DEVELOPMENT OF PEDIATRIC ANTI-HIV FORMULATIONS WITH IMPROVED DISSOLUTION CHARACTERISTICS

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DEVELOPMENT OF PEDIATRIC ANTI-HIV FORMULATIONS WITH IMPROVED DISSOLUTION CHARACTERISTICS

BY

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ABSTRACT

The human immunodeficiency virus (HIV) impacts up to 37 million people globally. Although not fatal on its own, HIV can develop into acquired immunodeficiency disease (AIDS), in which a person’s immune system becomes compromised. To date, there is no cure for HIV, although many treatment options are available. Despite their effectiveness, these treatments are commonly plagued by their inherent complexity. Factors such as doing regimen, pill burden, and undesirable side effects all contribute to variability in patient compliance, particularly in pediatric populations. Currently, there is no anti-HIV drug product readily available for pediatrics, despite close to 1.8 million children living with HIV. This is partially due to a diverse patient population (ranging from birth till adolescence age) with specific needs for various dosage forms and dosing unit size. In addition, taste preferences and toxicity of excipients and may differ in children compared to adults.

In the present study, we aimed to develop a pediatric-friendly formulation for anti-HIV therapeutics. Two protease inhibitors, lopinavir (LPV) and ritonavir (RTV) (commercially available as Kaletra®), were chosen as model drugs. Kaletra® is a fixed-dose combination (FDC) of LPV and RTV (4/1, w/w) in either a tablet or an oral solution form. However, neither of these dosage forms is suitable for children. The tablet is large, and therefore can be difficult to swallow for young children, especially for children under four years who generally cannot swallow tablets. In addition, the excipients used in the tablet formulation have been shown to induce adverse events in a pediatric population. On the other hand, the oral solution contains upwards of 40% ethanol and is not suitable for children. Both of these drugs exhibit very bitter taste
profiles, which children are very sensitive to. In addition, both LPV and RTV, are inherently poorly water-soluble and suffer from low bioavailability. In order to develop a pediatric-friendly formulation for FDC of LPV and RTV, it is critical to improve dissolution and palatability of the therapeutics using safe excipient(s).

Cyclodextrins (CD) are cyclic oligosaccharides that can form water-soluble complexes with hydrophobic drugs, and potentially enhance solubility and mask taste of the therapeutics. In this study, two CD derivatives, 2-hydroxypropyl-β-CD (HP-β-CD) and 2-HP-γ-CD were investigated. Phase solubility, isothermal titration calorimetry (ITC), nuclear magnetic resonance (NMR) and molecular modelling studies were conducted to determine interactions between them and the two anti-HIV drugs, LPV and RTV. The results showed that complexes can be formed between drug and CD and the optimal complexion ratio of drug/CD is 1:1. The results from each study showed that RTV is capable of forming more stable complexes than LPV, with both types of CD. Stability constant values calculated via phase solubility studies indicated that β-CD formed more stable complexes with the drugs than γ-CD. However, a different trend was obtained from the NMR and molecular modelling studies, which showed that γ-CD formed more stable complexes. This suggested that non-inclusion complex formation was favored, which NMR and modelling are less sensitive to detecting, over traditional inclusion complex formation. These studies also showed that the specific interactions that occurred between LPV and CD, and RTV and CD, such as hydrogen bonding and hydrophobic interactions, were different, as each drug has a fundamentally unique molecular structure.
Following this interaction analysis, formulation optimization of drug:CD complexes was conducted. The prepared drug:CD complexes were spray dried to obtain a final dry powder formulation. Solid state characterization of the spray-dried complexes was performed to determine physicochemical characteristics such as thermal profile, crystallinity, and morphology. Results showed that the spray-dried complexes did not exhibit a melting temperature, and were comprised of drug in an amorphous state, based on differential scanning calorimetry (DSC), X-ray diffraction (XRD) and polarized light microscopy (PLM) data. In addition, scanning electron microscopy (SEM) images showed that the spray-dried complexes exhibited a corrugated, raisin-like morphology. In vitro dissolution studies showed that RTV in an amorphous state exhibits a faster release profile than crystalline RTV. Spray-dried HP-β-CD/RTV complexes showed the most favorable dissolution profile, as 100% RTV was released in 45 minutes. Unexpectedly, converting LPV from crystalline to amorphous via spray-drying resulted in lower dissolution rate and extent. In addition, spray-dried CD/LPV complexes did not exhibit favorable dissolution characteristics, compared to the physical mixture of LPV, polymer, and CD.

Overall, interactions between both drugs and both CDs were characterized, and CD/drug complexes were successfully prepared. Further studies will be conducted to assess taste masking effect and in vivo bioavailability of the prepared drug/CD complexes. In addition, other strategies such as freeze drying and kneading will be investigated in the future to further optimize a suitable formulation with improved dissolution characteristics for LPV.
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Working under the guidance of two advisors has taught me that everyone is capable of bringing something different to the table, especially terms of research. They are both experts in their respective fields, and reaffirm that science is truly an interdisciplinary field. With that said, I would like to thank our collaborators in the Department of Chemistry, Dr. Brenton DeBoef and Dr. Ashvin Fernando for their expertise in NMR and molecular modelling. I would also like to thank Dr. Bongsup Cho and Rachel Carley for their expertise in ITC. In addition, I would like to thank my committee members, Dr. Jyothi Menon, Dr. Daniel Roxbury, and Dr. Brenton DeBoef for chairing my defense. Thank you to Dr. Irene Andreu Blanco and RIN², and the RI-INBRE Centralized Research Core Facility for allowing me access to vital pieces of
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PREFACE

This thesis was written and formatted in accordance with guidelines provided by the University of Rhode Island Graduate School and contains four chapters: Introduction, Materials and Methods, Results and Discussion, and Conclusion and Future Work. I hope you enjoy reading about my work completed over the past year.
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CHAPTER 1

INTRODUCTION

1.1 Background and Treatment of HIV

The human immunodeficiency virus (HIV) is a retrovirus that can lead to a chronic, incurable disease that impacts upwards of 37 million people around the world, of which approximately 1.8 million are children.\(^1\) Despite recent advancements in anti-HIV therapeutics, HIV remains a major public health concern. In particular, the virus remains an epidemic in regions of the world where resources are scarce. Countries like Botswana and South Africa are plagued by the virus, and they represent two of the regions in the world that have the largest population of HIV-infected patients.\(^2\)

HIV is capable of binding to various types of white blood cells, but most commonly attaches to T lymphocytes through the CD4 receptor. Figure 1.1 illustrates the replication process of HIV, where once the virus is inside the cell, it releases its RNA and begins converting RNA to DNA using the enzyme reverse transcriptase.\(^3\) The conversion of RNA to DNA is inherently error prone, and therefore mutations easily occur. After this step, newly synthesized viral DNA enters the cell nucleus and becomes integrated with the cell’s DNA via the enzyme integrase. New viral RNA and proteins are then produced from the DNA of the infected cell, and eventually a new HIV virus is created. Finally, the virus buds through the cell membrane as the enzyme HIV protease cleaves newly synthesized polyproteins, effectively creating a mature
infectious virus.\textsuperscript{4} Ultimately, the virus is capable of spreading and infecting other cells, destroying white blood cells, and weakening the body’s immune system. As a result, the body is less able to fight off infections and can eventually succumb to one if left untreated.

**Figure 1.1.** Schematic of an overview of the HIV replication process.\textsuperscript{5}

The current standard in the treatment of HIV involves the use of antiretroviral therapy (ART). ART is the combination of antiretroviral agents that target different processes in the HIV replication cycle.\textsuperscript{6} This type of combination therapy has been
shown to be effective in dramatically suppressing viral replication and reducing HIV plasma concentrations to below detectable limits. ART regimens vary based upon factors including viral load, CD4 cell count prior to treatment, the results of the HIV genotypic drug resistance test, and/or anticipated adherence. The World Health Organization (WHO) has recommended a combination of tenofovir (TDF), emtricitabine (FTC), and efavirenz (EFV) for those patients just beginning ART.

Despite the effectiveness of combination therapy, major concerns remain regarding patient adherence to such therapy. Since ART targets various underlying mechanisms of HIV replication, numerous drugs are needed to ensure its effectiveness, and as a result, the overall complexity of ART regimens has increased. Factors such as dosing complexity, pill burden, dietary restrictions, and undesirable side effects such as gastrointestinal (GI) intolerance including diarrhea, nausea, and vomiting can contribute to the challenge of maintaining patient adherence to ART. The potential alternative, fixed-dose combinations (FDC), are advantageous as they have been shown to improve patient adherence by reducing the pill burden faced by those on a strict ART regimen. Therefore, the importance of developing FDC formulations cannot be overlooked. Recently, scientists developed a formulation containing rilpivirine (RPV), a non-nucleoside reverse transcriptase inhibitor (NNRTI), and cabotegravir (CAB), an investigational new drug that serves as an integrase inhibitor. Phase II clinical trials were completed, and it was shown that taking this formulation either once a month or once every two months, via intramuscular injection, could effectively maintain viral suppression. This type of
formulation is clearly advantageous, as most ART therapies require daily dosing to achieve adequate viral suppression.

Kaletra® is an FDA-approved FDC product containing both lopinavir (LPV) and ritonavir (RTV) available in a tablet form (400 mg/100 mg and 100 mg/25 mg LPV/RTV) or as an oral solution (80 mg/mL LPV: 20 mg/mL RTV in 42.3% ethanol and 15.3% propylene glycol). The structures of LPV and RTV are shown in Figure 1.2. LPV is a selective inhibitor of type 1 HIV (HIV-1) protease that works by preventing viral maturation that ultimately results in the spread of infection. Administered alone, LPV has insufficient bioavailability due to extensive metabolism by the cytochrome P450-3A4 (CYP3A4) enzymes, which is the primary class of enzymes present in the liver responsible for metabolizing protease inhibitors. RTV is a protease inhibitor, albeit a much less potent one than LPV, and is capable of inhibiting CYP3A4 enzymes, and therefore aids in boosting the plasma concentration of and increasing the bioavailability of LPV. A recent study investigated the interaction between RTV and CYP3A4 enzymes and concluded that CYP3A4 inactivation results from RTV binding to Lys257 of the CYP3A4 apoprotein. Therefore, formulations containing RTV may require less frequent dosing due to the protease inhibitor boosting effect, and may result in a reduction in side effects.
1.2 Pediatric Anti-HIV Formulations

Regardless of the disease, developing an oral pediatric formulation is often more complex than developing one for adults, as there are more confounding variables that impact overall performance. It has been shown that children exhibit fundamental pharmacokinetic differences compared to adults, including gastrointestinal permeability, rate of gastric emptying, and surface area available for drug absorption. In addition, a child’s size, age, and taste preference can play a key role in determining medication adherence, and ultimately overall effectiveness. A recent review highlighted the challenges and potential solutions to developing pediatric formulations. With respect to oral dosage forms such as pills and tablets, size is a very important parameter when considering whether or not the formulation is safe for pediatric patients. Children aged two-six years old can swallow mini-tablets (two mm in diameter), however, larger tablets present a greater challenge.

Although various treatments options exist for adults, there have been limited efforts devoted to developing anti-HIV drug products suitable for pediatric populations. Anti-HIV drugs are listed on the NIH’s Best Pharmaceuticals for
Children’s Act (BPCA) Priority Needs List for 2018-2019, which cites the specific need to develop heat and light stable anti-HIV formulations with taste masking abilities.\textsuperscript{22} Despite the effectiveness of combination therapy with RTV and LPV, these two therapeutics have disadvantages such as poor water solubility and hence low bioavailability, and bitter taste profiles.\textsuperscript{23} Drug products such as Kaletra\textsuperscript{®} suffer from drawbacks including large tablet size, making swallowing them extremely difficult for children. In addition, the oral solution contains a large volume of ethanol, which is especially unsuitable for pediatric patients. Furthermore, both tablet and solution formulations are often associated with unwanted dose-dependent side effects such as diarrhea, nausea, and vomiting.\textsuperscript{24}

According to the Biopharmaceutics Classification System (BCS), both LPV and RTV are characterized as Class II drugs, exhibiting low solubility and high permeability, and hence variable bioavailability and high doses.\textsuperscript{25} There are a variety of strategies that are commonly used to enhance dissolution, and hence oral bioavailability, of poorly water-soluble drugs including micronization, micellar formation, and complex formation.\textsuperscript{26} Recent studies have shown improved efficacy of LPV and RTV using poly(lactic-co-glycolic acid) (PLGA) nanoparticles as the delivery carrier, in addition to improved LPV absorption using an electrospray encapsulation technique.\textsuperscript{27,28} Micronization techniques such as jet-milling and homogenization result in an increased surface area to volume ratio, thus allowing for more interaction between the drug particle surface and solvent and hence improved dissolution.\textsuperscript{29} In the formation of micelles, amphiphilic molecules arrange themselves into a spherical form, and hydrophobic drugs can form molecular interactions with the
inner hydrophobic core of micelles, thus enhancing the overall solubility of the drug-micelle system.\textsuperscript{30} In addition, lipophilic drugs can form complexes with carbohydrate polymers such as cyclodextrins, thereby enhancing dissolution rate and solubility of poorly water-soluble drugs.\textsuperscript{31}

### 1.3 Cyclodextrin Complexes

Current challenges involved in the development of anti-HIV pediatric formulations include the need to improve drug solubility and dissolution, improve product stability, and provide taste masking effects. One potential approach aimed at addressing these issues is the development of drug-cyclodextrin complexes. Shown in Figure 1.3, cyclodextrins are six-, seven-, or eight-membered oligosaccharides formed from the enzymatic degradation of starch.\textsuperscript{32} These supramolecular structures are cone-shaped, exhibiting a hydrophilic outer shell comprised of primary and secondary alcohol groups, and a hydrophobic core that can accommodate many non-polar therapeutics by forming inclusion and non-inclusion complexes.\textsuperscript{33}

**Figure 1.3.** Schematic diagrams of different types of cyclodextrin including α-, β-, and γ-cyclodextrins.\textsuperscript{34}
Drug-cyclodextrin complex formation arises when a drug is introduced to an aqueous cyclodextrin solution, resulting in the removal of enthalpy-rich water molecules from the hydrophobic cyclodextrin core. As water molecules are removed, non-polar drug molecules are able to maneuver into the center of cyclodextrin cavities, and form molecular interactions including hydrophobic, van der Waals, and electrostatic interactions.\textsuperscript{31} No covalent bonds are formed or broken during this process and bound drug molecules inside the cyclodextrin molecules are in dynamic equilibrium with free drug molecules.\textsuperscript{35} The simplest and most common type of complexation that can occur results in a binary system, in which only two different types of molecules are used (i.e. drug and cyclodextrin). As shown in Figure 1.4, drug and cyclodextrin molecules can form inclusion as well as non-inclusion complexes. As seen in this figure, not all drug molecules will form inclusion complexes with cyclodextrin molecules and it provides a much more realistic representation of possible interactions between drug and cyclodextrin molecules. Despite this complexity, drug-cyclodextrin complexes remain relatively simple to characterize. Analytical techniques such as nuclear magnetic resonance, molecular modeling, and isothermal calorimetry can be used to help explain the interactions between guest and host compounds.
Cyclodextrins have been used in various applications including use as food additives, cosmetics, and pharmaceutical drug carriers.\textsuperscript{35} The toxicity of cyclodextrins has been studied in recent years, and studies have shown no apparent toxicity from cyclodextrin when developed as an oral formulation, mainly due to lack of absorption in the GI tract.\textsuperscript{37} Native cyclodextrins (without modification) have limited aqueous solubility, and therefore in recent years they have been chemically modified to improve their aqueous solubility. 2-hydroxypropyl-beta-cyclodextrin (HP-\(\beta\)-CD) and 2-hydroxypropyl-gamma-cyclodextrin (HP-\(\gamma\)-CD) are two examples of cyclodextrin derivatives that have enhanced solubility profiles compared to parent cyclodextrins.\textsuperscript{38} These modified cyclodextrins may be ideal candidates for pediatric formulations due to their impressive safety profile, as recent studies have shown no apparent toxicity in juvenile or adult rats.\textsuperscript{39}
Some work has previously been done to investigate techniques to improve the solubility and dissolution profiles of LPV and RTV. A recent study showed that cyclodextrin-LPV complexes can improve the *in vitro* dissolution profile of LPV, and that more research is needed to utilize their full potential.\(^{40}\) However, in the described study, dissolution studies were performed under sink conditions using 0.06 M polyoxyethylene 10 lauryl ether, which does not accurately reflect the conditions of the GI tract. Additionally, the study investigated the use of cyclodextrins on the enhancement of LPV alone and did not investigate solubility enhancement strategies for RTV.

Cyclodextrins not only aid in the enhancement of stability and solubility in formulations, but they are also commonly employed for their taste masking abilities. This is significant attribute because a major concern in developing pediatric formulations is the palatability of the formulation. Studies have shown that children have a low tolerance for poor-tasting medications, which may negatively impact patient compliance, and overall medication effectiveness.\(^{20}\) One study concluded that more than 90\% of pediatricians reported that a drug’s taste and palatability presented the largest obstacle to overcome for children completing treatment.\(^{41}\) Additionally, many therapeutics, including LPV and RTV, have been reported as having very bitter, unpleasant taste profiles.\(^{42}\) Cyclodextrins can act as taste masking agents by forming complexes with bitter drugs, effectively shielding this property of the drug. A recent study investigated the taste masking effect of HP-\(\beta\)-CD on ranitidine hydrochloride, a common therapeutic used to treat a variety of GI diseases such as duodenal ulcer, reflux oesophagitis, and Zollinger-Ellison Syndrome. It was concluded through the use
of electronic taste sensing systems that increasing the molarity of cyclodextrin in a formulation resulted in the reduction of bitter taste.\textsuperscript{43}

In some instances, the solubility of binary drug-cyclodextrin complexes is rather low, leading scientists to look for additional strategies to further enhance drug solubility. It has been shown that the addition of water-soluble polymers, forming a ternary formulation (i.e. drug-cyclodextrin-polymer), can enhance the traditional drug-cyclodextrin complexation process, resulting in a system with improved water solubility and stability.\textsuperscript{44} Other studies have corroborated this effect, showing that the addition of water-soluble polymers to a drug-cyclodextrin system resulted in higher drug solubility than when using cyclodextrin or polymer alone.\textsuperscript{45} Water-soluble polymers can also aid in the overall stability of the final formulation, acting as drug crystallization inhibitors.\textsuperscript{45} Despite these advantages, ternary systems are much more difficult to analyze in terms of identifying specific molecular interactions occurring between the drug, polymer, and cyclodextrin.
CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Methanol (Chromasolv™, ≥ 99%), hydrochloric acid, trifluoroacetic acid (TFA), hydroxypropyl cellulose (HPC) and polyvinyl alcohol (PVA) were purchased from Sigma Aldrich (St. Louis, MO). Phosphate buffered saline (PBS) and Hydranal KF reagent were purchased from Fisher Scientific (Waltham, MA). 2-Hydroxypropyl-β-cyclodextrin (HP-β-CD, MW: 1,400 Da) and 2-hydroxypropyl-γ-cyclodextrin (HP-γ-CD, MW: 1,540 Da) were generously provided by Wacker Chemical Corporation (Adrian, MI). Lopinavir (LPV, MW: 628.80 Da) was purchased from AK Scientific (Union City, CA). Ritonavir (RTV, MW: 720.94 Da) was purchased from eNovation Chemicals (Bridgewater, NJ). Soluplus® and Kollidon® were kindly gifted by Badische Anilin-und Soda-Fabrik (BASF) (Ludwigshafen, Germany). Polyvinylpyrrolidone (PVP, K30), PVP VA 64, and hydroxypropyl methylcellulose (HPMC E15) were kindly gifted by JRS Pharmaceuticals (Patterson, NY). Deuterated methanol (methanol-d₄), deuterium oxide (D₂O), and trimethylsilane (TMS) were purchased from Cambridge Isotope Laboratories (Tewksbury, MA).

2.2 Phase Solubility Studies

Phase solubility studies of LPV and RTV were carried out in aqueous solutions containing either HP-β-CD or HP-γ-CD from 1.25 mM to 20 mM. Samples were prepared in triplicate and shaken at room temperature for 48 hours. The amount of
LPV and RTV in solution was determined using high performance liquid chromatography (HPLC, Shimadzu Nexera I 2040c, Japan) with a Phenomenex C18 column (4.6 × 250 mm, 5 µm). The mobile phase was composed of methanol/0.1% TFA in water (85/15, v/v) at a flow rate of 1 mL/min and the injection volume was 20 µL. Retention times were approximately 4.7 min for LPV and 3.9 min for RTV. A phase diagram was created by plotting the concentration of cyclodextrin versus the resulting drug solubility. For each drug:CD combination, these data were fit to a linear regression. Stability constants (K_{1:1}) were then calculated using the linear portion of the phase solubility diagram according to the Higuchi-Connors equation below:

\[ K_{1:1} = \frac{slope}{S_0 \left(1 - \frac{slope}{S_0}\right)} \]

where \(S_0\) is the solubility of drug in water, and the slope comes from the linear fit of the plotted data.

### 2.3 Isothermal Titration Calorimetry (ITC)

ITC was used to analyze the thermodynamic parameters of interactions between drug and CD at 298.15 K via a VP-ITC Microcalorimeter (MicroCal, Northampton, MA, USA). A typical titration consisted of 20 consecutive injections of 12 µL of a titrant with an interval of 210 s into a titration cell (containing CD). The CD solution in the cell was stirred continuously at 307 rpm. Samples were prepared as follows: stock solutions of LPV and RTV were made in methanol (1 mg/mL) and diluted down in water to a final concentration of 0.04 mM. Stock solutions of HP-β-CD and HP-γ-CD were made in DI water (1 mg/mL) and diluted down in water to a final
concentration of 0.004 mM. The samples were degassed for 20 min under vacuum using a ThermoVac (MicroCal, Northampton, MA, USA) prior to each experiment. Data were analyzed using nonlinear regression with a single-site binding model in VP Viewer 2000, using the scientific plotting software, ORIGIN 7 (Origin Lab. Corp., Northampton, MA, USA). In particular, the data were used to determine the number of binding sites \((n, \text{binding stoichiometry})\), inclusion enthalpy \((\Delta H)\), inclusion entropy \((\Delta S)\), and Gibb’s free energy \((\Delta G)\) related to the drug:cyclodextrin binding. The relationship between \(\Delta S\), \(\Delta H\), and \(\Delta G\) can be described using the following equation:

\[
\Delta G = \Delta H - T \Delta S
\]

where \(T\) is the temperature of the system.

2.4 Nuclear Magnetic Resonance (NMR) Analysis

All NMR titrations, \(^1\text{H}-\text{NMR}\) and 2D NMR, were performed in a Bruker 400 MHz NMR spectrometer at room temperature. Methanol-d\(_4\) and D\(_2\)O were used as solvents to determine any solvent effects. TMS was used as an internal standard. NMR titrations were performed in two different directions to determine whether higher order drug/CD complexations such as 1:2 or 2:1 occurred. First, the host molecule (i.e. HP-\(\beta\)-CD and HP-\(\gamma\)-CD) concentration was held constant (2 mM) while the guest (i.e. LPV or RTV) concentration was varied from 0 to 25 mM. Then the host concentration was varied from 0 to 60 mM while the guest concentration was held constant (6 mM). The chemical shift data obtained from the NMR titration study was used to plot NMR titration curves as described in Thordason's work.\(^{47}\) The software used for the non-
linear curve fit model was BINDFIT, which is offered as a freeware from supraolecualr.org.

Based on the change in chemical shift values as drug concentration increased, stability constants based on 1:1 guest-host stoichiometry were calculated using the following equations:

\[
K_a = \frac{[HG]}{[H][G]}
\]

\[
= \frac{\left[ \frac{HG}{H_0} \right]}{H_0}
\]

Where \( \Delta \delta \) is the change in chemical shift value to concentration of complexes formed ([HG]), \([H_0]\) is the initial host concentration, and \( \delta_{HG} \) is the chemical shift at a particular complex concentration. \( K_a \) is the stability constant to the concentration of complexes formed ([HG]), concentration of free host ([H]), and concentration of free guest ([G]).

2.5 Molecular Modelling

Molecular modelling was performed using Spartan16 and Molecular Operating Environment (MOE) software. The models were first constructed in Spartan and were then subjected to energy minimizations using molecular mechanics and semi-empirical level calculations. Once the energy was minimized, further docking studies were performed using MOE, where the host molecule (i.e. HP-β-CD and HP-γ-CD) was treated as the receptor and the drugs were treated as ligands. AMBER force field was used to perform the docking studies. The receptor and ligands were set for flexible
alignment to ensure free movement. Once the docking was completed, docking scores were tabulated, and ligand interactions were obtained to visualize the binding inside the cavity of cyclodextrins.

2.6 Formulation optimization of drug-CD complexes

2.6.1 Binary Complex Formation

Binary drug-CD complexes were initially formed at a 1:1 molar ratio of drug/CD. According to the phase solubility diagrams, 5 mM was chosen as an appropriate concentration to use for the formation of drug/CD complexes. A 10 mM stock solution of each drug was prepared in methanol and a 10 mM stock solution of each CD was prepared in DI water. 25 mL of 10 mM drug solution was slowly added to a beaker containing 25 mL of CD solution under continuous stirring at 360 rpm for 6 hours at room temperature. An overview of the complexation process can be seen in Figure 2.1. The mixture was filtered, and spray-dried using a Buchi B-290 spray dryer coupled with a B-295 inert loop (Büchi Labortechnik, AG, Switzerland) in closed mode as seen in Figure 2.2. The following spray dryer parameters were kept constant: 0.7 mm nozzle diameter, inlet temperature of 100°C, pump flow rate of 3 mL/min, gas flow rate of 414 L/h, nozzle cleaner rate of 0, and aspiration rate of 40 m³/h. The dry samples were collected and stored at -20°C for further use.
**Figure 2.1.** Schematic overview of the drug:cyclodextrin (CD) complexation process, where drug was dissolved in methanol and CD was dissolved in water, followed by the combination of these two solutions to allow for drug:CD complex formation.

**Figure 2.2.** Schematic of overview of spray drying process, where feed solutions were added to the spray dryer to be dried and collected as micron-size particulates in the particle collection chamber.
2.6.2 *Ternary Complex Formation*

The effect of various hydrophilic polymers (e.g. PVP K30, PVP VA 64, HPMC E15, Soluplus®, and Kollidon®) on the solubility improvement of LPV and RTV in the presence of HP-β-CD, and HP-γ-CD was investigated. The polymer showing the greatest enhancement was used to form ternary drug-CD-polymer complexes. Ternary complexes consisting of drug-CD-polymer were produced in a similar manner as the binary complexes as described in Section 2.6.1 above. The final complex formulation contains 1% (w/v) polymer.

2.7 *Physicochemical Characterization of the Complexes*

2.7.1 *Solubility Study*

Solubility studies of final complex formulations were performed in millipore water. Saturated solutions were prepared by adding each formulation into vials until visible precipitate was observed (indicating saturation). Samples were prepared in triplicate and shaken 37°C for 48 hours. The amount of LPV and RTV in solution was determined using HPLC, as described in Section 2.2.

2.7.2 *Differential Scanning Calorimetry (DSC)*

Thermal behavior of the raw compounds and spray-dried ternary drug/CD complexes were analyzed using a TA Q10 DSC system (TA Instruments, New Castle, DE) connected to an RSC-90 cooling accessory. Approximately 5 mg of each sample was hermetically sealed in an aluminum pan and analyzed at 10°C/min from 0 to 200°C. An empty, sealed aluminum pan was used as reference.
2.7.3 X-Ray Diffraction (XRD)

The crystallinity of the raw compounds and spray-dried ternary drug/CD complexes was determined using a Rigaku Multiflex X-ray diffractometer (The Woodlands, TX) with a Cu Kα radiation source of 40 kV and 44 mA. Samples were placed on a 3 mm horizontal quartz glass holder prior to analysis. Scans were taken from 5-60° in 2θ with a step width of 0.2 and a scan rate of 2°/min.

2.7.4 Polarized Light Microscopy (PLM)

Samples of raw drugs and spray-dried ternary drug/CD complexes were obtained and analyzed for birefringence activity using an AmScope polarized microscope (Irvine, CA). Samples were mounted onto microscope slides, dispersed in mineral oil, and imaged at 10x magnification at room temperature.

2.7.5 Scanning Electron Microscopy (SEM)

The morphology of the raw drugs and spray-dried ternary drug/CD complexes were analyzed via a Zeiss Sigma VP Field Emission-SEM (FE-SEM, Germany). Dry powder samples were placed on aluminum SEM stubs (Ted Pella, Inc., Reading, CA) via adhesive carbon tabs. Samples were sputter coated with a film of gold/palladium alloy using a BIO-RAD sputter coating system at a 20 μA for 75 seconds under argon. Images were taken at 7.0 kV and 7,500x, 15,000x, and 28,000x.

2.7.6 Moisture Content
The moisture content of the spray-dried ternary drug/CD complexes was measured via Karl Fischer (KF) titration using a 737 KF coulometer (Metrohm, Riverview, FL). Approximately 3-5 mg of sample was dissolved in anhydrous methanol before being injected into the reaction cell filled with Hydranal® reagent. Water content was then quantified using anhydrous methanol as the reference sample.

2.8 In Vitro Dissolution Studies

In vitro dissolution studies were carried out using a USP apparatus II method (Sotax AT Xtend apparatus, Allschwil, Switzerland) at 37°C with a paddle speed of 75 rpm. 100 mg LPV and 25 mg RTV pure drugs or drug/CD complexes were transferred to dissolution vessels containing release media i.e. 0.1 N HCl (pH 1.09) and 10 mM PBS (pH 6.8). At predetermined time intervals, release media were withdrawn and replenished with fresh media. The dissolution samples were filtered using a 0.45 µm syringe filter (polyvinylidene fluoride, PVDF) and analyzed via HPLC.
CHAPTER 3

RESULTS AND DISCUSSION

The present study investigated the use of two CD molecules (i.e. HP-β-CD and HP-γ-CD, Figure 1.3) and evaluated their impact on enhancing the solubility and dissolution profiles of two anti-HIV model therapeutics, lopinavir (LPV) and ritonavir (RTV). Initial phase solubility studies were performed to determine the effect of cyclodextrin concentration on drug solubility. Interactions between the drugs and cyclodextrin molecules were investigated via analytical techniques such as NMR, ITC, and molecular modeling. A water-soluble polymer, Soluplus®, was also included in the formulation in an effort to further enhance drug solubility and dissolution profiles. Final formulations were created following drug:CD:Soluplus® complexation and spray drying to produce micro-sized dry particulates, which were characterized for their morphology, thermal stability and crystallinity, water content, and in vitro dissolution.

3.1 Phase Solubility Studies

One of the major challenges of the described study was the need to improve the water solubility of two poorly-soluble small molecule drugs, LPV and RTV. Table 1 shows the molecular weight and solubility of LPV and RTV in water and in 0.1 N HCl, where both drugs exhibited aqueous solubilities below 10 µM. The solubility of RTV in 0.1 N HCl was significantly higher than LPV, mainly as a result of the protonation ability of thiazole groups present in RTV (Figure 1.2). In addition, LPV
solubility decreased nearly 10-fold in acidic conditions (compared to water), whereas RTV increased 585-fold.

Table 1. Molecular weight and solubility values of lopinavir (LPV) and ritonavir (RTV) in water and 0.1 N HCl.

| Drug   | Drug MW (Da) | Drug Solubility (37°C) (µM) Water | Drug Solubility (37°C) (µM) 0.1 N HCl |
|--------|--------------|-----------------------------------|---------------------------------------|
| LPV    | 628.81       | 6.66 ± 0.05                       | 5.57 ± 0.50                           |
| RTV    | 720.95       | 0.87 ± 0.19                       | 512 ± 16                              |

A solubility enhancement technique that is commonly used for poorly-soluble drugs is the cyclodextrin complexation approach, in which drug molecules can interact with cyclodextrin molecules to form either inclusion or non-inclusion complexes (Figure 1.4). In an initial attempt to increase LPV and RTV solubility, phase solubility studies of LPV and RTV were carried out in aqueous solutions containing increasing concentrations of HP-β-CD or HP-γ-CD from 1.25 mM to 10 mM. This initial study was vital in that it helped elucidate the effect of CD concentration on drug solubility. Figure 3.1 shows that both CDs enhanced the solubility of LPV and RTV with increasing CD concentration. These data were fitted to a linear equation to indicate the linear relationship between CD concentration (x) and drug solubility (y). Based on the Higuchi-Connors classification of phase solubility profiles (Figure A.1), the A-type phase solubility profiles were obtained for each system, indicating that solubility of the substrates increased (i.e. drug) with increasing ligand (i.e.
cyclodextrin) concentration. More specifically, the relationship between each drug and CD were found to exhibit A_l-type phase solubility profiles (Figure A.1), in which the complex is first-order with respect to the ligand and first or higher order with respect to the substrate. While phase solubility studies cannot be used to determine the formation of inclusion complexes, they can be used to show the influence of increasing CD concentration on drug solubility. In addition, it is most often the case that A_l-type phase solubility profiles result in complexes occurring in a 1:1 molar ratio.

**Figure 3.1.** Phase solubility analysis of lopinavir (LPV) and ritonavir (RTV) in the presence of varying concentrations of 2-hydroxypropyl-β-cyclodextrin (β-CD) or 2-hydroxypropyl-γ-cyclodextrin (γ-CD).
Stability constants (K1:1) were calculated from the functions generated in Figure 3.1 using the Higuchi-Connors equation, assuming 1:1 drug:CD stoichiometry. K1:1 values depend on the solubility of a drug in water, and are often calculated using the intrinsic solubility (Sint) from phase solubility diagrams, where Sint is where the linear plot passes through the y-axis. The calculated Sint values often do not reflect the true solubility of a drug, since they are based upon extrapolation. As a result, K1:1 values were also calculated based on the experimental solubility data (So) for both drugs (Table 1).

K1:1 can be used to determine the strength of interactions between a drug and CD. Based on the values in Table 2, the strength of interactions between β-CD and LPV and γ-CD and LPV were similar, as evidenced by their comparable K1:1 values. Despite this similarity, the K1:1 for β-CD and LPV was higher than γ-CD and LPV, indicating that β-CD may form stronger complexes with LPV than γ-CD. This has been previously shown in literature, with the explanation that β-CD has a slightly smaller cavity than γ-CD, allowing for a better fit and intermolecular interactions between β-CD and LPV.37 The interactions between β-CD and RTV were much higher than those between γ-CD and LPV, as indicated by the nearly 3-fold increase in K1:1, which is likely due to the smaller β-CD cavity as discussed above.

With respect to how So and Sint impact K1:1, the binding constants based on So for β-CD or γ-CD and LPV were similar to the binding constant values calculated from Sint. The K1:1 for γ-CD and RTV based on Sint were higher than the K1:1 values based on So. The binding constant based on Sint for β-CD and RTV was unable to be calculated due to a negative y-intercept value from the phase stability diagram,
however, $K_{1:1}$ was successfully calculated using $S_0$. Overall, the calculated $K_{1:1}$ values were between 250 and 150 M$^{-1}$, with the β-CD resulting in the highest $K_{1:1}$ values for both drugs. The reported trends are in good agreement with similar studies conducted on guest-host interactions involving β- and γ-CD.$^{37}$

Table 2. Phase solubility analysis of lopinavir (LPV) and ritonavir (RTV) with 2-hydroxypropyl-β-cyclodextrin (β-CD) or 2-hydroxypropyl-γ-cyclodextrin (γ-CD). Stability constant ($K_{1:1}$) values were calculated based on both the experimental drug solubility in water ($S_0$) and γ-intercept value ($S_{int}$) from the phase solubility data from Figure 1.

|          | $S_0$ ($\mu$M) | $S_{int}$ ($\mu$M) | $K_{1:1}$ (from $S_0$) (M$^{-1}$) | $K_{1:1}$ (from $S_{int}$) (M$^{-1}$) |
|----------|----------------|-------------------|----------------------------------|----------------------------------|
| β-CD+LPV | 6.66           | 6.5               | 316                              | 324                              |
| γ-CD+LPV | 6.6            | 6.6               | 286                              | 288                              |
| β-CD+RTV | 0.87           | ---               | 1490                             | ---                              |
| γ-CD+RTV | 0.7            | 0.7               | 572                              | 715                              |

3.2 Isothermal Titration Calorimetry (ITC)

ITC was used to confirm the binding stoichiometry between each drug and cyclodextrin. ITC is an analytical technique used to determine the stability and thermodynamics of cyclodextrin inclusion compounds in solution.$^{39}$ It can be used to determine parameters such as number of binding sites ($n$, binding stoichiometry),
inclusion enthalpy ($\Delta H$), inclusion entropy ($\Delta S$), and Gibb’s free energy ($\Delta G$) related to the drug:cyclodextrin binding. ITC allows for the measurement of the heat absorbed or released during complex formation as a drug is titrated into a CD solution. Typically, for guest-host (drug-CD) interactions, the complexation process is exothermic, as enthalpy-rich water molecules evacuate the CD cavity, making room for the more hydrophobic guest molecules. The amount of heat released due to complexation is proportional to the binding enthalpy of the system and to the number of complexes formed. Non-linear curve fitting is used to generate the binding isotherm for each system, as depicted in Figure 3.2.
Figure 3.2. Isothermal titration calorimetry (ITC) binding isotherms generated via non-linear curve fitting for each drug:cyclodextrin system (RTV = ritonavir, LPV = lopinavir, β-CD = 2-hydroxypropyl-β-cyclodextrin, and γ-CD = 2-hydroxypropyl-γ-cyclodextrin).
Based on the values of $n$ listed in Table 3, it is evident that the binding stoichiometry between each drug and each CD is 1:1, since all values are near 1, confirming the results of the phase solubility analysis. The $\Delta H$ and $\Delta S$ values can be used to determine the specific types of interactions that occur during drug:CD complexation. In particular, the binding enthalpy reflects the guest-host interactions in terms of van der Waals interactions and hydrogen bonding, whereas the entropy is a reflection of hydrophobic interactions between the guest and host. Based on the results, each system exhibited a negative binding enthalpy and a negative change in entropy, indicating that both van der Waals interactions and hydrophobic interactions were involved in the complexation processes.

The Gibb’s free energy value for each system was found to be negative, indicating that complexations were exothermic, spontaneous processes. $\Delta G$ values for RTV for both CD were higher than $\Delta G$ values calculated for LPV systems. This indicates that RTV can form more stable complexes than LPV, which is supported by phase solubility studies that showed increased binding constant values for RTV than LPV. Overall, these data show that the binding stoichiometry for each system was 1:1 (drug:CD) based on the calculated $n$ values, and that multiple types of interactions play a role in the complexation processes. The binding stoichiometry between drug and CD is a vital piece of information needed during formulation development. Lastly, the calculated $\Delta G$ values for each system were found to be negative, indicating spontaneous complex formation.
Table 3. Isothermal titration calorimetry data showing binding stoichiometry ($n$), enthalpy contribution ($\Delta H$), entropy contribution ($T\Delta S$), and calculated Gibb’s free energy ($\Delta G$) of each system (RTV = ritonavir, LPV = lopinavir, $\beta$-CD = 2-hydroxypropyl-$\beta$-cyclodextrin, and $\gamma$-CD = 2-hydroxypropyl-$\gamma$-cyclodextrin).

|        | $n$ | $\Delta H$ (kcal/mol) | $T\Delta S$ (kcal/mol) | $\Delta G$ (kcal/mol) |
|--------|-----|-----------------------|------------------------|-----------------------|
| $\beta$-CD+LPV | 1.00 | -123                  | -115                   | -8.0                  |
| $\gamma$-CD+LPV | 1.08 | -95                   | -87                    | -8.0                  |
| $\beta$-CD+RTV | 1.09 | -140                  | -131                   | -9.0                  |
| $\gamma$-CD+RTV | 0.97 | -228                  | -219                   | -9.0                  |

3.3 Nuclear Magnetic Resonance (NMR)

NMR was used to investigate the changes in chemical shifts of particular protons present in CD molecules when exposed to varying drug concentrations, which can then be used to generate predicted stability constant value and binding stoichiometry of each drug:CD system. NMR titration experiments were performed two different ways. The first method involved keeping the host (CD) concentration constant and varying the guest (drug) concentration, and the other method involved keeping the guest concentration constant and varying the host concentration. This allows for the prediction of stability constants associated with higher order complexes (1:2 and 2:1) based on mathematical equations that relate the concentration of the complexes formed, initial host concentration, and changes in chemical shift values. Chemical shift values are directly proportional to the concentration of host-guest complexes and original host concentration (as shown in Figures A.2-A.9). In this study, changes in chemical shift values ($\Delta \delta$) were not found to be consistent with the formation of
higher order complexes, as indicated in Table 4, as no stability constants were available for 1:2 and 2:1 complexations.

**Table 4.** Stability constant values calculated for drug:cyclodextrin interactions using changes in chemical shift values obtained via NMR titration studies (RTV = ritonavir, LPV = lopinavir, β-CD = 2-hydroxypropyl-β-cyclodextrin, and γ-CD = 2-hydroxypropyl-γ-cyclodextrin).

|            | Stability Constant (M⁻¹) | Binding stoichiometry |
|------------|--------------------------|-----------------------|
|            |                          | 1:1 | 1:2 | 2:1 |
| β-CD + LPV | 308                      | N/A | N/A | N/A |
| γ-CD + LPV | 669                      | N/A | N/A | N/A |
| β-CD + RTV | 1320                     | N/A | N/A | N/A |
| γ-CD + RTV | 1590                     | N/A | N/A | N/A |

Figure 3.3 represents the NMR titration curves obtained using the method where the CD concentration was kept constant and the drug concentrations were varied. The chemical shift value that corresponds to a particular drug molecule is on the x-axis, and the concentration of drug is denoted on the y-axis. For β-CD+LPV, the chemical shift values present (6.91 to 7.00 ppm) correspond to the aromatic ring structure protons containing two methyl groups in LPV. For LPV+γ-CD, the chemical shift values present (7.26 to 7.28 ppm) correspond to one of the two benzene rings in LPV. For both RTV+β-RTV and RTV+γ-CD, the chemical shift values (7.21 to 7.22 ppm) correspond to the benzene structure present in RTV. For all drug:CD complexations, as the concentration of LPV increased, the chemical shift values increased and shifted.
to the right, which indicates that these protons are being shielded, an event that commonly occurs during complexation between guest-host molecules.\textsuperscript{52} These results indicate that successful complexation occurred between LPV or RTV and the two CD.

\textbf{Figure 3.3.} NMR titrations for (A) lopinavir (LPV) and 2-hydroxypropyl-β-cyclodextrin (β-CD), (B) LPV and 2-hydroxypropyl-γ-cyclodextrin (γ-CD), (C) RTV and β-CD, and (D) RTV and γ-CD. Analysis was performed while holding the host (CD) concentration constant (2 mM) while the drug concentration ranged from 0-25 mM.

The stability constants ($K_a$) were calculated from the NMR data were slightly different from the experimental data. The $K_a$ values listed in Table 4 indicate that γ-CD has the potential to form more stable complexes than β-CD for both LPV and RTV. These results are contrary to those discovered during the phase solubility
studies, which indicated that β-CD forms were more stable complexes than γ-CD with the two drugs. This may be explained by the fundamental differences in the way the stability constant is calculated for each experiment. In the phase solubility study experiment, the stability constant is calculated based on regression analysis of experimental solubility data, and this study involves saturating each aqueous solution with pure, undissolved drug. In contrast, in NMR experiments the trials were performed in deuterated methanol, allowing both drugs to remain in solution for the entirety of the titration. These experimental differences may affect the trends associated with guest-host interactions, and may therefore impact the calculated stability constant values. Overall, the NMR studies were able to confirm 1:1 stoichiometry between the guest and host during complexation.

### 3.4 Molecular Modelling

Molecular modelling was performed to determine the most stable conformation (pose) for each drug:CD pairing. Each drug was constructed in the software, and energy minimization and docking studies were performed, where the host molecule (β-CD or γ-CD) was treated as the receptor and the guest molecule (LPV or RTV) was treated as the ligand. The five most stable conformations, based on drug:CD interactions, were obtained. The docking scores, which represent the predicted values of the free energy of binding between drug and CD, are recorded in Table 5. Based on these data, γ-CD exhibited lower free energy values than β-CD upon interacting with either LPV or RTV, indicating that γ-CD can form more stable complexes with these two drugs compared to β-CD. These results do not correlate well with phase solubility
results, in which β-CD was found to have higher binding constants for both drugs compared to γ-CD. One reason for this could be that in modeling studies only the lowest free energy conformations are examined, which are based on ideal conditions in the computational method. In addition, molecular modelling studies do not take into account non-inclusion based phenomena, in which drug and cyclodextrin complexes interact with one another to further aid in solubilization. **Figure 1.4** shows a more realistic scenario involving a variety of interactions between the drug and CD that can involve inclusion complexes, non-inclusion complexes, and CD-based micellar-like structures that can surround and encapsulate drug molecules. Despite this, the most stable conformations exhibit docking scores between -7 and -9 kcal/mol, which correlates well with the ΔG values calculated in the ITC studies. The docking scores that correspond to the most stable conformations for β-CD + LPV, γ-CD + LPV, β-CD + RTV, and γ-CD + RTV are -7.29, -7.54, -7.12, and -8.73 kcal/mol, respectively. Negative docking score values indicate that complexation can occur spontaneously and is an exothermic process, which supports the data from ITC studies.
Table 5. Docking scores of the five most stable conformations (poses) of β-CD + LPV, γ-CD + LPV, β-CD + RTV, and γ-CD + RTV (RTV = ritonavir, LPV = lopinavir, β-CD = 2-hydroxypropyl-β-cyclodextrin, and γ-CD = 2-hydroxypropyl-γ-cyclodextrin).

| Conformer | β-CD+LPV | γ-CD+LPV | β-CD+RTV | γ-CD+RTV |
|-----------|----------|----------|----------|----------|
| Pose 1    | -7.2895  | -7.5365  | -7.1190  | -8.7313  |
| Pose 2    | -7.2343  | -7.4682  | -7.1106  | -8.0085  |
| Pose 3    | -7.0827  | -7.2982  | -6.9980  | -7.7874  |
| Pose 4    | -6.9578  | -7.2553  | -6.9615  | -7.6955  |
| Pose 5    | -6.9211  | -7.2239  | -6.9473  | -7.6637  |

Following the docking score evaluation, the most stable conformation of each system was selected in order to visualize the potential interactions between each drug and CD, as shown in Figure 3.4. From these models, it is possible to investigate the specific interactions involved in the complexation process, and is particularly helpful in gaining insight into the interactions that occur in the CD cavity. In addition, in each two-dimensional figure, the blue shaded regions represent the portions of the drug molecule that are exposed (not located inside of the CD cavity).
Figure 3.4. Molecular modeling studies investigating interactions between β-CD + LPV, β-CD + RTV, γ-CD + LPV, and γ-CD + RTV (RTV = ritonavir, LPV = lopinavir, β-CD = 2-hydroxypropyl-β-cyclodextrin, and γ-CD = 2-hydroxypropyl-γ-cyclodextrin).

For β-CD+LPV it appears that one of the aromatic regions of LPV is able to insert itself into the CD cavity. This conformation likely occurs because the interior of the CD cavity is hydrophobic relative to the exterior, and the drug aromatic rings are inherently hydrophobic due to the C-C and C-H bonds that comprise their structure. It is also likely that hydrogen bonding, in addition to van der Waals interactions play a key role in complex stabilization, as denoted by the blue and yellow dashed lines, respectively. It appears that hydrogen bonds can form between the hydroxyl groups located on the wider rim of the CD molecule and the double-bonded oxygen carbonyl groups found on LPV. For β-CD+RTV the structure of RTV is inherently different than the structure of LPV, which results in different interactions between each drug and CD molecule. RTV contains two thiazole groups, which can act as hydrogen
acceptors that can readily participate in hydrogen bonding. This phenomenon can be confirmed from the modeling simulation, where the sulphur atom in the thiazole group interacts with exterior rim hydroxyl groups on the CD molecule. In addition, the terminal isopropyl group present in RTV is capable of inserting itself into the CD cavity, a process most likely driven by hydrophobic interactions, as high energy water molecules release from the CD cavity, making room for hydrophobic moieties. Additional hydrogen bonding occurs between CD hydroxyl groups and other protons present in RTV, which further aids in complex stabilization.

\( \gamma \)-CD+LPV interactions were similar to \( \beta \)-CD+LPV interactions. From the model, extensive hydrogen bonding occurs, especially between the carbonyl located on the pyrimidine on LPV and protons located on the outer CD rim. In addition, hydrogen bonding occurs between the amine group of the pyrimidine on LPV and the hydroxyl group on the CD rim. For \( \gamma \)-CD+RTV one of the thiazole groups in RTV appears to insert itself into the CD cavity and extend straight through to the back of the CD molecule. It is like that hydrogen bonding occurs between the inserted RTV moiety and hydroxyl groups present on the narrow rim of the CD. This phenomenon may be due to the increased size and internal volume of the \( \gamma \)-CD cavity, as a result of it being comprised of an additional glucopyranose unit, in comparison to \( \beta \)-CD. These phenomena may also explain why \( \gamma \)-CD+RTV exhibits the lowest docking score, and thus results in the most stable conformation. In particular, more of the drug molecule is able to insert itself into the CD cavity, and interactions occur with the narrow rim of the CD molecule. In addition, the other thiazole group present on RTV appears to hydrogen bond to exterior protons on the CD rim. Overall, these molecular modeling
studies were able to provide a unique look into how drug and CD molecules form complexes. Many interactions can form between drugs and CD, however, extensive hydrogen bonding between the drug and exterior rim hydroxyl groups appears to provide a significant stabilizing effect. In addition, hydrophobic interactions appear to play a key role in forming stable complexes.

3.5 Binary and Ternary Complex Formation

Phase solubility studies were initially conducted to investigate the influence of increasing CD concentration on drug solubility. Based on these values, further studies were performed to enhance the solubility of the drugs in solution with CD through the addition of a hydrophilic polymer. Various water-soluble polymers such as PVP, Soluplus®, Kollidon®, HPC, PVA, and HPMC were investigated. Figure 3.5 shows that Soluplus® was the polymer that resulted in the greatest enhancement in solubility for both LPV and RTV. Upon Soluplus® exposure, LPV solubility increased 146-fold and RTV solubility increased 17-fold in comparison to solubility analysis in only water. For both LPV and RTV, the addition of Soluplus® enhanced their solubility in water. Moreover, the addition of Soluplus® enhanced the solubility further than CD alone.
Figure 3.5. Solubility analysis of (Top) lopinavir (LPV) and (Bottom) ritonavir (RTV) in the presence of various polymers (0.5 weight%) in aqueous media.

A follow-up solubility study was conducted to determine the optimal concentration of Soluplus® to use in final formulations containing drug, CD, and the polymer. Soluplus® is a poly(ethylene glycol)-polyvinyl acetate-polyvinylcaprolactame-based grafted copolymer that is amphiphilic, containing both hydrophilic and hydrophobic moieties, which provides an ideal platform to form interactions with hydrophobic drugs while maintaining water solubility. Soluplus® has
been shown to not only improve solubility of poorly water-soluble drugs, but also aids in the stability of formulations, acting as a crystal growth inhibitor for drugs in amorphous solid dispersions.\textsuperscript{53} As seen in Figure 3.6, 1 wt\% of Soluplus\textsuperscript{®} resulted in the maximum enhancement in solubility for both drugs. The addition of Soluplus\textsuperscript{®} had the greatest impact on the solubility of LPV in comparison to RTV. Based on these results, ternary formulations were prepared that include drug, CD, and Soluplus\textsuperscript{®}. Following mixing and inclusion formation, these solutions were spray dried to form dry micron-based particles that represent to the final formulation products, denoted as $\gamma$-CD/LPV/SOL, $\beta$-CD/LPV/SOL, $\gamma$-CD/RTV/SOL, and $\beta$-CD/RTV/SOL.
**Figure 3.6.** Solubility analysis of (Top) lopinavir (LPV) or (Bottom) ritonavir (RTV) in water, 5 mM 2-hydroxypropyl-β-cyclodextrin (β-CD), or 5 mM 2-hydroxypropyl-γ-cyclodextrin (γ-CD) in the presence of varying amounts (weight %) of Soluplus®.

As shown in **Figure A.10**, ternary complexes showed significantly higher solubility compared to the pure drugs ($p<0.05$ for β-CD/LPV/SOL, β-CD/RTV/SOL, and γ-CD/RTV/SOL, and $p<0.001$ for γ-CD/LPV/SOL).
3.6 Thermal Analysis of Spray-Dried Microparticles

Thermal analysis of the spray dried formulations and raw compounds was performed using differential scanning calorimetry (DSC). As shown in Figure 3.7, a strong endothermic peak was evident at 113°C for raw LPV, which indicates the melting temperature of LPV. Similarly, a strong endothermic peak was present at 130°C for raw RTV, which indicates the melting temperature of RTV. These results are in agreement with previously reported LPV and RTV melting temperatures. Raw β-CD exhibited a glass transition near 149°C, and γ-CD exhibited a glass transition near 156°C. The sharp, endothermic peaks that correspond to raw LPV and RTV disappear after spray-drying each drug, which is an indication that the drugs transition from crystalline to amorphous states during the spray-drying process.

Spray-drying is a process where dry powders are created from a feed solution containing the formulation components. The feed solution is fed through a small diameter nozzle, forming atomized droplets of solution. Depending on temperature and solvents used, most of the solvent is quickly vaporized, and the droplets quickly condense into a dry powder for collection. Since the solution is heated, drug molecules that are initially arranged in an orderly, crystalline structure become excited and rearrange themselves into a more unstable, higher energy amorphous arrangement. This amorphous solution solidifies as it is quickly cooled. As a result, when pure drug is spray dried, its crystalline properties are lost, and thus its melting temperature disappears. The molecules no longer exhibit long order arrangement, and so the bonds
between drug molecules have different strengths. Therefore, there is no single amount of heat required to break the bonds; instead it requires a range of energies.

Figure 3.7. Differential scanning calorimetry thermograms indicating thermal transitions corresponding to (Top) lopinavir (LPV) samples and (Bottom) ritonavir (RTV) samples. β-CD = 2-hydroxypropyl-β-cyclodextrin, γ-CD = 2-hydroxypropyl-γ-cyclodextrin, and SOL = Soluplus®. β-CD/drug/SOL and γ-CD/drug/SOL refer to the final formulation samples.
Thus, the DSC thermograms indicate amorphous drug formation in both spray-dried LPV and RTV. Similarly, the final spray-dried formulations displayed amorphous characteristics. These results make sense, as β-CD, γ-CD, and Soluplus® are originally amorphous, and spray-dried drugs also exhibit amorphous characteristics.

3.7 Crystallinity Analysis of Spray-Dried Microparticles

The crystallinity of pure compounds as well as spray-dried formulations was further examined using X-Ray diffraction (XRD). It can be seen in Figure 3.8, both raw β-CD and γ-CD exhibited smooth diffractograms, with an absence of strong peaks. These results, in agreement with the DSC data, indicate that these compounds are amorphous. On the contrary, raw LPV and RTV exhibit a multitude of strong peaks between 8-25 degrees. These sharp peaks indicate that LPV and RTV are crystalline, which is supported by the DSC data. The final spray-dried formulations exhibit broad peaks around 18 degrees. These broad peaks indicate that the compounds present in the sample are amorphous, as the molecules exhibit a random, disordered molecular arrangement.
Figure 3.8. X-ray diffraction patterns of (Top) lopinavir (LPV) and (Bottom) ritonavir (RTV) samples. β-CD = 2-hydroxypropyl-β-cyclodextrin, γ-CD = 2-hydroxypropyl-γ-cyclodextrin, and SOL = Soluplus®. β-CD/drug/SOL and γ-CD/drug/SOL refer to the final formulation samples.

3.8 Polarized Light Microscopy (PLM)

Polarized light microscopy (PLM) was used to confirm the phases of the spray-dried formulations and their constituents. PLM is a type of optical microscopy technique in which light is passed through a polarized lens, only allowing light traveling along a particular plane through to the specimen. Samples that exhibit birefringence (materials that have refractive indices dependent on plane-polarized
light) can be observed using this technique. Materials that are crystalline often exhibit birefringence, which can be confirmed via PLM. Figure 3.9 contains representative PLM images for raw compounds and spray-dried formulations containing LPV. Raw β-CD and γ-CD appear amorphous by the lack of birefringence, which is supported by DSC and XRD data. It is evident that pure LPV is crystalline, as shown by the visible crystal-like structures exhibiting birefringence. Spray-dried LPV shows no birefringence and supports the claim that spray drying can convert crystalline drug to an amorphous form. Four sets of physical mixtures (PM) were imaged, including PM β-CD+LPV, PM γ-CD+LPV, PM β-CD+LPV+SOL, and PM γ-CD+LPV+SOL. These samples all exhibited some degree of birefringence, indicating that LPV remained in a crystalline form when mixed with CD and Soluplus® during simple physical mixing. The final spray-dried formulations were also imaged, including the binary formulations β-CD/LPV and γ-CD/LPV, in addition to the ternary formulations β-CD/LPV/SOL and γ-CD/LPV/SOL. These samples exhibited a lack of birefringence and visible dark spots, indicating that they were amorphous.
**Figure 3.9.** Polarized light microscopy images obtained for lopinavir (LPV) samples. \( \beta \)-CD = 2-hydroxypropyl-\( \beta \)-cyclodextrin, \( \gamma \)-CD = 2-hydroxypropyl-\( \gamma \)-cyclodextrin, and SOL = Soluplus\textsuperscript{®}. \( \beta \)-CD/drug/SOL and \( \gamma \)-CD/drug/SOL refer to the final formulation samples. Scale bar = 25 \( \mu \)m.

Corresponding RTV samples were imaged, as shown in **Figure 3.10.** The same trends occurred for RTV samples as did for LPV samples. Pure RTV was crystalline, whereas spray-dried drug appeared amorphous. All four physical mixtures containing RTV exhibited some degree of birefringence, indicating that RTV remained in its crystalline form. On the contrary, spray-dried formulations containing RTV appeared amorphous, which is consistent with orthogonal solid-state characterizations performed on these samples. Overall, the results obtained from PLM were consistent with other experiments conducted in this study and no contradictions were observed.
Figure 3.10. Polarized light microscopy images obtained ritonavir (RTV) samples. β-CD = 2-hydroxypropyl-β-cyclodextrin, γ-CD = 2-hydroxypropyl-γ-cyclodextrin, and SOL = Soluplus®. β-CD/drug/SOL and γ-CD/drug/SOL refer to the final formulation samples. Scale bar = 25 μm.

3.9 Scanning Electron Microscopy (SEM)

SEM images were taken of raw and spray-dried drugs and final ternary formulations to investigate their size and morphology. As seen in Figure 3.11, pure LPV appears crystalline and rectangular in nature, exhibiting a size close to 20 μm. Analogously, raw RTV exhibits a very ordered structure, with narrow, stick-like morphology, with crystals greater than 10 μm in length. The final formulations (i.e. β-CD/LPV/SOL, γ-CD/LPV/SOL, β-CD/RTV/SOL, and γ-CD/RTV/SOL) were imaged,
and consisted of microparticles with predominantly raisin-like morphology. The morphology of spray-dried particles is dependent on a number of process parameters such as inlet temperature and feed concentration.\textsuperscript{55} A recent study showed that increasing the spray drying inlet temperature from 70°C to 140°C resulted in particles transitioning from spherical, smooth morphology to displaying raisin-like morphology.\textsuperscript{56} Therefore, it is not unexpected that formulations spray-dried at an inlet temperature of 100°C and with relatively low feed concentration (20 mg/mL) would exhibit raisin-like morphology. A high inlet temperature and low feed concentration causes fast vaporization of the solvent from solution and subsequent, internal bubble nucleation, particle inflation and particle surface deformation during drying, thereby preventing droplets from forming spherical particles.

![Scanning electron microscopy images showing the morphology of raw lopinavir (LPV), raw ritonavir (RTV), spray-dried (SD) LPV, SD RTV, and the four final ternary complexes. β-CD = 2-hydroxypropyl-β-cyclodextrin, γ-CD = 2-hydroxypropyl-γ-cyclodextrin, and SOL = Soluplus®.](image)

\textbf{Figure 3.11.} Scanning electron microscopy images showing the morphology of raw lopinavir (LPV), raw ritonavir (RTV), spray-dried (SD) LPV, SD RTV, and the four final ternary complexes. β-CD = 2-hydroxypropyl-β-cyclodextrin, γ-CD = 2-hydroxypropyl-γ-cyclodextrin, and SOL = Soluplus\textsuperscript{®}. 48
3.10 Karl Fischer Titration

Karl Fischer titration was used to determine the water content of the final ternary formulations. As seen in Table 6, water content ranged from 1.06 to 1.24 wt%. The moisture content of formulations is an important characteristic relating to product stability, where low moisture content can reduce particle agglomeration as well as prevent crystal growth formation from amorphous drug in a formulation.\(^{57}\) These values indicate that dry microparticles were in fact produced, and that the spray-drying process is capable of removing most of the moisture from the samples.

Table 6. Water content values corresponding to the final formulations determined via Karl Fischer Titration. LPV = lopinavir, RTV = ritonavir, β-CD = 2-hydroxypropyl-β-cyclodextrin, γ-CD = 2-hydroxypropyl-γ-cyclodextrin, and SOL = Soluplus®.

| Formulation     | Water Content (weight %) |
|-----------------|--------------------------|
| β-CD/LPV/SOL    | 1.24 ± 0.51              |
| γ-CD/LPV/SOL    | 1.06 ± 0.35              |
| β-CD/RTV/SOL    | 1.19 ± 0.38              |
| γ-CD/RTV/SOL    | 1.21 ± 0.23              |

3.11 In Vitro Dissolution Analysis

In vitro drug dissolution characteristics of the final formulations, their physical mixtures, and the corresponding raw and spray-dried drugs were tested using a USP II apparatus. Dissolution media was prepared under two different pH values, 1.1 and 6.8, to simulate acidic conditions present in the stomach and more neutral conditions in the
small intestine, respectively. Paddle speed was set to 75 rpm and temperature was maintained at 37°C. In addition, non-sink conditions were used in this experiment because in order to satisfy sink conditions, an excessive quantity of surfactant (0.06 M polyoxyethylene 10 lauryl ether) would have been required, which does not replicate the actual in vivo environment, and therefore may not accurately reflect the “true” dissolution profile of the samples tested.

**Figure 3.12A** shows the release profiles of LPV controls and formulations containing LPV in 0.1 N HCl. Pure LPV exhibited a cumulative release of approximately 4% over the course of 120 minutes. The dissolution profile of spray-dried LPV was analyzed to investigate the effect of crystallinity on dissolution rate. Unexpectedly, the cumulative release of the spray-dried LPV was around 1.3% over 120 minutes, which is significantly lower than that of the pure LPV. This result indicates that transitioning LPV from crystalline to amorphous does not necessarily result in improved dissolution rate and extent. This was consistent with a recently reported study, in which different metastable forms of LPV were studied. Considering that LPV has four crystalline forms, it may be possible that amorphous LPV recrystallizes to form a less soluble crystalline form of LPV under super saturated conditions (non-sink conditions). Further studies need to be conducted to elucidate this phenomenon. Ternary formulations (i.e. β-CD/LPV/SOL and γ-CD/LPV/SOL) exhibited enhanced dissolution rates over the pure drug and spray-dried drug (**Figure 3.12A**). This is most likely due to the effects of CD complexation aided by the addition of water-soluble polymer Soluplus® rather than LPV being amorphous. It is worthy to note that large pieces of solid aggregates were observed
during the dissolution testing of the ternary formulations, indicating that a significant amount of drug may not have access to the dissolution medium and hence hampered dissolution. In addition, the physical mixtures of CD, LPV, and SOL resulted in an enhanced dissolution profile with a decrease in dissolution at 90 minutes followed by a sharp increase in dissolution. This indicates that polymorphic transition may have occurred during the dissolution testing process. It may be possible that LPV recrystallized to form a less soluble form at 90 minutes. However, a thorough solid state characterization study of LPV under supersaturated conditions needs to be conducted.

A similar trend was observed when testing the physical mixture and formulations in neutral condition (pH 6.8), as shown in Figure 3.12B. The cumulative release of LPV from both physical mixture samples peaked around 25% after 8 hours, whereas both formulations containing LPV exhibited a release around 9% within 8 hours. Again, this indicates that the conversion of LPV from crystalline to amorphous form may actually be detrimental to the dissolution rate of LPV under current testing conditions.

The dissolution profiles of RTV controls, and formulations containing RTV in 0.1 N HCl are shown in Figure 3.12C. The cumulative release of pure RTV (in a crystalline form) was around 32% after 2 hours. RTV has a much higher solubility in 0.1 N HCl than LPV due to protonation of the thiazole groups present in RTV. The dissolution rate increased in spray-dried RTV, as a cumulative release of 58% was observed after 2 hours. This result makes sense intuitively, as a metastable (amorphous) form of a drug usually results in an enhanced dissolution rate compared
to its crystalline form. Both physical mixtures containing RTV exhibited the lowest
dissolution rate out of all samples tested. In addition, both spray-dried formulations
exhibited enhanced dissolution profiles than the pure drug and physical mixtures,
although β-CD/RTV/SOL showed the fastest release profile. Based on the NMR
studies, β-CD + RTV has a lower association constant than γ-CD + RTV, and that
would allow RTV to evacuate the CD cavity quicker, leading to an enhanced release
profile. After 15 minutes, 80% of RTV was released from β-CD/RTV/SOL and 100%
drug was released after 45 minutes. A similar trend was observed when samples were
analyzed in neutral condition (pH 6.8), as shown in Figure 3.12D. Both physical
mixtures resulted in a cumulative release of less than 1%. β-CD/RTV/SOL exhibited a
cumulative release of approximately 16%, whereas γ-CD/RTV/SOL exhibited a
cumulative release of around 10%. Similarly, the formulation containing β-CD
showed a quicker dissolution, indicating that drug may be able to come out of the CD
cavity in less time than that out of the γ-CD.
**Figure 3.12.** *In vitro* dissolution profiles of lopinavir (LPV)-contained samples in (A) 0.1 N HCl and (B) phosphate buffered saline (PBS), and ritonavir (RTV)-contained samples in (C) 0.1 N HCl and (D) PBS. β-CD = 2-hydroxypropyl-β-cyclodextrin, γ-CD = 2-hydroxypropyl-γ-cyclodextrin, and SOL = Soluplus®.

Overall, these results indicate that LPV and RTV exhibit fundamentally different physiochemical characteristics and hence different outcomes of dissolution enhancement by forming complexes with CD. β-CD/RTV/SOL complexes showed the most profound dissolution enhancement.
CHAPTER 4

CONCLUSIONS AND FUTURE WORK

The interactions between two types of cyclodextrin (i.e. HP-β-CD and HP-γ-CD) and two different anti-HIV model drugs (i.e. LPV and RTV) were thoroughly investigated. Phase solubility studies confirmed that as CD concentration increases, there is a corresponding increase (linear relationship) in drug solubility for both LPV and RTV. It appears that HP-β-CD has a more significant impact on enhancing solubility. This can be due to the smaller size of the CD cavity itself, increasing the likelihood of interactions forming between drug and CD. In addition, it is possible that the drugs are forming non-inclusion complexes, and LPV and RTV are interacting with the functional groups (e.g. hydroxypropyl) on the outside of the CD and not just with the hydrophobic interior. ITC studies were performed mainly to investigate the binding stoichiometry between drug and CD. This information is extremely important when deciding on the ratio of drug:CD to use in the actual formulations. It was found that for both types of CD and both drugs, a 1:1 interaction was favored based on their thermodynamic behavior. The negative Gibb’s free energy values indicate that the complexation is a spontaneously exothermic process. NMR titration and molecular modeling studies were then performed to further confirm these findings, and to closely examine the particular the interactions that occur between the drugs and the CD. It was shown that multiple types of intermolecular forces such as hydrogen bonding and hydrophobic interactions play a role in the formation and drug:CD complexes.
To further enhance the dissolution characteristics of the two model compounds, a variety of polymers were studied. It was found that 1% Soluplus® (SOL) provided the greatest increase in solubility for both drugs, and therefore this polymer was chosen for further studies. Ternary complexes were prepared using drug, CD, and Soluplus®, and then spray-dried to yield dry powder microparticles. Various solid-state characterization techniques were used to analyze the dry complexes. SEM imaging revealed that microparticles exhibited mainly raisin-like morphology, with sizes ranging from 2-4 microns. DSC results showed that LPV and RTV had melting temperatures of 113°C and 130°C. Data also suggests that spray-dried formulations may contain amorphous drug, as indicated by the disappearance of melting temperature peaks. XRD results confirmed this finding, as both pure LPV and pure RTV exhibited shark, distinct peaks that disappeared in all complex formulations. PLM images were obtained to provide visual evidence of this phenomenon. Both drugs showed a significant degree of birefringence, indicative of crystalline structures. On the contrary, spray-dried drug as well as binary and ternary formulations exhibited a lack of birefringence, indicating that both drugs converted from crystalline to amorphous during the microparticle formulation process. In this study, we were able to successfully create spray-dried microparticles and characterize them based on their solid-state properties.

In vitro dissolution study showed that LPV and RTV had vastly different release profiles in both conditions, and that the effect of crystallinity had a different impact on each drug. For LPV, the physical mixture of LPV, CD, and SOL provided the greatest release profile. It was also found that spray-dried LPV did not offer any dissolution
enhancement, and in fact resulted in a lower release profile. On the other hand, the release rate of RTV was drastically enhanced in both formulations containing RTV, CD, and SOL, when compared to the controls. In addition, it was found that spray-dried RTV exhibited a better dissolution profile than crystalline drug, therefore indicating that spray-drying has a positive effect on overall RTV dissolution kinetics. Ternary RTV:CD complexes were able to successfully enhance dissolution rate and extent when compared to the controls.

Based on these findings, a different formulation technique will be required to enhance solubility and dissolution profile of LPV. It appears that converting LPV from crystalline to amorphous form via spray drying adversely affected dissolution characteristics of LPV, and therefore other methods such as kneading and lyophilization that do not involve high temperature will be studied. The taste assessment study of the optimized formulations will be performed to assess taste-masking capability of CD. In addition, stability studies will be conducted to analyze the effects of CD and SOL on the stability of both drugs. Following these studies, oral bioavailability study of the optimized formulations will be performed using a rat model.
APPENDIX

Figure A.1. Schematic showing the phase solubility profiles and classification of complexes according to Higuchi and Connors.\textsuperscript{36}

Figure A.2. NMR titration spectra for lopinavir (LPV) and 2-hydroxypropyl-\(\beta\)-cyclodextrin (\(\beta\)-CD).
Figure A.3. NMR titration spectra for lopinavir (LPV) and 2-hydroxypropyl-\(\gamma\)-cyclodextrin (\(\gamma\)-CD).

Figure A.4. NMR titration spectra for ritonavir (RTV) and 2-hydroxypropyl-\(\gamma\)-cyclodextrin (\(\beta\)-CD).
Figure A.5. NMR titration spectra for ritonavir (RTV) and 2-hydroxypropyl-γ-cyclodextrin (γ-CD).

Figure A.6. Stacked NMR spectra of lopinavir (LPV), 2-hydroxypropyl-β-cyclodextrin (β-CD), and a 1:1 molar solution of β-CD:LPV.
Figure A.7. Stacked NMR spectra of lopinavir (LPV), 2-hydroxypropyl-\(\gamma\) -cyclodextrin (\(\gamma\)-CD), and a 1:1 molar solution of \(\gamma\)-CD:LPV.

Figure A.8. Stacked NMR spectra of ritonavir (RTV), 2-hydroxypropyl-\(\beta\) -cyclodextrin (\(\beta\)-CD), and a 1:1 molar solution of \(\beta\)-CD:RTV.
Figure A.9. Stacked NMR spectra of ritonavir (RTV), 2-hydroxypropyl-γ-cyclodextrin (γ-CD), and a 1:1 molar solution of γ-CD:RTV.

Figure A.10. Solubility analysis of (Top) pure lopinavir (LPV) and ternary complexes including β-CD/LPV/SOL and γ-CD/LPV/SOL and (Bottom) pure RTV and ternary complexes including β-CD/RTV/SOL and γ-CD/RTV/SOL (* p<0.05, *** p<0.001).
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