = \log X_{\text{ideal}} - \log \gamma \quad \text{Eq. 4.}$$

Where (χ) is the measured aqueous solubility under nonionizing conditions, (X_{ideal}) is the calculated ideal solubility and (γ) the activity coefficient of the solute in water.

X_{ideal} can be approximated from following equation:

$$\log X_{\text{ideal}} = \frac{-\Delta H_m}{2.303R} \left(\frac{T_m - T}{T_m T} \right) \quad \text{Eq. 5.}$$

Where ΔH_m , T_m , R and T are melting enthalpy, melting temperature, the ideal gas constant and the experimental temperature (310K), respectively. Equation 5 is based on the assumption that the heat capacity change upon melting is zero and the enthalpy of the solution is equal to ΔH_m . Fig. 2 summarizes the contribution of the crystal packing, and the activity coefficient as barriers to solubility. ATV has a higher crystal packing contribution, and a lower $\log \gamma$, explaining why it has a higher amorphous solubility as compared to RTV. The lower solubility of crystalline ATV relative to RTV is explained by the high T_m and ΔH_m .

Fig. 2. The crystal ($\log X_{\text{ideal}}$) and solvation energy ($\log \gamma$) contributions to the experimental aqueous solubility of RTV and ATV. The grey area represents the activity coefficient calculated from Eq. 4. The black area of the bars represents ideal solubility calculated from Eq. 5.

3.2 ATV and RTV miscibility

The miscibility of RTV and ATV in the amorphous phase is expected to influence the solution thermodynamics. The miscibility of RTV and ATV was studied by investigating the T_m depression of RTV as function of the mole ratio of amorphous ATV in a physical mixture. ATV resulted in melting point depression of crystalline RTV, indicating that the two molecules are miscible in the molten state [31]. The resultant melt-quenched samples displayed a single T_g event at all ATV: RTV ratios (Fig. 3 and Fig S3). The change in T_g for different mixtures was well described by the Fox equation [32].

$$\frac{1}{T_g} = \frac{x_1}{T_{g(RTV)}} + \frac{x_2}{T_{g(ATV)}} \quad \text{Eq. 6}$$

Where T_g is the mixture T_g , $T_{g(RTV)}$ is the T_g of RTV, and x_1 is the weight fraction of RTV, $T_{g(ATV)}$ is the T_g of ATV and x_2 is the weight fraction of ATV.

The dispersions, which were confirmed to be amorphous by PXRD and DSC, showed only a single T_g event (Fig. S3 and S4). RTV has previously been observed to be miscible with PVP [33]. The T_g was found to be in the range 139-160°C, reflecting the low drug loading.

A)

B)

Fig. 3. A) Melting point depression of crystalline RTV as function of mole fraction of ATV. B) Glass transition temperature as a function of RTV weight fraction. Predicted line for T_g of a miscible system of RTV and ATV determined by the Fox equation (Red line).

3.3 Powder dissolution of ATV and RTV amorphous dispersions

Dissolution studies on amorphous solid dispersion were performed under non-sink conditions to evaluate the impact of second component on the maximum supersaturation achieved. Dissolution profiles of ASDs containing only one drug (PVP-ATV or PVP-RTV dispersions), those containing a 1:1 molar ratio of ATV and RTV formulated as a single phase system (PVP-ATV-RTV), and a 1:1 physical mixture prepared by mixing the PVP-ATV dispersion with the PVP-RTV dispersion (PVP-ATV:PVP-RTV physical mixture), are shown in Fig. 4.

A)

B)

Fig. 4. Powder dissolution profile of formulations containing 90% PVP and 10% total drug(s). ATV or RTV alone (green $\diamond$), 1:1 molar ratio of ATV:RTV (blue $\circ$), and 1:1 physical mixture of formulations containing either ATV or RTV (maroon Δ). Error bars represent standard deviation, $n=3$. Dashed black line and solid black line represent the amorphous and crystalline solubility values respectively. A) Concentration of ATV. B) Concentration of RTV.

The PVP-ATV and PVP-RTV dispersions dissolved rapidly to attain the amorphous solubility and maintained supersaturation for the duration of the experiment (120 min). For the formulation containing both drugs (PVP-ATV-RTV), the maximum solution concentration achieved for each compound was only approximately 50% of the corresponding value for the single drug component dispersions. This was despite the fact that sufficient powder was used to achieve a concentration greater than the amorphous solubility. Additional experiments were conducted with double the amount of powder, measuring the amount of dissolved compounds at 60 min. Similar results were obtained, with the maximum solution concentration being only approximately 50% relative to the dispersions containing only one drug. Interestingly, when the 1:1 physical mixture of the PVP-ATV and PVP-RTV dispersions was dissolved, a similar result was obtained (Fig. 4). In other words, it made no difference to the maximum observed

solution concentrations if the drugs were combined as a single phase in one amorphous formulation or added separately from two individual dispersions; the maximum solution concentration was reduced in both instances. These results, where reduced supersaturation is observed, are in contradiction with the current paradigm of formulating drugs together as an amorphous mixture or producing them as cocrystals for the purpose of improved dissolution and increased supersaturation.[7, 13-15, 34] Therefore, the maximum achievable supersaturation for various ATV:RTV molar ratios was investigated in order to better understand this phenomenon.

3.5 Supersaturation for drug combinations generated by amorphous dispersions and antisolvent addition

The maximum free drug solution concentration achieved upon dissolution of ASDs containing nine different ATV:RTV molar ratios, at a total drug loading of 10%, was determined and results are shown in Fig. 5.

Fig. 5. Concentration of ATV (blue ○) and RTV (green Δ) in drug-poor phase (supernatant) following dissolution of amorphous solid dispersions containing both compounds as a function of the mole fraction of RTV in the formulation. Dashed line represents the predicted concentration of each drug using equation 5.

When the drug concentration in the bulk aqueous phase was plotted against the mole fraction ratio of the drug in the formulation, a linear relationship was observed. Hence, the maximum free drug concentration of a given component appears to be directly related to the mole fraction of that component present in the dispersion.

Fig. 6. Concentration of ATV (Blue ○) and RTV (Green Δ) in the drug-poor phase (supernatant) as function of mole fraction of RTV in the drug-rich phase. Dashed line represents the predicted concentration of each drugs using equation 7.

To further evaluate these findings, a drug-rich phase was formed *in situ* using antisolvent addition, followed by determination of the free drug concentration. The mole fraction ratio of each drug in the drug-rich phase was then calculated from mass balance. From Fig. 6, it is apparent that there is a linear relationship between the free drug concentration of a given component and its mole fraction in the drug-rich phase. Thus the concentration of the drug in

the bulk aqueous phase is directly proportional to the mole fraction of that drug in the drug-rich phase. The relationship between the concentration of ATV in the bulk aqueous phase to that of RTV in the bulk aqueous phase is shown in Fig. 7.

Fig. 7. Concentration of ATV as a function of RTV concentration in the poor drug-phase when a drug-rich phase is present. Concentrations measured after dissolution of solid dispersions (Black Δ) and from antisolvent addition of drugs (Green $\circ$), followed by ultracentrifugation and analysis of the supernatant. Green dashed line is the LLPS predicted line (i.e. the predicted amorphous solubility change) using equation 7. The highlighted regions in blue are the concentration regions where each drug is undersaturated with respect to the crystal solubility. The black region indicates where both drugs are mutually undersaturated with respect to the crystal solubility.

The concentration of each component added to solution was above the LLPS concentration and under these conditions, ATV maximum solution concentration decreases as function of the maximum achievable RTV concentration. The formation of a drug-rich phase when a certain concentration was exceeded was confirmed by nanoparticle tracking analysis (Fig. 8). It was found that no scattering species were generated at concentrations below the amorphous solubility, whereas as the concentration increased beyond the amorphous solubility, an

increase in the number of scattering species was observed. This is consistent with the concept that once LLPS occurs, a maximum in the free drug concentration is reached and addition of more drug leads to the formation of more of the drug-rich phase. The particle size analysis further supports the finding and also shows that the size increases with increased drug concentration (Table S1).

Buffer only 4.74×10^6 particles/mL	20 µg/mL RTV 1.52×10^7 particles/mL	40 µg/mL RTV 6.00×10^7 particles/mL	50 µg/mL RTV 5.88×10^8 particles/mL

55 µg/mL ATV 6.54×10^6 particles/mL	110 µg/mL ATV 2.15×10^8 particles/mL	150 µg/mL ATV 4.15×10^8 particles/mL	55 µg/mL ATV and 20 µg/mL RTV 1.08×10^6 particles/mL

Fig. 8. Scattered light images from nanoparticle tracking analysis of solutions containing different concentrations of ATV, RTV, and their mixtures created by anti-solvent addition. Number of particles per mL is the average of three runs.

While ATV or RTV alone, below the amorphous solubility, did not show formation of a drug-rich phase, a mixture of ATV and RTV below their individual amorphous solubilities showed formation of a scattering phase. This confirms the formation of a drug-rich phase at a lower supersaturation when two solutes are present.

3.6. Membrane Transport

The free drug available for absorption has been found to reach a plateau when the compound is at or above the amorphous solubility [24]. The cell-based study was conducted to find out if the reduced maximum free drug concentration in the presence of a second compound will lead to reduced membrane transport across a biologically relevant system. Table 3 shows results for the flux of ATV and RTV from solutions containing each compound alone, at a concentration equivalent to the amorphous solubility, relative to the flux of each compound in the presence of the other compound at a (1:1) molar ratio. It is clear that the cell flux value was reduced to ~50% for each component in the binary solution, relative to the flux of each drug alone, in excellent agreement with the dissolution data. It was not possible to detect the drugs when a solution concentration equal to equilibrium solubility was used as it was below the limit of detection of the analytical method used (2.7×10^{-7} mg/mL).

Table 3. Flux measurements of different formulations (n=3)

Solution	Flux (mg/min/cm ²)
ATV amorphous solubility	$(10.5 \pm 0.1) \times 10^{-5}$
ATV from ATV-RTV (1:1) molar ratio	$(5.12 \pm 0.3) \times 10^{-5}$
RTV amorphous solubility	$(9.1 \pm 0.7) \times 10^{-5}$
RTV from ATV-RTV (1:1) molar ratio	$(4.3 \pm 0.7) \times 10^{-5}$

4. Discussion

When LLPS occurs, a metastable equilibrium is established between the drug-poor aqueous phase and the water saturated drug-rich phase. The system is metastable in comparison to the crystalline form(s) and can therefore crystallize. Hence the longevity of the two phase system will depend on the crystal nucleation kinetics at the particular experimental conditions. The maximum supersaturation that can be maintained in the system prior to crystallization is dictated by the concentration at which the drug-rich phase forms, and will be constant as long as the two phase system persists. The concentration in the drug-poor phase following phase

separation of the drug-rich phase has been found to be the same as the predicted amorphous solubility [23].

Scheme 3. Schematic presentation of LLPS and formation of a drug-rich phase in a multicomponent system with drug 1 and drug 2.

To understand the behavior of multicomponent supersaturated solutions, Scheme 3 can be considered. Scheme 3 shows a metastable equilibrium between each solute dissolved in the bulk aqueous phase with each compound mixed together in the drug-rich phase. This scenario occurs when an ASD containing two drugs dissolves in aqueous media, or high supersaturation of each drug is created via antisolvent addition, for systems where both drugs are miscible in the amorphous state. If mixing in the drug-rich phase is ideal, the mole fraction of each drug in the drug-rich phase will determine the resultant concentration of that component in the bulk aqueous phase [26, 27]. Consequently, the concentration of each

compound in the bulk aqueous phase will depend on the composition of the drug-rich phase. Fig. 7 shows the predicted phase boundary for the ATV and RTV systems. The line was generated by considering the changes in aqueous solubility as a function of the mole fraction of each drug in the drug-rich phase assuming ideal mixing between the two components in this phase [27]:

$$S_1 = S_{A1} \cdot X_1 \quad \text{Eq. 7}$$

Where S_1 is the amorphous solubility of component 1 in the bulk aqueous phase in the presence of a drug-rich phase containing a second component, S_{A1} is the amorphous solubility of component 1 alone in water and X_1 is the mole fraction of component 1 in the drug-rich phase. Similarly, using equation 7 the reduction in the maximum achievable supersaturation of ATV and RTV was predicted with results shown in Fig. 5 and 6 where it can be seen that the model predicted the experimental data very well. When there is a potential for supersaturated solutions to be generated, it is useful to consider different regions of the phase diagram for a binary solute system. In Fig. 7, three regions can be distinguished. Region I (black filled area) is where both drugs are undersaturated with respect to their crystalline solubility. Region II shows concentrations where both drugs are below their amorphous solubility (no LLPS) but are supersaturated with respect to their crystalline solubility. In region III both drugs are above their amorphous solubility and the drugs will phase separate to form a drug-rich phase until the metastable equilibrium, shown by the green dashed line in Fig. 7, is established. Eventually crystallization will occur, and solution concentrations will be reduced to the crystal solubility. Further, Fig. 7 clearly shows that the maximum free drug concentration of ATV can only be achieved in solutions where there is a low concentration of RTV and vice versa. Thus, at the recommended dosage combination of the drugs, a 1:3 weight ratio of RTV:ATV (which is approximately equal to a 1:3 molar ratio), it can be expected that the maximum achievable RTV free drug concentration will be only ~25% of the

amorphous solubility of the drug in the absence of ATV. Interestingly, a concentration equivalent to RTV crystalline solubility is achieved when the RTV concentration in the drug-rich phase is around 0.1. These observations clearly show the impact of combining these drugs on the achievable free drug concentration, which may in turn impact the absorption *in vivo*.

Supersaturated solutions contain a solute with higher thermodynamic activity relative to a saturated solution, and it has been documented that this in turn translates to a higher membrane transport rate, using artificial membrane [24, 35]. This was further confirmed by our results where solutions at the crystalline solubility had lower flux compared to supersaturated solutions at the amorphous solubility. It is also known that because the flux is linearly related to the free drug available for absorption, the flux will reach plateau at the amorphous solubility, which represents the maximum in the free drug concentration [24, 36]. Hence, when ATV and RTV were in solution together, the membrane transport was reduced as compared to the single component systems, which can be explained by reduced maximum free drug concentration available for absorption, due to the reduction in the amorphous solubility of each component. If the maximum flux can be obtained at the amorphous solubility, the reduced flux for a multidrug formulation can be predicted from equation 8:

$$J_1 = J_{A1} \cdot X_1 \quad \text{Eq. 8}$$

Where J_1 is the the flux at the amorphous solubility of component 1 in the bulk aqueous phase in the presence of a drug-rich phase containing a second component, J_{A1} is the flux at the amorphous solubility of component 1 alone and X_1 is the mole fraction of component 1 in the drug-rich phase. Equation 8 provides a good estimate of the flux of ATV and RTV across Caco-2 cells in a binary formulation containing a (1:1) molar ratio of the drugs which was observed to be approximately 50% of the flux values obtained for the solutions containing only one solute at the amorphous solubility (Table 3). Based on these observations, awareness

of the impact of multidrug combinations on solution thermodynamics is essential as reduced supersaturation can result. The reduced thermodynamic activity of each component will in turn lead to decreased membrane transport and hence is anticipated to negatively impact bioavailability.

The risk for reduced achievable supersaturation is exacerbated when more than two miscible compounds are combined, since the mole fraction of each component will be reduced by the presence of multiple other compounds. Thus Eq. 7 can be further extended for multicomponent systems (again assuming ideal mixing in the drug-rich phase) where X_1 is the mole fraction of drug 1 in the multicomponent drug-rich phase calculated from:

$$X_1 = \frac{n_1}{\sum n} = \frac{n_1}{(n_1+n_2+n_3+\dots)} \quad \text{Eq. 9}$$

The phenomenon of reduced achievable supersaturation, as well as the applicability of Eq. 7 was demonstrated for a formulation containing a 1:1:1 molar ratio of RTV, ATV and LPV. As expected, the maximum concentration achieved for each drug was about one third of its original amorphous solubility (Fig. 9).

A)

B)

C)

Fig. 9. Dissolution profiles of formulations containing 90% PVP and 10% total drug for a ternary drug system consisting of a 1:1:1 mole ratios of ATV:RTV:LPV. ATV or RTV alone (green $\diamond$) and 1:1:1 mole ratios of ATV:RTV:LPV (blue $\circ$). Error bars represent standard deviation, $n=3$. Dashed black lines represent the amorphous solubility values. A) Concentration of ATV. B) Concentration of RTV. C) Concentration of LPV. The amorphous solubility of LPV was determined by anti-solvent method and agreed with earlier work [27].

Given that many fixed dose combinations contain structurally similar compounds, the likelihood of at least partial miscibility in the amorphous state and the drug-rich phase formed upon LLPS is quite high. Thus ASD formulations containing multiple APIs are clearly at risk of a reduced achievable supersaturation relative to the individual dosing of single component drug formulations. In a recent study, coamorphous systems gave rise to a lower than expected dissolution rate and extent of supersaturation [16]. The current work shows the underlying thermodynamic basis for this type of behavior in binary and multicomponent ASDs. Furthermore, our data show that the issue with coformulation cannot be overcome by simultaneous dosing of the individual formulations. Indeed, co-administration also reduces the maximum achievable supersaturation through the formation of a new phase (i.e. the drug-rich phase that contains both drugs) following dissolution. It should be noted that the

behavior observed herein is mechanistically different from salting out or cocrystallization, where the components precipitate out to form a new crystal form (salt or cocrystal) with well-defined stoichiometry [37, 38]. In the case of LLPS, a wide range of supersaturations can be generated based on the composition of the drug-rich phase, which in turn will depend on the dose ratios of the various compounds. Moreover, for multicomponent systems, it is possible that a component present at a low mole fraction in the drug-rich phase could even have a lower solubility than the crystalline phase. Finally, it is worth noting that reduced achievable supersaturation is relevant not only for amorphous solid dispersions. Similar structures may form *in vivo* as a result of LLPS occurring when e.g. weak bases that supersaturate upon gastric emptying into the small intestine (in response to the pH change) are co-administered.

5. Conclusions

In this work, the impact of additional hydrophobic solutes on maximum achievable supersaturation of each drug was investigated, focusing on multicomponent ASDs. It was found that the maximum achievable supersaturation of the drugs evaluated decreased in the presence of one or more additional compounds. This was applicable when the drugs were formulated together or separately. The extent of decrease was proportional to the molar ratio of each component in the drug-rich phase that formed following dissolution of the dispersion. A simple thermodynamic model was employed to model the data, and was found to have good predictive power for these miscible systems. The drug combinations resulted in reduced membrane transport as compared to the drug alone. The decrease in maximum achievable supersaturation is likely to be of importance for the dissolution and absorption of some fixed dose combination formulations, or when certain drugs are co-administered.

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Graphical abstract

This study shows that a fixed dose combination strategy undermines the amorphous solubility advantage and provides a mechanistic explanation of why this occurs.

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