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Author: Sophia Yui Kau Fong Asiye Ibisogly Annette Bauer-Brandl Professor

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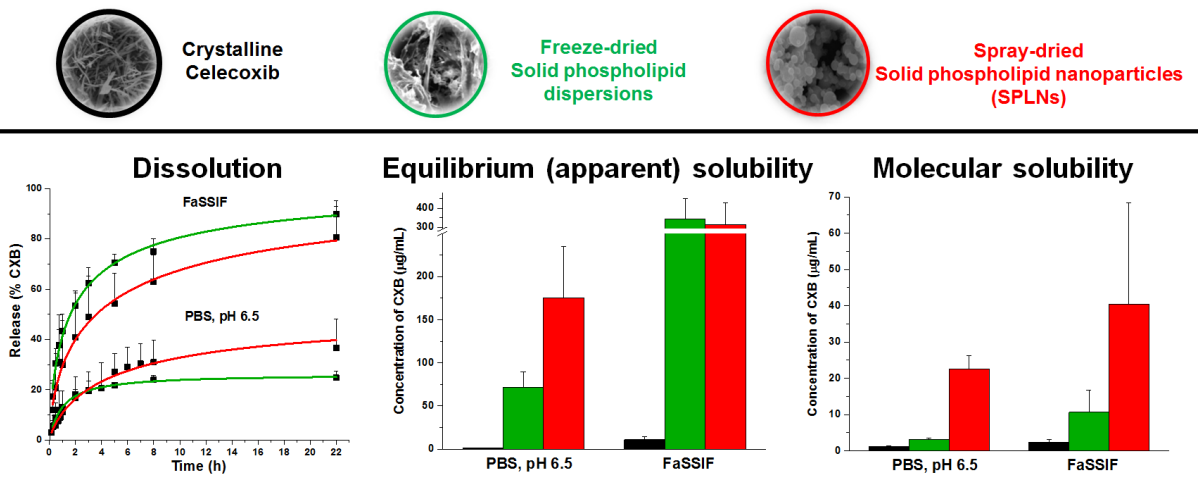
Solubility enhancement of BCS Class II drug by solid phospholipid dispersions: spray drying *versus* freeze-drying

Sophia Yui Kau Fong, Asiye Ibisogly, Annette Bauer-Brandl Professor* annette.bauer@sdu.dk

Department of Physics, Chemistry and Pharmacy, University of Southern Denmark, Campusvej 55, 5230 Odense M, Denmark

*Corresponding author at: Department of Physics, Chemistry and Pharmacy, University of Southern Denmark, Campusvej 55, 5230 Odense M, Denmark, Tel.: +45 6550 4497, Fax: +45 6615 8780.

Graphical Abstract



Abstract

The poor aqueous solubility of BCS Class II drugs represents a major challenge for oral dosage form development. Using celecoxib (CXB) as model drug, the current study adopted a novel solid phospholipid nanoparticle (SPLN) approach and compared the effect of two commonly used industrial manufacturing methods, spray- and freeze-drying, on the solubility and dissolution enhancement of CXB. CXB was formulated with Phospholipoid E80 (PL) and trehalose at different CXB:PL:trehalose ratios, of which 1:10:16 was the optimal formulation. Spherical amorphous SPLNs with average diameters <1 µm were produced by spray-drying; while amorphous 'matrix'-like structures of solid PL dispersion with larger particle sizes were prepared by freeze-drying. Formulations from both methods significantly enhanced the dissolution rates, apparent solubility, and molecularly dissolved concentration of CXB in phosphate buffer (PBS, pH 6.5) and in biorelevant fasted state simulated intestinal fluid (FaSSIF, pH 6.5) (p<0.05). While similar dissolution rates were found, the spray-dried SPLNs had a larger enhancement in apparent solubility (29- to 132-fold) as well as molecular solubility (18-fold) of CXB at equilibrium (p<0.05). The strong capability of the spray-dried SPLNs to attain 'true' supersaturation state makes them a promising approach for bioavailability enhancement of poorly soluble drugs.

Abbreviations
CXB: Celecoxib
FaSSIF: Fasted state simulated intestinal fluid
FD: Freeze-dried
PBS: Phosphate buffer
PB: Physical blend
PL: Phospholipid (Lipoid E80)
SD: Spray-dried
SPLN: Solid phospholipid nanoparticles

Keywords: Solid phospholipid nanoparticles; amorphous solid dispersion; solubility; spray-drying; freeze-drying; 36 celecoxib

1. Introduction

Recent reports estimate that at least 40% of the marketed drugs and 60-70% of the candidate compounds in pipeline belong to BCS Class II (*i.e.* having low solubility and high permeability) (Babu and Nangia, 2011). The poor aqueous solubility of these drugs remains a major challenge for oral dosage development and various kinds of 'enabling formulations' and solubilization technologies have been developed to increase the dissolution rate and/or dissolved-drug levels in an attempt to achieve the desired extent and rate of oral absorption.

Phospholipid (PL)-based drug delivery system, for instance, is among the most promising approaches for enhancing oral bioavailability. Being biocompatible, biodegradable, and having an amphiphilic character that allows PLs organized themselves as lipid bilayers when placed in water with the hydrophobic tails lines up against one another and the hydrophilic head-group facing the water on both sides, PLs are considered as suitable excipients for poorly water soluble drugs (Fricker et al., 2010). Solid PL-based formulations, in particular, have attracted increasing attention since they have distinct advantages over other PL-containing formulations for oral drug delivery, one of which is that they may have improved physical and chemical stability during storage. Another advantage is that they (the powder) can be incorporated into solid dosage forms with relatively straightforward preparation processes (Betageri, 2008). From our recent meta-analysis which included a selected range of BCS class II and III drugs, an average increase of up to 127.4% of solubility (95% CI [86.1, 168.7]), 59.6% of permeability (95% CI [30.1, 89.0]), and 18.5% of oral bioavailability (95% CI [10.1, 26.9]) was observed by the use of solid PL-based formulations (Fong et al., 2015). The potential candidate-enabling capability of this approach thus appears to be promising. Recently introduced by our group, 'solid phospholipid nanoparticle' (SPLN) represents a novel approach to enhance the bioavailability of

BCS Class II drugs. We have developed a high yield spray-drying technique to create SPLN (in nanometer and low micrometer range) and had successfully applied it to a BCS Class II drug griseofulvin with demonstrated superior dissolution rates (Brinkmann-Trettenes et al., 2014; Brinkmann-Trettenes and Bauer-Brandl, 2014). In the current study, we extended the application of the SPLN approach to a different BCS Class II model drug in order to evaluate the generalized applicability of this approach. In addition to spray-dried SPLN, we also produced PL-based solid dispersion by freeze drying and the effects of these two formulations on solubility enhancement were compared. Since both spray-drying and freeze-drying are commonly used industrial manufacturing methods, a comparison of these methods would provide useful information for scaling-up to industrial production.

Existing dissolution or solubility studies for evaluating enabling formulations often have the following limitations: (1) biorelevant media are not used as the dissolution/dispersion media; and (2) the molecular solubility of a drug in 'true' supersaturation condition is not determined. The use of biorelevant media for solubility and dissolution determination of poorly soluble drugs had been proposed since the late 90s and was considered important because these media are adapted to human small intestinal fluids in terms of pH, buffer capacity, osmolality as well as the bile salt and PL concentrations. As a result, the solubility/dissolution determination using biorelevant media better reflects the physiological solubility of poorly soluble drugs, and this is especially important for early formulation studies of BCS Class II drugs (Butler and Dressman, 2010; Galia et al., 1998). Current practice of assessing the influence of enabling formulations on drug solubility is by the "apparent" solubility, *i.e.* the maximum concentration of "dissolved" drug and it includes both molecularly dissolved drug molecules and colloidal drug (drug incorporated in carrier, micelles, *etc.*). However,

recent and growing evidence has suggested that "apparent" solubility in many cases is not a reliable predictor of oral absorption for enabling formulations (Buckley et al., 2013). On the other hand, "molecular" solubility, defined as the maximum concentration of "truly" dissolved drug, that is, a single drug molecule surrounded by a hydration shell, is recently proposed as the key to "true" solubility measurement for enabling formulations (Buckley et al., 2013; Frank et al., 2012a). For instance, (Miller et al., 2012) postulated that amorphous solid dispersions induce 'true' supersaturation (*i.e.* of molecularly dissolved drug) that improve oral bioavailability; while (Frank et al., 2012b) proved that it is the "molecular" solubility but not the "apparent" solubility that demonstrated a good correlation with permeation rate of a poorly water soluble drug formulated as amorphous solid dispersion.

Using celecoxib (CXB) as a model BCS Class II drug, the current study aimed (1) to develop an optimal PL-based solid dispersions of CXB using the novel SPLN approach by spray-drying; and (2) to compare the solubility and dissolution enhancement effect of the spray-dried SPLN with that of the freeze-dried PL-based solid formulation of CXB. A biorelevant medium, fasted state simulated intestinal fluids (FaSSIF) which contains phosphate buffer pH 6.5, lecithin and sodium taurocholate, was employed for the current solubility and dissolution studies. Furthermore, both the apparent and molecular solubility of CXB from the enabling formulations were determined.

2. Material and Methods

2.1 Materials

Lipoid E80 phospholipid (PL), an egg lecithin containing 80-85% phosphatidylcholine and 7.0-9.5% phosphatidylethanolamine according to the specification from manufacturer, was donated from

Lipoid GmbH (Ludwigshafen, Germany). Celecoxib (CXB) and D(+)–trehalose dihydrate (TRE) of analytical grade were purchased from Selleck Chemicals (TX, USA) and VWR International bvba (Leuven, Belgium) respectively. SIF Powder Origin for preparing FaSSIF media was obtained from biorelevant.com (Surrey, UK). Ethanol (96%), methanol (HPLC grade), formic acid, sodium chloride, sodium hydroxide, sodium phosphate monobasic monohydrate and tertiary butanol, were purchased from Sigma-Aldrich (St. Louis, USA). Purified water was supplied from Milli-Q® Millipore (Merck, Germany).

2.2 Methods

2.2.1 Preparation of formulations

2.2.1.1 Compositions

The compositions of the different formulations studied in the current experiments are summarized in **Table 1**. A total of nine formulations were prepared: three fixed mass combinations of excipients CXB + PL + TRE (1 + 2.5 + 5, 1 + 5 + 8, 1 + 10 + 16; m + m + m) were prepared by three production methods: (1) physical blends of raw materials, (2) co-spray-dried formulation, and (3) co-freeze-dried formulation. For processing reasons as previously evaluated by our group (Brinkmann-Trettenes et al., 2014), the PL-to-TRE ratio in all compositions are fixed at 1:1.6. Ethanol in water (80:20, w/w) and tert-butanol in water (60:40, w/w) were the solvents used for dissolving the formulations prior to spray-drying and freeze-dried respectively.

2.2.1.2 Physical blend

Physical blends of raw materials were prepared by mixing crystalline CXB, PL and crystalline TRE using a mortar and pestle, in 3 different compositions according to **Table 1**. These formulations were prepared freshly prior to use.

2.2.1.3 Spray-drying

Spray-drying was performed using Büchi B-90 Nano Spray Dryer with inert loop B-295 dryer (Büchi Laboretechnik AG, Flawil, Switzerland). According to the compositions described in **Table 1** and with a total solute concentration of 4%, CXB and PL were dissolved in ethanol while TRE was dissolved in water. The two solutions with a final solvent ratio of 80:20 (ethanol:water) were mixed under magnetic stirring for 30 min before spray-drying. The conditions for spray-drying were set as follows: air inlet temperature at 80 °C, inert loop temperature at 10 °C, air flow rate of 140 L/min, speed of the peristaltic pump at 12.5 mL/min, and nozzle mesh size of 5.5 µm. Nitrogen was used as the drying gas and the maximum oxygen concentration was set to 4%. The yields of the collected powder were all above 85%. The spray-dried formulations were stored at room temperature in a desiccator above calcium chloride until analysis.

2.2.1.4 Freeze-drying

Freeze-drying was performed with Christ Gamma 2-16 LSC Freeze Dryer (Martin Christ GmbH, UK). Tert-butanol was first liquefied in water at a ratio of 60:40 (w/w). CXB, PL, and TRE were then dissolved in the solvent mixture according to their individual composition described in **Table 1**, followed by mixing under magnetic stirring for 30 min. The formulations were then frozen at -80 °C for 24 h before being placed in the pre-cooled freeze dryer at -60 °C. Freeze-drying was performed

according to the following program: a main drying phase with shelf temperature at 25 °C and pressure of 0.1 mbar for 24 h; followed by a final drying phase with shelf temperature at 25 °C and pressure of 0.01 mbar for 4 h. The residual content of organic solvent was controlled by weighing the sample vials prior to and after freeze drying and was found to be acceptable (<1%). The vials were sealed and stored at room temperature in a desiccator above calcium chloride until analysis.

2.2.2 Structural analysis

The structural analyses of CXB crystalline and the different formulations were carried out by an X-ray Diffractometer (Rigaku®, MiniFlex 600, Japan) with Cu Kα radiation (λ = 1.5418 Å). The powder samples were measured within an angular range of 14-27° 2θ, with a step size of 0.02 under the following conditions: current 10 mA, voltage 30 kV, and scanning speed 10° 2θ/min.

2.2.3 Particle morphology

The particle morphologies of CXB crystalline and different formulations were analyzed using a scanning electron microscopy (LEO 435 VP, Zeiss, Germany) with the following parameters: probe current at 50 pA and voltage at 20,000 eV. A thin layer of gold (20 nm) was coated over the sample to enhance the electrical conductivity by using an ion sputter coater of JEOL (Ion Sputter JFC-1100).

2.2.4 Physical stability

Formulations SD3 and FD3 were stored in glass vials for a period of up to 2 months at room temperature (25 °C) in a desiccator. On day 1, day 8, day 15, day 30 and day 60 after their preparations,

these samples were subjected to X-ray diffraction as described in **Section 2.2.2** to examine their re-crystallization tendency upon storage.

2.2.5 Solubility and dissolution studies

2.2.5.1 Preparation of media

Phosphate buffer (PBS, pH 6.5) and FaSSIF buffer (pH 6.5) were used as media for solubility and dissolution studies. Both of them were prepared in accordance with the procedures suggested by the manufacturer (biorelevant.com, UK). For the preparation of PBS, 0.42 g of sodium hydroxide, 6.19 g of sodium chloride, and 3.95 g of sodium phosphate monobasic monohydrate were dissolved in 1 L of purified water. The pH of the PBS buffer was adjusted to 6.5 with 1 M of sodium hydroxide or hydrochloric acid. FaSSIF buffer was prepared by dissolving 2.24 g of SIF powder in 1 L of PBS buffer (pH 6.5). The freshly prepared FaSSIF was allowed to stand at room temperature for 2 h before use.

2.2.5.2 Dissolution studies

Dissolution test was performed using an USP type 1 apparatus (DT-70, Pharma Test, Germany) in 500 mL of the respective dissolution media at 37(±1) °C at 50 rpm. Circular paper filters were put in the bottoms of the basket before the respective formulations were weighed in. The total amount of CXB content in each formulation was equal (approximately 5 mg). 5 mL of sample was withdrawn at regular time intervals (0.25, 0.5, 0.75, 1, 2, 3, 5, 8, and 22 h) and was filtered through 0.45 µm filter (Frisenette ApS, Denmark). The withdrawn volume was replaced by fresh PBS buffer or FaSSIF buffer pH 6.5 respectively to keep the total volume constant. The samples were stored at room temperature within one day before analysis. All experiments were performed in triplicates.

2.2.5.3 Thermodynamic and apparent solubility studies

The (thermodynamic) solubility of crystalline CXB in PBS buffer pH 6.5 and the apparent solubilities of CXB, physical blends, spray-dried and freeze-dried formulations in the respective media (PBS buffer pH 6.5 and FaSSIF buffer pH 6.5) were determined by the shake flask method. Excess (10 mg of total CXB) of CXB, physical blend, or formulation were dispersed in 5 mL of the respective medium. The samples were placed onto a 25 °C shaking water bath (Julabo SW23, Buch & Holm, Denmark) and mechanical shaking were allowed at 100 rpm until equilibrium was reached. Upon equilibrium (on Day 7 according to preliminary study), the samples were centrifuged at 11,000 rpm for 1 h to separate the undissolved solid phase. The supernatant was filtered through a 0.45 µm filter before analyzing by HPLC. All experiments were performed in triplicates.

2.2.5.4 Quantification of molecularly dissolved drug

To analyze the amount of molecularly dissolved CXB in the optimal formulations (SD3 and FD3) in PBS and FaSSIF buffer pH 6.5, the method described by (Frank et al., 2012b) was used with slight modification. In brief, a dialysis kit with 6-8 kDa cut-off (Mini Pur-A-Lyzer, Sigma-Aldrich, USA) was employed. The Pur-A-Lyzer tubes were first filled with 250 µl of purified water and incubated for 5 min. The tubes were then emptied, filled with 200 µL of PBS buffer pH 6.5, set into a sponge-like floating device and placed into a 50 mL glass vial containing the respectively dispersed formulation (in excess) and media. The vials were placed onto a shaking water bath at 25 °C with a shaking speed of 100 rpm. Samples were drawn from inside the dialysis tubes under equilibrium

condition (on Day 7), diluted with methanol and analyzed by HPLC. All experiments were performed in triplicates.

2.2.6 Analytical method

High-performance liquid chromatography/ultraviolet detection (HPLC/UV, 2487 Dual Absorbance, Waters, USA) was employed for the quantification of CXB in all samples. chromatographic separation was achieved by a reverse-phased Acclaim® C18 column (150 mm × 4.6 mm i.d., 3 μm particle size, Thermo Fisher) equipped with a Acclaim® guard filter (5 μm, Thermo Fisher). According to our validated method (data not shown), isocratic elution with mobile phase consisting of 0.1% formic aicd:methanol (16:84) at a flow rate of 1 mL/min was employed, and the oven temperature was set at 30 ℃. The ultraviolet detection wavelength of CXB was set at 254 nm.

2.2.7 Statistical analysis

Data are presented as mean ± standard deviation. For the comparison of dissolution/solubility performance between each formulation group with that of the crystalline CXB, and between spray-drying and freeze-drying groups, statistical analyses were performed using one-way analysis of variance (ANOVA) with Tukey's post-hoc by SPSS® Statistics 16.0 (SPSS Inc.), with $p<0.05$ as the criterion of significance.

3. Results and Discussion

3.1 Structural analysis

By formulating crystalline CXB with PL and TRE using spray- and freeze-drying, upon complete dissolution of the compound it is anticipated that amorphous state would be generated. X-ray powder diffraction was employed to examine the degree of crystallinity of the raw and formulated CXB. As presented in **Figure 1(a)**, the X-ray diffractorgram of the raw CXB shows highly characteristic peaks at 2θ ranged from 15-25°, indicating the crystalline nature of CXB. Upon spray- and freeze-drying with PL and TRE at different drug-to-excipient ratios, the X-ray diffractograms exhibited characteristic peak-less patterns (**Figure 1(b)** and **1(c)**). This indicated that CXB was molecularly dispersed within the PLs in these formulations, thereby creating amorphous solid dispersions in all cases.

3.2 Particle morphology

Scanning electron microscopy was carried out to examine the surface morphology of raw CXB, spray- and freeze-dried formulations with CXB:PL:TRE compositions of 1:10:16 (*i.e.* SD3 and FD3). From **Figure 2(a)**, the raw CXB appeared to be crystalline and needle-shaped with a length of approximately 100 µm. The observed crystallinity of CXB is in agreement with our results from the X-ray diffractogram and with the observation by (Lee and Lee, 2013). The spray-dried formulation SD3 exhibited spherical morphology with diameters ranging from several micrometers down to the nano-range (<1 µm) (**Figure 2(b)**). Amorphous SPLNs were thus successfully created by formulating CXB with PL and TRE using spray-drying approach, as recently described by our group (Brinkmann-Trettenes and Bauer-Brandl, 2014). Freeze-dried CXB with PL and TRE (FD3), on the other hand, appeared to have an amorphous ‘matrix’-like structure with much larger particle sizes (**Figure 2(c)**).

3.3 Physical stability

A known phenomenon of amorphous solid dispersions is their tendency to re-crystallize upon storage. Therefore the physical stabilities (or the re-crystallization) of the formulations with the highest PL content (*i.e.* SD3 and FD3) were studied after storage in dehumidified room temperature (25 °C) for various durations. Results from X-ray diffraction suggested that both formulations were stable and remained in their amorphous state for up to 60 days, according to the peak-less X-ray diffractograms (figures not shown). In parallel, the physical stabilities of the formulations in ambient storage condition (*i.e.* 25 °C and with uncontrolled relative humidity of approximately 25-35%) were also evaluated. According to the X-ray diffractograms, the formulations showed a tendency to re-crystalize after 7 days under ambient conditions. This reveals that air moisture accelerates the re-crystallization of CXB in amorphous solid dispersions and shortened the storage period. Therefore, the optimal storage condition and duration of these spray-dried SPLNs and freeze-dried solid dispersions are recommended to be at 25 °C in a dry environment for 2 months.

3.3 Dissolution experiments

Dissolution studies were performed to evaluate the release profiles of CXB from formulations with different PL-to-drug ratios and to compare the dissolution enhancement effects by the two preparation methods (spray- *vs.* freeze drying).

3.3.1 Dissolution studies in PBS buffer pH 6.5

The dissolution study with crystalline CXB was not carried out since CXB crystals, particularly those of micronized quality, are poorly wettable. It appears that reproducible dissolution experiments in

pharmacopoeia apparatus type 1 or 2 are difficult. Furthermore, dissolution experiments of crystalline CXB in distilled water using 8 station USP 23 dissolution testing apparatus had been described by (Gupta et al., 2007). The amount of CXB released was below 1.8% of their preparation (which contained approximately 10-fold higher total amount of CXB compared to the present study) after 3 h. Therefore, it is expected that the total % release of CXB is less than 1% in PBS buffer pH 6.5. This expectation is confirmed by our results from the physical blend dissolution experiments. The total amount of CXB dissolved in PBS buffer pH 6.5 from the physical blend formulations was below 1% after 5 h (PB3) and 22 h (PB1 and PB2) respectively (**Table 2(a)**). With the addition of the largest PL content (PB3), the % release of CXB in 22 h increased to 10.31 ± 1.41%. According to the dissolution rate equation proposed by (Noyes and Whitney, 1897), dissolution of a substance can be increased by three mechanisms: (1) increasing the surface area of the solid particles (smaller particles), (2) reducing the thickness of the diffusion layer (stirring), or (3) increasing the (apparent) solubility of the solid (via alternative particle structure, composition or different dissolution medium). In this case of PB3, the increasing amount of PLs from formulation provides more liposomes in the aqueous medium that help solubilizing CXB. Therefore, with the increased apparent solubility of CXB (mechanism number (3)), the dissolution extent of CXB in PB3 is increased.

Spray- and freeze-drying CXB with PL and TRE significantly improved the release performance of CXB in PBS buffer pH 6.5. As shown in the dissolution profiles (**Figure 3**) and **Table 2(a)**, formulation with the highest PL content (SD3 and FD3) resulted in the fastest initial release and the highest % of CXB release. In general, the release appeared to be slow. Even with the largest amount of excipients, the highest release of the drug substance after 22 h was less than 35% (**Table 2(a)**). Formulations with smaller fractions of ingredients (CXB+PL+TRE 1+2.5+4 and 1+5+8) resulted

in even less release (2-19% release of CXB after 22 h for both formulations). Since the % of drug release (even in the presence of formulations) is considerably low, the quantified CXB amounts in the dissolution samples are very low and are sensitive to variabilities. Therefore, large relative standard deviations are observed (up to 70%). Nevertheless, considering the very low reference value (*i.e.* <1% release of CXB from PB), the improvement by solid PL dispersion is prominent since 2- to 34-fold increases in dissolution were achieved over 22 h. Furthermore, the extent of CXB release by SD3 and FD3 in PBS buffer was demonstrated to be comparable or superior to other CXB enabling formulations with liposomes (Deniz et al., 2010) or agglomerates with 10% polyvinylpyrrolidone (Gupta et al., 2007) respectively. The improvement of CXB dissolution by our formulations is attributed to the increased apparent solubility of CXB achieved by amorphous solid dispersion with PLs (mechanism (3)). In addition, a comparison between the dissolution enhancement of formulations produced by the two manufacturing method was made. It was found that there was no significant difference between the formulations produced by spray-drying and freeze-drying in terms of rate and extent of CXB dissolution enhancement (p>0.05, **Table 2(a)**).

3.3.2 Dissolution studies in FaSSIF buffer pH 6.5

Being a biorelevant medium that reflects the fasted state gastrointestinal tract fluid, FaSSIF was used in the current study as a dissolution medium in order to mimic the dissolution processes in the gastrointestinal tract with regards to the intended oral administration. Compared to the dissolution profiles in PBS buffer, the total release of CXB in FaSSIF buffer from physical blends of drug and excipients was largely increased (**Table 2(b)**). While the initial dissolution rate **(**% drug release over first hour) was still very low (≤1%), the % CXB released after 22 h reached 12% (PB1) and 43-48% (PB2 and PB3) respectively. Since FaSSIF contains sodium taurocholate and lecithin, a higher extent of

dissolution of CXB was expected due to increased apparent solubility by formation of mixed micelles (*i.e.* mechanism (3) from Noyes-Whitney equation). It should again be noted that for those formulations with % of release below 15%, large relative standard deviations (as high as 89%) are observed since the quantified CXB amounts in the dissolution samples are very low and are sensitive to variabilities.

Since the % of drug release (even in the presence of formulations) is considerably low, the quantified CXB amounts in the dissolution samples are very low and are sensitive to variabilities. Therefore, large relative standard deviations are observed (up to 70%).

Spray- and freeze-dried CXB with PL and TRE markedly enhanced the release behavior of CXB in FaSSIF buffer pH 6.5. The respective dissolution profiles are depicted in **Figure 4(a)** (spray-dried formulations) and **Figure 4(b)** (freeze-dried formulations). In both preparation methods, the initial dissolution rate and the extent of CXB released after 22 h directly depended on the excipient composition (*i.e.* higher PL content resulted in better dissolution behavior). At the optimal formulation (CXB + PL + TRE 1 + 10 + 16), the initial dissolution rates of SD3 and FD3 were 26.91 ± 9.55%/h and 34.09 ± 0.81%/h respectively, which were significantly increased compared with their corresponding PB3 dissolution rate (1.08 ± 0.45%/h) (p<0.05, **Table 2(b)**). The maximal % release of CXB after 22 h reached 80.60 ± 14.68% (SD3) and 89.79 ± 3.13% (FD3) respectively, which were significantly higher than that released from PB3 (48.07 ± 23.95%) (p<0.05). Overall, the dissolution extent of CXB was enhanced respectively by 1.7- to 3.2-fold by the formulations over 22 h. Similar to the observation as discussed in **Section 3.3.1**, spray- and freeze-dried formulations behaved similarity in terms of dissolution rate and extent improvement in FaSSIF medium (p>0.05).

3.4 Solubility studies

The (thermodynamic) solubility of crystalline CXB and the apparent solubilities of physical blends, spray- and freeze-dried formulations in PBS buffer pH 6.5 were determined after equilibrium condition was reached on Day 7. The time for CXB to reach equilibrium in aqueous medium is longer than those reported previously, which ranged from overnight (Morgen et al., 2012) to 24 h (Gupta et al., 2007) and to 3 days (Abu-Diak et al., 2011). The poor wettability of the crystalline CXB powder used in the current study may partly account for the longer equilibrium time, since part of the powder samples were clearly floating on top of the medium during the shaking process. The solubility of crystalline CXB in PBS buffer pH 6.5 at equilibrium was determined to be 1.33 ± 0.27 µg/mL (at 25 °C), which is comparable (1.58 µg/mL) or slightly lower than the literature reported values (2-4 µg/mL) (Abu-Diak et al., 2011; Gupta et al., 2007; Liu et al., 2010). The higher temperature condition used by the latter three studies (at 37 °C) for the determination of CXB solubility may explain the higher solubility values. The apparent solubilities of CXB in the physical blend formulations were proportionally increased in accordance to their PL content (**Figure 5(a)**): PB2 (4.66 ± 2.43 µg/mL) < PB3 (8.13 ± 4.57 µg/mL) < PB4 (15.94 ± 7.02 µg/mL). Taking the crystalline CXB and the physically blended CXB solubility values as references, the apparent solubilities of the spray-dried and freeze-dried formulations were significantly increased (**Figure 5(a)**, $p<0.05$). Independent of the preparation method, the formulation with the highest excipient content exhibited the highest apparent solubility (i.e. CXB + PL +TRE 1 + 10 + 16). Compared to the solubility of crystalline CXB (1.33 ± 0.27 µg/mL), the solubility of CXB in the freeze-dried formulations apparently increased to 9.54 ± 5.77 µg/mL (FD1), 24.39 ± 4.87 µg/mL (FD2), and 71.28 ± 18.09 µg/mL (FD3) respectively. At all three compositions, the spray-dried SPLN formulation demonstrated a significantly higher apparent solubility than that of their corresponding freeze-dried formulations (**Figure 5(a)**, $p<0.05$). The measured apparent solubility of

SD1, SD2 and SD3 were 70.66 ± 19.66 µg/mL, 107.78 ± 15.53 µg/mL, and 175.34 ± 59.59 µg/mL, respectively. At the optimal composition (*i.e.* the formulation with the highest PL content), FD3 and SD3 enhanced the apparent solubility of CXB in PBS buffer by factors of 54 and 132 respectively.

Subsequently, the apparent solubilities of crystalline CXB and the different formulations were determined in FaSSIF buffer pH 6.5 upon equilibrium for 7 days. A similar trend was observed in which the apparent solubility of CXB was increased along with the PL content present in the formulation (**Figure 5(b)**). Physical blends of CXB with excipients significantly increased the apparent solubility of CXB in FaSSIF (10.83 ± 3.21 µg/mL) to 57.70 ± 4.60 µg/mL (PB1), 66.75 ± 6.98 µg/mL (PB2), and 164.02 ± 26.64 µg/mL (PB3) respectively. Spray-dried and freeze-dried formulations significantly further increased the apparent solubility of CXB by 10.7- (SD1) to 29- (SD3) folds and by 12.0- (FD1) to 31.7- (FD3) folds respectively. Unlike the findings observed in PBS buffer, no significant difference was found between the apparent solubilities of the products from the two manufacturing methods. It had been suggested that two independent yet co-existing mechanisms, one related to the amorphous solid dispersion and one related to the dispersion medium (FaSSIF), could contribute to the observed solubility enhancement (Frank et al., 2012a). The solubilizing effects of FaSSIF, possibly via generation of taurocholate-CXB micelles and taurocholate-lecithin-CXB mixed micelles, may play a more dominant role in enhancing the solubility of CXB than that exerted by the formulation. Therefore, the solubility enhancement differences between the two preparation methods were not observed in FaSSIF but only in PBS buffer medium. In order to evaluate the 'true' solubility enhancement effect produced by the formulations, the molecularly dissolved drug concentration should be determined and this would be discussed in the following section.

3.5 Molecularly dissolved drug

It had been recently suggested that molecular solubility reflects the 'true' solubility (or 'true' supersaturation) and it is the key factor to biopharmaceutical performance assessment of enabling formulations (Buckley et al., 2013). In the current study, a dialysis method was performed to determine the concentration of molecularly dissolved CXB. The cut-off (6-8 kDa) was chosen such that only molecularly dissolved CXB could pass, but not the micellular- or liposomal-bound ones. Based on the results from dissolution tests (**Section 3.3**) and solubility studies (**Section 3.4**), the composition with the highest excipient content (CXB + PL + TRE 1 + 10 + 16) have the highest potential for further formulation development. Therefore, the molecular solubilities of CXB in SD3 and FD3 were further evaluated, along with crystalline CXB and PB3 which served as controls. The results of the molecular solubility studies are summarized in **Table 3**, and the apparent solubility results are also given as references. As shown in **Table 3**, the values of the molecularly dissolved CXB are much smaller compared to the corresponding apparent solubility values, indicating that the majority of solubilized CXB in micelles, liposomes, *etc.* was unable to pass through the dialysis tube and therefore this is a reliable method for measuring molecularly dissolved drug concentration. It should however be noted that in the presence of biorelevant medium, micelles/liposomes exist in dynamic equilibrium with free unassociated surfactant (bile salts) and phospholipid molecules at equilibrium state. These free surfactants may also be able to pass through the dialysis membrane and reform micelles inside the dialysis device, thus manipulating the "molecular" solubility values. While this is a potential limitation of the dialysis method in the presence of biorelevant medium, our earlier studies using dynamic light scattering method had provided evidence that supramolecular structures were absent in the dialysate (Frank, 2012). We thus hypothesize that while the unassociated surfactant and phospholipid molecules

are freely permeating the membrane, their concentrations are below the critical micelles concentrations such that micelles are not reformed inside the dialysis device.

The molecular solubility of crystalline CXB in PBS buffer was determined to be 1.23 ± 0.16 µg/mL using the dialysis setup, which is not different from its equilibrium solubility using classical shake flask method (1.33 ± 0.27 µg/mL). The presence of FaSSIF medium did not significantly increase the molecularly dissolved CXB concentration (2.41 ± 0.63, $p>0.05$), unlike the observation found from apparent solubility (**Table 3**). Similarly, dialysis of the physical blend PB3 dispersed in PBS buffer did not reveal an increase in molecularly dissolved CXB concentration (1.83 ± 0.53 µg/mL *vs.*1.23 ± 0.16 µg/mL). However, considerable 'true' supersaturation of CXB was achieved by spray-dried SPLNs. In the case of SD3 dispersed in PBS buffer, the molecular solubility of CXB increased by 18-fold in comparison to that of crystalline CXB (22.58 ± 3.62 µg/mL, $p<0.05$). The above observations indicate that supersaturation is not related to the mere presence of the excipients (from FaSSIF or physical blend PL excipients) but the amorphous state of CXB in the spray-dried formulation. The current findings are in agreement with that reported by (Frank et al., 2012a) using a poorly soluble drug ABT-102. Interestingly, about the same extent of 'true' supersaturation (*i.e.* enhanced molecular solubility of CXB by SD3) was observed in FaSSIF (17-fold increment) as compared to that in PBS (18-fold increment). Similar observations were applied to freeze-dried formulation, in which the molecular solubility of CXB increased by a factor of 3 and 4 in PBS and FaSSIF media respectively. This indicates that 'true' supersaturation was obtained irrespective of the dispersion medium. It is known that supersaturation is a physically unfavorable state and the molecularly dissolved drug will precipitate from its metastable supersaturation state over time. In the current study, the experimental time for CXB to reach its equilibrium solubility was 7 days. It is surprising that supersaturation could be maintained for such a long time for the SPLNs and freeze-dried

formulations. Without the addition of precipitation inhibitor, the sustained supersaturation state achieved by the formulations is clearly an advantage.

Comparing the two manufacturing methods, the extent of molecular solubility enhancement by spray-drying was significantly much higher than that by freeze-drying (p<0.05 in PBS buffer, **Table 3**). In FaSSIF medium, although a significant difference was not found (p=0.09) due to their large standard deviations, the molecular solubility of spray-dried SD3 was found to be 4 times higher than that of the freeze-dried FD3 (40.46 ± 27.91 *vs.* 10.61 ± 6.11 µg/mL). The higher molecular solubility achieved by spray-drying may probably be due to the spherical morphology of the spray-dried SPLNs and/or other factors attribute to the processing method. The distinct small particle structure (in lower micrometer and nanometer range) of the spray-dried SPLNs as observed by the scanning electron microscopy may also play a role in its higher molecular solubility. It should however be noted that while scanning electron micrographs identify apparent morphology differences, a quantitative analysis of *e.g.* particle size distribution or surface area measurement is necessary for confirming the difference between SPLNs and freeze-dried materials.

To our knowledge, the current study is the first to report a difference between the two preparation methods in terms of molecular solubility; while existing studies only compared the dissolution and/or apparent solubility enhancement (Dontireddy and Crean, 2011; Singh et al., 2013). It has been suggested that only molecularly dissolved drug can cross the intestinal barrier and be absorbed *in vivo*, independent of the high solubilization state (Frank et al., 2012b). Therefore, the stronger capability of the spray-dried SPLNs to attain 'true' supersaturation state makes spray-drying a more promising method than freeze-drying for bioavailability enhancement of poorly soluble drugs. Apart from having the superiority in achieving higher 'true' supersaturation, spray-drying has other

advantages over freeze-drying, such as lower operation cost and better powder processability (Desobry et al., 1997; Singh et al., 2013). Taken these issues together, spray-dried SPLNs appeared to be an attractive candidate-enabling formulation for BCS class II drugs.

4. Conclusion

Solid phospholipid nanoparticles (SPLNs) demonstrated a promising candidate-enabling approach for drugs with poor aqueous solubility. In terms of surface morphology, particle size and solubility enhancement, spray-drying appeared to be a better preparation method than freeze-drying. 'True' supersaturation was achieved by SPLNs and they have a great promise in increasing the oral bioavailability of BCS Class II drugs.

Acknowledgement

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Figure Captions

Figure 1. X-ray diffractograms of (a) crystalline CXB, (b) spray-dried formulations and (c) freeze-dried formulations.

Figure 2. Scanning electron microscopy micrographs of (a) crystalline CXB (scale bar 100 µm), (b) formulation SD3 (spray-dried CXB + PL + TRE 1 + 10 + 16, m/m, scale bar 10 µm), and (c) formulation FD3 (freeze-dried CXB + PL + TRE 1 + 10 + 16, m/m, scale bar 100 µm).

Figure 3. Dissolution profiles of CXB in (a) spray-dried and (b) freeze-dried formulations with PL and TRE at different ratios in PBS buffer pH 6.5. Studies were performed using USP type I apparatus at 50 rpm in 500 mL PBS buffer pH 6.5, with a total amount of 5 mg CXB in each vessel. Data represent mean ± standard deviation (n=3).

Figure 4. Dissolution profiles of CXB in (a) spray-dried and (b) freeze-dried formulations with PL and TRE at different ratios in FaSSIF buffer pH 6.5. Studies were performed using USP type I apparatus at 50 rpm in 500 mL FaSSIF buffer pH 6.5, with a total amount of 5 mg CXB in each vessel. Data represent mean ± standard deviation (n=3).

Figure 5. Equilibrium solubilities of crystalline CXB and apparent solubilities of physical blends (PB1, PB2, PB3), freeze-dried formulations (FD1, FD2, FD3), and spray-dried formulations (SD1, SD2, SD3) in (a) PBS buffer pH 6.5 and (b) FaSSIF buffer pH 6.5 at 25 °C. Data represent mean ± standard deviation (n=3).

Figure 1

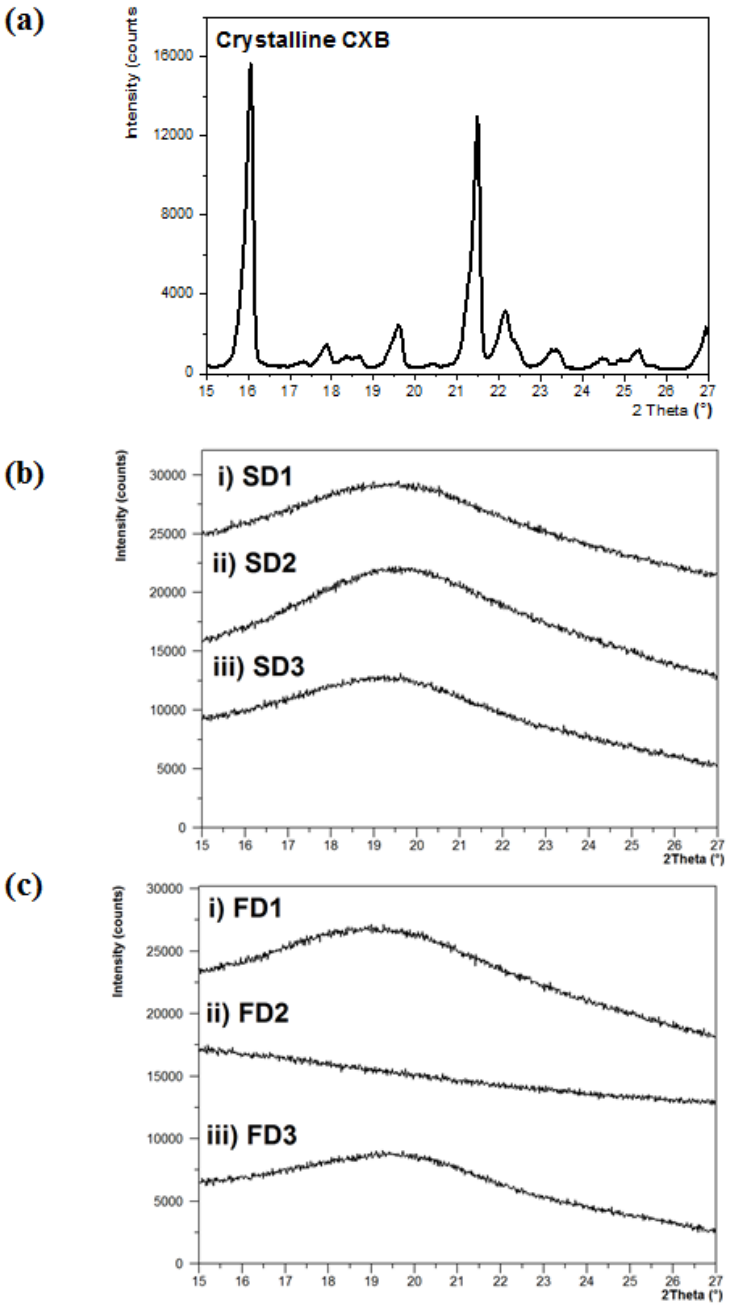


Figure 2

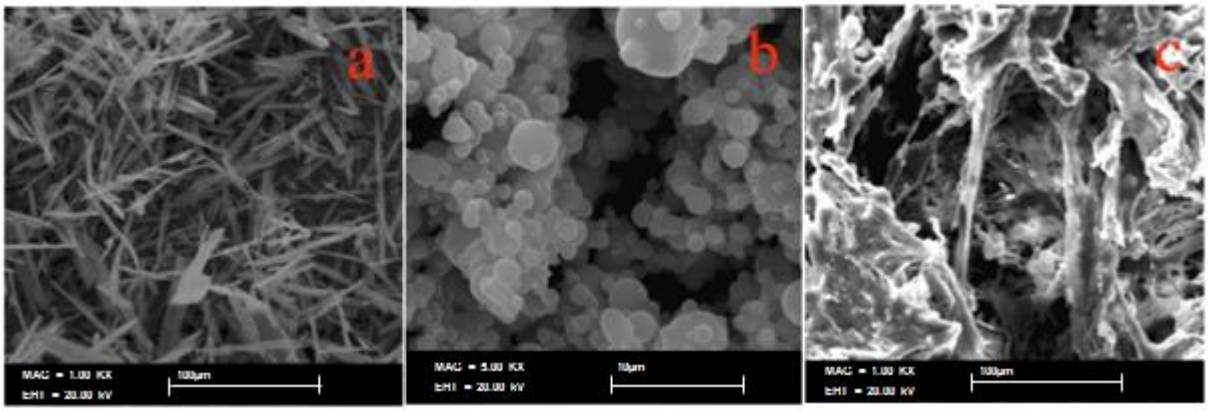
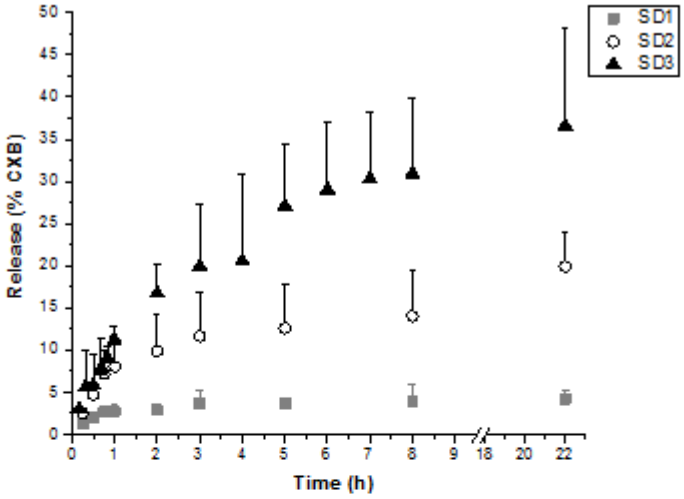


Figure 3

(a)



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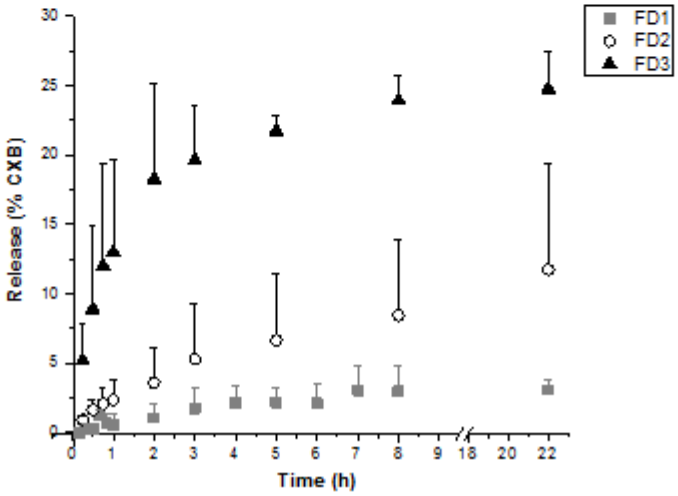
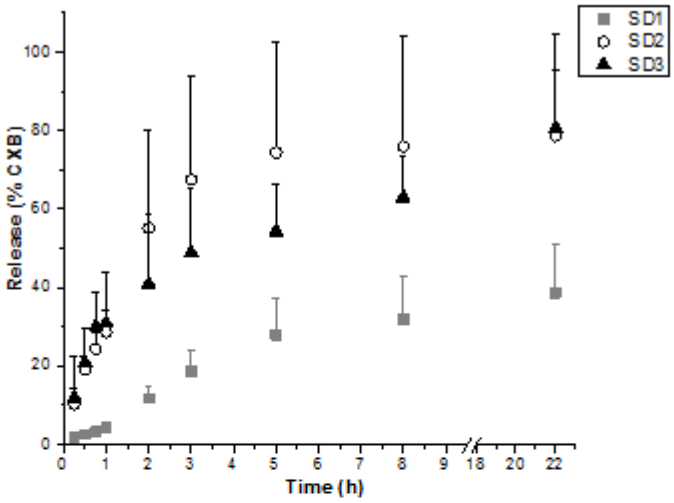


Figure 4

(a)



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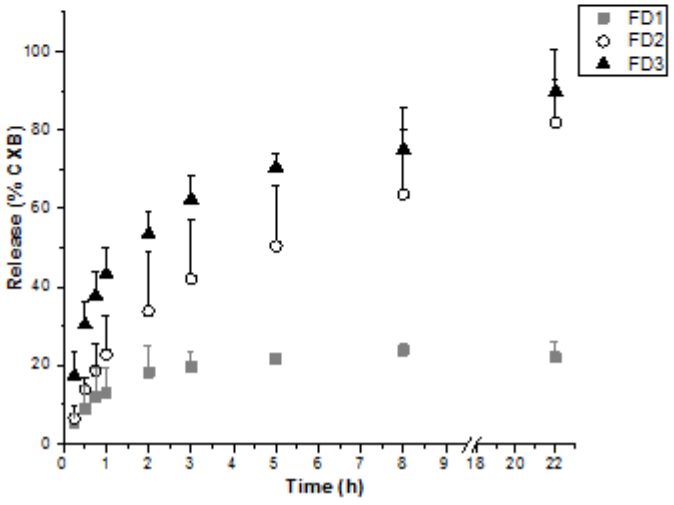
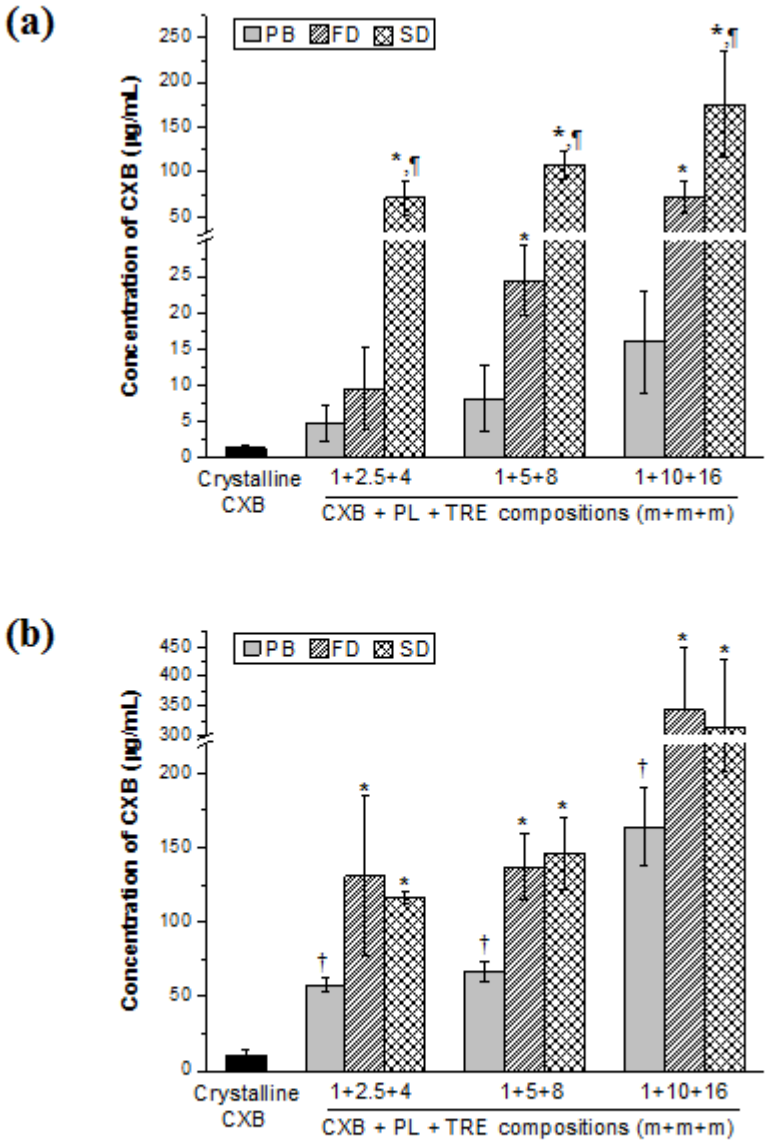


Figure 5



† $p<0.05$ compared to crystalline CXB
*$p<0.05$ compared to both crystalline CXB and corresponding PB group with the same composition
¶ $p<0.05$ compared to corresponding FD group with the same composition

Tables

Table 1. Compositions of the studied formulations.

Table 2. Initial dissolution rate and % CXB released after 5 and 22 h, from the nine studied formulations in (a) PBS and (b) FaSSIF buffer pH 6.5. Data presented as mean ± standard deviation, *n*=3.

Table 3. Apparent solubility and molecularly dissolved concentration of CXB in (a) PBS buffer pH 6.5 and (b) FassIF buffer pH 6.5, in physical blend, freeze-drying and spray-drying formulations with composition of 1+10+16 (CXB+PL+TRE). Data represent mean ± standard deviation (*n*=3).

Tables

Table 1. Compositions of the studied formulations.

Formulation code	Production method	Composition (mass)			Solvent
		CXB	PL	TRE	
CXB crystalline	N/A[a]	1	0	0	N/A
PB1	Physical blend	1	2.5	4	N/A
PB2		1	5	8	N/A
PB3		1	10	16	N/A
SD1	Spray drying	1	2.5	4	Ethanol in H_2O
SD2		1	5	8	(80:20, w/w)
SD3		1	10	16	
FD1	Freeze drying	1	2.5	4	Tert-butanol in H_2O
FD2		1	5	8	(60:40, w/w)
FD3		1	10	16	

[a]N/A: not applicable

Table 2. Initial dissolution rate and % CXB released after 5 and 22 h, from the nine studied formulations in (a) PBS and (b) FaSSIF buffer pH 6.5. Data presented as mean ± standard deviation, *n*=3.

Formulation	Composition (CXB + PL + TRE)	Initial dissolution rate (% drug release over first h)	% CXB released after 5 h	% CXB released after 22 h
(a) PBS buffer pH 6.5				
Crystalline CXB	N/A[a]	Not studied[b]	Not studied	Not studied
PB1	1 + 2.5 + 4	n.d.[c]	<1	<1
PB2	1 + 5+ 8	n.d.	<1	<1
PB3	1 + 10 + 16	n.d.	<1	10.31 ± 1.41
SD1	1 + 2.5 + 4	2.04 ± 0.95	3.68 ± 0.20	4.20 ± 1.13
SD2	1 + 5+ 8	7.64 ± 4.97	12.60 ± 5.11[†]	19.39 ± 3.98
SD3	1 + 10 + 16	10.56 ± 2.10	27.07 ± 7.24[†]	36.58 ± 11.50[†]
FD1	1 + 2.5 + 4	1.22 ± 0.40	2.14 ± 1.05	3.07 ± 0.67[†]
FD2	1 + 5+ 8	2.02 ± 1.45	6.65 ± 4.78	11.74 ± 7.62
FD3	1 + 10 + 16	10.63 ± 5.49	21.73 ± 1.15[†]	24.75 ± 2.70
(b) FaSSIF buffer pH 6.5				
Crystalline CXB	N/A	Not studied	Not studied	Not studied
PB1	1 + 2.5 + 4	n.d.	5.60 ± 2.29	12.22 ± 1.21
PB2	1 + 5+ 8	n.d.	12.03 ± 10.76	43.11 ± 9.05
PB3	1 + 10 + 16	1.08 ± 0.45	10.41 ± 6.08	48.07 ± 23.95
SD1	1 + 2.5 + 4	3.59 ± 0.95	28.03 ± 9.22[†]	38.69 ± 12.21[†]
SD2	1 + 5+ 8	24.10 ± 9.72	74.42 ± 28.11[†]	78.65 ± 25.70
SD3	1 + 10 + 16	26.91 ± 9.55[†]	54.25 ± 12.09[†]	80.60 ± 14.68[†]
FD1	1 + 2.5 + 4	10.63 ± 5.49	21.73 ± 1.15[†]	22.12 ± 3.96
FD2	1 + 5+ 8	12.75 ± 4.92	50.44 ± 15.66	81.99 ± 18.56
FD3	1 + 10 + 16	34.09 ± 0.81[†]	70.49 ± 3.31[†]	89.79 ± 3.13[†]

[a]N/A: not applicable; [b]Not studied since the total % release of CXB is expected to be <<1%; [c]n.d.: cannot be determined since the % release of CXB in the first hour is <<1%; [†]$p<0.05$ compared to corresponding PB group with the same composition

Table 3. Apparent solubility and molecularly dissolved concentration of CXB in (a) PBS buffer pH 6.5 and (b) FaSSIF buffer pH 6.5, in physical blend, freeze-drying and spray-drying formulations with composition of 1+10+16 (CXB+PL+TRE). Data represent mean ± standard deviation (*n*=3).

Formulation	Apparent solubility (μg/mL)	Molecular solubility (μg/mL)
(a) PBS buffer pH 6.5		
CXB crystalline	1.33 ± 0.27	1.23 ± 0.16
PB3	15.94 ± 7.02	1.83 ± 0.53
FD3	71.28 ± 18.09*	3.14 ± 0.43
SD3	175.34 ± 59.56*,¶	22.58 ± 3.62*,¶
(b) FaSSIF buffer pH 6.5		
CXB crystalline	10.83 ± 3.21	2.41 ± 0.63
PB3	164.02 ± 26.64†	5.11 ± 1.93
FD3	343.35 ± 105.03*	10.61 ± 6.11
SD3	314.32 ± 113.83*	40.46 ± 27.91*

*p<0.05 compared to both crystalline CXB and corresponding PB group with the same composition

¶ p<0.05 compared to corresponding FD group with the same composition

† p<0.05 compared to crystalline CXB