Development of Binary Composite Solid Microparticles for Improved Intranasal Absorption of Low Permeation Drug Molecules

Dipy Madhukar Vasa

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DEVELOPMENT OF BINARY COMPOSITE SOLID MICROPARTICLES
FOR IMPROVED INTRANASAL ABSORPTION OF LOW PERMEATION
DRUG MOLECULES

A Dissertation
Submitted to the Graduate School of Pharmaceutical Sciences

Duquesne University

In partial fulfillment of the requirements for
the degree of Doctor of Philosophy

By
Dipy M. Vasa

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Dipy M. Vasa

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ABSTRACT

DEVELOPMENT OF BINARY COMPOSITE SOLID MICROPARTICLES
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May 2017

Dissertation supervised by: Peter L.D. Wildfong, Ph.D.

The objective of this work was to introduce a novel formulation strategy for intranasal delivery of two low permeation candidates: levodopa (L-DOPA; antiparkinsonism drug) and ribavirin (RBVN; an antiviral drug). The central hypothesis states that preparation of binary composite microparticles with Pluronic® triblock copolymers (polyethylene glycol-polypropylene glycol-polyethylene glycol) should allow increased drug transport across nasal mucosal tissue relative to drug alone. Testing the hypothesis necessitated execution of specific aims directed to addressing issues inherent to the proposed approach. The work herein is presented with respect to two separate subjects: (1) material understanding and (2) formulation development. In the first two chapters, the feasibility of direct deposition of dry, solid drug microparticles on intranasal
permeation was explored. Further, specific emphasis was placed on selection of the most suitable solid form which can sustain the stresses of the chosen manufacturing processes.

The concluding part of this work is devoted to illustrating the use of binary composite polymeric microparticles, ultimately stemming from the materials and methodologies developed in the initial stages. The rate and extent of ribavirin transport was significantly higher from solid polymeric microparticles compared to standalone drug powder and its aqueous solution. Increased drug transport was attributable to permeation enhancement afforded by Pluronic® accompanied with a high initial concentration gradient established across the model tissue.
FOR MUMMA AND PAPA
Imagine being 21 and confronted with the toughest decision of your life – move half way across the world to pursue the dream of doctoral studies at the cost of leaving behind family, friends and familiarity. That was me seven years ago....When I set foot on this adventurous journey and came to Duquesne University, I did not have the faintest idea of the challenges ahead. Navigating through the success and failure in graduate school while grappling with the responsibilities of adulthood was difficult, at best. Fortunately, I was surrounded by many influential individuals who helped me get through the various stages of graduate school. I want to take this opportunity to acknowledge them for their phenomenal contribution.

I express sincere gratitude:

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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>API</td>
<td>Active pharmaceutical ingredients</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-Brain Barrier</td>
</tr>
<tr>
<td>BCRP</td>
<td>Breast Cancer Resistant Proteins</td>
</tr>
<tr>
<td>BCS</td>
<td>Biopharmaceutics Classification System</td>
</tr>
<tr>
<td>BET</td>
<td>Brunauer, Emmett and Teller</td>
</tr>
<tr>
<td>CM</td>
<td>Cryogenic milling</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal Fluid</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential Scanning Calorimetry</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>HSM</td>
<td>Hot-stage Polarized-light microscopy</td>
</tr>
<tr>
<td>IC</td>
<td>Intracranial</td>
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<tr>
<td>IM</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>IN</td>
<td>Intranasal</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>Levodopa</td>
</tr>
<tr>
<td>MRP</td>
<td>Multidrug Resistance-associated Protein</td>
</tr>
<tr>
<td>NCE</td>
<td>New Chemical Entity</td>
</tr>
<tr>
<td>PDF</td>
<td>Pair distribution function</td>
</tr>
<tr>
<td>PEG4000</td>
<td>Polyethylene glycol 4000</td>
</tr>
<tr>
<td>POX188</td>
<td>Poloxamer® 188</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>PSD</td>
<td>Particle Size Distribution</td>
</tr>
<tr>
<td>PXRD</td>
<td>Powder X-ray Diffraction</td>
</tr>
<tr>
<td>RBVN</td>
<td>Ribavirin</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscope</td>
</tr>
<tr>
<td>TGA</td>
<td>Thermogravimetric Analysis</td>
</tr>
<tr>
<td>VM</td>
<td>Vibratory impact Milling</td>
</tr>
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<td>WHO</td>
<td>World Health Organization</td>
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Chapter 1: Introduction

1.1. Introduction

The selectively permeable nature of the blood-brain barrier (BBB) presents a considerable challenge for effective delivery of drug molecules to the central nervous system (CNS). According to one estimate, < 2% of all small molecules and virtually no large molecules reach the CNS after systemic administration.\(^1\) Several potential neurotherapeutic drug candidates that are efficacious \textit{in vitro} fail during the clinical phase owing to the inability to enter the CNS. In general, the BBB limits substrate transport based on lipophilicity, molecular size, and specificity for a variety of ATP-dependent transport systems. Although there is a positive correlation between lipophilicity and the BBB permeability,\(^2\) many of these substrates and their metabolites are removed almost instantly by the efflux transporters (such as P-glycoprotein; P-gp) expressed in the endothelial cells of the BBB. In addition to Pgp, the ATP binding cassette transporters of the multidrug resistance protein (MRP) family and the breast cancer resistance protein (BCRP) have a role in central drug uptake.\(^3^\)\(^-^\)\(^5\)

Early assessment of the ability of a drug candidate to cross the BBB is critical. Over the past several years, academia and industry have invested significant effort in the development and optimization of screening procedures, including \textit{in vitro} assays and computational models to evaluate CNS penetration. This information can be used to discover new chemical entities (NCEs) amenable to BBB transport, however, the
financial burden that it puts on a pharmaceutical company often questions the feasibility of this seemingly straightforward choice. The decline of “blockbuster” drug development over the last decade further echoes the increasing need for exploring alternate routes of administration and/or innovative formulations to effectively deliver extant therapeutic molecules to the brain.

A key focus of intranasal (IN) drug delivery research is to afford direct entry of drug molecules into the CNS using the unique anatomy and physiology of the nasal cavity.\textsuperscript{6-10} The olfactory bulb is the only region of the CNS in direct contact with the nasal cavity, which potentially allows molecules to bypass the BBB. Additionally, IN delivery offers avoidance of first-pass hepatic metabolism along with local intestinal metabolism/toxicity/efflux/degradation. Recent patents detailing IN delivery for the treatment of Parkinson’s disease, multiple sclerosis, Alzheimer’s disease,\textsuperscript{11-14} dementia\textsuperscript{15} and migraine\textsuperscript{16} support ongoing interest in this route of administration. Large biomolecules, including cytokines and growth factors,\textsuperscript{17} have also shown promise for CNS entry through IN administration. From a quantitative standpoint, however, IN administration has shown relatively poor accumulation of drug in the CNS.\textsuperscript{6,18,19} According to recent reports, the average fraction of nasally administered dose that reached the CNS was < 1 \%.\textsuperscript{20,21,22} This is primarily attributed to (1) inadequate drug deposition in the desired region of the nasal cavity which facilitates direct CNS access i.e., the olfactory region and (2) rapid removal of drug from the site of deposition via mucociliary clearance.
Optimizing drug deposition in the olfactory region for targeted nose-to-CNS delivery is rather complicated. The convoluted geometry and narrow passages of the nasal cavity make access to the olfactory region challenging. Nonetheless, there are some unique delivery devices, such as OptiNose® and ViaNase™ that have effectively administered drugs deep into the nasal cavity, with considerable exposure in the olfactory region.\textsuperscript{23,24} Impel NeuroPharma® has also designed a novel nasal delivery technology to provide targeted olfactory deposition via a pressurized aerosol device.\textsuperscript{24} These reports represent an important step towards the ultimate goal of nose-to-CNS delivery and invigorate pharmaceutical investment in this area.

Conversely, mucociliary clearance, which leads to rapid elimination of IN-administered products in the gastrointestinal tract (GIT), cannot be effectively addressed via change of delivery device. Briefly, the nasal mucus layer is renewed every 10-20 min,\textsuperscript{25} which reduces total absorption of the deposited drug. To overcome this limitation, an increased residence time (t\textsubscript{res}) of drug preparations in the nasal cavity is desirable. This, in turn, is heavily dependent on the selection of formulation design and the components. Reports suggest that drug administered as solid powder is likely retained at the deposition site longer than corresponding aqueous solution. Prolonged t\textsubscript{res} increases the contact time between the formulation and the nasal mucosa, which can result in increased absorption and enhanced bioavailability. Use of mucoadhesive polymers has also shown promise in increasing nasal retention time. The cross-linking between polymer and components of nasal mucus presumably increases the viscosity of the mucus, and eventually decreases the rate of clearance. This, in turn, may enhance nose-to-CNS drug uptake.\textsuperscript{26-29}
Several mucoadhesive polymers are commonly used as permeation enhancers. These can act by one or more of the following mechanisms:

- Alteration of the physical properties of the olfactory membrane
- Transient opening of tight junctions between epithelial cells
- Inhibition of P-gp drug efflux transporters
- Increasing membrane fluidity by extraction by creating disorder in the phospholipid domains of the membrane

For targeted nose-to-CNS delivery, these excipients may be most favorable for the molecules that lack adequate permeability (BCS Class III, IV) and are often dismissed as viable development candidates.

Other than increase in the residence time, nasal powders offer the advantages of stability (chemical and microbiological) and patient safety (lack of preservatives). There is a great deal of interest in the pharmaceutical industry to develop solid dosage forms that can be administered nasally with a state-of-the-art delivery device. Currently, we are far from having a well-optimized solid drug product with exclusive delivery to the olfactory region. But some underlying trends suggest that specific control over particle size distribution (PSD) could be significant. Typically, microparticles in the size range of 10 – 45 μm provide nasal deposition profiles favorable for nose-to-CNS delivery. Proper identification of manufacturing processes which can easily and reproducibly generate microparticles in the desired size range is crucial to the success of this formulation platform.
Traditionally, dry powder formulations with nasal or pulmonary therapeutic application are prepared by mixing micronized drug particles (1 – 5 μm) with larger carrier particles.\textsuperscript{31} The inter-particle force is relatively weak which may promote segregation of components upon insufflation. To avoid this, it is imperative to combine the ingredients of the formulation as solid composites that can potentially withstand the agitation of pressurized delivery. Often, binary solid composites such as eutectics and amorphous solid dispersions, are considered as enabling technologies for low solubility drug candidates.\textsuperscript{32} However, with adequate modifications, the application of these solid composites can be expanded to low permeation molecules that necessitate specialized route of administration.

1.2. Hypothesis and research objectives

The central hypothesis of this dissertation is that direct deposition of solid microparticles containing a low permeation drug alone or as a composite with a permeation-enhancing polymer, will enhance mass transport across olfactory epithelial tissue relative to aqueous drug solution.

In order to test this hypothesis, the following research objectives were proposed and executed:

1. An excised epithelial tissue permeation model that is simple yet translatable to humans was identified. To test the usability of this model, L-DOPA epithelial transport was measured when given as aqueous solution and standalone solid microparticles.
2. Consistent with regulatory guidance on the use of polymorphic solid materials in drug products, a thorough characterization of the relevant solid forms of ribavirin was undertaken. It was expected that this would enable selection of appropriate micronization equipment and procedures to avoid process-induced polymorphic conversion during composite microparticle preparation.

3. Binary composite solid microparticles of ribavirin and a permeation-enhancing polymer were prepared, characterized and evaluated for toxicity, drug release and permeation in vitro. Collectively, these data provided sufficient evidence regarding effective IN delivery of ribavirin.

1.3. Nasal cavity

Drug deposition and clearance are heavily influenced by nasal anatomy and physiology. The anatomical features of the olfactory mucosa important for nose-to-CNS delivery are detailed throughout the literature.

The mammalian nasal cavity has three distinct functional regions: vestibular, respiratory, and olfactory. The vestibular region opens to the outside at the nares and provides protection by filtration of airborne particles. The respiratory region constitutes the inner part of each nasal cavity, including three lateral turbinates (or conchae) on the lateral sides of the cavity and the nasal septum on the medial side of each nostril (Figure 1.1).
Figure 1.1. Schematic representation of the lateral wall of the human nasal cavity. The boxed region delineates the olfactory region.\textsuperscript{30}

### 1.3.1 Respiratory region

The respiratory epithelium covers approximately 50\% of the nasal cavity in rats and 80–90\% in humans.\textsuperscript{34} The human respiratory epithelium is comprised of goblet cells, ciliated cells, intermediate cells, and basal cells. Primarily, goblet cells secrete mucus, which covers the entire respiratory region (~10 \(\mu\)m thick) with a lower sol layer and an upper gel layer. The normal pH range of nasal mucus is approximately 5.5-6.5; however, the nasal mucosa tolerates solutions having a pH range of 3-10.\textsuperscript{35} The ciliated cells project hair-like protrusions from the surface, which beat very rapidly in synchronized fashion. In humans, the ciliary beat frequency is 1000 strokes per minute resulting in an average nasal clearance rate of 8 mm/min.\textsuperscript{36} Consequently, nasal mucus is propelled towards the nasopharynx where it is either swallowed or expectorated. The presence of
dense microvilli on the epithelial cells, combined with high vascularization of this region, makes it suitable for systemic drug absorption.

1.3.2 Olfactory region

The olfactory region (also known as the olfactory mucosa) is located within the recesses of the skull, just under the cribriform plate of the ethmoid bone, approximately 7 cm from the nostril, at the roof of the nasal cavity, lying partly on the nasal septum and partly on the superior turbinate (delineated in Figure 1.1). The surface area of the olfactory mucosa in humans is estimated at 10 cm². Conversely in dogs, this region constitutes 150 cm², indicating the importance of olfaction in the daily functions of dogs, unlike humans.

The olfactory mucosa is comprised of three cell types: olfactory receptor cells (also called olfactory receptor neurons, or ORN), supporting epithelial cells, and basal cells (Figure 1.2). The ORN are bipolar neurons with a spherical cell body. A single dendritic process extends from the cell body to the free apical surface (lumen of the nasal cavity) where it terminates as a small knob-like swelling. This, in turn, bifurcates into numerous long fibers and modified cilia. On the end of CNS, the ORN taper into an unmyelinated axon, which penetrates the basal membrane to join other axons and form large bundles in the lamina propria. The axons cross into the cranial cavity through small holes in the cribriform plate and synapse in the olfactory bulb, thereby establishing a direct anatomical connection between these two regions.
Figure 1.2. Anatomical representation of olfactory epithelium. Yellow dashed line (----) indicates the paracellular pathway, purple dashed line (---) indicates the transcellular pathway and the red dashed line (---) represents the transneuronal pathway.\(^{30}\)

The cilia on the ORN are different from those on the respiratory epithelium,\(^{36}\) and exist as three distinct, ciliated sub-regions: mobile, non-mobile, and intermingled. The combination of ciliated sub-regions disturbs the systematic beating of the cilia, thereby reducing the mucus clearance rate to 1-2 mm/h, which is \(~240\) times slower relative to the respiratory region.\(^{9,36}\) This potentially allows for greater retention of IN formulations in the olfactory region.

Previously, it was believed that drug transport from the nasal cavity to the CNS was mediated directly by the ORNs (neuronal pathway), through the supporting epithelial cells (epithelial pathway), or between the epithelial cells in the olfactory region (paracellular pathway) (Figure 1.2). However, CNS entry may also occur through cells outside of the olfactory region, such as the trigeminal nerves that innervate different
regions of the nasal mucosa. It has also been shown that transporter systems in the olfactory region have a role in drug uptake. Co-administration of transporter-efflux inhibitors resulted in enhancement of drug absorption in the CNS. Other plausible mechanisms for nose-to-CNS transport are discussed in the comprehensive work by Dhuria et al. It is likely that multiple pathways are responsible for drug uptake into the CNS, although one pathway may predominate depending on the properties of the therapeutic, characteristics of the formulation, and delivery device used.

1.3.3. Evidence of nose-to-CNS pathway

It is well known that the euphoria derived from sniffing cocaine occurs rapidly (within 3–5 min). It has been suggested that the reason, apart from a rapid nasal absorption, is the presence of a direct pathway from the nasal cavity to the CNS and the ability of the drug to concentrate selectively in specific regions in the brain. Various animal studies have confirmed that, at early time points after nasal administration, the concentration of cocaine in the brain was higher with IN administration relative to IV, thereby showing the existence of the pathway from nose-to-brain.

Okuyama was the first to attempt a direct assessment of nose-to-brain transport of compounds in humans. Using a radiotracer $^{99m}$-technetium-labelled diethylenetriamine-penta acetic acid ($^{99m}$Tc-DTPA) mixed with hyaluronidase sprayed deep into the nasal cavity; gamma-scintigraphy was used to record the amount of activity appearing in the intracranial space. A significant rise in cerebral radioactivity was observed 5 min after introduction of the nasal spray. In another example, it was shown that 27-times higher [3H]-dopamine reached the olfactory lobe after nasal administration relative to intravenous injection in rodent model. Fehm reported a significant
accumulation of insulin in the CSF after a single nasal administration of 40 IU insulin, whereas no increase was seen in insulin plasma levels.

Evidence of direct nose-to-brain transport of drugs has also been gathered in humans, mostly in terms of pharmacodynamic effects on the CNS, comparing drug administration via nasal and intravenous routes of delivery. A list of the drugs studied in humans is given in Table 1.1.

Table 1.1. Drugs used in nose-to-CNS drug transport studies in humans.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Number of Participants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginin-vasopressin (n=15)</td>
<td></td>
</tr>
<tr>
<td>Cholecystokinin-8 (n=20)</td>
<td></td>
</tr>
<tr>
<td>Angiotensin II (n=12)</td>
<td></td>
</tr>
<tr>
<td>Adrenocorticotropic 4-10 (n=54)</td>
<td></td>
</tr>
<tr>
<td>Insulin (n=12)</td>
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</tbody>
</table>

Functional evidence in man of facilitated transport to the brain was provided by changes in event related potential during performance of an oddball task.

1.4. Factors influencing nasal absorption

Whether a drug is nasally administered to induce systemic or CNS effects, absorption from the nasal mucosa is a pre-requisite for successful delivery. Several physiological and physicochemical factors play a crucial role in determining the rate and extent of nasal absorption.

1.4.1. Physiological considerations

1.4.1.1. Targeted deposition

For desirable therapeutic outcomes, it is essential to target an appropriate deposition site for inhaled particles. Locally acting drugs are best targeted to the middle meatus, while the turbinates and septum wall are best for systemic delivery owing to efficient drug absorption. Deposition on the anterior portion of the nose typically
provides a longer nasal residence time; however, this region is not amenable to
permeation and thereby nasal absorption. In contrast, deposition of formulations in the
posterior chamber of the nasal cavity enables greater uptake but is subjected to rapid
mucociliary clearance, which ultimately limits nasal absorption. For direct nose-to-CNS
transport, the olfactory region is the best target.\textsuperscript{47}

From a mechanistic standpoint, inertial impaction and gravitational sedimentation
predominantly govern deposition of pharmaceutical materials.\textsuperscript{48-52} Briefly, turbulent
airflow is needed to displace the relatively stagnant air volume in the upper nasal cavity
in order to allow impaction of mucosa with drug particles.\textsuperscript{53} Reports suggest that the
choice of dosage form (drops versus sprays) and the physical properties (particle size and
size distribution, plume angle, and plume velocity) play a crucial role in determining the
extent of nasal deposition targeted specifically to the olfactory region. These are
discussed in later sections.

1.4.1.2. Mucociliary clearance

Mucociliary clearance is a natural phenomenon that protects the lungs from
foreign particles. When allergens, microbes, toxins, \textit{etc.} adhere to, or dissolve in the
mucus of the nasal cavity, they are transported towards the nasopharynx for eventual
discharge into the gastrointestinal tract (GIT). Mucociliary clearance results from the
coordination between the overlying mucus and the wavelike movement of the cilia,
therefore, the efficiency depends on the length, density and beat frequency of cilia as well
as the amount and viscoelastic properties of the mucus. Clearance rates may vary owing
to any environmental or pathological factor that alters mucus production, mucus viscosity
or ciliary beat frequency. Increase in body temperature and cigarette smoking has shown
decrease in clearance rates possibly from dehydration of mucus layer and/or impaired cilia. The effects of disease conditions on normal mucociliary clearance rates are summarized in Table 1.2.

**Table 1.2.** Pathological conditions and their impact in nasal mucociliary clearance.\(^{54,55}\)

<table>
<thead>
<tr>
<th>Pathological conditions</th>
<th>Effect on mucociliary clearance and reason</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciliary dyskinesia</td>
<td>Impaired: absence or dyskinetic beating cilia</td>
</tr>
<tr>
<td>Asthma</td>
<td>Increased: inflammatory process and irritation</td>
</tr>
<tr>
<td></td>
<td>Decreased: epithelial damage</td>
</tr>
<tr>
<td>Cystic fibrosis</td>
<td>Decreased: dehydration of mucus</td>
</tr>
<tr>
<td>Viral and bacterial infections</td>
<td>Impaired: loss of cilia and change of mucus properties</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>Decreased: dehydration and microvascular damage</td>
</tr>
</tbody>
</table>

Theoretically, the mucus layer is transported at 5 mm/s and renewed every 10-20 min,\(^{37}\) which can reduce total absorption of the intranasally deposited drug. Slow clearance allows increased time of contact between drug molecules and the mucosal layer and subsequently enhances nasal absorption.

Clearance of a drug formulation can be influenced by its deposition site. Fortunately, ciliated epithelium is not uniformly located across the nasal cavity. While the middle and posterior turbinates are covered with ciliated epithelium, cilia are mostly absent from the anterior regions. Differences in ciliation significantly changes the clearance kinetics for particles deposited in different regions, and in turn alters \(t_{\text{res}}\). This is perhaps the reason for the biphasic clearance pattern observed for inhaled particles in human studies.\(^{49,56,57}\) Initially, particles deposited in the ciliated regions of the posterior nasal cavity were rapidly cleared, followed by a comparatively slower secondary clearance attributable to particles deposited on the non-ciliated anterior region of the nose. While ciliary movement is the primary mechanism for clearance from the
respiratory region, the presence of non-mobile cilia in the olfactory region makes it most likely that the upright position of the human head results in the removal of mucus in this region due to gravity.\textsuperscript{26} This may provide an opportunity for decreased mucociliary clearance and enhanced direct nose-to-CNS uptake for a drug formulation targeted for deposition in the olfactory region.

1.4.1.3. Enzymatic degradation

Even though nasally administered drugs are thought to avoid first-pass hepatic metabolism, the bioavailability of nasally administered drug molecules and proteins can be limited by a spectrum of metabolic enzymes localized in the nasal mucosa. Carboxyl esterases, aldehyde dehydrogenases, epoxide hydrolases and glutathione S-transferases have been found in nasal epithelial cells.\textsuperscript{58,59} Cytochrome P450 isoenzymes expressed in the nasal mucosa are also reported to limit absorption of drugs such as cocaine, nicotine, alcohols, progesterone and decongestants.\textsuperscript{59} Likewise, proteolytic enzymes (aminopeptidases and proteases) reported to impede intestinal uptake of peptides such as calcitonin, insulin and desmopressin are also expressed in nasal cells.\textsuperscript{59} Xenobiotic metabolizing enzymes may affect the pharmacokinetic and pharmacodynamic profiles of drugs administered intranasally. It is important to acknowledge that several pathological conditions, including the common cold, nasal polyps, and rhinitis may alter the relative expression of xenobiotic enzyme, thereby influencing nasal absorption.
1.4.1.4. Nasal permeation

For successful nose-to-CNS delivery, it is of utmost importance that the drug molecule demonstrates a balance of solubility in the mucus layer and permeation across the olfactory epithelium. Lipophilic drugs such as propranolol, progesterone and fentanyl are, in general, well absorbed from the nasal cavity, presenting pharmacokinetic profiles similar to those obtained after intravenous administration with a nasal-to-systemic uptake near to 100%. However, it is important to note that this is predominantly true for lipophilic compounds presenting a molecular weight <1 kDa. The extent of nasal absorption for lipophilic drugs >1 kDa is significantly reduced. The nasal membrane is predominantly lipophilic, hence, drug absorption is expected to diminish with a decrease in lipophilicity. The rate and degree of nasal absorption of polar drugs with molecular weight higher than 300 Da is notoriously low.

For some small polar molecules, a bioavailability of only 10% may result. Furthermore, polar drugs are more likely to be affected by mucociliary clearance, owing to greater solubility in the nasal fluid. Thus, it is evident that polar drugs are not easily transported across nasal mucosa, and necessitate formulation strategies that reduce clearance rates. The apparent distribution coefficient (K') is also an important factor influencing the permeability through nasal mucosa. This depends on both the drug pKₐ and the pH at the absorption site (estimated to be 5.0-6.5 in human nasal fluid). According to the pH-partition theory, a biological membrane is more permeable to the non-ionized fraction of a drug relative to the ionized fraction, therefore, it can be assumed that the nasal absorption of weak electrolytes depends on the extent of their ionization, with the most rapid absorption occurring for the unionized species. It is notable, however,
that drugs such as acetylsalicylic acid and bucolome have been observed to be well absorbed across the nasal membrane, even in environments in which they were expected to be primarily ionized. Further, multidrug resistance transporters have been identified in human nasal respiratory and olfactory mucosa, which may be involved in the transport of a wide variety of hydrophilic and amphiphilic drugs.

1.4.2. Formulation / Dosage form considerations

1.4.2.1. Nasal drops and sprays

Liquid formulations are commonly used for IN delivery. Common drawbacks associated with liquid formulations, such as drug substance aqueous solubility, chemical stability in a liquid vehicle, and formulation sterility should, however, be considered. Additionally, most liquid formulations require preservatives, which have demonstrated ciliotoxic effects in animal models following chronic use. Nonetheless, controlled volumes of formulated solutions can be easily delivered into the nasal cavity as sprays or drops, making them attractive as a delivery system. The differences in deposition pattern and clearance kinetics for these dosage forms merit particular attention.

The type of liquid dosage form selected heavily influences deposition and clearance. Drops are one of the oldest delivery methods for nasal administration of liquids. Despite being relatively inexpensive and easy to manufacture, their main disadvantage stems from the complex maneuvers of the delivery device for effective administration. Several papers have highlighted the difficulty of identifying a single “best technique” for the administration of nasal drops. There is tremendous variability in deposition and clearance owing to varied administration procedures, anatomy, and head position. Generally, drops tend to be deposited in the posterior region over a larger area.
in the respiratory epithelium, with minimal (or no) deposition in the olfactory region. As a result, drops are cleared more rapidly than sprays.\textsuperscript{56,69-71} Moreover, slight changes in dosing volume in nasal drops can lead to erratic pharmacokinetic profiles and strict patient compliance becomes necessary.\textsuperscript{67}

Given the advantages of dosing precision and reproducible droplet size characteristics, nasal sprays are most widely used for IN delivery of solutions and suspensions. The diversity of pump choices and actuator fittings makes these sprays suitable for targeted, region-specific nasal delivery. Mechanistically, metered sprays build hydraulic pressure in the metering chamber upon compression of the pump, which when it reaches an adequate level, pushes the liquid formulation out of the small orifice at the tip of the actuator. Shear forces and surface tension act upon the formulation, converting the liquid stream into a plume with unique geometric and droplet properties.

1.4.2.2. Nasal powders

Although dry powders benefit from improved chemical and microbiological stability relative to solutions, they are less frequently used for IN drug delivery. In addition to better stability, nasally administered solid particles may also result in better patient compliance, especially for children if the smell and taste of the drug in solution or suspension is unacceptable. Most powder formulations do not require preservatives, which have demonstrated ciliotoxic effects in animal models following chronic use.\textsuperscript{64,65} Furthermore, powders allow delivery of the highest mass of active ingredient without the necessity of a liquid vehicle or propellant, making for simpler formulations. Nonetheless, nasal powder formulations should allow for reproducible dose delivery, which is ultimately governed by several physical attributes such as particle shape, particle size and
size distribution, bulk density, hygroscopicity and flow properties. Furthermore, these formulations should also be appropriate for aerosolization to allow targeted nasal deposition and distribution.

Through *in vitro* and *in vivo* comparisons of IN delivered dry powder and liquid formulations, it is generally held that solid particles deposit locally in the nasal cavity, providing higher concentrations at the deposition loci, which combined with improved residence time, affords higher bioavailability relative to liquids. The reason for this advantage may be associated with the un-hydrated state of any solid dosage form. At the deposition sites, the powder is hydrated owing to the presence of water in the nasal mucus, but at the cost of dehydration of the latter. Consequently, sporadic regions having increased local viscosity are generated, which may increase resistance to the ciliary beat, in comparison to an equivalent solution form. Results of studies comparing drug uptake/bioavailabilities from different dosage forms are summarized in Table 1.3. Since most of these studies were pursued for systemic delivery, additional research efforts focusing on nose-to-CNS transport are warranted to determine full potential of solid nasal carriers.
Table 1.3: Comparison of liquid and powder formulations for nasal deposition and clearance.

<table>
<thead>
<tr>
<th>Drug of interest</th>
<th>Model</th>
<th>Observation and inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin&lt;sup&gt;72&lt;/sup&gt;</td>
<td>Beagle dogs</td>
<td>Crystalline insulin provided higher hypoglycemic effect relative to insulin suspension (pH 5.8) at a similar dose of 5 IU/kg</td>
</tr>
<tr>
<td>Insulin + dimethyl-β-cyclodextrin (permeation enhancer – 1.9 µmol/dose)&lt;sup&gt;73&lt;/sup&gt;</td>
<td>Rabbits</td>
<td>Use of freeze dried powder formulation resulted in higher systemic bioavailability of insulin (~13%) compared to same formulation as solution (~1%) at a consistent dose of 4 IU/rabbit</td>
</tr>
<tr>
<td>Calcitonin and Glucagon&lt;sup&gt;74&lt;/sup&gt;</td>
<td>Human</td>
<td>Administration of spray solution and powder did not give significantly different (p &lt; 0.001) absorption profiles</td>
</tr>
<tr>
<td>Dihydroergotamine (DHE) combined with randomly methylated-β-cyclodextrin (solubilizer – 10 mg/kg)&lt;sup&gt;75&lt;/sup&gt;</td>
<td>Rabbit</td>
<td>DHE serum bioavailability with nasal powder (56%) was not significantly enhanced (p &gt; 0.05) relative to liquid spray form (44 - 50%). DHE was administered at 0.1 mg/kg for all formulations</td>
</tr>
<tr>
<td>Ribavirin&lt;sup&gt;22&lt;/sup&gt;</td>
<td>Rat</td>
<td>Concentration in the olfactory bulb was &gt; 3-fold higher for dry powder relative to aqueous solution</td>
</tr>
<tr>
<td>Cromoglycolate loaded in polymeric microsphere system&lt;sup&gt;76&lt;/sup&gt;</td>
<td>Human</td>
<td>Small volumes of liquid or powder particles have almost the same clearance rate in the absence of bioadhesive excipients. However, in the presence of bioadhesive excipients, solid formulations cleared slower.</td>
</tr>
<tr>
<td>Chitosan&lt;sup&gt;57&lt;/sup&gt;</td>
<td>Sheep</td>
<td>Clearance time of chitosan solution and chitosan microspheres was 43 min and 115 min respectively.</td>
</tr>
</tbody>
</table>

1.4.2.3. Physical properties

Although estimates of particle sizes (PS) have shown that droplets or particles having aerodynamic diameters of ~5 µm typically deposit in the upper respiratory tract, the latest FDA guidance for nasal devices recommends minimizing the fraction of...
respirable particles below 9 μm in order to avoid lung inhalation. With PS increased >10 μm, total nasal deposition was shown to effectively increased to ~100%. More detailed analyses of sub-regional nasal deposition (especially olfactory region) are less common, but can be helpful for optimizing drug product characteristics.

Physical properties of IN liquid formulations, particularly droplet size, have a significant influence on the deposition pattern, with droplet diameters >20 μm resulting in ~100% nasal deposition. Analysis of sub-regional deposition suggests that larger droplets primarily deposit in the anterior or vestibular regions (D_{50} = 40 μm), leaving a significant portion of the nasal cavity unexposed to the drug. Among other relevant physical properties, plume angle and spray velocities have shown to heavily influence sub-regional nasal deposition.

For powder formulations, a study was attempted to identify the optimum particle size range needed to maximize total and regional deposition in the nasal cavity. Briefly, different size ranges of β-cyclodextrin powder were released in the silicone nasal cast and evaluated for distribution upon aerosolization. In these experiments, particles up to 45 μm provided a more uniform covering of the nasal surface, whereas larger diameter particles (125-250 μm) were found to be sparsely distributed.

Computational fluid dynamic (CFD) modeling has been used to augment inertial impaction and gravitational sedimentation, and help identify factors that potentially affect nasal deposition. Garcia and Kimbell developed a CFD model of a rat nose for quantifying the deposition of inhaled nanoparticles into the rat olfactory region at different particle sizes (1-100 nm) and airflow rates. The simulations indicated a strong influence on PS with a 6-9% of the inhaled dose as the maximum deposition achieved in
the olfactory region for 3-4 nm particles. Although this study focused on pharmaceutically impractical PS range, this study suggests that human CFD models could be generated for predicting the fraction of olfactory deposition as a factor of physical properties.

1.4.2.4. Mucoadhesives

As discussed earlier, MCC is one of the most important factors limiting nasal absorption owing to reduction in the time allowed for drug uptake. Intuitively, therefore, nasal absorption can be improved by prolonging the contact time between drug and mucosal membrane. In this regard, mucoadhesive drug delivery systems have been developed for IN delivery. Use of polymeric ingredients, among others, in the dosage form markedly reduces the rate of MCC. Several theories have been proposed to explain the mechanism of polymer–mucus interactions that lead to mucoadhesion. The most commonly accepted theory includes intimate contact between polymer and the biological tissue followed by wetting of the polymer chains. Subsequently, cross-linking between polymer and mucus occurs. This increases the viscosity of the mucus, and eventually decreases the rate of mucociliary clearance. Therefore, it is logical that changes in polymer properties, such as surface roughness, work of adhesion, hydration potential, molecular mass, cross-linking density, ionic strength, pH, and surface charge can considerably alter the extent of mucoadhesion.

In an recent example, Charlton et al. compared the abilities of several polymeric formulations to reach the olfactory region and increase the residence time on the epithelium. Different bioadhesive formulations were prepared using polymers such as pectin and chitosan, the amount of which was determined based on ease of delivery of the
final solution from the nasal delivery devices and adequate gelling characteristics at nasal physiological conditions. Distribution and clearance of the formulation from the olfactory region was assessed by taking photographs over time using endoscopy. From these studies, the authors concluded that all bioadhesive formulations were retained on the olfactory epithelium for more than four times longer than relative control solutions (See Figure 1.3).

**Figure 1.3.** $T_{res}$ for each formulation averaged over the whole test group. Error bars represent the S.E.M., $n = 12$. There was no statistical difference between the average $T_{res}$ values for each polymer formulation. Reprinted from European Journal of Pharmaceutical Sciences, 30, S. Charlton, N.S. Jones, S.S. Davis, L. Illum, Distribution and clearance of bioadhesive formulations from the olfactory region in man: Effect of polymer type and nasal delivery device, pp:295-302, Copyright 2007, with permission from Elsevier.

Likewise for polymeric solid microparticles, Tafaghodi et al. compared the clearance rates to highlight the importance of selecting the correct polymer for an optimum nasal absorption. Briefly, technetium-labeled microspheres of PLGA, alginate, Sephadex® and lactose were prepared and administered to healthy human volunteers. The clearance rates were compared using two regions of interest; the initial site of deposition
and the nasopharynx. Alginate and PLGA microspheres showed the lowest clearance rate compared to lactose powder (p < 0.0001 and p < 0.001) respectively.\textsuperscript{85}

In a recent clinical study, the plasma area under the curve (AUC) of desmopressin acetate following administration of a nasal powder formulation was increased 3-fold in the presence of sodium starch glycolate (SSG).\textsuperscript{86} The drug was homogenously distributed on SSG microparticles and, owing to complete hydration of this carrier material, increased release and absorption of the active drug was expected. In another study, it was concluded that a lyophilized insulin powder combined with Carbopol\textsuperscript{®} 934 resulted in the highest systemic absorption of insulin relative to other formulations.\textsuperscript{87} The authors explained that the gelling of Carbopol\textsuperscript{®} 934 following hydration may have led to longer adhesion with the nasal mucosa, thereby allowing more time for enhanced insulin absorption.

1.4.2.5. Permeation enhancers

The key to successful nose-to-CNS delivery, however, is not only an increase in nasal residence time but also increased CNS uptake of drugs. Hydrophilic drug molecules may poorly permeate the nasal epithelium and, thereby, exhibit insufficient bioavailability. It is possible to greatly improve their absorption by combination with absorption enhancers which induce transient modifications on the structure of epithelial barrier. In IN delivery, the most widely used absorption enhancers include surfactants (laureth-9), bile salts, fatty acids (taurodihydrofusidate) and polymers (chitosan, cyclodextrins, poly-L-arginine, polyethylene glycol and polyoxyethylene).\textsuperscript{26,88-90} The mechanism of action of these permeation enhancers is not well known but they typically alter the permeability of epithelial cell layer by modifying the phospholipidic bilayer,
increasing membrane fluidity or opening tight junctions between epithelial cells and, thus, increasing paracellular transport.\textsuperscript{91} Although the absorption promoters enhance drug bioavailability, chronic use may damage the integrity of the olfactory membrane which should not be overlooked.

Recently, Vaka \textit{et al.} demonstrated that formulations containing chitosan glutamate enhanced the hippocampal bioavailability of IN administered nerve growth factor (NGF) solution up to 14-fold.\textsuperscript{92} This observation was attributable to the permeation enhancement of chitosan, which transiently opens tight junctions between the olfactory epithelial cells. In another study, an alternative strategy involved conjugation of therapeutic agents with receptor-targeting moieties, which showed promising results for direct CNS uptake.\textsuperscript{93} For example, the olfactory bulb concentration of 1.0\% horseradish peroxidase (HRP) protein was almost 700 times (\(p < 0.001\)) higher when conjugated with wheat germ agglutinin (WGA) relative to HRP itself. It was hypothesized that WGA conjugation provided accessibility to receptor mediated endocytosis into olfactory sensory neurons. Subsequently, a fraction of the internalized protein transcytosed to axon terminals in the olfactory bulb. Such strategies can open possibilities of nose-to-CNS transport for other therapeutic agents used in neurodegenerative diseases. Use of chitosan glutamate (CG) as both a mucoadhesive agent and a permeation enhancer has shown exciting results for nose-to-CNS delivery of zolmitriptan, an anti-migraine drug. Microparticles (2.7 – 9.4 \(\mu\)m) of 1:1 drug:polymer ratio were prepared through spray drying process and assessed for \textit{ex vivo} permeation enhancement and adhesiveness.\textsuperscript{94} Although, the formulation showed comparable mucoashesion relative to drug alone (\(p < 0.05\)), the drug permeation was increased by 28\% for the CG containing microparticles as
compared to the spray dried drug (p < 0.05). When the authors compared the CSF concentrations of zolmitriptan following nasal administration as suspension versus drug-loaded chitosan-based microparticles, the solid formulation resulted in a modest 1.6-fold increase in drug in the CSF, verifying that polymers helped enable CNS targeting of the drug. In a similar study, the CSF-to-blood ratio of an anti-ischemic agent was respectively 4.3 and 57.5 for mannitol-lecithin and chitosan-loaded microparticles. The CSF concentration of the same drug through nasal suspension, however, was undetectable at the same dose.\textsuperscript{95}

1.4.3. Delivery device considerations

There are a wide variety of delivery devices available for IN delivery of powders. Correct choice of delivery device is important because differences in actuation pressure and device tip dimensions can affect both the volume of the emitted dose and its nasal deposition pattern.\textsuperscript{96-100} Pringels et al. studied the effect of delivery devices on deposition patterns of dry powder formulations.\textsuperscript{101} In their work, a silicone human nose model was insufflated with freeze-dried, radiolabeled lactose powder from three different devices: the Monopowder P\textsuperscript{®} system, the Pfeiffer system, and an experimental device developed in-house. Of these, the Monopowder P\textsuperscript{®} system showed the greatest deposition near the upper turbinate region, whereas the experimental device resulted in highest deposition in the anterior regions of the nasal cavity. Administration using the Pfeiffer system resulted in homogenous distribution throughout the nose, which led to faster clearance. The authors controlled both size fraction and bulk density of the powders; however, device-specific comparisons of airflow velocity at the time of actuation, administration angle, and device insertion depth were less clear. Additionally, an explanation for the
differences in deposition behavior between delivery devices was not provided, making it difficult to identify the particular device attributes that favor site-specific deposition. Such parameters can alter the deposition site, as described in a study investigating overall nasal deposition of budesonide powder from a unique breath actuated delivery device (Turbuhaler®). In 2006, Djupesland et al. developed the OptiMist™ device, a modified version of the traditional nasal spray that allowed penetration of the emitted dose beyond the anterior region of the nasal cavity, which had previously been a substantial challenge. This device utilizes the positive dynamic pressure of exhalation, which expands the narrow nasal passages, allowing the inhaled particles to reach the posterior regions of the nasal cavity. The results of a study comparing nasal deposition and clearance of drug administered from the OptiMist™ device with a traditional nasal spray were highly promising. Human volunteers were administered technetium labeled saline using both devices on separate days. The devices were characterized for the reproducible dose volume and droplet size (48 μm). Both delivery technique and head position of the volunteers were kept constant for all administrations. Regional deposition analysis indicated that the OptiMist™ device delivered more than 70–80 % of the dose beyond the anterior region when compared with the traditional nasal spray device. Additionally, the OptiMist™ device provided ~3× more deposition in the upper posterior region of the nasal cavity, which houses the olfactory region (p < 0.004). Although a major fraction (about 50%) of the OptiMist™ deposited in this region was cleared within 6 min of administration, it can be anticipated that the use of bioadhesive components in the
formulation will increase the residence time in the olfactory region to improve CNS uptake.

Recently, the regional human nasal deposition and clearance patterns of lactose powder delivered by the breath actuated bi-directional powder device (OptiNose™) were compared with those from a traditional liquid nasal spray (droplet size = 48.5 μm). Radiolabeled lactose powder ($d_{10} = 3$ μm, $d_{50} = 15$ μm, $d_{90} = 38$ μm) was delivered through the breath-actuated powder device. The method of administration and head position of the volunteers were kept consistent between two methods. Upon administration, gamma scintigraphy images were collected for studying nasal deposition and clearance. Results showed that both powder and spray were distributed to all nasal regions. However, in the upper posterior region, which is of concern to nose-to-CNS transport, the initial deposition of OptiNose™ powder was approximately nine times higher than the traditional spray (See figure 1.4).
Figure 1.4. Gamma camera image information from the nasal cavity is superimposed on the corresponding sagittal MRI section. The images are from the same subject and present deposition 2 min after delivery using (a) a traditional liquid spray, (b) the breath-powered Opti-Nose™ powder device, and (c) the breath-powered Opti-Mist™ liquid spray device incorporating the same spray pump as used in a. The initial deposition following traditional spray was greatest in the lower anterior regions of the nose, whereas deposition with the breath-powered delivery devices was greatest in the upper posterior regions of the nose. The less broad distribution in b following breath-powered Opti-Nose™ powder device is believed to be due to the slower clearance for powder the first 6–8 min, reflecting the dissolution of the powder into the mucosal layer. 23
Currently, it is difficult to make conclusions regarding the relative performance of breath-actuated novel devices, namely, OptiMist™ and OptiNose™, despite similar underlying mechanisms. Due to the inconsistency of formulation ingredients and essential physical properties such as particle size and particle density in the present data, supplementary studies to overcome these differences can greatly substantiate it. Nevertheless, it is encouraging to observe that the remarkable influence of delivery devices on olfactory deposition. Presently, this novel device is being studied for delivery of sumatriptan and fluticasone for migraine and chronic rhinosinusitis, respectively.

In another study, a pressurized olfactory device (POD) consistently delivered a majority of its dose to the olfactory region of adult rats. Pressurized nitrogen was mixed with a dye solution to create an energetic aerosol directed towards the roof of the nasal cavity. The insertion depth and dose volumes were well controlled from animal-to-animal. Qualitative deposition analysis suggested that the POD results in preferential localization in the olfactory region. To monitor nose-to-brain delivery, the dye solution was replaced with either radiolabeled mannitol (hydrophilic) or radiolabeled nelfinavir (lipophilic) liquid as model solutions. Following nasal administration, the radioactivity of samples was determined in excised brain tissues and blood. Using the POD, mannitol concentrations increased 25.6-fold (p < 0.05) in the olfactory bulb when compared with administration of drops. Similarly, nelfinavir concentrations were increased at the olfactory bulb (5.8-fold) and cortex (13.8-fold, p < 0.05). Further, the cortex-to-blood ratio for mannitol (3.6-fold, p < 0.05) and nelfinavir (13.6-fold, p < 0.05) increased with POD administration. These results support the possibility of enhanced nose-to-brain transport with increased deposition in the olfactory region.
1.5. Chemical profile

1.5.1. Levodopa

Levodopa (L-DOPA; L-3,4-dihydroxyphenylalanine) is an amino acid and precursor of dopamine, which remains the mainstay of treatment for Parkinson’s disease (PD). The molecular formula of L-DOPA is C₉H₁₁NO₄ and its molecular weight is 197.2 g/mol. L-dopa exists as zwitterions in solution, owing to the presence of an amine group (pKₐ 8.11) and a carboxylic acid group (pKₐ 2.30). The cLogP of L-DOPA is -2.19 and this molecule demonstrates good aqueous solubility at 1.65 mg/ml (20 °C).

L-DOPA crosses the BBB and is rapidly taken up by dopaminergic neurons and converted to dopamine (Figure 1.5) by the enzyme aromatic L-amino acid decarboxylase (DOPA decarboxylase). Consequently, moderate increases in the levels of dopamine alleviate the complications associated with PD.

![Chemical reaction showing (a) conversion of L-DOPA to dopamine (b) oxidation of L-DOPA to L-dopaquinone and (c) zwitterionic nature of L-DOPA between pH of 3 to 9.](image)

Figure 1.5. Chemical reaction showing (a) conversion of L-DOPA to dopamine (b) oxidation of L-DOPA to L-dopaquinone and (c) zwitterionic nature of L-DOPA between pH of 3 to 9.
Traditional peroral L-DOPA therapy is limited by poor relative bioavailability of approximately 5-15%, with less than 1% of the administered dose reaching the brain.\textsuperscript{108} For this reason, large peroral doses of L-DOPA are required for adequate therapeutic effect, which may often be accompanied with painful side effects such as motor fluctuations, dyskinesia, nausea and vomiting, some of which are attributable to dopamine formed in extracerebral tissues.\textsuperscript{109,110} Potential reasons for the low bioavailability of orally administered L-DOPA therapy include site-specific absorption of the drug in the duodenum, carrier-mediated active transport from the gut wall, extensive metabolism in the gastrointestinal tract and inter-individual variability in the degree of first-pass metabolism.\textsuperscript{108,111}

Recently, CNS targeting of L-DOPA was investigated in an aqueous solution through IN versus IV administration (\textbf{Figure 1.6}).\textsuperscript{20} The pharmacokinetic analysis showed that the amount of L-DOPA that reached the CNS was therapeutically ineffective.
Figure 1.6. Time courses of plasma and brain l-dopa concentrations following intranasal administration of l-dopa (2.5 mg/kg) in the presence and absence of C-dopa (0.63 mg/kg) (mean ± SD, n = 6). Reprinted from European Journal of Pharmaceutical Sciences, 38, Tae Kyung Kim, Wonku Kang, In Koo Chun, Seaung Youl Oh, Yeon Hong Lee, Hye Sun Gwak, Pharmacokinetic evaluation and modeling of formulated levodopa intranasal delivery systems, pp:525-523, Copyright 2009, with permission from Elsevier.

This was attributable to the low aqueous solubility of L-DOPA, which restricted the amount of drug that could be loaded per dose volume. Furthermore, the fluctuations in CNS concentration of the drug suggested inconsistent delivery from liquid vehicles potentially due to rapid removal of the drug from the absorption site initiated by mucus turnover. In another study, water-soluble prodrugs of L-DOPA in liquid vehicles were formulated and the issue of solubility was minimized, however, IN administration of the prodrugs showed nearly complete absorption of the drug in the systemic circulation, defeating the purpose of nose-to-brain transport. This study also suggested the potential role of mucociliary clearance in limiting the nasal retention of aqueous formulations. Additionally, the chemical instability of the prodrugs in aqueous media, similar to the drug itself, limited the usefulness of this strategy. Based on these observations, it can be speculated that the use of solid platforms for intranasal delivery of this drug may be beneficial in addressing the aforementioned issues.
1.5.2. Ribavirin

Ribavirin \( (1\beta-D-\text{Ribofuranosyl-1H-1,2,4-triazole-3-carboxamide}) \), an anti-viral drug, is a synthetic nucleoside analog structurally related to guanine. The molecular formula of ribavirin is \( \text{C}_8\text{H}_{12}\text{N}_4\text{O}_5 \) and molecular weight is 244.2 g/mol. The structure of ribavirin is shown in figure 1.7.

![Chemical structure of Ribavirin](image)

*Figure 1.7. Chemical structure of Ribavirin*

Ribavirin is a polar compound with \( \text{clog}P \) of -3.23 and an experimental \( \text{log}P \) (MlogP) reported as -1.85.\(^{112}\) Ribavirin is an ampholyte having both an acidic and a basic group, the \( pK_a \) values of which are reported as 12.95 and 1.00 respectively for the acid and base.\(^ {112}\) These values indicate that at most physiologically relevant pH values, the compound is unionized.

Ribavirin is typically administered orally in doses of 400, 500, or 600 mg twice daily. It is rapidly absorbed into the systemic circulation, with the uptake mechanism being active transport by the human concentrative nucleoside transporter 2 (hCNT2) in the proximal small bowel. Saturable uptake of ribavirin in the human intestine is the most likely explanation for the observation that, at very high doses (1200-2400 mg), the maximal plasma concentration of ribavirin after oral administration of the drug does not
increase in proportion with the dose.\textsuperscript{113,114} The combined use of ribavirin and pegylated interferon-\(\alpha\) is approved by the US Food and Drugs Administration (FDA) for treatment of hepatitis C in normal and HIV-infected individuals.\textsuperscript{115} According to the Essential Medicines List of the World Health Organization, ribavirin can also be used to treat viral hemorrhagic infections, such as Lassa fever and Crimean-Congo fever.\textsuperscript{116} Further, it is also used to treat respiratory syncytial virus infections in moderately to severely immunocompromised patients.\textsuperscript{117}

Despite demonstrating high antiviral activity \textit{in vitro} against CNS-borne pathogens,\textsuperscript{118-120} the \textit{in vivo} effectiveness of RBVN is restricted by its inability to cross the BBB. Studies reveal that intraperitoneal (IP), subcutaneous (SC) and intramuscular (IM) injections of RBVN were ineffective against several encephalitis viruses in mice.\textsuperscript{121,122} The drug improved the survival of subacute sclerosing panencephalitis (SSPE) in measles virus-infected hamsters only when administered intracranially (IC),\textsuperscript{122} which, though effective, is not a route of administration amenable to patient self-administration. For this reason, alternative means of administering RBVN or innovative formulation strategies are sought in order to increase the extent of drug that reaches the CNS.

A recent study investigated distribution of ribavirin in the CNS after IN administration in the form of aqueous solution or powder at the same dose. IV solution was used as control group. It was found that 20 min after administration, the accumulation of ribavirin in olfactory bulb was comparable between IV and IN solutions, whereas the powder led to significantly higher levels (\textbf{figure 1.8}).\textsuperscript{22} The extent of drug accumulation, however, was not enough to be efficacious. This supports the rationale that
solid nasal dosage forms have the ability to potentially deliver therapeutic quantities of drug to the CNS if formulated appropriately with permeation enhancing excipients.

**Figure 1.8.** (a) Ribavirin brain concentrations after in vivo nasal administration of an aqueous solution (white bars) versus intravenous injection (gray bars). (b) Ribavirin brain concentrations after in vivo nasal administration of an aqueous solution (white bars) versus ribavirin powder (black bars). The measured values were normalized by the dose administered. OB: olfactory bulb; CTX Ant and CTX Post: anterior and posterior cerebral cortex; BG: basal ganglia; Hip: hippocampus; CSF: Cerebrospinal fluid. Reprinted from Antiviral Research, 92(3), Gaia Colombo, Luca Lorenzini, Elisa Zironi, Viola Galligioni, Fabio Sonvico, Anna Giulia Balducci, Giampiero Pagliuca, Alessandro Giuliani, Laura Calzà, Alessandra Scaglarini, Brain distribution of ribavirin after intranasal administration, pp: 408–414, Copyright 2011, with permission from Elsevier.
1.5.3. Poloxamers

Poloxamers, also known by trade names Pluronics® or Kolliphor®, are tri-block copolymers of poly(oxyethylene)–poly(oxypropylene)–poly(oxyethylene). They are predominantly used as surfactants, solubilisers, wetting agent for microemulsions and as drug reservoir post micellization. All Pluronics® have similar chemical structures but with different molecular weights and composition of the hydrophilic PEO block (a) and hydrophobic PPO block (b) (shown in figure 1.9).

![General chemical structure of Poloxamers® or Pluronics®](image)

**Figure 1.9.** General chemical structure of Poloxamers® or Pluronics®.

For pharmaceutical applications, two of the most commonly used grades of Pluronics® are F-68 (also referred as Poloxamer 188; a=80, b=27) having an average molecular weight of 8400 Da, and F-127 (or Poloxamer 407; a=101, b=56) having an average molecular weight of 12200 Da. These polymers exhibit inverse thermosensitivity; therefore, they are soluble in aqueous solutions at low temperature, but gel at higher temperatures.

Pluronics® are widely used in drug delivery owing to the relatively low toxicity and potential for permeation enhancement. For hydrophilic drug molecules, these copolymers have been shown to promote permeation via inhibition of the P-gp and MRP mediated efflux. For lipophilic molecules, however, the process of permeation may
be facilitated by adsorption of copolymer on the cell membrane, followed by translocation of drugs molecules across lipid membranes.\textsuperscript{127} Pluronics® in this regard, offer universal applicability of BCS class II, III and IV molecules.

According to a recent report,\textsuperscript{128} CNS delivery of curcumin (a form of turmeric and naturally occurring food color) was significantly enhanced in the presence of poloxamers. In the rodent model, nasal \textit{in-situ} hydrogels comprising of Pluronic F-68 and F-127 exhibited higher nasal retention time resulting from possible gelation at the physiological temperature, thereby promoting curcumin uptake into the CNS. Results from \textit{in vivo} mucoadhesion tests revealed that yellow color in the oropharynx appeared 67 min after IN administration of hydrogels. This was almost 10-fold longer than the mucociliary transit time recorded for curcumin aqueous solution (see figure 1.10).

\textbf{Figure 1.10.} \textit{in vivo} mucociliary transit time recorded for curcumin loaded in aqueous solution and poloxamer containing hydrogel. Reprinted from Journal of Pharmacy and Pharmacology, 65, Xi Chen, Feng Zhi, Xuefeng Jia, Xiang Zhang, Rohan Ambardekar, Zhengjie Meng, Anant R. Paradkar, Yiqiao Hu, Yilin Yang, Enhanced brain targeting of curcumin by intranasal administration of a thermosensitive poloxamer hydrogel, pp:807-816, Copyright 2013, with permission from John Wiley and Sons.
The superiority of intranasally administered curcumin hydrogels was also reflected in the pharmacokinetic evaluations where the drug targeting efficiency (DTE; calculated as $\text{AUC}_{\text{brain part}}/\text{AUC}_{\text{plasma}}$) in the olfactory bulb, cerebellum and hippocampus was significantly higher ($p < 0.01$) than the equivalent dose of i.v. solution. Authors deduced that curcumin may be absorbed in the cerebral tissues via olfactory pathway.

In another study,$^{129}$ the use of Pluronics® improved the delivery of zidovudine to the brain. This is an antiviral drug which chemically resembles the structure of ribavirin. Briefly, a 20% w/v solution of F-127 was prepared in phosphate buffer (pH 5.5). The solution exhibited desired gelation characteristics between 27–30 °C. *In vitro* permeation experiments performed in rabbit nasal mucosa using side-by-side diffusion cells showed a 53% rise through incorporation of copolymers. These results also confirmed that entrapment of zidovudine in a hydrogel network does not inhibit the diffusivity of the molecule. It was concluded that the absorption of zidovudine may not be limited by the rate of drug release from the gel rather governed by the rate of drug diffusion through the biological membrane. The pharmacokinetic and brain distribution studies further supported the observation *in vitro*. Almost 56 times higher amounts of zidovudine accumulated in the brain and cerebrospinal fluid (CSF) from nasal application of the *in situ* gelling formulation when compared with i.v. dose. At any rate, this study shows promise that IN administration of a polar compound such as zidovudine could also preferentially enter into the CNS possibly via the olfactory route.
1.6. Summary

Intranasal drug delivery systems have shown variable and limited success in direct CNS uptake. Hence, the clinical usefulness of this pathway is a topic of current discussion. Targeted olfactory deposition and adequate residence time of the formulation within this region are essential to successful IN delivery. Currently, an ideal formulation or delivery device that enables exclusive drug delivery to the olfactory region has yet to be identified; however, research continues to maximize the fraction of dose delivered to this location. Generally, solid powder dosage forms show greater site-specific nasal deposition and reduced clearance relative to liquid dosage forms. Combining a well-optimized solid drug product with a state-of-the-art nasal delivery device can provide adequate drug targeting to the olfactory epithelium. Even though there are a number of challenges in IN delivery, the potential of this route to provide direct access to the CNS continues to encourage academic and industrial research. Exciting developments in formulation design and delivery device can enhance clinical developments and eventually time-to-market of new products.

1.7. Acknowledgments

Chapter 2: Improved flux of levodopa via direct deposition of solid microparticles on nasal tissue

2.1. Introduction

Parkinson’s disease (PD) is a progressive neurodegenerative disorder resulting in tremors, physical rigidity, and difficulty with walking, balance and coordination. Currently, 1 million Americans are affected with PD, with an estimated 60,000 new diagnoses each year. As of 2012, the Center for Disease Control (CDC) ranks the complications of PD as the 14th leading cause of death in the United States. Although the average age of onset is 60, the Parkinson’s Disease Foundation estimated that 4% of PD patients experienced "early-onset," prior to age 50. The US Census Bureau indicates that the fastest growing segment of the U.S. population is individuals over the age of 65, suggesting the potential for a commensurate increase in the incidence of PD in the near future.

Descriptions of the origin and progression of PD are reviewed in detail elsewhere. In brief, PD motor symptoms manifest when approximately 60–80% of the dopamine-producing cells are damaged in the substantia nigra. If left untreated, PD progresses to the point at which the patient becomes totally disabled, leading to a decline in all brain functions and an early death. Direct dopamine supplementation as an approach for symptomatic relief in PD patients is ineffective owing to the high ionizability of the molecule, which limits transport across the blood-brain-barrier (BBB). Therefore, oral administration of the metabolic precursor of dopamine i.e., levodopa (L-DOPA, L-3,4-dihydroxyphenylalanine) has been the mainstay of PD management for
decades.\textsuperscript{133-136} Currently marketed peroral formulations contain carbidopa (SINEMET\textsuperscript{®} and STALEVO\textsuperscript{®}), a DOPA decarboxylase enzyme inhibitor which has shown remarkable improvement in the oral bioavailability of L-DOPA (> 70\%).\textsuperscript{137} However, the problems associated with erratic absorption, pulsatile delivery, and rapid rise and fall in CNS levels of the drug still persist.\textsuperscript{137,138}

Innovation in L-DOPA formulations and delivery is an active area of research, aimed at accumulating consistent drug concentration in the brain. Although continuous intravenous infusion of L-DOPA alone or in combination with a decarboxylase inhibitor demonstrably reduces the frequency of motor fluctuations,\textsuperscript{139} and provides significant improvement in mobility,\textsuperscript{140} it is impractical and inconvenient for routine patient self-administration. Solution-based IN administration of both dopamine\textsuperscript{141} and many water soluble prodrugs, including L-DOPA has been demonstrated.\textsuperscript{20,106} Unfortunately, low brain levels of L-DOPA accompanied with dramatic fluctuations in drug concentration suggest inconsistent delivery from liquid vehicles. Further, these formulations were confronted with the issue of rapid mucociliary clearance and oxidation of L-DOPA in solution limiting the effective dose delivered.

While liquid formulations offer a potentially easy method of IN delivery (via nasal pump or spray), it is well established that liquid vehicles are most likely to have to contend with rapid clearance from the nasal cavity, highlighting one of the major factors that limits direct nose-to-CNS drug uptake. Normal mucociliary transit time in humans has been reported to be between 10–20 min, during which the mucus lining moves towards the nasopharynx for eventual drainage to the gastrointestinal tract. As a consequence, the majority of the administered dose is cleared from the nasal mucosa,
which is exacerbated by the drainage of solution droplets via the same channel owing to gravity. In contrast, semisolid platforms (such as gels) and direct deposition of solid particles (via insufflation) provide longer retention times in the nasal cavity, making them increasingly favorable approaches for IN delivery. It is anticipated that these dosage forms increase the viscosity of nasal mucus, leading to reduced clearance rate. At the very least, they are less prone to gravitational drainage than their liquid counterparts.

To take advantage of IN delivery, while circumventing stability and clearance issues, this work evaluates permeation of L-DOPA from solid microparticles placed in direct contact with nasal epithelial tissue relative to permeation from solution formulation. It is expected that IN delivered solid particles will be retained on olfactory epithelial tissue longer relative to solution droplets, resulting in greater cumulative mass transport. Further, it is hypothesized that L-DOPA molecules delivered from solid particles deposited directly in contact with the mucosal membrane will result in more rapid flux relative to L-DOPA in solution, due to an increase in localized concentration in nasal fluid, resulting in a higher concentration gradient, which leads to a more rapid diffusion rate. Ultimately, this research finding can provide a foundation for development of innovative sustained release solid dosage forms for IN application and potentially minimize problems associated with fluctuations in drug levels, in PD patients.

2.2. Experimental Section

2.2.1. Chemicals and Reagents

L-DOPA, > 99% pure, was obtained from Acros Organics™, (New Jersey, USA) and stored in a low relative humidity desiccator over anhydrous CaSO₄ (Drierite™) prior
to experimentation. Solubility studies were conducted by preparation of simulated nasal electrolyte solution (SNES), which combined 8.77 g NaCl, 2.98 g KCl, 0.59 g CaCl\(_2\) anhydrous in 1000 mL of deionized water.\(^{142}\) The pH of the solutions was adjusted between 5.5 and 6.0 with 1N HCl to simulate the pH of nasal fluid. Kreb’s Ringer Buffer (KRB) was used for permeation studies, and was prepared by adding 0.05 g MgCl\(_2\), 0.34 g KCl, 7.0 g NaCl, 0.18 g Na\(_2\)HPO\(_4\), 0.1 g NaH\(_2\)PO\(_4\), 1.8 g D-Glucose, 0.28 g CaCl\(_2\), and 1.26 g NaHCO\(_3\) to 1000 mL deionized water. Both buffer solutions contained 0.021 g/L sodium metabisulphite to control oxidation of L-DOPA.\(^{143}\) All reagents were purchased from Sigma Aldrich (Saint Louis, MO, USA).

2.2.2. L-DOPA solid microparticles

2.2.2.1. Solid-state characterization

Powder X-ray diffraction (PXRD) patterns were obtained using an X’Pert Pro MPD system (PANalytical B.V., Almelo, the Netherlands), equipped with a copper anode (Cu K\(\alpha\), \(\lambda = 1.5406\) Å), an auxiliary elliptical mirror, and X’Celerator\(^{\text{TM}}\) detector. L-DOPA powder was loaded between two layers of Kapton\(^{\circledR}\) film (ChemPlex, Palm City, FL) and spun in a vertical sample stage; consistent irradiation times of 135 s per step with an angular step size of 0.017 °2\(\theta\) were used for all diffraction experiments. PXRD data were collected over the range of 5–50 °2\(\theta\), using an operating voltage and amperage set, respectively, to 45.0 kV and 40.0 mA.

Thermogravimetric analysis (TGA) was performed in an open Pt pan using a TA instruments TGA (Model Q50, New Castle, DE). Approximately, 10 mg of sample was accurately weighed and placed in the pan. The furnace temperature was increased at a constant rate of 5 °C/min from 40 °C to 400 °C, as precise sample weights were recorded.
Differential scanning calorimetry (DSC) was performed using a TA instruments DSC (Model Q100, New Castle, DE). Accurately weighed samples (4.0–5.0 mg) were hermetically sealed in Al pans, and the furnace temperature was increased at a constant rate of 5 °C/min from 0 °C to 350 °C under constant 50 mL/min dry N\textsubscript{2} gas purge. A three-point enthalpy-temperature calibration was performed at 10 °C/min using o-terphenyl, In, and Sn standards. Measurements were performed in triplicate and the mean ± standard deviation is reported.

2.2.2.2. Particle Size analysis

Sieve fractionation (Performer III Model: SS-3, Gilson Company, Lewis Center, OH) was used to separate different size fractions of 5 g L-DOPA powder (Table 3.1). Collected fractions were weighed at intervals ranging from 15–30 min until the measured weight change was less than 0.1 g. The fine sieve cut representing 20–53 μm of L-DOPA was retained and stored for use in subsequent experimentation. Additionally, L-DOPA fines were analyzed using an Olympus BX-51 optical microscope equipped with polarizing filter, under 10x and 25x magnification. Photomicrographs were obtained to evaluate morphology and measure Feret diameter for approximately 300 particles.

2.2.3. L-DOPA solution

2.2.3.1. Assessment of chemical stability

To ensure chemical integrity during the course of tissue experiments, the concentration of L-DOPA in aqueous solution was determined using HPLC. Briefly, solid L-DOPA was dissolved in water and vortexed until no undissolved particles were observed. The solutions were analyzed for decreased potency under the conditions summarized in Section 2.3.2. Triplicate samples of each test combination were removed
from stock solutions following storage for 0, 1, 2, 3 and 7 days, and L-DOPA concentration was assayed in every sample. Previous reports indicate that aqueous stability of L-DOPA is unaffected by light, however, care was taken to avoid over-exposure, and all samples were stored in the dark.

L-DOPA concentration in all samples was determined using a Waters Alliance 2690 HPLC system (Millford, MA) equipped with photo diode array (PDA) detector using a Lichrospher 100 RP-18 column (25 cm x 4 mm with 5 µm particle size). Previously filtered and degassed mobile phase comprising of 97:3 mixture of monobasic potassium phosphate (0.01 M, pH = 2 adjusted with phosphoric acid) and acetonitrile was used. It has been previously demonstrated that L-DOPA remains chemically stable in the presence of strong acids. The mobile phase was pumped continuously at a flow rate of 1.0 mL/min with an injection volume of 20 µL. Standard solutions and test samples were analyzed at the detector wavelength of 280 nm. All solutions were diluted in mobile phase. Data were analyzed using one way analysis of variance (ANOVA) model. Post-hoc comparisons of individual time points were conducted using paired t-test statistics with a Bonferroni corrected significance level of 0.05 (two-tailed).

2.2.3.2. Measurement of L-DOPA saturation solubility

Equilibrium solubility of L-DOPA was determined by placing excess solid in SNES containing antioxidant in glass vial enclosures. Vials were stirred mechanically (200 rpm) in an orbital water bath shaker (Lab-Line instruments Inc., Melrose Park, IL, Model No. 3540) maintained at a constant temperature (25 °C or 37 °C). At predetermined time intervals, aliquots of 10 mL were withdrawn, filtered using 0.45 µm nylon filter (Millipore), and measured spectrophotometrically (UV Spectrophotometer
8453, Hewlett Packard, Germany) at \( \lambda = 280 \) nm. Experiments were carried out in triplicate; therefore, only mean values with S.D. error bars are reported.

### 2.2.4. L-DOPA Transport in Nasal Mucosa

Bovine olfactory mucosa was used as a model tissue for permeation studies, where L-DOPA permeability was measured by adapting the methods published by Chemuturi et al.\textsuperscript{143} Briefly, cow heads were obtained from Bud’s Custom Meats (Riverside, IA). The olfactory turbinates, which were covered by a pale yellow olfactory mucosa, were removed within 15 min of decapitation. The excised tissues were thoroughly rinsed with KRB and transported in fresh KRB maintained on ice; all studies were conducted within 4 h of procurement to ensure tissue viability.

The permeability of L-DOPA molecules through the bovine olfactory mucosa was measured during experiments that used NaviCyte vertical chambers (Harvard Apparatus, South Natick, MA). Tissue was carefully removed from the underlying cartilage and affixed with the mucosal side facing the donor compartment, between the opposing faces of the two half-chambers (donor and receiver), using a series of pins that surrounded the opening. L-DOPA was loaded into the donor compartment either as a solution in KRB (1 mL of 0.75 mg/mL) or as solid microparticles (1.5 mg L-DOPA powder), which were evenly sprinkled directly on the mucosal side of the tissue during apparatus assembly. It should be noted that the mass of L-DOPA microparticles deposited on tissues was 2-fold greater than that in solution samples to enable accurate weighing of the solid and reproducible transfer, while minimizing powder loss. The temperature of the diffusion cells was maintained using a heating block (Brinkmann Instruments Co., New York, NY). Tissue samples were equilibrated for 30 min in KRB at 37°C, followed by
replacement of donor and receiver with L-DOPA solution and fresh KRB, respectively. Alternatively, the equilibration step was performed prior to assembly of the chambers that were loaded with L-DOPA microparticles. The cells were aerated by constant carbogen flow (95% O₂ + 5% CO₂) at a rate of 3–4 bubbles/s. The pH of the donor and receiver solutions was adjusted to 5.7 ± 0.2 with 1 N HCl. Every 15 min, 200 µL samples were withdrawn from the receiver compartment and replaced with fresh, pre-warmed KRB. L-DOPA permeation was measured from the receiver compartment aliquots using the previously described HPLC method. Tests were performed in triplicate; results are expressed as mean ± SEM. At the end of each transport study, tissue viability was confirmed by measurement of the electrical resistance across the mucosal membrane, where non-viable tissue samples were considered those having measured control trans-epithelial resistance values (approximately 180 ohm/cm²) that dropped by more than 20%. Based on the TEER measurements, the viability of all tissue explants was maintained during the course of all transport studies. It is noted, however, that a paracellular transport marker (Lucifer yellow, LY) or histopathology tests could be used as an additional tool to verify tissue integrity.

Calculations of steady-state and apparent permeability coefficients were based on Fick’s First Law of diffusion. Steady-state flux \( J_{ss} \) was calculated according to Equation 2.1:

\[
J_{ss} = \frac{dM}{dt} \frac{1}{A}
\]

(2.1)

where \( M \) is the cumulative amount of drug in the receiver cell at time \( t \), \( dM/dt \) is the mass transport rate obtained from linear regression of the initial steady-state portion (µg/min)
and $A$ is the area of the exposed membrane, i.e., diffusion area ($0.64 \text{ cm}^2$). The apparent permeability coefficients ($P_{\text{app}}$) were calculated using Equation 2.2:

$$P_{\text{app}} = \frac{J_{\text{ss}}}{C_0} \times 60$$

(2.2)

where $C_0$ is the initial donor concentration ($\mu g/mL$), resulting in units for $P_{\text{app}}$ of cm/s. The underlying assumption of these equations is that the donor concentration of analyte remains constant for the period of steady state.

2.3. Results and Discussion

2.3.1. L-DOPA Solid

As with many other dosage forms involving crystalline particles, solid state characterization of the drug substance is necessary for development of dry powder inhalation products. In that regard, it is important to identify the presence of ordered/disordered phases that may significantly impact the reproducibility of manufacturing and therapeutic performance. L-DOPA powder was characterized using PXRD, DSC and TGA. The PXRD pattern of the vendor-supplied L-DOPA is shown in Figure 2.1, and corresponds well with the respective calculated reference pattern (Cambridge Structural Database refcode: LDOPAS03) indicating the presence of a single solid phase.\textsuperscript{146}
Figure 2.1. Experimental PXRD pattern for vendor-supplied L-DOPA powder (black solid line) is compared with simulated diffraction pattern (ICDD reference code: LDOPAS03 grey solid line). The data indicate that the powder sample is comprised of a single phase solid form.

In Figure 2.2, the DSC data show a single endotherm attributable to melting with an onset temperature \( (T_m) \) of 276.6 (1.3) °C, \( n = 3 \), which agrees closely with reported values (30, 31). Melting was followed immediately by rapid thermal degradation of L-DOPA at approximately 298.3 (3.8) °C \( (T_d) \), \( n = 3 \), which was consistent with the rapid 12.90% weight-loss observed in the TGA data. This suggested that proper care should be taken in all preparation and experimental procedures to avoid temperatures exceeding \( T_d \). Neither TGA nor DSC data showed signal consistent with desorption of water, suggesting that the applied storage conditions were appropriate for solid L-DOPA.
Figure 2.2. Experimental DSC thermogram of L-DOPA (solid line) showing $T_m = 276.6$ °C. Post-melting baseline is indicative of rapid thermal degradation. Superimposed TGA thermogram (dashed line) represents 12.90% weight loss beginning immediately at $T_d$, confirming degradation at $T>T_m$.

Particle size (PS) is an important parameter that is known to influence nasal delivery, therefore, sieve fractionation of L-DOPA powder was performed and the particle size distribution (PSD) was represented as cumulative percent undersize (Table 2.1).

**Table 2.1.** Particle size distribution of L-DOPA determined by sieve analysis.

<table>
<thead>
<tr>
<th>L-DOPA</th>
<th>Cumulative Percentage Undersize</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt; 20 µm</td>
</tr>
<tr>
<td></td>
<td>4.2</td>
</tr>
</tbody>
</table>
Recognizing that optimum *in vivo* olfactory deposition requires particles sized approximately 10–45 µm, the fraction of L-DOPA fines collected on the 20 µm sieve were retained for mass transport studies involving solid microparticles. It is acknowledged that, although sieve fractionation yielded a sufficient quantity of L-DOPA fines for the present studies, larger scales, and undoubtedly, manufacturing, would likely require comminution to homogenize L-DOPA particle size to this specification. In those cases, solid state characterization would be essential to ensure that the much harsher processing did not deleteriously change the L-DOPA solid. In the present study, the selected sieve fraction was characterized for surface and bulk morphology, with photomicrographs showing that the majority of L-DOPA crystallites exhibited a plate-like morphology (*Figure 2.3a – 2.3c*). The median primary particle size ($d_{50}$) was 20.93 µm, with $d_{10}$ and $d_{90}$ respectively measuring 9.5 µm and 43.7 µm (See *Fig. 2.3d*).
Figure 2.3. (a) Micrograph depicting morphology of L-DOPA primary particles immersed in oil, observed through a 25× objective; (b,c) typical micrographs used to measure Feret diameter (n = 300), 4x objective; (d) particle size distribution of primary particles observed in (b and c).

Although the morphology of particles obtained from analytical samples may not be particularly favorable for reproducibly emitted doses from nasal devices, it is expected that the size reduction step necessary at larger scales will likely make the particle morphology more regular; making it suitable for insufflation and nasal deposition.
2.3.2. L-DOPA Solution

The potency of L-DOPA solutions was evaluated when stored in combination with an antioxidant (0.021 g/L sodium metabisulphite). To simulate both storage and physiological conditions, 0.4 mg/mL L-DOPA solutions were held at 25 °C and 37 °C, respectively. The % recovery vs. time profiles of L-DOPA, subjected to experimental conditions are shown in Figure 2.4.

**Figure 2.4.** Effect of temperature and presence of antioxidant on the concentration of L-DOPA in aqueous solution. Data are shown as mean ± SD. Bonferroni multiple comparison (α = 0.05) between groups is shown to denote the extent of loss in L-DOPA concentration on day 7. The rate constants for degradation are summarized in Table 2.2.

Generally, it was noted that the extent of L-DOPA degradation increased with experimental temperature, as expected. In the solutions containing antioxidant, however, the L-DOPA content was less significantly reduced at both temperatures, suggesting that the sodium metabisulfite inhibited oxidative degradation in solution. After 7 days, a
maximum of 3.8% and 7.7% decrease in the amount of L-DOPA was recorded respectively for solutions maintained at 25 °C at 37 °C. In contrast, solutions without antioxidant resulted in greater L-DOPA degradations of 18.3 % (at 25 °C) and 31.4 % (at 37 °C). A moderate green discoloration in these samples was observed, suggesting solution-mediated oxidation of L-DOPA to L-dopaquinone as a primary degradation pathway. The data are in agreement with earlier reports and show goodness of fit to pseudo first-order kinetics.

The degradation rate constants were determined using Equation 2.3:

\[
-\frac{dC}{dt} = k(C) \leftrightarrow \ln \frac{C_0}{C} = kt
\]  

(2.3)

where \( k \) is the pseudo first-order rate constant and \( C_0 \) and \( C \) represent L-DOPA concentrations at time zero and \( t \), respectively. If the results of Figure 2.4 are plotted in the form of Equation 2.3, \( k \) values can be computed from the slopes of the straight lines, which is summarized in Table 2.2.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Rate constant (( k )) min(^{-1})</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 °C with antioxidant</td>
<td>( 0.7 \times 10^{-2} )</td>
<td>0.99</td>
</tr>
<tr>
<td>25 °C without antioxidant</td>
<td>( 3.4 \times 10^{-2} )</td>
<td>0.99</td>
</tr>
<tr>
<td>37 °C with antioxidant</td>
<td>( 1.5 \times 10^{-2} )</td>
<td>0.99</td>
</tr>
<tr>
<td>37 °C without antioxidant</td>
<td>( 9.7 \times 10^{-2} )</td>
<td>0.99</td>
</tr>
</tbody>
</table>
These results were important in deciding a rigorous timeline for the permeation studies and subsequent quantitative analyses. Accordingly, L-DOPA solutions containing antioxidant were prepared on the day of permeation tests, thereby minimizing degradation to < 1%. Although it is recognized that chronic use of metabisulphites can result in cellular injury,\textsuperscript{149} the time of antioxidant exposure in these permeation tests is not expected to be particularly damaging to the nasal olfactory tissue.

The saturation solubility of L-DOPA microparticles isolated through sieve fractionation (see \textbf{Section 2.3.1}) was evaluated prior to tissue permeation experiments. \textbf{Figure 2.5} plots the concentrations obtained in the dissolution medium for crystalline L-DOPA microparticles as a function of time, in the presence of excess solid phase.

\textbf{Figure 2.5.} Saturation solubility of L-DOPA microparticles. Closed grey squares (■) and closed black circles (●) represent solubility profile at 25° and 37°C, respectively. In 12 h, the measured saturation concentration (Cₜ) of L-DOPA was almost 1.6 times higher at 37°C (p <0.001, Student’s \textit{t}-test).
At equilibrium, the measured saturation concentration \((C_s)\) of L-DOPA was, respectively, \(1.73 \pm 0.15\) mg/mL and \(2.77 \pm 0.16\) mg/mL at \(25\) °C and \(37\) °C. The data corroborated earlier reports indicating slight solubility of L-DOPA in aqueous medium.\(^{150,151}\) These measurements were not atypical, owing to the zwitterionic nature of the L-DOPA molecule at pH values between 3–9, having an amine group \((\text{pK}_a 8.11)\) and a carboxylic acid \((\text{pK}_a 2.30)\). Consequently, increased solution potencies are possible only when the pH of the solution is altered dramatically. For example, the solubility of L-DOPA is reported to be 10 mg/mL in 0.01 M maleic acid \((\text{pH} = 2)\).\(^{20}\) Considering that such extreme conditions do not accurately simulate the physiological environment of the nasal cavity, and could potentially affect the integrity of nasal tissue, using dissolution media having such a low pH is not recommended. In the present experiments, PXRD data obtained for solid residue in contact with KRB at either \(25\) °C or \(37\) °C was consistent with the L-DOPA Cambridge Structural Database powder pattern, suggesting that the solid phase remained unchanged during the experiments (data not shown).

### 2.3.3. L-DOPA transport studies

Permeation of L-DOPA through bovine olfactory tissue was evaluated in vitro. Since liquid formulations are the most commonly developed dosage forms for IN delivery, 1 mL of a 0.75 mg/mL L-DOPA solution in KRB was chosen as a comparator for mass transport resulting from the 1.5 mg of L-DOPA solid microparticles directly deposited on the epithelial tissue surface. The amount of L-DOPA measured in the receiver compartment over time was used to calculate \(J_{ss}\) and \(P_{app}\) as described above (Equations 2.1 and 2.2). Figure 2.6(a) demonstrates the permeation profiles across nasal mucosa, when L-DOPA was loaded as either solution or solid microparticles. After 120
min of contact with nasal mucosa, the cumulative amount of L-DOPA that permeated from solution, per unit area tissue, was 225.13 ± 38.31 µg cm$^{-2}$ (n = 3). In contrast, a maximum of 501.07 ± 105.63 µg cm$^{-2}$ mass transport was measured when the donor side was loaded with L-DOPA solid microparticles (n = 2).

This was also reflected in the average $J_{ss}$ values, where approximately 3-fold higher flux was measured in the diffusion cells loaded with L-DOPA solid when compared with those containing L-DOPA solution (See Table 2.3). The linear region in all permeation profiles, chosen based on goodness-of-fit ($R^2 >0.97$), was used to estimate mass transport under steady-state conditions (shown in dashed line).

**Table 2.3.** Comparison of average *in vitro* nasal epithelial flux of L-DOPA from aqueous solution and solid microparticles.

<table>
<thead>
<tr>
<th>L-DOPA</th>
<th>Average steady state flux ($J_{ss}$) µg.cm$^{-2}$.min$^{-1}$ (correlation) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>aqueous solution $^a$</td>
<td>2.13 (0.95) 0.97</td>
</tr>
<tr>
<td>solid microparticles $^b$</td>
<td>6.08 (0.97) 0.69</td>
</tr>
</tbody>
</table>

$^a$ number of replicates = 3  
$^b$ number of replicates = 2
Figure 2.6. *in vitro* permeation profiles of L-DOPA across bovine olfactory mucosa from an aqueous solution (empty circles, $n = 3$) and solid particles (filled circles, $n = 2$). (a) Data are expressed as cumulative L-DOPA permeated with time (mean ± SD). The solid connecting line represents the initial steady-state portion that was chosen to calculate $J_{ss}$ according to Eq. 1; (b) data normalized with respect to initially loaded mass of L-DOPA *i.e.*, 0.75 mg in 1 mL donor solution and 1.5 mg as solid microparticles.
It should be noted that no additional dissolution medium was added to the donor compartments containing the solid microparticles, which were directly deposited on tissue surfaces. It was expected that dissolution of the L-DOPA in the naturally moist surface of olfactory mucosal membrane in these experiments would occur similarly in vivo following insufflation.

Despite a low hydration volume, the relatively rapid dissolution of L-DOPA from microparticles in the nasal mucus is proposed to occur by two mechanisms. Given that the average size of the primary L-DOPA microparticles was approximately 20 µm, a fraction of the solid was expected to rapidly dissolve in the small volume of nasal fluid, owing to an increase in the surface energy and thereby improved wettabili

152 The other contribution to rapid dissolution includes favorable interactions forming between the overall negative charge, typical of nasal cell surfaces (resulting from the presence of sialic acid in nasal mucin), and the positively charged L-DOPA molecules expected at experimental pH.\textsuperscript{153} As a consequence, the L-DOPA microparticles were expected to saturate the mucus layer, the concentration of which was expected to be close to \( C_s \) (2.77 ± 0.16 mg/mL at 37°C). Such a strong driving-force can potentially justify the rapid mass transport observed within the first 60–75 min of contact with nasal mucosa. The subsequent reduction in L-DOPA flux may be attributed to removal of fine solid particles away from the diffusion area under gravity.

The average apparent permeability coefficient (\( P_{\text{app}} \)) of L-DOPA was calculated to be \( 4.73 \times 10^{-5} \text{ cm/s} \) from solution samples. As the chemical structure of the molecule was not altered in this study, it was assumed that \( P_{\text{app}} \) should remain consistent for L-DOPA solid samples, even when the measured membrane flux (\( J_{ss} \)) was considerably
different. Using **Equation 2.2**, the apparent initial concentration \( (C_o) \) for solid samples was calculated to be \( 2.14 \pm 0.24 \) mg/mL. The closeness of this value to the experimentally measured \( C_s \) (2.77 mg/mL) supports that the claim the L-DOPA solid particles saturated the nasal mucus (refer to **Figure 2.7**).

To calculate \( P_{app} \), it was taken the donor concentration remains constant for the steady state region (dashed line). In order to test the validity of this assumption for the solid microparticle group, the cumulative L-DOPA transport at 60 min is used as an example. The mean cumulative L-DOPA permeated at 60 min is \( 220.31 \pm 50.98 \) μg. If L-DOPA trapped within the olfactory tissue is insignificant, the amount of drug remaining on the donor side is about 1280 μg which is still expected to saturate the nasal mucus. Therefore, it supports the assumption of steady donor concentration. Beyond this time point, it can be seen that the permeation profile levels off which may be attributable to slow drifting away of particles from the diffusion area under gravity.
Figure 2.7. Schematic representation of the L-DOPA permeation of nasal epithelial cells. Green closed circles represent directly deposited L-DOPA microparticles on the donor side of tissue, which saturate the mucus layer ($C_s = 2.77$ mg/mL). This contrasts the dark blue shaded region denoting L-DOPA solution contact with the mucus layer at a constant concentration throughout the entire donor chamber (0.75 mg/mL). As depicted, saturation of the mucus layer by L-DOPA dissolved from solid microparticles results in a much higher steady-state concentration gradient that explains the 3-fold increase in flux relative to L-DOPA solution experiments.
It is acknowledged that the starting mass of L-DOPA solid was almost double the quantity loaded in solution, which was necessitated in order to minimize weighing errors. Therefore, the comparison between the mass transport from either physical form is incomplete if the cumulative L-DOPA transport values are not normalized with respect to initial concentration. The adjusted data showed that approximately 11.79% of the initial amount was permeated from L-DOPA microparticles within 30 min. In contrast, only about 7.84% of the initial concentration of L-DOPA was permeated from molecules in solution, as shown in figure 2.6(b). It is clear that, despite normalization of mass transport values, the solid microparticles demonstrated greater L-DOPA permeation (up to 75 min), which can be related to the higher concentration gradient across the epithelial tissue. Notably, the maximum % of L-DOPA transported after 120 min was comparable, with 30.1% and 33.4% respectively for the solution and powder samples, however, the present data still represent a significant finding in that the delivery of L-DOPA from solid microparticles will not be restricted by low dissolution volume in the nasal cavity. Moreover, the residence time for solid microparticles is expected to be much greater relative to solution, which is rapidly drained away from the delivery site by normal mucocilliary clearance and flow due to gravity. As such, the more extensive initial permeation from the microparticles suggests that delivery in this physical form may provide the opportunity for greater drug absorption.

2.4 Conclusions

Deposition of solid microparticles on nasal mucosa via insufflation is a potentially viable, yet relatively unexplored means of IN drug delivery. These data showed that total
in vitro nasal permeation of L-DOPA dissolved from solid microparticles was comparable with aqueous solution. Almost three-fold greater nasal epithelial steady-state flux was observed for L-DOPA absorbed from powder experiments, indicating rapid mass transport. Furthermore, well known issues related to chemical stability of aqueous solution of L-DOPA as well as potential tissue injury owing to the use of sodium metabisulfite in solution formulations were successfully avoided by deposition of solid microparticles, which required neither an aqueous vehicle nor antioxidant. Collectively, the data demonstrated that dry, solid powder dosage forms could be developed for IN delivery of L-DOPA. In the pharmaceutical field, such evidence could be particularly important in treatment of other neurological disorders where no straightforward, non-invasive therapies exist in current clinical practice.

2.5 Acknowledgments

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Chapter 3: Solid-state transformations of ribavirin as a result of high shear mechanical processing

3.1. Introduction

Ribavirin is predominantly administered via solid oral dosage forms. Some marketed products include Copegus® (200 mg film coated tablets; Hoffman La-Roche), Rebetol® (200 mg hard capsules and 40 mg/mL oral solution; Merck), Ribasphere® (200, 400 and 600 mg hard capsules; Three Rivers Pharmaceuticals), and Moderiba™ (200, 400, 600 mg film coated tablets, AbbVie). Additionally, it is also given as a powder for inhalation solution (Virazole® 6 g vials; Valeant Pharmaceuticals). Regardless of the delivery system, it is likely that solid ribavirin will be subject to some degree of high-shear mechanical processing, including milling, granulation and compaction, making unanticipated solid-state transformations, including crystal-to-crystal and crystal-to-glassy solids, a possibility during manufacturing.

Over the last few decades, numerous examples of process-induced phase changes of pharmaceutically relevant materials have been reported. Some estimates suggest that approximately 30% of active pharmaceutical ingredients (API) are susceptible to solid form changes during mechanical processing. The impetus to identify potential process-induced transformations during processing, followed by thorough characterization and elucidation of the underlying mechanisms is important for product development, and consistent with the materials understanding at the heart of worldwide regulatory guidances.
Ribavirin was previously reported as solidifying as one of two unique crystalline polymorphs, conventionally named R-I and R-II, which respectively represent the high-melting and low-melting temperature enantiotropes. Careful selection of a consistent solid form during pre-formulation, which persists in the final drug product, is, therefore, warranted. In addition, the impalpable nature of ribavirin powder, accompanied with low and variable tapped densities (reported range 0.32-0.45 g/mL) pose considerable challenges to material handling. While flow properties and processability of ribavirin can be improved through secondary manufacturing procedures, such as pelletization, roller compaction and wet granulation; the potential for concurrent solid-state transformations during manufacturing has not been investigated. It seems likely that, consistent with other small molecule crystalline materials, unanticipated partial or complete phase conversions can alter the mechanical properties, processability, and performance attributes of the drug substance in the final drug product.

Polymorphic transformations of pharmaceutically relevant solid materials are a known potential consequence of milling, which can affect the quality and performance of the final drug product. When a crystalline API is milled, the excessive shear stress accompanied by other manifestations of very high mechanical energy (such as heat), elicit structural changes to the crystal, while introducing substantial lattice defects that alter the physical and chemical properties of the solid. While empirical models of mechanical activation potential exist, their utility is restricted to specific experimental conditions such as temperature, equipment dimensions, and sample size, making universal predictions elusive. Experimental observation of mechanically induced
transformations, therefore, remains important, especially if a range of temperatures, processing stresses and other conditions are expected.

In the present work, the influence of thermal and mechanical stresses on ribavirin was investigated as they pertained to the potential to induce solid-state phase transformations as a consequence of processing. Following the observation that the kinetics of a temperature-mediated enantiotropic conversion was prohibitive for unprocessed ribavirin, it was hypothesized that transitions between polymorphs could be observed in shorter timeframes when defects resulting from pulverization enabled more rapid transformations at elevated temperatures. To test the hypothesis, phase pure samples of each ribavirin enantiomere were generated, and the enantiotropic transition temperature ($T_{tr}$) was determined. Samples were milled, with and without temperature control, and the outcomes of mechanical and/or thermal stimuli were recorded. Particle size reduction, commensurate with increased specific surface area, and the extent of crystal damage were measured to identify which of these factors predominate in the observed phase transformations. This additional effort was expected to provide a better understanding of the likelihood of phase conversion during other manufacturing steps (such as compaction, which also involves particle fracture and crystal damage). Reversibility of the polymorphic transition (R-I $\rightarrow$ R-II) was also investigated by isothermal exposure of the phase-pure metastable polymorphic form to varied conditions of relative humidity. Ultimately, these observations serve as a reference for materials that may be similarly susceptible to mechanically induced phase changes.
3.2. Materials and Methods

Ribavirin was purchased from Jinan Jiaquan Chemicals Co. Ltd. (Jinan, China, Lot number 02110330). Samples were stored in a desiccator containing phosphorous pentoxide (P₂O₅) at ambient temperature prior to use.

3.2.1. Recrystallization of Ribavirin Polymorphs

Phase-pure samples of ribavirin polymorphs were prepared by recrystallization from solvents, using previously reported methods with slight modification. To obtain pure R-II, a slight excess of vendor supplied ribavirin (R-RW) was dissolved in water at 80 °C to form a supersaturated solution. The suspension was filtered and left to cool slowly to room temperature. The resulting crystals were vacuum dried at 25 °C overnight and stored for further characterization.

Pure R-I was prepared by fractional recrystallization, where a supersaturated solution of R-RW was formed in methanol (> 99% HPLC grade, Fisher Scientific) at 60 °C and filtered. The filtrate was dried under a current of air, which facilitated rapid crystallization, owing to rapid evaporation of organic solvent, accompanied by a temperature drop. Upon appearance of the first few crystals, the mother liquor was re-filtered to harvest pure R-I; phase purity was confirmed by rapid differential scanning calorimetry (DSC) and peak-by-peak comparison of powder X-ray diffraction (PXRD) patterns with the known crystal structure (CSD refcode: VIRAZL). Experimentation demonstrated that the re-filtration to harvest R-I crystals was required to prevent recrystallization of a mixture of phases. Isolated R-I crystals were dried in a vacuum oven, and used as seeds for producing subsequent batches (0.5-1.0 g) of pure R-I.
3.2.2. Generation of Amorphous Ribavirin

Amorphous ribavirin was prepared in situ during DSC experiments. An accurately weighed sample of R-II was heated to \( T_m + 10^\circ C \) at a rate of 5 \( ^\circ C/\text{min} \), and held isothermally for 10 min. The molten sample was rapidly cooled at 40 \( ^\circ C/\text{min} \), with no observation of recrystallization, and the \( T_g \) was determined during the subsequent DSC heating cycle.

3.2.3. Isothermal heating experiments

Conversion to the high-temperature stable enantiotrope, R-I, was attempted using a method similar to that reported for chlorpropamide enantiotropes.\(^{172}\) Approximately 500 mg of R-II powder was placed in glass petri dishes, which were held isothermally at 150\(^\circ\)C (\( T < T_m \) for either polymorph) in an Isotemp 13-246-506GA gravity convection oven (Fisher Scientific, Dubuque, IA). Thermal exposure was maintained as long as the sample did not exhibit signs of thermal degradation. Samples were periodically characterized to identify the emergence of the R-I phase, by comparison of experimental diffraction patterns with the calculated PXRD patterns of each enantiotrope.

3.2.4. Milling experiments

R-II was milled at frequency of 30 Hz in a vibratory impact ball mill (Mixer Mill MM200, Retsch GmbH & Co., Germany) for milling durations of 60, 180 and 270 min. A 0.5 g powder sample was placed in a 25 mL volume stainless steel jar containing one 12 mm diameter stainless steel ball. Milled samples were immediately characterized using PXRD and DSC. Although care was taken to avoid excess heat accumulation, by intermittently opening the milling jar for short time intervals, it is acknowledged that the procedure does not allow rigorous temperature control.
In contrast, cryogenic grinding of R-II was accomplished using a SPEX Certiprep 6750 Freezer Mill (SPEX Certiprep, Metuchen, NJ) in which powder samples weighing approximately 1.0 g were placed in a polycarbonate milling vessel. A stainless steel cylindrical milling rod was placed in the vessel, and samples were allowed to equilibrate for 3 min following immersion in a liquid nitrogen bath prior to milling. Temperature control during processing was enabled by consistently maintaining the liquid nitrogen bath during the experiments, allowing continuous immersion of the samples and milling medium throughout comminution. Similar to a previously published method, materials were ground for 2 min intervals at a frequency of 10 Hz, separated by 2 min cool-down periods, allowing minimization of local sample temperature increases during experimentation. Upon completion of milling, triplicate samples were analyzed using PXRD and DSC.

The particle size and size distributions of unmilled R-II and all milled samples were determined using an Olympus BX-51 optical microscope equipped with polarizing filter, at 10x magnification. Primary particles were separated by preparing a dilute suspension in immersion oil, which was placed between microscope slides and a coverslip. Photomicrographs for each sample were collected to measure the Feret diameter for approximately 300 particles.

3.2.5. Influence of storage conditions

Recrystallized R-I was observed for re-conversion to the stable polymorph under varied conditions of relative humidity at ambient room temperature (22 ± 2°C). Each 300 mg sample was equilibrated at 0% RH by storage in a desiccator with P2O5, or at % RH of 33%, 60%, and 75% by equilibration over saturated solutions, respectively containing
MgCl₂, NaBr, or NaCl. Repeated hygrometer measurements ensured that each humidity condition was maintained at ± 3% of the desired values. Samples were evaluated for phase changes using PXRD at regular intervals for up to 60 days.

3.2.6. Characterization

3.2.6.1. Thermogravimetric Analysis (TGA)

Samples were evaluated for the possibility of solvate formation using a thermogravimetric analyzer (Model Q500, TA Instruments, New Castle, DE). Samples weighing approximately 10 mg were placed on an open platinum pan and heated at 2°C/min under constant nitrogen purge at 60 mL/min. Subsequent thermograms were examined for stepwise weight loss commensurate with desolvation.

3.2.6.2. Differential Scanning Calorimetry (DSC)

Conventional and modulated temperature DSC analyses were performed using a TA Instruments Q100 differential scanning calorimeter (TA Instruments, New Castle, DE) with an attached refrigerated cooling accessory. Dry nitrogen gas (50 mL/min) was allowed to constantly purge the DSC cell during all experiments, while a three-point temperature and enthalpy calibration was accomplished using Sn ($T_m$ 231.9 °C), In ($T_m$ 156.6 °C), and o-terphenyl ($T_m$ 55-56 °C) standards. Conventional DSC measurements were made to determine the melting temperatures ($T_m$) of ribavirin polymorphs. Accurately weighed samples (5.0 ± 0.5 mg) were hermetically sealed in an Al sample pan and heated at constant rates of 0.5, 2, 5, 10, 20, or 40°C/min to a temperature beyond the reported $T_m$. All measurements were performed in triplicate and reported as mean values ± standard deviation.
The heat capacities of R-I, R-II and amorphous ribavirin were determined in the MDSC (modulated temperature) mode, using experimental parameters carefully selected to produce accurate and artifact-free data. A modulation amplitude of ± 0.256 °C, period of 100 s, and an underlying heating rate of 1 °C/min were used to allow 4-5 modulation cycles over the critical temperature range of transition (55-65 °C) while minimizing the thermal gradient across the sample. Samples weighing approximately 10 mg were loaded and tightly packed in standard Al pans, which were subsequently crimped to ensure good sample-to-pan contact. Background noise was minimized by ensuring that sample and reference pan weights matched within ± 0.02 mg. Sample heat capacities were then obtained by deconvoluting the non-reversing and reversing signals from the total heat flow, using instrument software (Universal Analysis 2.0, TA Instruments). The heat capacities of both the crystalline and amorphous forms of ribavirin were measured over temperatures ranging from \( T_g - 50 \) °C to \( T_g + 50 \) °C. The heat capacity constant (\( K_{Cp} \)) was previously calibrated using a sapphire disc weighing approximately 25 mg. Additionally, vacuum dried samples of crystalline sucrose were used as external standard to verify the precision of the instrument, and measured heat capacities were compared with the literature reported values.

3.2.6.3. X-ray powder diffraction (PXRD)

Powder X-ray diffraction (PXRD) patterns were obtained using an X’Pert Pro MPD diffractometer (PANalytical B.V., Almelo, the Netherlands), equipped with an auxiliary elliptical mirror and X’Celerator™ detector. Powder samples were placed between two layers of Kapton® film (ChemPlex, Palm City, FL) and spun in a vertical sample transmission stage. Irradiation with X-rays from a copper source (Cu K\( \alpha \), \( \lambda = \))
1.5406 Å) for a duration of 135 s per step, and incremental angular steps of 0.017 °20 were used for all diffraction experiments. PXRD data were collected over the range of 5–50 °20, using an operating voltage and amperage set, respectively, to 45.0 kV and 40.0 mA.

3.2.6.4. Solution Calorimetry

The heats of solution ($\Delta H_{\text{sol}}$) at 25 °C were determined for ribavirin polymorphs using an isothermal heat-conduction microcalorimeter (Model 7211, Calorimetry Sciences Corp., Lindon, UT). Solid samples of phase pure polymorphs were accurately weighed (25±1 mg), and the integral heat of solution was measured as each was dissolved into 25.0 mL of distilled water. The dissolution medium was stirred at 50 rpm by a paddle. Triplicate measurements were made and reported as an average ± standard deviation.

3.2.6.5. Measurement of aqueous solubility

The aqueous solubility of ribavirin polymorphs was determined by placing excess solid in screw-capped glass vials containing distilled water. Vials were stirred mechanically in an orbital water bath shaker (Model 3540, Lab-Line Instruments Inc., Melrose Park, IL) maintained at a constant temperature of 25°C and 200 rpm speed. At appropriate time intervals, 10 mL aliquots were withdrawn, filtered using 0.45 µm nylon filter and measured spectrophotometrically (UV Spectrophotometer 8453, Hewlett Packard, Germany) at $\lambda = 207$ nm. Experiments were carried out in triplicate; therefore only mean values with S.D. error bars are reported.
3.2.6.6. Surface area determination (BET)

Brunauer, Emmett and Teller (BET) surface area quantification was performed using a Flow Sorb II 2300 (Micromeritics Corp., Norcross, GA) instrument and a five-point BET N\textsubscript{2} adsorption protocol. Samples weighing approximately 200 mg were degassed under dry nitrogen for 12 h prior to analysis. Room temperature was used to try and avoid any possible phase transformation. Replicate measurements were performed to verify the precision of each measurement.

3.2.6.7. High performance liquid chromatography (HPLC)

Ribavirin was assayed using a published stability-indicating HPLC method with slight modifications.\textsuperscript{175} The analysis employed a Hypersil C18 reverse phase column (5 \(\mu\)m, 250 mm \(\times\) 4.6 mm i.d., Waters, MA, USA) and a Waters 2690 LC system equipped with a Waters 996 photodiode array detector and an autosampler (Waters Corp., MA, USA). The mobile phase, comprised of 0.01 M potassium dihydrogen phosphate solution and pure methanol (95:5, v/v), was eluted isocratically at a flow rate of 1 mL/min, while the detector response was set at \(\lambda = 207\) nm. Ribavirin was eluted as a single peak in the chromatogram at approximately 4.55–4.65 min.

R-RW, R-II and R-I samples were separately dissolved in distilled water to a final concentration of 50 \(\mu\)g/ml, of which 20 \(\mu\)L was injected into the column. A calibration curve constructed using standard ribavirin solutions within the appropriate concentration range (10 – 90 \(\mu\)g/ml) displayed excellent linearity with an \(R^2\) larger than 0.99. All measurements were performed in triplicate.
3.3. Results and Discussion

3.3.1. Physical characterization of ribavirin polymorphs

The PXRD pattern for ribavirin obtained from the vendor (R-RW) was compared with simulated reference patterns obtained from the Cambridge Crystallographic Data Centre (Figure 3.1). All peaks for R-RW corresponded with the reference pattern for R-II (CSD refcode: VIRAZL01) with no discernable peaks attributable to R-I (CSD refcode: VIRAZL).176
Figure 3.1. The experimental PXRD pattern for vendor-supplied ribavirin powder R-RW (red) is compared with recrystallized ribavirin R-I (green) and recrystallized ribavirin R-II (blue). Simulated diffraction patterns for R-I (CSD reference code: VIRAZL, in grey) and R-II (CSD reference code: VIRAZL01, in black) are also provided. The data indicate that R-RW corresponded entirely with the R-II pattern, and did not contain discernable quantities of R-I.
DSC thermograms for R-RW collected at 2 °C/min exhibited a melting endotherm at 166.05 °C for R-II; followed by partial recrystallization and subsequent melting at 174.9 °C for R-I (Figure 3.2). This thermal behavior was in close agreement with that reported in the literature,\textsuperscript{162,163} and not unexpected for enantiotropes characterized by DSC. To ensure phase purity of the starting materials used for subsequent experimentation, ribavirin polymorphs were recrystallized from solvent, as described in Section 3.2.1. As shown in Figure 3.2b, the DSC thermograms of the isolated samples of R-I and R-II exhibited no thermal features identifiable with the other solid form, suggesting that recrystallization had resulted in pure phases. Additionally, PXRD patterns for samples of R-I and R-II recrystallized from solvent (Figure 3.1), were consistent with the respective calculated reference patterns obtained from the CCDC. TGA data for each recrystallized sample did not show weight loss prior to melting, suggesting that residual solvent from recrystallization was removed during vacuum drying, and confirmed that neither crystalline form was solvated.
Figure 3.2. Comparison of DSC thermograms of R-RW (red) with recrystallized phase pure samples of R-I (green) and R-II (blue). R-RW melted at 166 °C consistent with R-II ($T_m = 168.1 \pm 0.7$ °C) followed by recrystallization and subsequent melting at 174 °C, consistent with R-I ($T_m = 177.4 \pm 0.2$ °C). The superimposed TGA thermogram (dashed line) shows no weight loss until approximately $T_m R-I + 30$ °C, confirming that both solid forms of ribavirin were thermally stable, and essentially devoid of residual solvent.
Comparison of relevant crystallographic and thermal properties of ribavirin polymorphs, as listed in **Table 3.1**, revealed that the higher $T_m$ form had a lower enthalpy, indicating enantiotropic polymorphism, per the heat of fusion rule.\textsuperscript{177} The reported crystallographic densities of R-I and R-II (at ambient temperature) appear to violate the density rule;\textsuperscript{176} however, these observations are consistent with other small molecule solids at room temperature, including hydroquinone\textsuperscript{178} and resorcinol.\textsuperscript{179}

**Table 3.1.** Summary of crystallographic and thermal properties of ribavirin solid forms\textsuperscript{177}

<table>
<thead>
<tr>
<th>Properties</th>
<th>R-II</th>
<th>R-I</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Crystallographic properties</strong></td>
<td>Unit cell dimensions (Å)</td>
<td>Unit cell dimensions (Å)</td>
</tr>
<tr>
<td></td>
<td>$a = 25.034$</td>
<td>$a = 14.863$</td>
</tr>
<tr>
<td></td>
<td>$b = 7.719$</td>
<td>$b = 7.512$</td>
</tr>
<tr>
<td></td>
<td>$c = 5.289$</td>
<td>$c = 8.788$</td>
</tr>
<tr>
<td>Density (g/cm$^3$) = 1.587</td>
<td>Space group = P2$_1$2$_1$2$_1$</td>
<td>Space group = P2$_1$2$_1$2$_1$</td>
</tr>
<tr>
<td>$Z = 4, Z' = 1$</td>
<td></td>
<td>$Z = 4, Z' = 1$</td>
</tr>
<tr>
<td><strong>Thermal properties</strong></td>
<td>Melting temperature - $T_m$</td>
<td>Melting temperature- $T_m$</td>
</tr>
<tr>
<td></td>
<td>$168.1 \pm 0.7 , ^\circ$ C</td>
<td>$177.4 \pm 0.2 , ^\circ$ C</td>
</tr>
<tr>
<td>Enthalpy of melting – $\Delta H_m$</td>
<td>$44.1 \pm 0.8 , $kJ/mol</td>
<td>Enthalpy of melting – $\Delta H_m$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$41.3 \pm 0.1 , $kJ/mol</td>
</tr>
</tbody>
</table>
3.3.2. Estimation of transition temperature ($T_{tr}$) and enthalpy ($\Delta H_{tr}$)

The determination of $T_{tr}$ for ribavirin was important in order to identify the circumstances under which its enantiotropes were subject to conversion. For enantiotropically related systems, such as carbamazepine$^{180,181}$ or sulfamerazine$^{182}$, identification of $T_{tr}$ allowed assessment of the risks associated with conversions to different forms by temperature excursions during processing.$^{183}$ In the present work, two independent methods were used to determine this value: 1) interpolation of a $G_c$ phase diagram and 2) solubility extrapolation.

1) $G_c$ phase diagram method: Configurational thermodynamic quantities have traditionally been used to estimate the molecular mobility and physical stability of amorphous solid forms.$^{184,185}$ More recently, however, this has been applied to estimate $T_{tr}$. $^{186,187}$ Calculations of thermodynamic parameters, such as configurational enthalpy ($H_c$) and configurational entropy ($S_c$) were used to plot $G_c$ versus $T$ using the following equations:

$$H_c(T) = H_{am}(T) - H_{xtal}(T) = \Delta H_m + \int_T^{T_m} C_{pconf} dT$$  \hspace{1cm} (3.1)

$$S_c(T) = S_{am}(T) - S_{xtal}(T) = \frac{\Delta H_m}{T} + \int_T^{T_m} \frac{C_{pconf}}{T} dT$$  \hspace{1cm} (3.2)

$$G_c(T) = H_c(T) - S_c(T) \cdot T$$  \hspace{1cm} (3.3)

where $C_{pconf}$ is the reversing heat capacity (Rev $C_p$) difference between the amorphous and crystalline forms (Refer Figure 3.3).

$$C_{pconf(R-I)} = C_p^{am} - C_p^{R-I}$$  \hspace{1cm} (3.4)

$$C_{pconf(R-II)} = C_p^{am} - C_p^{R-II}$$  \hspace{1cm} (3.5)
Figure 3.3. (a) Reversible heat capacity as a function of temperature for crystalline forms of ribavirin; R-II in blue and R-I in green, respectively. Amorphous ribavirin is shown in black. (b) Configurational heat capacity for R-I and R-II shown in green and blue respectively.
The point of intersection of the $G_c$ curves for the two ribavirin solid forms was taken as the thermodynamic transition temperature ($T_{tr}$), which is shown in Figure 3.4 ($T_{tr} = 68.4 \, ^\circ\text{C}$).

![Figure 3.4](image)

**Figure 3.4.** Configurational free energy ($G_c$) phase diagram for ribavirin polymorphs. $G_c$ of R-I is denoted by green dashed line and of R-II is denoted by blue solid line. Intersection ($T = 341.6 \, \text{K}$) represents $T_{tr} = 68.4 \, ^\circ\text{C}$.

Although this technique only provides an estimate of $T_{tr}$, the enthalpy associated with the polymorphic transition ($\Delta H_{tr}$) was calculated from the difference between the experimentally obtained heats of fusion,¹⁸⁸ and determined to be 2.8 kJ/mol. While the parameters of melting temperatures and heats of fusions derived from thermoanalytical techniques are routinely used for estimation of $T_{tr}$, if concurrent thermal events occur (e.g., commensurate melting and recrystallization or melting with decomposition), then the reliability of the data may be questionable.
2) **Solubility extrapolation:** Determination of solution enthalpies ($\Delta H_{\text{sol}}$) is also a widely accepted technique used to differentiate between polymorphs.\textsuperscript{181,182,187} In order to confirm the values obtained by interpolation of the $G_c$ data, a complementary method that used the heat of solution and solubility data was performed for estimation of $T_{tr}$ (Equation 3.6):

$$T_{tr} = \left( \frac{2.303 \cdot R \cdot (\log S_{II,T} - \log S_{I,T})}{\Delta H_{tr,T}} + \frac{1}{T} \right)^{-1} \quad (3.6)$$

Above, the logarithmic solubility of R-I and R-II were represented as $\log S_I$ and $\log S_{II}$, respectively, at temperature $T = 25 \, ^\circ\text{C}$. $\Delta H_{tr,T}$ is the heat of transition corresponding to the difference in the heat of solution at temperature, $T = 25 \, ^\circ\text{C}$. Although the heat of solution varies with the choice of solvent, the heat of transition corresponding to the difference in the heat of solution is theoretically equal. In distilled water, the heat of solution for R-I and R-II was measured to be $-29.4 \pm 0.8$ kJ/mol and $-31.2 \pm 0.1$ kJ/mol, respectively, while $\Delta H_{T,25 \, ^\circ\text{C}}$ was estimated to be $1.8 \pm 0.35$ kJ/mol.

**Figure 3.5** plots the concentrations of each form of ribavirin in distilled water as a function of time, in the presence of excess solid phase. As shown, both solid forms reached a plateau of maximum solubility within 30 min, at which the measured saturation concentrations at 25 °C of R-I and R-II were, respectively, $162.5 \pm 3.6$ mg/mL and $149.0 \pm 1.7$ mg/mL.
Figure 3.5. Aqueous saturation solubility of ribavirin polymorphs. Closed green circles (●) and open blue circles (○) represent solubility profile of R-I and R-II, respectively. Data are represented as mean ± standard deviation, n = 3.

In all cases, it was confirmed by DSC and PXRD that the residue did not convert to another solid form during the course of the experiment (data not shown), confirming that these values are specific to R-I and R-II. When these measured values were substituted into Equation 3.6, the $T_{tr}$ for R-I and R-II polymorphs was determined to be 71.3 °C, which agrees well with the value obtained by interpolation of the $G_c$ diagram (68.4 °C). The slight difference in values is likely attributable to the underlying assumptions of each technique, but suggests a good approximation of the actual $T_{tr}$. Both methods employed here assume that the heat capacity difference between the two polymorphs is independent of temperature. Further, the melting method assumes that enthalpy of fusion is independent of temperature. Likewise, the solubility – heat of solution method assumes the difference between the activity coefficients of two
polymorphs is negligible. Ultimately, since this polymorphic pair has a $T_{tr}$ that is both realistic (less than either of the melting points of the polymorphs), and mutually supported by independent techniques, their enantiotropism was confirmed.

3.3.3. Exposure of R-II to $T > T_{tr}$

Following determination of R-I and R-II enantiotropism, and the $T_{tr}$, it was reasonable to expect that temperature-mediated conversion directly from R-II to R-I should be observed upon heating of R-II to $T > T_{tr}$ without exceeding the $T_m$ for either form. R-II was heated in situ in DSC pans at different rates ranging from 2-40 °C/min; ultimately the slowest DSC data were selected for their resolution, and the potentially reduced likelihood of instrumental artifacts. Additionally, it was expected that the slower heating rate would enable conversion of R-II to R-I without melting during the experiments. However, all DSC thermograms for R-II showed only a single endothermic event with an onset temperature of $169.29 \pm 2.72$ °C and an enthalpy of $44.32 \pm 1.56$ kJ/mol. The absence of any notable endothermic transition prior to melting, even at the slowest heating rates, suggested that the kinetics of the R-II to R-I solid state conversion were much slower than those afforded by even the slowest DSC experiment.

To address the need for longer exposure time at $T > T_{tr}$, R-II samples were held isothermally at 150 °C, with care taken not to expose samples to temperatures that might melt either polymorph. Against expectations, samples of R-II held at 150 °C for 7 days persisted without conversion to R-I, as evidenced by characterization of these samples using PXRD and DSC. It was also noted that isothermal storage at this temperature beyond 7 days resulted in brown discoloration of portions of the samples, suggesting thermal instability. This was confirmed via HPLC by a decrease in ribavirin potency to
less than 70 % when compared with the USP standard, for samples held at 150 °C for longer than 7 days. Unlike other examples of temperature induced solid-state changes, the present work demonstrated that, although enantiotropically related transitions are thermodynamically reversible with temperature and pressure, the kinetics may prohibit interconversion within reasonable (or applicable timeframes). It is important that new chemical entities (NCEs) demonstrating enantiotropism are also tested for kinetics of thermally mediated transitions, as shown here.

### 3.3.4. Exposure of R-II to high-shear mechanical energy

Although the R-II to R-I conversion kinetics upon exposure to $T > T_{tr}$ appear to be much slower than the kinetics of chemical degradation at the same temperature, it is anticipated that during typical manufacturing operations, ribavirin will be exposed to mechanical stress conditions potentially capable of accelerating the kinetics of solid state transformations. Milling of R-II in a vibratory impact mill prior to isothermal temperature experiments was expected to damage crystallite surfaces to the extent that a commensurate increase in molecular mobility around damage sites would enable either a sufficiently rapid polymorphic transformation that observation is possible prior to extensive chemical degradation, or, potentially facilitating a direct conversion during prolonged mechanical treatment.

**Vibratory impact ball milling:** Samples of R-II were extensively milled in a vibratory impact mill without temperature control. In a previously published paper, bulk ribavirin that was ball milled for 50 min resulted in only 0.642 % w/w conversion to R-I, as estimated using only DSC. Therefore, in the present work, R-II was milled for 60 min, with the intent of creating surface defects on ribavirin crystallites without causing
detectable phase change. This was confirmed using PXRD patterns of milled R-II samples, which showed that, even after 60 min milling, the crystallites still diffracted at 20 angles entirely consistent with the R-II reference pattern (Figure 3.6).

![Figure 3.6. PXRD patterns of R-II milled for various time intervals in a vibratory impact ball mill. Note: Eventual conversion from R-II to R-I at 270 min of milling.](image)

The diffraction peaks were observed to broaden, relative to the starting material, as evidenced in the full width at half maximum (FWHM) of characteristic peaks, suggesting a potential reduction in R-II crystallinity as a consequence of impact milling (See Table 3.2).
Table 3.2. Comparison of full width half maximum (FWHM) between diffraction patterns of R-II as a function of milling time in vibratory impact ball mill.

<table>
<thead>
<tr>
<th>Miller indices (hkl)</th>
<th>Position (°2θ)</th>
<th>Full width at Half Maximum – FWHM (°2θ)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>R-II (before milling)</td>
</tr>
<tr>
<td>(110)</td>
<td>11.92</td>
<td>0.0836</td>
</tr>
<tr>
<td>(210)</td>
<td>13.43</td>
<td>0.0836</td>
</tr>
<tr>
<td>(310)</td>
<td>15.59</td>
<td>0.0836</td>
</tr>
<tr>
<td>(201)</td>
<td>18.15</td>
<td>0.1171</td>
</tr>
<tr>
<td>(420)</td>
<td>27.12</td>
<td>0.1506</td>
</tr>
<tr>
<td>-</td>
<td>30.56</td>
<td>0.1171</td>
</tr>
<tr>
<td></td>
<td>30.82</td>
<td>0.1171</td>
</tr>
<tr>
<td>-</td>
<td>32.04</td>
<td>0.1506</td>
</tr>
<tr>
<td></td>
<td>32.35</td>
<td>0.1004</td>
</tr>
</tbody>
</table>

*double peaks merge into a single peak

Although particle size (PS) reduction can lead to similar PXRD pattern broadening, optical microscopy of the R-II samples taken before milling (\(d_{10} = 8.1 \, \mu\text{m}; d_{50} = 14.8 \, \mu\text{m}; d_{90} = 30.4 \, \mu\text{m}\)) and after milling (\(d_{10} = 5.4 \, \mu\text{m}; d_{50} = 11.2 \, \mu\text{m}; d_{90} = 32.9 \, \mu\text{m}\)) did not show significant reduction in the PS of R-II primary particles. These data suggest that much of the mechanical energy was used to induce defects, resulting in dislocated molecules that are lost as contributors to diffracting planes, thereby broadening the diffraction pattern. Similarly, the BET results for R-II exhibited a comparable trend with PS data, in which the specific surface area (SSA) did not change significantly for samples measured following milling (1.4 m²/g) relative to those measured prior to milling (1.1 m²/g). These results are similar to those from a published study in which it was reported that cryogenic grinding of ketoconazole particles caused nominal fracture, which ceased after 5 min of milling time. In this same study, milling for 10 and 30 min did not result in continuous particle size reduction, possibly due to sintering or fusion of
fines onto larger particles, which was reflected in BET measurements for milled ketoconazole in which the increase in SSA (relative to unmilled samples) after 30 min of milling was $\leq 1.0 \text{ m}^2/\text{g}$. It is important to consider that a tiny fraction of amorphous ribavirin (if formed) could remain undetected; hidden under the strong Bragg peaks of the milled crystalline ribavirin. To eliminate this ambiguity, DSC analysis of ground R-II was performed at multiple heating rates. Slow heating rates of up to 5 °C/min resulted in thermograms having a single melting endotherm, attributable to R-I ($T_m = 176.09$ °C; $\Delta H_m = 38.4 \text{ KJ/mol}$) without a recrystallization exotherm or glass transition prior to this event. At first, these data seemed to contradict the PXRD observations, which showed no detectable R-I in the milled samples. Close inspection of the thermograms, however, showed a small endotherm between 144–146 °C, having an enthalpy of 1.9 kJ/mol, suggesting that the milled R-II converted to R-I in situ during DSC experiments, owing to the imposition of a temperature ramp on the samples during the experiment (Figure 3.7a). In contrast, DSC measurements conducted at more rapid ramp rates (10, 20 and 40 °C/min), showed two endothermic events, corresponding to both R-II and R-I, where the area of the peak attributable to R-II progressively increased with increasing experimental heating rate, while the onset and peak temperatures for R-II showed a 15-20% decrease with milling time (Figure 3.7b). These data suggested that the milled R-II samples underwent partial in situ conversion to R-I during the DSC temperature ramp, to an extent commensurate with longer times spent at higher temperatures. As before, the PXRD data for milled R-II showed no R-I formation, while the DSC data for unmilled R-II, showed no in situ conversion to R-I. The data collectively suggest that milling of R-II
resulted in the induction of crystal defects, which facilitated rapid conversion to R-I when those samples were exposed to $T > T_{tr}$.

Figure 3.7. (a) DSC thermogram of R-II milled for 60 min a vibratory impact ball mill is compared with the untreated polymorph samples. The dotted circle highlights the endotherm associated with R-II to R-I transition in situ (See inset). (b) DSC thermograms of 60 min milled R-II, recorded at various heating rates.
Solid-state transformations caused *in situ* during DSC experiments have been reported for several pharmaceutically relevant materials.\textsuperscript{180,195,196} In the example of carbamezapine enantiotropes, DSC measurements at 2 °C/min resulted in an endothermic event attributable to form III $\rightarrow$ I transition, followed by melting of form I. It was only at higher heating rates (10, 20, 30 and 40 °C/min) that the melting of form III prior to melting of form I was observable. Furthermore, the melting of form III in every experiment was immediately followed by a distinct exothermic event attributed to formation of form I via sublimation. In the present case, the higher relative stability of R-I near the melting temperature of R-II, and the apparent ease of nucleation and crystallization of R-I make direct assessment of the extent of polymorphic conversion impossible from a single DSC experiment, conducted at a single heating rate.

Use of PXRD to characterize processed ribavirin was particularly beneficial in the present work. Had the conclusions been based only on DSC measurements, the data recorded at slow heating rates might have suggested complete transition to R-I as a consequence of milling, while those recorded at higher heating rates would have suggested a partial enantiotropic conversion, despite a consistent milling duration. As it turned out, neither interpretation would have been accurate, as demonstrated by the PXRD data which showed no conversion to R-I during milling. To further examine the effects of milling on the facilitation of R-II to R-I solid state conversion, milled R-II was held isothermally at 150 °C in a convection oven. In contrast to the unmilled R-II, which remained physically stable for 7 days before eventually chemically degrading, the milled R-II samples completely converted to R-I within 15 min isothermal storage.
Additionally, when the milled R-II samples were annealed at \( T < T_{tr} \) (50 °C), no conversion to R-I was observed. These data collectively suggest that the induction of defects in R-II crystallites during milling is needed in order to accelerate the conversion kinetics sufficiently that complete conversion to R-I at \( T > T_{tr} \) can be observed.

It seems likely that the commercially available ribavirin products (all of which contain R-II), or any innovative new formulations will utilize pulverization during primary and/or secondary manufacturing. If the milled R-II particles later experience temperature excursions that exceed \( T_{tr} \), during storage or transport, a relatively rapid conversion to R-I becomes much more likely. Should such an unanticipated change result in significant alterations in either dosage form performance or downstream material handling, a company that is unaware of this possibility may run into major regulatory challenges.\(^{197}\)

Additional impact milling experiments were conducted, which involved durations beyond 60 min. As shown in Figure 3.6, when R-II was comminuted for 270 min in the uncontrolled temperature impact mill, complete conversion to R-I occurred in the milling vessel, as evidenced by the PXRD data. The DSC data for the same samples, showed only a single melting endotherm, observed at all experimental heating rates (Figure 3.8), which was unambiguously attributable to R-I. Although there was no evidence in these samples of persistent amorphous ribavirin, it is likely that regional disordering during pulverization, by the accumulation of defects, provided sites of increased mobility that allowed rapid recrystallization to R-I, driven by localized heating inside the milling jar to temperatures potentially exceeding both \( T_g \) (~ 61 °C) and \( T_{tr} \).
Figure 3.8. DSC thermograms of 270 min milled R-II measured at different heating rates. Complete R-II $\rightarrow$ R-I conversion can be seen based on the single melting endotherm attributable to high $T_m$ enanti trope (R-I). DSC data recorded at 2 and 20 °C/min are not shown.

In order to evaluate whether extensive comminution caused any chemical degradation, all milled ribavirin samples were analyzed using HPLC. Despite simultaneous exposure to high-shear mechanical energy and likely increased localized temperatures, the concentration of ribavirin recovered in all samples was >99%. These data suggested that, although extensive processing under these conditions elicited a physical change in the ribavirin solid, the molecules remained chemically unaltered.

**Cryogenic impact milling:** The observations described above starkly contrast the results of temperature controlled, cryogenic milling, which maintained samples, medium, and milling environment in a constant liquid nitrogen bath throughout processing. In order to isolate, as best as possible, the role of mechanical energy in defect formation,
from localized temperature build-up, the relative fraction of sample volume in the milling jar was kept consistent relative to the vibratory ball milling experiments.

Characterization of cryomilled samples using PXRD and DSC showed progressive reduction of long-range lattice periodicity, resulting in the formation of completely disordered ribavirin after 120 min. As shown in Figure 3.9, diffraction patterns for these milled samples have a prominent X-ray amorphous halo, while DSC data showed an observable $T_g$ (61-65 °C), consistent with the measured value for ribavirin. These results suggest that the limited molecular mobility attributable to the very low milling temperature ($T << T_g$) likely inhibited recrystallization of the ribavirin to either crystalline solid form. Additionally, the difference in particle size and morphology as a result of cryogenic grinding was negligible with all milled samples. Instead, micrographs revealed considerable de-agglomeration, irregular morphology, and surface crack formation, but little change in primary particle size. This suggests that the incoherent scattering of X-rays from the 120 min cryomilled samples was due to complete loss of lattice periodicity, as opposed to a PS effect, confirming that the mechanical energy was used to disorder the crystalline solid particles rather than consumed by sequential fracture events.
Figure 3.9. (a) PXRD patterns of R-II milled for various time intervals in a cryogenic impact mill. Note the halo pattern following 120 min cryomilling, suggestive of complete loss of lattice periodicity (b) DSC thermogram of R-II milled for 60 and 120 min a cryogenic impact mill is compared with the unprocessed starting material (i.e. R-II). The inset shows observed glass transition of ribavirin after 120 min of cryomilling, confirming transformation to persistent amorphous ribavirin. Data was recorded at 20 °C/min.

The results of cryomilling ribavirin indicated that the solid form of the “end product” was directly influenced by the temperature experienced during milling. These data follow nicely with the published observations for fananserine,\textsuperscript{198} where the milling temperature relative to the $T_g$ of the material being milled determined the solid form of the end product. Fananserine milled above $T_g$ (19 °C) resulted in transformation to its
metastable polymorph, while milling under liquid nitrogen temperatures resulted in complete conversion to the amorphous phase. This is because the milling temperature was well below \( T_g - 50 \) °C, a temperature at which mobility is expected to be too low to support recrystallization. Even if localized temperatures exceeded cryogenic conditions, continuous maintenance of the liquid nitrogen bath, accompanied by regular cool down cycles during the milling protocol, are expected to keep the powder bed well below \( T_g \) for the material. Based on the observations for fananserine and the \( T_g \) for ribavirin (61-65 °C), milling of R-II might have been expected to convert to the amorphous state as a result of either technique used in the present work, given that both the milling temperatures were \(< T_g \). The characterization data, however, show that continuous impaction without temperature control resulted in conversion to R-I, rather than formation of a persistent amorphous solid. This seems likely to have been the result of localized temperature excursions in the vibratory impact mill, which exceeded \( T_g \) and, possibly even \( T_{tr} \) for ribavirin. This appears to be consistent with the results of Chieng et al. who, using a similar impact mill, observed that the average temperature of a powder bed was increased by 30-35 °C within 60 min of milling and remained constant when milling was continued for 180 min. Such a prolonged elevation of the bed temperature for ribavirin would approach \( T_g \), allowing for local heating at surfaces to exceed it. Although persistent amorphous ribavirin was not observed in samples milled without temperature control, it is plausible that R-II \( \rightarrow \) R-I may have been the result of the formation of a transient amorphous intermediate, which rapidly recrystallizes to R-I at \( T > T_g \).

In the present work, the observed transformation of ribavirin is thought to be initiated when the long range order of R-II crystals was disrupted via application of high-
shear stress, without substantial particle size reduction. The resulting defect accumulation facilitated sufficient molecular mobility that, when exacerbated by temperatures exceeding $T_{g}$, allowed collective reorganization of ribavirin molecules and direct crystal-to-crystal transformation without the formation of a persistent amorphous intermediate. This type of mechanism has been observed in cold worked metals$^{200,201}$ and potentially supports the observation of a solid-state change in R-II tablets when compressed at high loads.$^{164}$

There are several theories in support of formation of amorphous solids as a result of milling. One hypothesis suggests that temperature elevation owing to continuous impact and attrition may sufficiently induce local hot points that exceed the $T_m$ of the API, whereupon subsequent returns to ambient temperature would quench the material allowing for formation of amorphous solid by a series of melt/quench events. In the present work, however, the cryogenic environment rigorously maintains the experimental temperature $\sim 360 \, ^\circ\text{C}$ below the $T_m$ of ribavirin, making this mechanism unlikely.

Alternatively, the model adapted by Wildfong et. al.$^{202}$ seems more potentially relevant. In that work, the free energy required for incorporation of a critical dislocation density was related to the free energy change needed to convert the crystalline material into an amorphous solid. At this critical density, the accumulated dislocations were thought to cause overall lattice perturbations such that the collective molecular bonds required to maintain periodicity of the lattice were lost. As with the present work, experiments conducted in the dislocation-based model, also rigorously controlled the milling temperature to inhibit recrystallization of any amorphous solid formed during milling, thereby facilitating persistence of the “end product” solid form.
Additional support for a dislocation-mediated transformation of R-II to amorphous ribavirin during cryogenic milling comes from considering the extension of Wildfong’s work by Lin et al.,\textsuperscript{171} who showed that the potential to become completely disordered as a result of continuous cryogenic milling was highly correlated to the combination of the $T_g$ of the material being milled and the molar volume ($M_v$) of the molecules comprising the solid. According to their work, values of $T_g$ and $M_v$ that exceed the bivariate decision boundary $g(T_g,M_v)$ correspond to materials that are predicted to form a persistent amorphous solid via continuous cryogenic milling. Calculation of the $M_v$ of R-II (147.64 cm$^3$/mol), in combination with its $T_g$, and application to the decision boundary function described in Lin et al.,\textsuperscript{171} results in a value that corresponds with a material predicted to become completely amorphous during extensive cryomilling, which was confirmed by characterization of the milled samples.

### 3.3.5. Recrystallization and kinetic irreversibility

The reversibility of enantiotropic polymorphism can be identified using DSC.\textsuperscript{189,203} Usually, the presence of an exothermic peak in a DSC thermogram occurring during the cooling cycle at a temperature close to that at which an endothermic transition occurs during heating, suggests that a reversible transition is observable. Accordingly, samples of phase pure R-I were allowed to melt in situ in DSC pans, followed by subsequent cooling at different rates (2 and 20 °C/min) in order to identify whether a distinct exothermic transition associated with re-conversion to R-II could be observed. Neither cooling rate, however, resulted in any observable thermal event surrounding the apparent transition temperature, suggesting that the high-melting enantiotrope of ribavirin, R-I, is kinetically irreversible, even if the temperature is decreased below the estimated $T_{tr}$. To
verify this observation, R-I samples were stored under various conditions of relative humidity (0%, 33%, 60% and 75%) at room temperature ($T << T_r$), for 60 days. Despite much longer storage at conditions that should be favorable to the conversion from R-I to R-II, the high temperature enantiotrope remained unchanged under all humidity conditions, as evidenced by PXRD devoid of peaks attributable to R-II, and DSC thermograms showing only melting associated with R-I. This is relevant to the scenario described above, in which unanticipated conversion of R-II to R-I occur as a result of applied high-shear stress and elevated temperatures during processing. The DSC data suggest that R-I would be kinetically trapped, potentially resulting in batches that contain a mixture of solid forms having variable performance characteristics. Although the high aqueous solubility of either form of ribavirin would likely offset any potential differences in bioavailability stemming from phase impurity induced during manufacturing, differences in material handling and mechanical properties are more likely to be impacted, potentially manifesting as product uniformity and other regulatory issues. Given the results detailed herein, further investigation of this material seems warranted.

3.4. Conclusion

Process-induced transformations of solid materials have received a great deal of attention in the pharmaceutical industry, where understanding the relationships and pathways between different solid forms plays a role in determining the most suitable solid phase for a formulation and manufacturing scheme. The enantiotropic relationship between ribavirin polymorphs R-II and R-I was previously reported, however, determination of the solid state transition temperature of ~70 °C, and characterization of
the very slow kinetics of $R-I \rightarrow R-II$ reconversion are expected to provide more information regarding how these polymorphs may interconvert. Additionally, solid-state transformations of ribavirin were examined under different thermal and mechanical stress conditions. Unprocessed $R-II$ did not transform into $R-I$, at $T > T_{tr}$, even after 7 days, however, following grinding, the same isothermal storage conditions resulted in rapid $R-II \rightarrow R-I$ conversion. This suggested that the energy barrier required to produce the high $T_m$ enantiotrope was reduced by generating high mobility defects during milling. Extensive milling also led to complete $R-II \rightarrow R-I$ conversion, when the milling temperature was not controlled, likely proceeding via formation of a transient disordered phase, which rapidly recrystallized at $T > T_g$ (See figure 3.10). In contrast, high shear cryogenic milling of $R-II$, conducted at $T << T_g$ resulted in complete transformation to persistent amorphous ribavirin.

These data suggest that the solid form that results from milling was determined by the interplay of two processes: (1) regional disordering of the sample due to continuous impact and attrition, and (2) thermally mediated recrystallization. Recrystallization to $R-I$ takes advantage of the higher molecular mobility afforded by amorphous regions, which at $T > T_g$ favors conversion to a crystalline phase. At $T << T_g$, however, continuous regional disordering eventually spreads throughout the sample as milling continues, and a completely disordered state can be achieved.
**Figure 3.10.** Schematic representation of solid state transformation in R-II.

### 3.5. Acknowledgments

This work was made possible by the generous funding from Boehringer-Ingelheim Pharmaceuticals, Inc., Ridgefield, CT, USA (G1400043). Reprinted from International Journal of Pharmaceutics, vol. 524, Dipy M. Vasa, Peter L.D. Wildfong, Solid-state transformations of ribavirin as a result of high-shear mechanical processing, pp. 339-350, Copyright (2017), with permission from Elsevier. The final publication is available at Elsevier via [http://doi.org/10.1016/j.ijpharm.2017.04.002](http://doi.org/10.1016/j.ijpharm.2017.04.002).
Chapter 4: Preparation, characterization and evaluation of ribavirin loaded polymer microparticles for intranasal uptake

4.1. Introduction

Ribavirin [RBVN, 1-(-d-ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide] is a water-soluble synthetic nucleoside (BCS class III drug) having broad spectrum antiviral properties. Although the activity of RBVN in vitro is promising, its in vivo effectiveness in the treatment of viruses affecting the central nervous system is reduced by its inability to cross the blood-brain-barrier (BBB). Previous studies reveal administration of RBVN via intraperitoneal, subcutaneous, and intramuscular injections proved ineffective against several encephalitis viruses in mice, and showed improved survival against subacute sclerosing panencephalitis (SSPE) in virus-infected hamsters only when administered intracranially. Pursuit of alternative routes of administration for RBVN that increases CNS bioavailability, with the potential for patient self-care, requires further research.

Intranasal (IN) drug delivery holds promise for CNS targeting, given its potential to bypass the BBB. Direct nose-to-CNS delivery occurs through the olfactory region of the nasal cavity, offering several advantages, including avoidance of hepatic first pass metabolism, rapid onset of action, and the potential for patient self-administration. Nonetheless, targeted deposition and adequate residence time in the nasal cavity remain as challenges. Currently, an ideal formulation or device enabling drug deposition exclusively to the olfactory region is not known; however, ongoing research
continues to improve the fraction of dose that is delivered to this location.\textsuperscript{24,30} The deposition pattern for liquid IN formulations is influenced, in particular, by droplet size, with diameters $>20 \, \mu m$ resulting in almost 100\% nasal deposition.\textsuperscript{70,75,79} Additionally, breath-actuated insufflation of solid particles ($D_{90} < 40 \, \mu m$) has indicated that preferential deposition of drug occurs on the olfactory tissue, while promoting longer retention of particles relative to liquid vehicle dosage forms such as metered sprays.\textsuperscript{98,103} This is suggested to be the result of slow hydration of powder particles in contact with the nasal mucus, which increases viscosity and provides greater resistance to mucociliary clearance.

The key to successful nose-to-CNS delivery, is not only increased nasal residence time, but also increased CNS uptake of the targeted drug. Certain polymers have demonstrated utility in this regard,\textsuperscript{76,84} including poloxamers, which are tri-block-copolymers consisting of polyethylene oxide (PEO) and polypropylene oxide (PPO) monomer units. Owing to the thermoreversible sol-gel transition at physiological temperatures, poloxamers are suited for mucosal adhesion following i.n delivery. Additionally, poloxamers have also been shown to enhance permeation across the nasal mucosa, possibly owing to a combination of the inhibition of efflux transporters,\textsuperscript{123} acceleration of solute diffusion within the lipid bilayer,\textsuperscript{127} and transient opening of the cellular tight junctions.\textsuperscript{205} One recent study showed that IN administration of the anti-retroviral drug zidovudine in adult rabbits resulted in nearly 4-fold greater CNS concentrations from formulations containing poloxamer, presumably due to longer retention in the nasal cavity and/or permeation enhancement of the drug.\textsuperscript{129}
Intranasal insufflation is less studied than instillation of droplets or inhalation of atomized liquid sprays. As a result, optimization of solid particle formulations intended for IN insufflation has received less attention in the literature. Formulation of very fine drug particles adsorbed onto the surfaces of larger inert carriers, analogous to dry powder inhalers, is unlikely to be successful for IN insufflation as the larger carrier particles would be more likely to settle under gravity and provide little or no deposition in the olfactory region. Physical mixtures of drug and polymer having comparable particle size ranges may be possible in some cases, however, segregation of components upon insufflation is undesirable. Consequently, in this study, formulation involved suspension of ultrafine drug particles in molten carrier polymer, followed by co-solidification upon cooling. Subsequent micronization allowed sizing of compositionally uniform particles according to IN delivery-specific recommendations.

In the present work, preparation and characterization of binary composite solid microparticles of RBVN in poloxamer allowed evaluation of their potential use in direct CNS targeting using intranasal drug delivery. It was hypothesized that deposition of these solid microparticles on the nasal mucosa would result in higher permeation of RBVN across epithelial cells relative to a RBVN solution. Solid drug-in-polymer microparticles were prepared, optimized, and characterized. Permeation of RBVN across olfactory mucosal tissue was studied in vitro and compared with permeation from liquid vehicle solutions of the drug.
4.2. Experimental Section

Ribavirin was purchased from Jinan Jiaquan Chemicals Co. Ltd. (Jinan, China, Lot number 02110330). Poloxamer 188 NF (POX188, also referred to as Pluronic F68) was purchased from Spectrum Chemicals, New Brunswick, NJ (CAS Number 9003-11-6). All powders were stored in a desiccator over P₂O₅ (~0% RH) for at least one week prior to experimentation, to remove environmental water.

4.2.1. Determination of RBVN solubility in POX188

Mixtures of RBVN and POX188 were manually prepared by geometric dilution at 10, 20, 30, 40, 50, 60, 70, 80 and 85 % w/w with respect to polymer. Each physical mixture was analyzed using a differential scanning calorimeter (DSC), equipped with a refrigerated cooling accessory (Model Q100, TA Instruments, New Castle, DE, USA). Three replicate DSC samples (5.0 ± 0.5 mg) were prepared at each composition placed in an Al sample pan that was hermetically sealed. Samples were heated from ambient temperature to 200 °C at a constant heating rate of 2 °C/min. Dry N₂ gas (50 mL/min) was allowed to constantly purge the DSC cell during all experiments, and measurements were subject to a three-point temperature and enthalpy calibration accomplished using Sn \((T_m 231.9 \degree C)\), In \((T_m 156.6 \degree C)\), and o-terphenyl \((T_m 55-56 \degree C)\) standards. The endotherm attributable to melting of RBVN in the presence of varying amounts of POX188 was carefully recorded, and the depression of its extrapolated onset of melting \((T_o)\), peak maximum \((T_p)\) and enthalpy of melting \((\Delta H_m)\) of RBVN were used to indicate solubility in molten POX188 which in turn would influence maximum drug loading.
4.2.2. Preparation of binary composite microparticles

Physical blends of RBVN and POX188 were prepared in 1:1 w/w ratio using a laboratory scale blender, followed by equilibration at ~0% RH for 24 h. These blends were assessed for content uniformity with a low variation (RSD <5%) chosen as the criteria for subsequent use. Physical mixtures were transferred to a crucible, which was immersed in a silicon oil bath maintained at 100 °C which was higher than the melting temperature of POX188 ($T_m = 55–60$ °C), but well below the melting temperature of RBVN ($T_m = 168–170$ °C). Crystalline RBVN was suspended in molten POX188 and the set temperature was maintained isothermally to allow homogeneous mixing. To minimize water vapor sorption, dry N$_2$ gas was continuously streamed over the crucibles during preparation. Following mixing, the 1:1 RBVN : POX188 suspensions were quenched by immersion of the crucible in liquid nitrogen, resulting in formation of a co-solidified disc that was removed for further processing.

To ensure no thermal degradation of the RBVN during suspension preparation, blends were held isothermally for 20-30 min in a thermogravimetric analyzer (TGA) at 100 °C, and <1% weight loss was recorded. Additionally, physical blends were heated in a DSC to 100 °C and held isothermally for 30 min to assess time-dependent drug solubility in polymer. Following these extended hold times samples were quenched in situ in DSC, before reheating. Recorded values for the melting parameters ($T_o$, $T_p$ and $\Delta H_m$) between heating cycles remained consistent with pure component values, indicating that the RBVN remained undissolved in POX188 during suspension preparation.

To make the co-solidified discs suitable for simulation of IN delivery, mechanical sizing was accomplished using a cryogenic impact mill (SPEX Certiprep 6750 Freezer
Mill, Metuchen, NJ). Approximately 1.0 g of RBVN : POX188 (1:1) solid suspension was manually broken down into coarse particles (~3-4 mm), which were placed in a cylindrical polycarbonate milling vessel. A stainless steel cylindrical milling rod was placed in the vessel, and the sample temperature was allowed to equilibrate for 3 min prior to milling, following immersion in the mill liquid nitrogen bath. The processing temperature was continuously maintained during comminution by regular replenishment of the liquid nitrogen bath. Similar to Crowley and Zografi, materials were ground for 2 min intervals at a frequency of 10 Hz, separated by 2 min cool-down periods, allowing minimization of local sample temperature increases. Upon completion of 20 min total milling, the ground sample was sieve fractionated (Performer III Model: SS-3, Gilson Company, Lewis Center, OH) and the fines that passed through the 53 µm sieve (US standard number 270) were collected and stored over P₂O₅. Subsequent characterization of milled particles was performed to verify maintenance of RBVN phase purity during manufacturing, sample potency, chemical stability, particle size, and morphology. All characterization experiments were repeated in triplicate.

4.2.3. Characterization

4.2.3.1. Ribavirin solid phase purity

Binary microparticles obtained through cryogenic grinding were characterized to ensure that processed RBVN maintained a single solid form, using powder X-ray diffraction (PXRD) and differential scanning calorimetry (DSC). PXRD patterns were obtained using an X’Pert Pro MPD system (PANalytical B.V., Almelo, the Netherlands), equipped with a Cu anode (λ = 1.5406 Å), an auxiliary elliptical mirror, and an X’Celerator™ detector. Powder samples were loaded between two layers of Kapton®
film (ChemPlex, Palm City, FL) and spun in a vertical sample stage. Consistent irradiation times of 135 s per step with an angular step size of 0.017 °2θ were used for all diffraction experiments. PXRD data were collected over 5–50 °2θ, using a respective voltage and amperage of 45.0 kV and 40.0 mA.

Conventional DSC measurements were made to determine the melting temperatures ($T_m$) of ribavirin polymorphs. Accurately weighed samples of $5.0 \pm 0.5$ mg were hermetically sealed in Al sample pans and heated at either 2°C/min or 40°C/min to 200 ºC. All measurements were performed in triplicate and reported as mean values ± standard deviation.

4.2.3.2. Assessment of Particle properties

The particle size and size distribution of sieve cuts containing 20–53 μm microparticles were analyzed at 10x magnification using an Olympus BX-51 optical microscope equipped with a polarizing filter. Photomicrographs were obtained to evaluate size and measure the Feret diameter for approximately 300 particles and crystallinity was confirmed through notable birefringence. Additionally, scanning electron microscopy (Hitachi S-3400N scanning electron microscope equipped with a Bruker Quantax model 400 energy dispersive spectrometer using an XFlash® 5010 EDS detector) was used to image particles and assess morphology. Samples were mounted on double-sided carbon tape affixed to an aluminum specimen holder. Images were collected using a working distance of 15 mm and an accelerating voltage of 2 kV.
4.2.3.3. Microparticle drug content and Content uniformity

Drug content was measured by dissolving a mass of RBVN : POX188 (1:1) composite microparticles equivalent to a dose of 20 mg RBVN in distilled water. The resulting solutions were filtered through 0.45 μm filter and diluted for HPLC analysis. Particle content uniformity was determined by randomly choosing 12 samples from the milled and sized powder. A low variation (RSD <5%) was chosen as the acceptance criteria for drug uniformity.

Finally, to ensure RBVN chemical stability during temperature-intense suspension preparation, and shear stress-intense particle sizing, the potency of RBVN extracted from microparticles was compared with untreated raw material. All measurements were reported as mean ± SD. Precise RBVN concentration measurements for all samples were made with a Waters Alliance 2690 HPLC system (Milliford, MA) equipped with photo diode array (PDA) detector using a Hypersil C18 reverse phase column (5 μm, 250 mm x 4.6 mm i.d., Waters, MA, USA). Previously filtered and degassed mobile phase comprising of 95:5 v/v mixture of 0.01 M potassium dihydrogen phosphate solution and pure methanol (pH adjusted to 5) was used. The mobile phase was pumped continuously at a flow rate of 1.0 mL/min with an injection volume of 20 μL. Standard solutions and test samples were analyzed at the detector wavelength of 207 nm. All sample solutions were diluted in mobile phase.
4.2.4. Evaluation of Microparticle Performance

4.2.4.1. in vitro Drug Release

The in vitro RBVN release studies were performed using Franz diffusion cells (PermeGear, Hellertown, PA). Regenerated cellulose dialysis membrane (Spectrum Laboratories Inc., Houston, TX) was mounted between the donor and receiver compartments of the diffusion cell. The receiver compartment was filled with 5 mL of simulated nasal electrolyte solution (SNES), thermostated at 37±1 ºC and gently agitated (200 rpm) by means of a magnetic stirrer. SNES was prepared with 8.77 g NaCl, 2.98 g KCl, 0.59 g CaCl₂ anhydrous in 1000 mL distilled water with the final pH adjusted between 6.0 and 6.5. Microparticles characterized in Section 4.2.3 containing no more than 5.0 mg of free drug (dose adjusted accordingly) were evenly sprinkled on the pre-hydrated dialysis membrane. Only 20 µL SNES containing 2% w/v mucin was added to the donor side in order to maintain a hydration environment similar to that in the nasal cavity.\textsuperscript{142}

At predetermined time intervals, 200 µL samples were withdrawn from the sidearm of the receiver compartment and replaced with fresh, pre-warmed dissolution medium. The amount of drug released was assessed spectrophotometrically (UV Spectrophotometer 8453, Hewlett Packard, Germany) at λ\textsubscript{max} of 207 nm (Linearity range = 5 µg/mL to 17.5 µg/mL, R\textsuperscript{2} = 0.9998). To ensure mass balance, the residual powder in the donor compartment was dissolved in 200 µL of fresh, pre-warmed SNES and assayed. Each release experiment was performed in triplicate.
4.2.4.2. *in vitro* Nasal Cell Toxicity

RPMI 2650 human nasal septal carcinoma cells (ATCC cat. no. CCL 30) cells, between passages 27 – 32, were grown in Eagle’s minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) 2 mM L-glutamine, 100 units/mL penicillin and 100 mg/mL streptomycin, in collagen coated T-75 flasks (Novagen Inc. Germany) humidified at 37 °C incubator with 5% CO₂. The medium was changed every 48–36 hours. Cells were trypsinized with a 0.1% trypsin-EDTA solution upon reaching 80-90% confluency. Cytotoxicity assays were conducted using cells that were passaged at a density of 3 x 10⁴ cells per well in 200 µL culture medium to collagen-coated 96-well plates (Novagen Inc. Germany), and incubated overnight for cell attachment. Subsequently, 100 µL of culture medium was removed from all wells and changed with appropriately diluted solutions of only RBVN, only POX188 or RBVN and POX188 mixture (1:1) in MEM to achieve final concentration spectrum of 0, 7.8, 15.6, 31.25, 62.5, 125, 250, 500, 1000 µg/mL of each ingredient and incubated for 24 h.

Cell viability was determined by adding the CellTiter-Glo® reagent (Promega). For this, 50 µL media containing treated cells was transferred to an opaque-walled multiwell plate. About 25 µL of CellTiter-Glo® reagent was added to every well. The contents were mixed for 15 minutes on an orbital shaker to induce cell lysis. The plate to incubated at room temperature for 10 minutes to stabilize luminescent signal and luminescence was measured using a microplate reader (Model 1420-051, Victor³, Perkin Elmer, Shelton, CT). Control wells were prepared by mixing 50 µL of media without cells and 25 µL of CellTiter-Glo® reagent to obtain a value for background luminescence.
4.2.4.3. *in vitro* Permeation Measurements

Bovine olfactory mucosa was used as a model tissue for permeation studies, where L-DOPA permeability was measured by adapting the methods published by Chemuturi *et al.* Briefly, cow heads were obtained from Bud’s Custom Meats (Riverside, IA). The olfactory turbinates, which were covered by a pale yellow olfactory mucosa, were removed within 15 min of decapitation. The excised tissues were thoroughly rinsed with KRB and transported in fresh KRB maintained on ice; all studies were conducted within 4 h of procurement to ensure tissue viability.

The permeability of RBVN molecules through the bovine olfactory mucosa was measured during experiments that used Franz diffusion cells displaying a permeation area of 0.64 cm². Tissue was carefully removed from the underlying cartilage and affixed with the mucosal side facing the donor compartment, between the opposing faces of the two half-chambers (donor and receiver), using a series of pins that surrounded the opening. Five mL of Krebs ringer buffer (KRB, pH 6.8), pre-warmed to 37 °C, was added to the acceptor chamber. To ensure oxygenation and agitation, a mixture of 95% O₂ and 5% CO₂ was bubbled through the system. The temperature within the chambers was maintained at 37 °C. After 30 min equilibration, RBVN was loaded either as a 5 mg/mL aqueous solution, 5 mg of solid particles, or 10 mg of 1:1 RBVN : POX188 microparticles. For the solid sample test groups, an additional 50 µL of KRB was added to the donor compartment to mimic the low hydration volume of a typical nasal cavity. At predetermined time points over a 2 h interval, 250 µL samples were withdrawn from the acceptor compartment, and replaced with an equal volume of fresh, pre-warmed KRB. The samples were appropriately diluted, filtered and used for quantification. The amount
of permeated drug was determined using UV-visible spectrophotometry at \( \lambda = 207 \) nm (Linearity range = 5 \( \mu \)g/mL to 17.5 \( \mu \)g/mL, \( R^2 = 0.9998 \)). The apparent permeability coefficient (\( P_{app} \)) (cm/s) was calculated according to the following equation.

\[
P_{app} = \frac{\left( \frac{dM}{dt} \right)}{A} \times 60
\]

(4.1.)

where \( M \) is the cumulative amount of drug in the receiver cell at time \( t \), \( dM/dt \) is the mass transport rate obtained from linear regression of the initial steady-state portion (\( \mu \)g/min), \( A \) is the diffusion area (0.64 cm\(^2\)) and \( C_o \) is the initial donor concentration (\( \mu \)g/mL).

### 4.3. Results and Discussion

#### 4.3.1. Preparation of Binary composite microparticles

Composite microparticles were designed to contain phase-pure inclusions of crystalline RBVN suspended by a POX188 matrix to enable intimate mixing between the two components and alleviate segregation issue likely with a simple physical mixture of RBVN and POX188. This was intended to be achieved by heating 1:1 RBVN : POX188 mixtures to 100 °C (\( T_{m, polymer} < 100 \) °C < \( T_{m, API} \)), allowing melting of the polymer and distributive mixing of the fine solid drug particles. Rapid solidification of the polymer would result in suspension of phase-pure API crystallites surrounded by a re-solidified polymer matrix, as demonstrated previously.\(^{206}\) Prior to this, the decision of drug loading was based on the DSC data taken for physical mixtures of RBVN and POX188 at various compositions (Figure 4.1). Each DSC thermogram was observed to have two distinct melting endotherms consistent with the values for POX188 (57.07 ± 1.31 °C) and the low \( T_m \) enantiotrope of RBVN, R-II (166.87 ± 2.03 °C).\(^{163}\) Of note, neither the onset of
melting \( (T_m) \) nor the peak maximum temperature \( (T_{\text{max}}) \) deviated significantly relative to either pure component, indicating that little to no drug dissolved in the molten polymer during preparation, confirming preparation of the desired suspension. Avoiding partial or complete dissolution of RBVN in molten polymer was deemed important to prevent heterogeneous formation of molecularly dispersed drug-polymer solid solution surrounded by variable degrees of suspended crystalline RBVN. It was thought that such systems would likely have highly varied delivery rates, the potential for non-uniform moisture uptake, and potentially issues with respect to insufflation, nasal deposition, dissolution and permeation profiles.
Figure 4.1. (a) DSC thermograms of physical mixtures of RBVN and POX188 in varying proportions of polymer measured at the heating rate of 2 °C/min. (b) Extrapolated onset of melting plotted as a function of polymer composition.
These data also suggested that a relatively high fraction of RBVN could be suspended in POX188, allowing the formulation to balance the need for reasonable dosing with the presence of sufficient polymer to promote permeation enhancement. As such, formulations containing 25% or 50% w/w RBVN were selected for subsequent experimentation.

**Figure 4.2** shows a schematic representation of the process by which 1:1 RBVN : POX188 binary composite microparticles were prepared. Solidified drug-polymer mixtures had to be milled in order to achieve nasally administrable particle sizes. A SPEX Certiprep freezer mill was chosen to micronize the samples, owing to cryogenic temperature control throughout the comminution process. In **Chapter 3**, it was noted that when RBVN was cryogenically milled for longer than 60 min, changes in solid form were observed. In the present work, therefore, total milling exposure was limited to shorter times (i.e. 10 or 20 min).

**Figure 4.2.** Schematic representation of RBVN : POX188 (1:1) binary composite preparation.
In this manner, a 2x2 full factorial design of experiments was created with drug loading (as factor 1) and milling time (as factor 2). The effect of these factors on physical transformation, particle size and size distribution and content uniformity was studied. The results are summarized in Table 4.1 and detailed discussion on the chosen formulation is provided in the later sections.

Table 4.1. Properties of BCM prepared using 2x2 full factorial design of experiments (DoE) with % w/w drug loading (as factor 1) and milling time (as factor 2).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Drug loading (% w/w)</th>
<th>Milling time (min)</th>
<th>% Yield (20 μm sieve cut)</th>
<th>Evidence of physical transformation</th>
<th>Drug content</th>
<th>RSD – Relative standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>50</td>
<td>20</td>
<td>48 %</td>
<td>No</td>
<td>RSD 9.8</td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td>50</td>
<td>10</td>
<td>42 %</td>
<td>No</td>
<td>RSD 3.0</td>
<td></td>
</tr>
<tr>
<td>F3</td>
<td>25</td>
<td>20</td>
<td>42 %</td>
<td>No</td>
<td>RSD 4.6</td>
<td></td>
</tr>
<tr>
<td>F4</td>
<td>25</td>
<td>10</td>
<td>30 %</td>
<td>No</td>
<td>RSD 2.6</td>
<td></td>
</tr>
</tbody>
</table>

As the data indicate, none of the four formulations showed evidence that milling caused a physical transformation of the RBVN; however the yield from milling varied, represented by the sieve fraction of interest, and the drug content. Based on these results, F2 (50% w/w drug loaded product milled for 10 min), which demonstrated moderate yield with very tight drug content and content uniformity, was used for subsequent experiments.
4.3.2. Characterization of Microparticles

4.3.2.1. Solid state properties

PXRD and DSC were used to characterize the microparticles following preparation and processing. The PXRD patterns of pure component RBVN powder, POX188 powder, and processed microparticles were compared as shown in Figure 4.3. As shown, the reference diffractograms for the RBVN and POX188 show characteristic Bragg peaks, respectively at 11.94, 18.15, 20.3, 20.6, 24.44, 27.1 °2θ and 19.1, 22.0, 23.2 °2θ, all of which were conserved in both physical mixtures as well as processed microparticles. These data indicate that the crystal form of both components was preserved throughout preparation.

![Figure 4.3. PXRD patterns of 1:1 RBVN and POX188 product as it progresses through the different stages of manufacturing scheme, PM – physical mixture, SS – solid suspension post cooling in ice water, BCM – Binary composite microparticles after sieve fractionation. PXRD patterns of pure components are provided for comparison.](image-url)
Figure 4.4 shows a comparison of DSC thermograms for pure components with those of a 1:1 physical mixture and 1:1 RBVN : POX188 microparticles. The DSC thermogram for pure RBVN showed only a single single sharp endothermic peak at 168.64 ± 0.21 °C, consistent with melting of the room-temperature stable polymorph, Form R-II.163 POX188 thermograms had an endothermic peak at 54.32 ± 1.39 °C, consistent with literature values for its melting.206 DSC data for the 1:1 composite microparticles showed two distinct endothermic peaks, one at 55.9 ± 1.59 °C and 168.2 ± 0.48 °C, which were attributable to POX188 and RBVN, respectively, while the thermograms for the microspheres, slow heating DSC curve (2 °C/min) was similar to that for physical mixture with no reduction in the melting of POX188 and a 3 °C depression in the onset of melting of RBVN. These differences could be the effect of particle size reduction that the microparticles were subjected to during milling. None of the microparticles showed any evidence of a solid form change, confirming that RBVN and POX188 remained phase pure. It is noted that high heating rate (40 °C/min) was applied for improving detectability of small quantities of amorphous RBVN/R-I, but no discernable changes were observed.
4.3.2.2. Particle properties

In the present study, the selected sieve fraction of composite microparticles was characterized for particle size distribution and bulk morphology (Figure 4.5a – 4.5b). The median primary particle size ($d_{50}$) was 20.6 µm, with $d_{10}$ and $d_{90}$ respectively measuring 6.03 µm and 48.1 µm (See Fig. 4.5c). When observed under polarized light, particles prominently exhibited birefringence suggesting that RBVN remains in crystalline state (data not shown).
Figure 4.5. (a and b) Micrograph depicting particle size distribution of 1:1 RBVN : POX188 binary composite microparticles. The particles were not immersed in oil, observed through a 10× objective. These micrographs were used to measure Feret diameter (n = 300) particles; (c) particle size distribution of primary particles observed in (a and b).

Scanning electron microscopy (SEM) technique was used to assess the bulk morphology of final microparticles. Relative to the agglomerated needle-like morphology of standalone RBVN and spherical shape of POX 188, the binary composite microparticles demonstrated irregular surface morphology (figure 4.6). Although the morphology of particles obtained from analytical samples may not be particularly favorable for reproducibly emitted doses from nasal devices, it is expected that the size reduction step necessary at larger scales will likely make the particle morphology more regular; making it suitable for insufflation and nasal deposition.
Figure 4.6. Scanning electron micrographs: (A) unprocessed ribavirin, (B) poloxamer 188 and (C) 1:1 (w/w) milled + sieved fractionated composite microparticles.

4.3.2.3. Drug content and uniformity

Samples were randomly withdrawn from the microparticles collected on the 20 μm sieve and assessed for drug content. Based on spectrophotometric analysis, the mean RBVN content was measured to be 50.1 %w/w with a relative standard deviation RSD of 3.0. This suggests that the two components of the microparticle system were adhered strong enough to overcome the mechanical stresses of milling and sieve fractionation. For comparison, a control group comprised of a 1:1 physical mixture of RBVN and POX188 (both components individually milled to 20 – 53 μm particle sizes) was prepared in lab scale V-blender. This mixture was exposed to mechanical sieve shaking similar to
composite microparticles. Interestingly, segregation into individual components was observed within 10 min of mechanical shaking, resulting in a mean RBVN content of 52.1 %w/w with poor RSD of 8.3, which worsened as shaking continued. Similar to this observation, segregation of RBVN and POX188 is likely to occur during transport and/or insufflation of a dry, solid, nasal drug product, potentially manifesting as inconsistent performance. As a result, physical mixtures of RBVN and POX188 were felt unlikely to be viable formulations; therefore only the binary composite microparticles were evaluated for *in vitro* performance.

4.3.3. Evaluation of formulation

4.3.3.1. *in vitro* Drug Release

Drug release from microparticles was evaluated *in vitro* using a semi-permeable regenerated cellulose membrane to separate donor and receiver compartments in a Franz diffusion cell. A 3000-3500 Da MWCO (molecular weight cut off) was used to facilitate selective permeation of dissolved RBVN molecules (molecular weight = 244.21 Da), while retaining the POX188 molecules (average molecular weight = 8400 Da) on the membrane surface. In contrast to typical diffusion experiments, the dissolution volume on donor side of the Franz cell was purposely maintained at 20 µL to mimic the hydration volume in a typical adult human nasal cavity. Consequently, *in vitro* release experiments were considered to consist of three-steps: (1) drug dissolution within the hydrated polymer matrix (2) drug diffusion through the polymer matrix into the donor fluid, and (3) accumulation of drug in the receiver side of the diffusion cell, after passing through the membrane.
It is acknowledged that the chosen release model only allows for multiple sampling from the receiver side which, in turn, provides information about the rate of drug accumulation in the receiver side. It does not, however, directly assess the first two processes i.e., drug dissolution and diffusion rates. Owing to the complexity associated with this setup, a control group of pure component RBVN microparticles was added to the experimental design to eliminate one of three abovementioned processes i.e., drug diffusion from the polymer matrix. Furthermore, it is conservatively assumed that RBVN permeation through the cellulose membrane is less likely to be rate-limiting as the pore size of the membrane (~1-10 nm reported by manufacturers) is considerably larger than the size of single drug molecule dissolved in water. By this argument, the observed “apparent release” profile from standalone RBVN microparticles (see figure 4.7) is an indirect estimation of drug dissolution in the donor side. It can be seen that dissolution of RBVN in the donor side is not instantaneous but proceeds gradually over the course of the experiment. Approximately 70% of the drug was released (or dissolved in this case) in 45 min, which reaches completion (~97%) within 180 min.
Figure 4.7. The apparent cumulative RBVN release from standalone solid drug particles (black circles) and binary composite microparticles (grey circles).

When these data are compared with the RBVN release rate from binary composite microparticles, the influence/effect of POX188 on drug release became clear. Although the extent of cumulative RBVN release from polymer microparticles remained comparable at 180 min, it is noteworthy that the initial release rate was slower relative to the pure component drug particles. This result was not unexpected because the RBVN particles have to not only undergo dissolution in the donor side but the dissolved RBVN molecules have to diffuse through the polymer matrix prior to permeation, collectively decreasing the rate at which drug molecules appear in the receiver compartment.

Poloxamers® are known to exhibit reversible thermal gelation and their effect on drug release is extensively studied. Evidence suggests that in an aqueous solution, the copolymer molecules aggregate as micelles when the temperature is increased. The
micellization is likely due to the dehydration of the hydrophobic PPO blocks with temperature. At sufficiently high concentrations and temperature, these micellar structures entangle to form rigid gels, which in turn produces a decrease in the number and size of water channels, leading to an increased path length for diffusion. A decrease in RBVN release rate from binary composite microparticles is attributed to poloxamer gelation in the donor compartment. As the release experiments progressed, rapid conversion of solid RBVN : POX188 composite microparticles into a thin film was observed; which supports this claim. According to the data, it took approximately 75 mins for 70% drug to release, which was 1.7 times longer than the control group. While it can be argued that such a release rate is not reflective of the human nasal clearance time (estimated at 10-20 min, and most drug may remain unabsorbed and lost, there is some literature data to suggest that poloxamer containing gel formulations can be retained in the nasal cavity for up to 100-110 min.\textsuperscript{128,129} This suggests that >90% drug release can be assumed in the nasal cavity from the microparticles formulated in this study.

\textbf{4.3.3.2. \textit{in vitro} Cytotoxicity}

During the development of drug-loaded formulations for IN administration, preclinical studies assessing transmucosal drug transport or toxic effects of excipients can be performed using excised tissue, primary cell cultures, or immortalized cell lines.\textsuperscript{211} Excised human tissue is not only difficult to obtain, but also presents inter- and intra-individual differences. As a consequence, drug permeation studies are usually performed in nasal mucosa excised from experimental or slaughtered animals, which introduce other drawbacks, such as species differences in tissue composition, transporter expression and
ethical concerns. Alternatively, in vitro human cell lines (primary and immortalized) can be used to evaluate drug formulations.

Immortalized cell lines from human origin are of great interest owing to good genetic homogeneity, data reproducibility and ease of culture maintenance. In particular, RPMI 2650, an immortalized cell line derived from an anaplastic squamous cell carcinoma of the human nasal septum, is the only commercially available cell line having morphological and biochemical resemblance with normal human nasal epithelium. These RPMI 2650 cells were investigated as a permeation model to evaluate RBVN : POX188 (1:1) solid microparticle formulations prepared and characterized in Section 4.3.1 and 4.3.2, respectively. Prior to the permeation experiments, it was imperative to assess the safety of nasal epithelium in the presence of the components of microparticles. For this reason, RPMI 2650 cell viability was studied after 24 h of incubation with pure RBVN, pure POX188 and 1:1 mixture of RBVN and POX188 over a spectrum of concentration and the results are captured in Figure 4.8.
Cytotoxicity assay using CellTiter Glo® reagent (reported as mean ± SD, n ≥ 3): RPMI 2650 cells were incubated with varying concentrations of POX188 only, RBVN only and RBVN: POX188 (1:1) mixture for 24 h at 37 °C and 5% CO₂ humidified atmosphere. 2 mg/mL stock solutions prepared in high-grade sterile water was sterile filtered and serially diluted with media to generate the spectrum of concentrations used here. For 1:1 physical mixtures, appropriate dose adjustment was done (i.e. 4 mg/mL stock) was prepared to obtain concentrations upon dilution that are comparable to the other test groups. Statistical differences within the same group are denoted as; **P < 0.01; ***P < 0.001 and between groups are denoted as #P < 0.05.

The effect of increasing ribavirin concentration on the viability of RPMI 2650 cells is shown here. As expected, higher concentrations of RBVN resulted in a greater reduction in cell viability. In the test group containing only RBVN, the measured cell viability was reduced by 25.7 %, 30.1 % and 36.8 % respectively, at concentrations 250, 500 and 1000 μg/mL. RBVN is known to inhibit the synthesis of DNA within cells, resulting in cell death and prevention of cell division.215,216
In contrast to the results above, pure component POX188 showed no significant cytotoxic effect over the entire concentration range studied, which was in close agreement with observations in other cell lines. Poloxamers® have been shown to have cytoprotective effect in vitro and in vivo. The most well-documented characteristic of POX188 is its ability to repair damaged cell membranes by mechanisms that, while not entirely clear, may be attributable to helping increase the lipid packing density by direct incorporation of POX188 into the phospholipid bilayer. In the present study, when the cells were exposed to RBVN in the presence of POX188, the viability was improved considerably, which was more prominent at higher concentration. At 1000 μg/ml, cells incubated with RBVN exhibited 63.2% viability, which was improved to 89.5% in the presence of POX188. It is plausible that poloxamer may have sequestered RBVN from entering into the cells through the ability to incorporate in lipid bilayer.

It is acknowledged that the time of incubation used in these experiments is not reflective of human nasal residence time; however, the extension of exposure time guaranteed an observable and quantifiable change in viability that enabled a comparison of the cytotoxicity between test groups. Based on these results, 1000 μg/mL initial RBVN concentration was used for in vitro permeation experiments using a nasal cell line, with an exposure period of only 4 h where no cytotoxicity was observed (data not shown). Using the method laid out by Bai et. al. and Goncalves et. al. attempted growth of RPMI 2650 monolayers (See figure 4.9a) at air-liquid interface was performed. The RPMI 2650 cells were seeded (at a density of 4 × 10⁵ /cm²) onto collagen-coated permeable polytetrafluoroethylene inserts and grown at an air–liquid interface for 8-10 days. Unlike Bai et. al., however, our repeated trials failed to produce polarized
monolayers with cells interconnected through tight junction proteins. Instead, clusters of cells and distinct open regions resulting in a ‘leaky’ permeation membrane were observed, as represented in **figure 4.9b**. Therefore, it was decided to incorporate freshly excised olfactory tissue from bovine source as the model for permeation.

**Figure 4.9.** (a) A representation of permeation barrier produced with RPMI 2650 nasal cell line uniformly grown at an air-liquid interface within the cell inserts. Reprinted from *International Journal of Pharmaceutics*, 515, Vanessa S.S. Gonçalves, Ana A. Matias, Joana Poejo, Ana T. Serra, Catarina M.M. Duarte, Application of RPMI 2650 as a cell model to evaluate solid formulations for intranasal delivery of drugs, pp:1-10, Copyright 2016, with permission from Elsevier. (b) A microscopic image of RPMI 2650 cells growing as clusters on the inserts, as observed on day 10 from the day of seeding. Media was changed on the basolateral side every 48 hours. Reprinted from *Journal of Pharmaceutical Sciences*, 97, Shuhua Bai, Tianzhi Yang, Thomas J. Abbruscato, Fakhrul Ahsan, Evaluation of human nasal RPMI 2650 cells grown at an air–liquid interface as a model for nasal drug transport studies, pp:1165-1178, Copyright 2008, with permission from Elsevier.

**4.3.3.3. in vitro Permeation**

The present work was designed to evaluate RBVN permeation when deposited as solid microparticles directly on nasal mucosal tissues. As such, a control group involving loading of RBVN in aqueous solution was introduced to compare how the permeation of
drug might be affected. It should be noted that the configuration of permeation cells used here was different from Chapter 2. In using the vertical tissue diffusion cells for the L-DOPA work, it was realized that solid microparticles may have drifted away from the diffusion area during the course of the experiment, which would manifest itself as a premature plateau in the drug transport profile. To avoid this potentially confounding variable and ensure that the solid RBVN microparticles remained deposited on the tissue throughout experiments, diffusion chambers allowing horizontal tissue mounting were used.

Figure 4.10. in vitro permeation profile of RBVN across bovine olfactory tissue, when loaded as aqueous solution. Data are expressed as mean ± SD, n=3. The dashed line denoted steady state region.
A 0.2 mL aliquot of a 5 mg/mL aqueous solution of RBVN was added to the donor compartment to expose (theoretically) 1 mg of RBVN to the mounted tissue. After 120 min of contact with the nasal mucosa, the cumulative amount of RBVN that permeated from solution, per unit tissue area, was 742.20 ± 16.92 µg cm⁻² (n = 3, see figure 4.10). The linearity of the profile (r² > 0.99), without significant lag-time, suggested that drug permeation occurred at a constant rate. The average flux in the steady-state region (Jss), calculated based on Equation 4.1, was 4.02 ± 0.51 µg/cm²-min, while the apparent permeability coefficient (Papp) was estimated to be (1.34 ± 0.17) x 10⁻⁵ cm/s based on Equation 4.1. This agreed closely with a previously reported value of Papp of RBVN across rabbit nasal mucosa (1.92 ± 0.18) x 10⁻⁵ cm/s).²² These preliminary experiments confirmed high permeability of RBVN across bovine olfactory tissue in vitro; however, considering the rapid mucociliary clearance of the nasal cavity, it is likely that aqueous solutions containing RBVN would not remain in residence for more than 10-20 min.

For a direct comparison with aqueous RBVN solution, direct deposition of 1.0-1.5 mg RBVN solid microparticles on the excised tissue was planned. Owing to the impalpable nature of the solid RBVN microparticles, it was difficult to manually load such small quantities of powder and maintain a dose precisely consistent with the solution group. Therefore, 5 mg of RBVN solid microparticles was directly deposited on the donor side of the tissue, while the dose of RBVN : POX188 (1:1) microparticles was adjusted to provide comparable drug exposure (i.e., 10 mg of 1:1 binary microparticles). The permeation profile for the two test groups of solid particles is shown in figure 4.11.
As shown, the cumulative amount of RBVN permeated from polymer microparticles (4332.95 ± 460.11 µg/cm²) was about 3.3 fold greater than that which permeated from pure component RBVN microparticles (1330.79 ± 182.83 µg/cm²). This was also reflected in the flux values, which were 18.72 ± 3.4 µg/cm²·min ($r^2 >0.92$) and 47.81 ± 22.42 µg/cm²·min ($r^2 >0.99$) respectively for RBVN particles and RBVN : POX188 (1:1) composite microparticles.

A comparison of the permeation profiles exhibited by each test group notes striking differences. The mass transport profile for RBVN loaded as standalone drug...
microparticles reaches a plateau after approximately 45 min, unlike the 1:1 RBVN : POX188 composite microparticles. Given the horizontal setup, loss of microparticles from the site of diffusion due to gravity was not expected. Additionally, under the parameters maintained during these experiments, the receiver volume was >10 times the saturation volume for RBVN ($C_s = 149$ mg/mL at 25 ºC) (Chapter 3, Section 3.3.2), suggesting that it was reasonable to expect that sink conditions were maintained throughout permeation measurements.

Careful examination of the literature offered some logical explanation of these data in relation to the mucosal uptake mechanism of ribavirin molecules. Briefly, it is known that ribavirin is absorbed into biological compartments (including intestinal cells, erythrocytes, striated muscles and neurons) by transporter-mediated uptake, particularly nucleoside transporters, CNT 2/3 and ENT 1/2. Owing to the high expression and localization of these transporters in the olfactory epithelium, as reported by Al-Ghabeish, it is safe assume that RBVN molecules predominantly underwent active uptake in these permeation measurements. Furthermore, the hydrophilic nature of ribavirin makes molecular RBVN unlikely to undergo significant passive transport.

It is well-known that transporter-mediated uptake mechanisms can be saturated if the substrate (drug) levels are sufficiently high. Once specific transporters are saturated, no additional drug can be absorbed, irrespective of the concentration available in the donor side. Reports indicate that ribavirin transport by human ENT1 in the erythrocytes is saturable at $420 \mu$m ($\sim 100 \mu$g/mL), and in intestinal lumen at $44.5 \mu$m ($\sim 11 \mu$g/mL). In the absence of direct measurements on olfactory epithelium, it can only be hypothesized that at 45 min almost all RBVN powder dissolved in the small volume of
donor fluid and saturated the transporters, which in turn would be consistent with the observed plateau beyond 45 min in the permeation profile of RBVN solid microparticles.

Alternatively, it can be speculated that the influx of RBVN molecules in the olfactory tissue was counteracted by efflux transporters, resulting in reduced overall mass transport. Evidence suggests that P-gp and BCRP efflux transporters are highly expressed in the olfactory tissues, which show affinity towards nucleoside analogs structurally related to RBVN. In contrast to the pure RBVN data, formulation of RBVN : POX188 (1:1) binary microparticles enabled unrestricted unidirectional transport of RBVN, as shown in figure 4.11. Poloxamers® have been widely studied for efflux pump inhibition in the areas of drug delivery beyond the blood brain barrier (BBB) and cancer therapy. Additionally, these polymers are known to have an effect on membrane fluidization and transient opening of tight junctions. It is speculated that a combination of these physiological changes enabled higher flux and greater RBVN mass transport in the presence of POX188. A schematic representation of the proposed mechanism is shown in figure 4.12.
Figure 4.12. Schematic representation of RBVN (blue closed circles) transport across the olfactory epithelium.

As depicted in the figure, the presence of POX188 enables greater RBVN permeation presumably via a combination of opening of tight junctions and inhibition of efflux transporters. This is supported by our experimental observations. These data confirm the superiority of the 1:1 binary composite microparticles in that it does not compromise the cumulative extent of RBVN release, potentially reduces the cytotoxic effect of RBVN and improves epithelial flux and overall mass transport. In contrast, the usability of aqueous RBVN solutions is likely restricted by long term chemical integrity of RBVN in dissolved state and rapid clearance from the site of deposition. Standalone RBVN microparticles surely circumvent the issue of chemical instability but their full potential may not be utilized due to saturable RBVN mucosal uptake.
4.4. Conclusion

In this work, solid microparticles comprised of a 1:1 suspension of fine crystalline RBVN in POX188 matrix were developed. Particles were milled to an average size of 20 μm to make them suitable for intranasal delivery. The final microparticles were physically and chemically stable, with a narrow particle size distribution and precise content uniformity. The extent of RBVN release from microparticles was comparable to standalone drug particles. Preliminary cytotoxicity tests indicated the effects of RBVN and POX188 on the viability of nasal cell lines when exposed individually and as a 1:1 combination. This helped establish dosing for tissue experiments. Permeation of excised bovine nasal tissue was evaluated in vitro, showing that 1:1 RBVN : POX188 composite microparticles allowed the highest mass transport of ribavirin relative to RBVN solid particles or RBVN solution. This was expected to be the result of transient opening of tight junction proteins along with inhibition of efflux transporters. These data suggest that formulation of a poorly permeating drug, such as RBVN with a permeation enhancer, such as POX188, can be advantageous to ultimate mass transport of drug. The effect of chronic dosing of permeation enhancer on the integrity of nasal epithelium, however, remains to be tested.
Chapter 5: Summary

Intranasal drug delivery has shown tremendous potential for direct entry of permeation rate limited molecules into the CNS, by allowing drugs to bypass the BBB. Research on nose-to-CNS uptake has revealed that deposition of formulation in the olfactory region of the nasal cavity, allows opportunity for drug to be directly absorbed to the CNS, providing the opportunity for treatment of various neurological conditions.

Research has suggested that deposition of solid particles resists mucociliary clearance, and can allow for greater retention time in the nasal cavity. Additional time at the deposition site results in increased absorption from the nasal cavity when compared with drug solutions or other liquid vehicle dosage forms. This was tested in the present work, first by measuring epithelial flux and permeability across bovine olfactory tissue for L-DOPA when loaded as either an aqueous solution or as dry, crystalline particles. As hypothesized, the average steady-state flux ($J_{ss}$) of L-DOPA from the solid microparticles directly deposited on nasal epithelial tissue was 3 times greater than the $J_{ss}$ measured for L-DOPA from solution. This was attributed to rapid dissolution of L-DOPA from the microparticles that ultimately saturated the mucus layer. Consequently, a high driving force was established across the nasal membrane, which enabled improved mass transport. These findings suggested that intranasal delivery of small molecule organic substances (SMOs) by the administration of solid microparticles might not only benefit from improved chemical and microbiological stability but do so without compromising the cumulative mass transport of drug across the olfactory membrane.
The antiviral compound RBVN has been investigated in the treatment of several diseases affecting the CNS, however, bioavailability to this compartment is extremely limited, owing to the poor lipid permeation of this molecule. To improve the cumulative extent of tissue transport in cases such as these, literature reports suggest the use of permeation-enhancing polymers. The present work explored the IN delivery of solid binary composite microparticles consisting of ultrafine crystalline RBVN suspended in a surrounding solid matrix of Poloxamer F-188 (POX188). Prior to the preparation of composite microparticles, it was crucial to investigate the effect of standard micronizing techniques on the physical and chemical stability of RBVN. Little has been done concerning susceptibility of this material to solid form changes during processing. In the present work, solid-state transformations of RBVN were examined under different thermal and mechanical stress conditions. Although the $T_{tr}$ for RBVN enantiotropes was measured at ~70 °C, unprocessed R-II did not transform into high melting enantiotrope R-I, at $T > T_{tr}$, even after 7 days. However, following grinding, the same isothermal storage conditions resulted in rapid R-II $\rightarrow$ R-I conversion. This suggested decrease in the energy barrier required to produce the high $T_m$ enantiotrope during pulverization; potentially by creating crystal defects during milling. Further, a comparison of two milling equipments revealed that extensive grinding led to complete R-II $\rightarrow$ R-I when the mill temperature was not controlled. In contrast, high shear cryogenic grinding of R-II, conducted at $T << T_g$ resulted in complete transformation to persistent amorphous ribavirin. Ultimately, the findings in this work facilitated intelligent selection of manufacturing equipment and process conditions for reproducibly generating composite microparticles of RBVN and POX188.
Co-solidified mixtures of 1:1 drug and polymer were micronized to dimensions between 10-45 µm to promote maximum nasal deposition. Consistent with the RBVN characterization work, it was confirmed that the final drug product demonstrated a consistent solid form, acceptable chemical stability, and drug content uniformity, even following comminution. Additionally, *in vitro* drug release studies revealed that the cumulative RBVN release from the polymeric microparticles was comparable with standalone drug microparticles. Although the initial release rate was slower in the presence of polymer, it was argued that the mucoadhesive property of POX188 will retain the drug product at the deposition site longer and not compromise the extent of RBVN release. Further, the presence of POX188 appeared to counteract the observed cytotoxic effect of RBVN *in vitro* by sequestering the latter. Lastly, a 3.3 fold higher amount of RBVN cumulatively permeated through excised olfactory tissue when deposited as 1:1 RBVN : POX188 solid microparticles relative to standalone RBVN solid particles. This was attributed to a combination of effects (1) transient opening of tight junctions between olfactory epithelial cells and (2) blockage of efflux transporters.

Ultimately, these data suggested a potential avenue for pharmaceutical formulation research and development that could be particularly important in the treatment of other neurological disorders. Indeed, drug molecules having limited CNS bioavailability may be subject to greater nasal retention and permeation when introduced as solid microparticles, providing the possibility of straightforward, non-invasive therapy for current clinical practice. Nonetheless, it will be crucial to investigate the effect of multiple dosing of microparticles on the integrity of nasal tissue – a compromised state of which may inadvertently provide CNS entry to toxins and allergens.
Appendix A: Physical characterization of drug and polymer dispersion behavior in PEG 4000 solid dispersions using a suite of complementary analytical techniques

A.1. Introduction

As poorly soluble new chemical entities enter drug development pipelines, different approaches for solubility enhancement may be attempted as they are considered for adoption as candidate molecules. Among these, preparation of solid amorphous dispersions has received considerable attention.\textsuperscript{32, 227-230} Solid dispersions are prepared by rapid co-solidification of an active pharmaceutical ingredient (API) and a carrier excipient (frequently a water soluble polymer), by one of several methods, including spray drying,\textsuperscript{231} super-critical fluid processing,\textsuperscript{232} lyophilization,\textsuperscript{233} hot-melt extrusion,\textsuperscript{234} and high shear co-trituration.\textsuperscript{235, 236} Solid dispersions can increase the apparent solubility of a material by allowing the drug to persist as an amorphous solid.\textsuperscript{237} Spontaneous recrystallization of the drug is inhibited by its physical intercalation within interstices formed by entanglement of the polymeric host.\textsuperscript{230, 238-240}

Since both formation and physical stability of solid dispersions relies on solidification from a mutually miscible API and polymer mixture, research in this area has focused on predicting how the interactions between API and carrier enable dispersability.\textsuperscript{241-244} Despite predictive advances, dispersion formulation still relies on analytical techniques to confirm that a true dispersion has been formed. Several different characterization approaches are reported throughout the literature, and include single-
instrument, two-instrument and multi-instrument methods, each aimed at demonstrating that the physical properties and microstructure of the binary solid, established by intimate, mutual dispersion of the components, are distinct from physical mixtures of the two solids. Moreover, as solid dispersions become viable formulation strategies for poorly soluble API, regulatory agencies will see an increased number of filings for these materials. At present, no standard guidance directs characterization of solid dispersions, leaving it to the literature to report best practices.

In this study solid dispersions were attempted using a modest library of 15 model API in polyethylene glycol 4000 (PEG4000). PEG4000 is a hydrophilic, semicrystalline polymer potentially useful as a solid dispersion carrier which has been shown to increase the dissolution rate of poorly water soluble drugs such as carbamezapine. Despite historical use, the phase behavior of API in PEG remains challenging to characterize. Upon co-solidification, PEG4000 has been shown to disperse API as pure amorphous solid, a combination of amorphous and crystalline phases, only crystalline solid, and form eutectic mixtures with API. PEG4000 was, therefore, chosen for this study as illustrative of resulting in very complex solid mixtures with API, providing analytically challenging signals for interpretation using individual techniques commonly employed in solid dispersion characterization. In this work, API:PEG4000 combinations will be classified in one of three categories, 1) Fully dispersed: quenching of a completely miscible molten mixture results in a single amorphous phase; 2) Partially dispersed: quenching results in a detectable amorphous phase, and recrystallized drug and/or polymer; 3) Fully phase separated: quenching results in complete recrystallization of the drug and polymer. A fourth potential outcome might be expected in some cases,
where both components are in the amorphous state, but they are not dispersed in one another. This possibility is unlikely with the polymer used here, and was not observed, most likely due to the propensity of PEG4000 to rapidly crystallize.

The key objective of this work was to demonstrate that comprehensive characterization is needed for PEG-based dispersions in order to properly classify API:polymer behavior. Each sample in the library was characterized using a suite of techniques including powder X-ray diffraction (PXRD), pair distribution function (PDF) analysis, hot-stage polarized-light microscopy (HSM), and differential scanning calorimetry (DSC). The dispersion behavior of each API in PEG4000 was inferred using this suite of techniques. In some instances, inferences drawn from single techniques resulted in inconsistent classifications, while complementary characterization allowed for better informed classification of the binary materials.

A.2. Materials and Methods

A.2.1. Materials

Chlorpropamide, indomethacin, tolbutamide, cimetidine, and griseofulvin were all purchased from MP Biomedicals (Solon, OH). Ketoconazole and ibuprofen were purchased from Spectrum (Gardena, CA). Quinidine and cloperastine·HCl were purchased from Sigma-Aldrich (St. Louis, MO). Terfenadine and sulfanilamide were purchased from Acros Organics (Geel, Belgium). Propranolol·HCl, nifedipine, and itraconazole were purchased from TCI Chemicals (Portland, OH). Melatonin and polyethylene glycol 4000 were purchased from Alfa-Aesar (Ward Hill, MA). The structures for all model drugs and PEG4000 are shown in Table A.1.
Table A.1. Molecular structures for model drugs and polymer used in this study.

**Model Compounds:**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorpropamide</td>
<td><img src="image1" alt="Molecular Structure" /></td>
</tr>
<tr>
<td>Nifedipine</td>
<td><img src="image2" alt="Molecular Structure" /></td>
</tr>
<tr>
<td>Quinidine</td>
<td><img src="image3" alt="Molecular Structure" /></td>
</tr>
<tr>
<td>Ibuprofen</td>
<td><img src="image4" alt="Molecular Structure" /></td>
</tr>
<tr>
<td>Propranolol</td>
<td><img src="image5" alt="Molecular Structure" /></td>
</tr>
<tr>
<td>Indomethacin</td>
<td><img src="image6" alt="Molecular Structure" /></td>
</tr>
<tr>
<td>Ketoconazole</td>
<td><img src="image7" alt="Molecular Structure" /></td>
</tr>
<tr>
<td>Cloperastine</td>
<td><img src="image8" alt="Molecular Structure" /></td>
</tr>
<tr>
<td>Sulfanilamide</td>
<td><img src="image9" alt="Molecular Structure" /></td>
</tr>
<tr>
<td>Itraconazole</td>
<td><img src="image10" alt="Molecular Structure" /></td>
</tr>
<tr>
<td>Terfenadine</td>
<td><img src="image11" alt="Molecular Structure" /></td>
</tr>
<tr>
<td>Tolbutamide</td>
<td><img src="image12" alt="Molecular Structure" /></td>
</tr>
<tr>
<td>Cimetidine</td>
<td><img src="image13" alt="Molecular Structure" /></td>
</tr>
<tr>
<td>Melatonin</td>
<td><img src="image14" alt="Molecular Structure" /></td>
</tr>
<tr>
<td>Griseofulvin</td>
<td><img src="image15" alt="Molecular Structure" /></td>
</tr>
</tbody>
</table>

**Polymer:**

![Molecular Structure](image16)  
PEG 4000
A.2.2. Co-solidification of API:PEG4000 Mixtures

Physical mixtures of each API and PEG4000 were manually prepared in a 3:1 (%w/w) API:polymer ratio, and equilibrated at 10%RH for 24 h. Physical mixtures were transferred to a crucible immersed in silicone oil maintained at $T_{m,API} +10^\circ C$. The molten mixture was stirred for 20-30 min to ensure that all solid had disappeared. Mixing times were determined based durations at $T_{m,API} +10^\circ C$ at which insignificant thermal degradation was observed. Each material was held isothermally in a thermogravimetric analyzer (TGA) at $T_{m,API} +10^\circ C$, and <2% w/w weight loss was set as a criterion for thermal stability at this temperature. Additionally each API was heated in a DSC to $T_m +15^\circ C$, and held isothermally for 30 min. After the extended isothermal hold, samples were quenched in the DSC, before reheating. The $T_g$ values all corresponded well with reported literature values, and the baseline heat flows following melting of these samples were steady, suggesting that each API was stable under the conditions used to make the dispersions. Additionally, the literature suggests that no significant chemical degradation should be expected at temperatures equal to or greater than those used in the present experiments for chlorpropamide,\textsuperscript{255} griseofulvin,\textsuperscript{256} indomethacin,\textsuperscript{257} itraconazole,\textsuperscript{258} ketoconazole,\textsuperscript{259} nifedipine,\textsuperscript{260} terfenedine,\textsuperscript{261} and tolbutamide.\textsuperscript{257}

Nitrogen gas was continuously streamed over the crucibles during preparation, to prevent moisture sorption. Molten mixtures were quenched by immersion of the crucible in liquid nitrogen. Pure component amorphous samples were prepared using the same method as the API:PEG4000 mixtures. All preparations were repeated in triplicate.
A.2.3. Differential Scanning Calorimetry (DSC)

Glass transition temperatures ($T_g$), melting temperatures ($T_m$) and recrystallization temperatures ($T_c$) were measured in triplicate for all samples using a Model Q100 DSC (TA Instruments, New Castle, DE), operated under a three-point temperature/enthalpy calibration using o-terphenyl, indium, and tin standards, and a cell constant calibration using indium. All experiments were conducted using 50 mL/min nitrogen purge to the cell. Sample “chips” of co-solidified mixtures (4-8 mg) were hermetically sealed in aluminum pans, and initially cooled at 20°C/min to -80°C. Following in situ equilibration at -80°C, each sample was heated at either 2°C/min or 20°C/min to $T_m,API +10°C$. All pure component drug and polymer samples (3-5 mg) were heated at 20°C/min from room temperature to $T_m +10°C$, held isothermally for 5 min, and then rapidly cooled to -80°C in situ. Quenched samples were then re-heated at 20°C/min to $T_m +10°C$.

The expected $T_g$ for drug:polymer mixtures (assuming intimate mixing of the two liquid phases) was calculated using the Couchman-Karasz equation:

$$T_g = \frac{w_{API}T_{g,API} + Kw_{p}T_{g,p}}{w_{API} + Kw_{p}}$$  \hspace{1cm} (A.1)

where $w_{API}$ and $w_{p}$ were the weight fractions of API and polymer, respectively, $T_{g,API}$ and $T_{g,p}$ are the glass transition temperatures of amorphous API and polymer, respectively, and $K=\Delta C_{p,p}/\Delta C_{p,API}$, where $\Delta C_{p,p}$ and $\Delta C_{p,API}$ are the changes in heat capacity through the glass transitions of the polymer and API, respectively. Calculated $T_g$ values were compared with observed $T_g$ values for mixtures, interpolated from the DSC heat flow signal, measured as the midpoint of the step change in heat capacity. It is important to
note that $T_{g,p}$ measured in these experiments was very low (-67.92°C),\textsuperscript{263-265} and PEG4000 rapidly recrystallized at room temperature. As such, pure amorphous PEG4000 could not be prepared as a pure component reference for use with any of the other characterization experiments.

**A.2.4. Optical Hot Stage Microscopy (HSM)**

Small, intact chips of each co-solidified API:PEG4000 mixture were placed on a microscope slide and heated/cooled using an Instec HCS 302 Pelletier heating stage with an STC 200 temperature controller (Instec, Boulder, CO). Samples were heated at 10°C/min from ambient temperature to $T_{m,API} +10^\circ$C, held isothermally for 5 min, and then cooled back to ambient temperature at 10°C/min. Observations were made using an Olympus BX-51 optical microscope equipped with a polarizing filter, under 10X magnification. Photomicrographs were obtained throughout the heating/cooling profile to observe phase changes in the solids as a result of heating, or to distinguish between crystalline polymer and crystalline API.

**A.2.5. Powder X-ray Diffraction (PXRD)**

PXRD data were collected in transmission geometry, by placing intact samples between two layers of Kapton® film (ChemPlex, Palm City, FL), spun (16 rpm) in a vertical sample stage. Sample positioning, rotation and transmission geometry allowed complete volume interrogation of all samples, therefore, diffraction data were collected from each solid, in its entirety. An X’Pert Pro MPD system (PANalytical B.V., Almelo, the Netherlands), equipped with a copper anode ($\lambda = 1.5406$ Å), an auxiliary elliptical mirror, and X’Celerator\textsuperscript{TM} detector was used to collect diffraction data at an operational voltage/amperage of 45.0 kV/40.0 mA. To maintain consistent counting statistics
between experiments, consistent irradiation times of 51.04 s per step (0.017° 2θ steps) were used to collect data over 2-100 °2θ.

**A.2.6. Pair Distribution Function (PDF) analyses of PXRD data**

The pair distribution function (PDF) is a total scattering method utilizing the Fourier relationship between reciprocal and real space. The PDF reports the probability of finding two atoms separated by a distance \( r \), and is obtained using

**Equation A.2:**

\[
G(r) = \frac{2}{\pi} \int_0^{Q_{\text{max}}} Q [S(Q) - 1] \sin Q(r) \, dQ
\]

\( S(Q) \) is the structure function obtained from a normalized PXRD pattern, and \( Q \) is the magnitude of the scattering vector. To enable correct calculation of \( S(Q) \), corrections were made to measured diffraction data, according to those outlined in Egami and Billinge. The integration limit, \( Q_{\text{max}} \), is the momentum transfer resolution of the diffraction experiment, which depends on both the maximum diffraction angle (100 °2θ) and the X-ray wavelength (\( \lambda = 1.5406 \) Å) used in PXRD data acquisition. Intensity corrections (e.g., background due to Kapton® film, absorption, etc.) and PDF calculations were performed in the MATLAB environment (v2012b, MathWorks, Natick, MA) using a graphical user interface programmed in-house.

PDF-transformed PXRD data from co-solidified samples has been shown to be useful in differentiating between phase-separated and completely dispersed samples. In the present work, PDF-transformed PXRD data for co-solidified samples (observed PDF) were compared to calculated PDFs generated by weighted linear combination of the PDF transforms for individual amorphous components (theoretical
PDF). Dispersion behavior was inferred using the following criteria: 1. Sum-of-squares difference between theoretical and observed PDFs (R-value); 2. agreement or deviation of scaling coefficients relative to known 3:1 experimental compositions of API:polymer; and, 3. observation of regions in the PDF difference plot that did not contain zero in the 3σ confidence interval (CI).

Cases in which the theoretical and observed PDFs were superimposable were concluded to be at least partially phase-separated. That is, the short-range order of the co-solidified product was adequately described by the interatomic distances of physical mixtures of API and polymer. Conversely, significant deviations between theoretical/observed PDFs were used to suggest that short-range order unique to intimate API/polymer mixing was present, suggesting complete dispersion following solidification from a miscible mixture.

It was noted above that, owing to rapid recrystallization of PEG4000, PXRD for pure amorphous polymer could not be obtained, but was required for calculation of theoretical PDFs. As an alternative, PXRD patterns of liquid PEGs (PEG 200, PEG 300, and PEG 400) were collected. The PXRD and PDF data for the three liquid PEGs were indistinguishable, suggesting that although the polymers differed in their degree of substitution, their disorder and aperiodicity were sufficiently similar that they could be reasonably substituted for higher molecular weight PEGs, such as amorphous PEG4000 in the PDF routine.
A.3. Results and Discussion

A.3.1. API:PEG4000 library

15 separate API:PEG4000 binary solids (n=3) were made. Following quenching, each sample was characterized using PXRD (with PDF-transformation, where appropriate), HSM, and DSC in order to classify co-solidified mixtures as either fully dispersed, partially dispersed or fully phase separated. Inferences made using each technique independent of the others are detailed below, followed by a summary that considers collective inferences made using complementary characterization.

A.3.2. Inferences from PXRD data

Pure PEG4000 recrystallized rapidly on solidification, consistent with literature reports for high molecular weight PEGs. PXRD patterns for PEG4000 were collected for both untreated solid, and polymer subjected to the same melt-quench cycle described for API:PEG4000 mixtures. Both PEG4000 diffraction patterns were superimposable, suggesting that polymer recrystallization following melt-quenching resulted in the same solid as received from the vendor. PXRD patterns for API:PEG4000 mixtures were, therefore, easily separated based on characteristic PEG4000 diffraction relative to peaks unique to the API.

PXRD data are summarized in Table A.2. Of the 15 API solidified with PEG4000, 10 mixtures showed evidence of some crystallinity. In most cases, characteristic peaks attributable to both drug and polymer were detectable, however, tolbutamide:PEG4000 was unique in that it only diffracted at angles consistent with the API, but not the polymer. In contrast, cloperastine·HCl:PEG4000,
itraconazole:PEG4000, and propranolol·HCl:PEG4000 solid mixtures all diffracted at angles consistent with only the polymer.
Table A.2. Summary of PXRD analyses. Cambridge Structural Database (CSD) reference codes for each API are indicated parenthetically below the compound name.\textsuperscript{271,272} CSD structures were used to identify diffraction peaks for crystalline API in API:polymer mixtures.

<table>
<thead>
<tr>
<th>API component (CSD Reference code)</th>
<th>Diffraction attributable to API (°2θ)</th>
<th>Diffraction attributable to PEG4000 (°2θ)</th>
<th>X-ray scattering attributable to some amorphous content</th>
<th>PXRD Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorpropamide (BEDMIG02)</td>
<td>Minute peaks at 11.7, 19.5, 21.6</td>
<td>Minute peaks at 15.0, 19.1</td>
<td>Yes</td>
<td>Partially dispersed</td>
</tr>
<tr>
<td>Cimetidine (CIMETD)</td>
<td>Not observed</td>
<td>Not observed</td>
<td>Yes</td>
<td>Fully dispersed</td>
</tr>
<tr>
<td>Cloperastine HCl (QAWNAD)</td>
<td>Not observed</td>
<td>Minute peaks at 18.6, 22.7</td>
<td>Yes</td>
<td>Partially dispersed</td>
</tr>
<tr>
<td>Griseofulvin (GRISFL01)</td>
<td>Minute peaks at 10.8, 26.7</td>
<td>Minute peak at 23.9</td>
<td>Yes</td>
<td>Partially dispersed</td>
</tr>
<tr>
<td>Ibuprofen (IBPRAC01)</td>
<td>Definite peaks at 6.1, 16.6, 17.7</td>
<td>Definite peaks at 22.7, 23.5</td>
<td>No</td>
<td>Fully phase separated</td>
</tr>
<tr>
<td>Indomethacin (INDMET03)</td>
<td>Not observed</td>
<td>Not observed</td>
<td>Yes</td>
<td>Fully dispersed</td>
</tr>
<tr>
<td>Itraconazole (TEHZIP)</td>
<td>Not observed</td>
<td>Definite peaks at 18.6, 22.7</td>
<td>Yes</td>
<td>Partially dispersed</td>
</tr>
<tr>
<td>Ketoconazole (KCONAZ)</td>
<td>Not observed</td>
<td>Not observed</td>
<td>Yes</td>
<td>Fully dispersed</td>
</tr>
<tr>
<td>Melatonin (MELATN01)</td>
<td>Minute peak at 10.6, unidentified peaks* at 18.6, 25.4</td>
<td>Minute peak at 23.6</td>
<td>Yes</td>
<td>Partially dispersed</td>
</tr>
<tr>
<td>Nifedipine (BICCIZ)</td>
<td>Not observed</td>
<td>Not observed</td>
<td>Yes</td>
<td>Fully dispersed</td>
</tr>
<tr>
<td>Propranolol HCl (FIDGAB)</td>
<td>Not observed</td>
<td>Minute peaks at 18.6, 22.7</td>
<td>Yes</td>
<td>Partially dispersed</td>
</tr>
<tr>
<td>Quinidine (BOMDUC)</td>
<td>Minute peaks at 8.0, 18.5, 33.0</td>
<td>Minute peaks at 19.1, 23.3</td>
<td>Yes</td>
<td>Partially dispersed</td>
</tr>
<tr>
<td>Sulfanilamide (SULAMD06)</td>
<td>Minute peaks at 15.7, 19.7, 35.5</td>
<td>Minute peaks at 22.7, 33.0</td>
<td>Barely</td>
<td>Partially dispersed</td>
</tr>
<tr>
<td>Terfenadine (XUHTID)</td>
<td>Not observed</td>
<td>Not observed</td>
<td>Yes</td>
<td>Fully dispersed</td>
</tr>
<tr>
<td>Tolbutamide Form I (ZZZPUS02)</td>
<td>Minute peaks at 11.9, 19.1, Some peaks attributable to Form II**</td>
<td>Not observed</td>
<td>Yes</td>
<td>Partially dispersed</td>
</tr>
</tbody>
</table>

*Peaks not consistent with MELATN01 structure; may be metastable form

**Peaks consistent with ZZZPUS03 structure; metastable to Tolbutamide (Form I)
Recrystallization of both drug and polymer during solidification was easily observed in all three ibuprofen:PEG4000 samples. Characteristic diffraction from both drug and polymer were detected in co-solidified mixtures (Figure A.1a), and significant X-ray scattering was not detectable in any of the diffraction patterns, suggesting absence of amorphous drug.

Figure A.1. Overlay of PXRD patterns. (a) Pure ibuprofen (IBPRAC01) is indicated in blue; co-solidified 3:1 w/w ibuprofen:PEG4000 is shown in black; pure PEG4000 is shown in red. Select characteristic diffraction peaks attributable to API and polymer are indicated as blue stars and red closed circles, respectively. (b) Pure cimetidine (CIMETD) is indicated in blue; co-solidified 3:1 w/w cimetidine:PEG4000 is shown in black; pure PEG4000 is shown in red. Characteristic diffraction from neither drug nor polymer was observed, suggesting no crystalline content.

Taken in isolation, the PXRD data indicate that ibuprofen and PEG4000 were completely phase separated, suggesting that ibuprofen did not form a stable dispersion with PEG4000 under these conditions. Of the 15 API tested, ibuprofen was the only API whose PXRD characterization demonstrated this phase behavior (Table A.2).

Each of the 6 solid mixtures (other than ibuprofen) whose PXRD patterns contained at least some diffraction attributable to the API also contained evidence of persistent amorphous content. These samples were largely X-ray amorphous, with diffuse scattering over all angles 2θ, which obscured all but the most intense Bragg
diffraction peaks, detectable for both the API and polymer (Table A.2). As such, PXRD could not determine whether the amorphous content was from the API, polymer, or both. Since PEG4000 was expected to rapidly recrystallize in all samples, and the drug was present in 3:1 w/w ratio, the inclination might have been to assume that only drug was present as amorphous solid. This would be inconsistent with the semicrystalline nature of PEG4000, which even when recrystallized, contributes minor diffuse scatter from the amorphous regions of the polymer. More strikingly, however, were the PXRD patterns for 5 of the mixtures (cimetidine, indomethacin, ketoconazole, nifedipine, and terfenadine) all of which were completely X-ray amorphous following solidification (Figure A.1b, Table A.2). This suggested that the high concentration of drug in these preparations inhibited PEG4000 recrystallization, contrary to its expected behavior. As such, it is likely that amorphous scattering observed in PXRD for other solids contained contributions from both drug and polymer. Had the PXRD data been interpreted independent of the other characterization data, these 5 materials would have been classified as fully dispersed, having solidified from fully miscible mixtures of API and PEG4000. The remaining 9 materials would have been classified as partially dispersed in PEG4000 (Table A.2), where the combination of API and polymer at this composition allows some amorphous solid to persist at room temperature, following quenching.

Using PXRD data in this way ignores some important limitations of the technique. X-ray scattering by most mixtures in these experiments suggested significant amorphous content. In highly amorphous samples, diffraction from very small crystallites of API or polymer may not be detectable, potentially leading to the assumption that samples contained no crystalline content. A study conducted by
Rumondor et al., demonstrated a 1.9-2.2% limit of detection for crystalline felodipine in a substantially amorphous background that included poly(vinylpyrrolidone). Assuming no crystalline content in X-ray amorphous patterns might lead to incorrect classification of systems such as cimetidine, indomethacin, ketoconazole, nifedipine, and terfenadine, since the presence of even small quantities of crystalline API suggests only partially dispersed samples at this composition. Mixtures solidified from PEG4000 and chlorpropamide, cloperastine, griseofulvin, itraconazole, melatonin, propranolol·HCl, quinidine, sulfanilamide and tolbutamide, were classified as partially dispersed, which did not change when data from complementary techniques were ultimately considered, however, the PXRD data alone were incapable of distinguishing whether persistent amorphous phase was due to API, PEG4000, or both. Identifying this particular phase behavior is important to suggest whether or not a different ratio of API and polymer might result in a fully dispersed solid, which might be useful to a formulator attempting to use PEG4000 as a carrier. Finally, scattering from a very small fraction of amorphous content may be undetected in highly crystalline PXRD patterns. This could potentially lead to an improper inference for systems such as ibuprofen:PEG4000, where the PXRD data suggested complete recrystallization of the drug. Even if undetected persistent amorphous ibuprofen was present, the results suggest insignificant ability to disperse it in PEG4000.

A.3.3. Inferences from PDF-transformed PXRD data

Of the 15 API:PEG4000 combinations in this study, 5 mixtures, cimetidine, indomethacin, ketoconazole, nifedipine, and terfenadine, resulted in completely X-ray amorphous diffraction patterns, which, based on previous research involving analysis of
PDF analysis\textsuperscript{17} were the only data suited to interpretation in this manner. Despite X-ray amorphous PXRD patterns, PDF analysis was not done for ketoconazole:PEG4000, owing to the inability to prepare a fully amorphous sample of pure ketoconazole for theoretical PDF calculation. A summary of the PDF data for the remaining 4 solids is shown in Table A.3.
Table A.3. Summary of PDF analyses. R-values were minimized by artificially scaling API:polymer w/w concentrations used to generate theoretical PDF.

<table>
<thead>
<tr>
<th>Solid Mixtures</th>
<th>R-valuea</th>
<th>Theoretical Drug Concentration (%)</th>
<th>Theoretical PEG4000 Concentration (%)</th>
<th>Observed regions in $r$ where 0 was not contained in the CI</th>
<th>PDF inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cimetidine:PEG4000</td>
<td>0.259 (0.022)</td>
<td>54 (1)</td>
<td>46 (1)</td>
<td>Yes</td>
<td>Fully dispersed</td>
</tr>
<tr>
<td>Terfenadine:PEG 4000</td>
<td>0.198 (0.007)</td>
<td>76 (2)</td>
<td>24 (2)</td>
<td>Yes</td>
<td>Fully dispersed</td>
</tr>
<tr>
<td>Indomethacin:PEG 4000</td>
<td>0.184 (0.039)</td>
<td>64 (3)</td>
<td>36 (3)</td>
<td>Yes</td>
<td>Fully dispersed</td>
</tr>
<tr>
<td>Nifedipine:PEG 4000</td>
<td>0.126 (0.010)</td>
<td>77 (2)</td>
<td>23 (2)</td>
<td>Yes</td>
<td>Fully dispersed</td>
</tr>
</tbody>
</table>

aReported values represent the average and standard deviation of three replicate preparations
PDF interpretation for cimetidine:PEG4000 suggests complete dispersion at this composition, resulting from solidification of a completely miscible mixture of molten drug and polymer. In this case, the observed and calculated PDFs were not superimposable (Figure A.2a), reflected in $R=0.259$, which was only minimized when the theoretical PDF drug:polymer concentration weightings were artificially adjusted to 54% and 46% (w/w), respectively (Table A.3). Additionally, the difference plot ($\Delta G(r)$ between predicted and observed data) for cimetidine:PEG4000 contained regions in $r$ where the confidence intervals did not contain zero (Figure A.2b). Taken together, this suggests that the PDF-transformed data for solidified cimetidine:PEG4000 contained new interatomic distances not explained by a physical mixture of the two amorphous solids, supporting its complete dispersion in PEG4000. Similar interpretations of the PDF patterns for indomethacin:PEG4000, nifedipine:PEG4000, and terfenadine:PEG4000 solids are also shown in Table A.3.

Figure A.2a and A.2b PDF analysis of 3:1 w/w co-solidified cimetidine:PEG4000 sample (as labeled).
This particular characterization approach, while useful, is the most ambiguous when interpreted without complementary techniques. Its inclusion in a characterization routine can provide additional information for PXRD data, but should not be done in lieu of other complementary techniques. To reinforce this, consider that the PXRD data suggested that cimetidine, indomethacin, nifedipine, and terfenadine had all formed completely amorphous binary dispersions, which was subsequently confirmed by the PDF analyses. PDF interpretations suggested that each of these systems formed intimate drug/polymer interactions unique to a dispersion. This interpretation required comparison between an observed and calculated PDF generated from PXRD data from amorphous pure components. Since amorphous PEG4000 could not be prepared, PXRD data from liquid PEG400 were used to generate the calculated PDF. It is easy to imagine that differences between liquid PEG400 and solid amorphous PEG4000 at the scale of drug/polymer interactions may be substantial enough that the unique separation distances interpreted in the data were manifestations of the theoretical PDF and not indicative of the actual dispersion. Ultimately, classifications of dispersion behavior made using the other techniques are much more compelling, suggesting that PDF analysis is inappropriate for PEG-based dispersions.

A.3.4. Inferences from HSM data

The use of polarized light microscopy in dispersion characterization is widespread, where observed birefringence indicates the presence of crystallinity in a sample. This technique is analytically challenging when characterizing systems comprised of a rapidly recrystallizing carrier polymer, such as PEG4000, as differentiating between birefringence from the polymer and API is required.
All samples were observed initially at room temperature, immediately following preparation, and the photomicrographs for each API:PEG4000 mixture are shown in Figure A.3. Observed birefringence was interpreted as recrystallization of either drug or polymer during quenching. Samples were heated and cooled, as described above, while observed using both polarized and un-polarized light to differentiate between crystalline polymer and API.

**Figure A.3.** Initial room temperature polarized light microscopy images of co-solidified 3:1 w/w samples containing PEG4000 and (a) chlorpropamide (b) cimetidine (c) cloperastine·HCl (d) griseofulvin (e) ibuprofen (f) indomethacin (g) itraconazole (h) ketoconazole (i) melatonin (j) nifedipine (k) propranolol·HCl (l) quinidine (m) sulfanilamide (n) terfenadine (o) tolbutamide. Image (p) represents only PEG4000 at room temperature. All scale bars (in yellow) correspond to 50 µm.
Extensive birefringence was observed throughout the ibuprofen:PEG4000 samples, from small crystallites not easily distinguished as either drug or polymer (Figure A.3e). As the solid was heated, the polymer began melting at \(\sim 45^\circ C\), resulting in disappearance of some of the birefringent crystallites, while the remainder persisted as crystalline ibuprofen, which melted beginning at \(\sim 70^\circ C\). Interpretation of HSM data alone indicated no detectable amorphous ibuprofen, suggesting complete phase separation when quenched in the presence of PEG4000.

The photomicrographs for mixtures containing cimetidine, indomethacin, ketoconazole, nifedipine, and terfenadine (all of which resulted in completely X-ray amorphous diffraction patterns), showed that only cimetidine:PEG4000 resulted in a dispersion having no birefringence when observed under polarized light. Moreover, this image showed a single, homogeneous sample, resembling a solidified glass (Figure A.3b), having no observed grains that might indicate trace phase separation. When the cimetidine:PEG4000 was heated to \(T_{m,API} +10^\circ C\), minor recrystallization of trace cimetidine was observed at 80\(^\circ\)C (in 1/3 preparations), which then melted at 160\(^\circ\)C. In the other 2 cimetidine:PEG4000 solids, neither recrystallization nor melting of either polymer or drug was observed. Taken alone, HSM suggests that cimetidine and PEG4000 were fully dispersed, having solidified from a completely miscible molten mixture at this composition. Without the complementary techniques, however, the question could certainly be raised whether or not the photomicrographs were representative of the entire sample, as the experiments focused on a single area during heating, making it possible to have missed subtle phase behavior outside the field of view. Here, the HSM inference for cimetidine:PEG4000 miscibility is made more
compelling when complemented with the PXRD data, which involved complete sample interrogation with X-rays.

Room temperature photomicrographs for the mixtures containing indomethacin, ketoconazole, nifedipine, and terfenadine clearly showed small, birefringent grains, representing a small fraction of the total sample, some of which melted at ~60°C (identifying them as PEG4000). The other grains melted at the respective $T_{m,API}$, suggesting that a small amount of the drug had recrystallized during quenching of each sample. These HSM data suggested that these 4 drugs were only partially dispersed with PEG4000, but that extensive recrystallization did not occur during quenching. It is important to note that this inference was potentially complicated by observed recrystallization of API from molten PEG at ~80°C in each of these samples. Since recrystallization began immediately following melting of the PEG4000, distinguishing between persistent API grains and those formed during the experiment was difficult, and could potentially vary from one field of view to another. Nonetheless, at room temperature most of the API and polymer was initially amorphous, and the solid that recrystallized during quenching likely fell below the detection limit of PXRD. This reinforces that classifying solids of indomethacin, ketoconazole, nifedipine, and terfenadine with PEG4000 as fully dispersed, made using only PXRD data were incorrect.

A.3.5. Inferences from DSC data

Of the various techniques employed in the characterization of solid dispersions, DSC is among the most frequently used. Fully dispersed systems should, in theory, result in thermograms containing a single $T_g$ intermediate to the $T_g$ of either pure
component amorphous phase, predictably weighted according to relationships such as the Couchman-Karasz Equation$^{262}$ or the Gordon-Taylor Equation.$^{275}$ Partially dispersed mixtures characteristically show two distinct $T_g$ values, plasticized or anti-plasticized relative to their pure component values, and typically crystalline API formed during solidification. While classifications of dispersion behavior can be straightforward for some API:polymer combinations, PEG4000 proved to be much more complicated.

DSC data for API:PEG4000 mixtures are summarized in Table A.4.
Table A.4. Summary of DSC analyses.

<table>
<thead>
<tr>
<th>Solid Mixture</th>
<th>Pure component T$_g$ (°C)</th>
<th>Calculated T$_g$ (°C) from Eq 1</th>
<th>Observed T$_g$ (°C) (20°C/min)</th>
<th>Observed PEG4000 T$_m$ (°C) (20°C/min)</th>
<th>Observed API T$_m$ (°C) (20°C/min)</th>
<th>DSC Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorpropamide</td>
<td>15.39</td>
<td>-1.29</td>
<td>-4.97 (0.78)</td>
<td>Not observed</td>
<td>105.21 (3.21)</td>
<td>Fully dispersed</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>57.38</td>
<td>39.87</td>
<td>30.24 (2.54)</td>
<td>58.38; n=1</td>
<td>Not observed</td>
<td>Fully dispersed</td>
</tr>
<tr>
<td>Cloperastine</td>
<td>41.47</td>
<td>11.05</td>
<td>-4.11 (0.07); n=2</td>
<td>50.76 (4.95)</td>
<td>144.40 (1.15)</td>
<td>Partially dispersed</td>
</tr>
<tr>
<td>HCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>69.95 (1.11)</td>
<td></td>
</tr>
<tr>
<td>Griseofulvin</td>
<td>98.68</td>
<td>67.75</td>
<td>30.65 (0.18)</td>
<td>Not observed</td>
<td>144.12 (1.17)</td>
<td>Fully dispersed</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>-35.35</td>
<td>-39.36</td>
<td>Not observed</td>
<td>44.86 (2.36)</td>
<td>161.88 (0.47)</td>
<td></td>
</tr>
<tr>
<td>Indomethacin</td>
<td>54.14</td>
<td>34.66</td>
<td>1.00 (0.70)</td>
<td>Not observed</td>
<td>144.12 (1.17)</td>
<td>Fully dispersed</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>66.17</td>
<td>45.35</td>
<td>-22.09; n=1</td>
<td>54.90 (3.82)</td>
<td>161.88 (0.47)</td>
<td></td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>53.97</td>
<td>27.62</td>
<td>-52.28 (0.12); n=2 1.24; n=1</td>
<td>55.47 (1.03); n=2 149.18 (0.31)</td>
<td></td>
<td>Partially dispersed</td>
</tr>
<tr>
<td>Melatonin</td>
<td>20.12</td>
<td>7.2</td>
<td>1.35 (0.34)</td>
<td>52.76 (1.30)</td>
<td>105.63 (0.67)</td>
<td>Partially dispersed</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>53.35</td>
<td>30.83</td>
<td>-0.63 (5.96)</td>
<td>Not observed</td>
<td>151.06 (8.13)</td>
<td>Fully dispersed</td>
</tr>
<tr>
<td>Propranolol</td>
<td>43.45</td>
<td>24.15</td>
<td>11.79 (1.31)</td>
<td>Not observed</td>
<td>157.43 (0.52)</td>
<td>Fully dispersed</td>
</tr>
<tr>
<td>HCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>166.40 (1.79)</td>
<td></td>
</tr>
<tr>
<td>Quinidin</td>
<td>68.35</td>
<td>51.57</td>
<td>10.43 (8.75)</td>
<td>Not observed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfanilamide</td>
<td>N/A*</td>
<td>N/A*</td>
<td>-0.74 (0.9); n=2</td>
<td>56.00 (0.98)</td>
<td>153.97 (4.27)</td>
<td>Partially dispersed</td>
</tr>
<tr>
<td>Terfenadine</td>
<td>69.05</td>
<td>39.65</td>
<td>-30.79 (3.72); n=2 7.95 &amp; 61.32*** (n=1)</td>
<td>T$_{C,API}$ = 86.14 (1.35) n=2</td>
<td>140.15 (0.59)</td>
<td>Partially dispersed</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>13.16</td>
<td>-4.10</td>
<td>Not observed</td>
<td>42.21 (0.62)</td>
<td>113.91 (0.57)</td>
<td>Fully phase separated</td>
</tr>
</tbody>
</table>

*Reported values represent the average and standard deviation of three replicate preparations unless otherwise mentioned

*Sulfanilamide pure component T$_g$ unable to be determined by DSC; rapid recrystallization during in situ quench

$T_{API}$ preceded by PEG4000 recrystallization  $T_{API}$ preceded by API recrystallization

**API recrystallization over expected T$_m$ range. PEG melting confirmed in Hot Stage Microscopy

***Two T$_g$’s, one attributable to PEG4000 and the other to API
Classification of ibuprofen:PEG4000 was very straightforward using PXRD and HSM. This continued with DSC, although the data suggested potential eutectic behavior between this drug and polymer with complete recrystallization upon cooling the preparation (Figure A.4).

![DSC thermograms](image)

**Figure A.4.** Overlay of DSC thermograms. Pure ibuprofen is indicated in blue, co-solidified 3:1 w/w ibuprofen:PEG4000 is shown in black; pure PEG4000 is shown in red. No $T_g$ was observed; only melt endotherms consistent with $T_{m,p}$ and $T_{m,API}$.

This interpretation is supported by observations made by Law *et al.* No $T_g$ was observed in these thermograms, however, distinct melting endotherms, having onsets at 44.86°C and 69.95°C were observed, corresponding with slightly depressed values for the respective $T_m$ for PEG4000 and ibuprofen. Neither $T_m$ were preceded by recrystallization, suggesting that both drug and polymer fully crystallized during quenching. Taken alone, the DSC data suggest that ibuprofen and PEG4000 completely phase separated during solidification. A similar inference could be made for
tolbutamide:PEG4000. No $T_g$ was observed for these solids, suggesting no amorphous content (Table A.4). Melting of the PEG4000 was clearly observed for tolbutamide:PEG4000, which was not preceded by recrystallization, suggesting that the polymer had recrystallized during solidification. Following $T_{m,p}$ however, the baseline in these thermograms did not re-establish, prior to the signal that was clearly interpreted as melting of the tolbutamide. In this case, the signals suggested that the API crystallized during solidification, and then dissolved/recrystallized in the molten PEG as the temperature was increased. The $T_g$ region of the DSC thermogram for cimetidine:PEG4000 is shown in Figure A.5, relative to pure component thermograms obtained, respectively from melt/quenched samples of cimetidine and PEG4000.

![Figure A.5](image)

**Figure A.5.** Overlay of $T_g$ regions in DSC thermograms from top to bottom: Pure cimetidine (in blue), co-solidified 3:1 w/w cimetidine:PEG4000 (in black) and pure PEG4000 (in red). A single $T_g$ was measured for the mixture, which fell intermediate to $T_{g,p}$ and $T_{g,API}$. 
A single $T_g$ was observed at 30.24°C for cimetidine:PEG4000, which was comparable with the theoretical $T_g$ of the mixture (39.87°C) predicted by Equation A.1 (Table A.4). In 1/3 replicates for cimetidine:PEG4000, a small endotherm at 58.38°C was observed, corresponding to $T_{m,p}$. None of the 3 cimetidine:PEG4000 replicates showed evidence of API recrystallization/melting during the temperature ramp. These data suggest that 1/3 cimetidine:PEG4000 solid mixtures had slight recrystallization of the polymer, although all three allowed persistence of amorphous cimetidine. Independent of the other techniques, the DSC data strongly suggest that a fully dispersed binary amorphous solid can form by solidification of a fully miscible 3:1 mixture of cimetidine and PEG4000. These data potentially illustrate a limitation to characterizing dispersions using only DSC. Given the limited sample size used in these experiments, sub-sampling from the co-solidified mixtures was unavoidable. It could be argued that DSC might miss recrystallized drug or polymer, if the “chips” taken from the overall glass were selected from a persistent homogeneous region, rather than a phase separated region. This reinforces the necessity of complementary techniques having greater (if not complete) sample interrogation for confirmation that sub-sampling doesn’t bias the inferences made from DSC alone.

Table A.4 also illustrates the complexity of the DSC data used to characterize solid mixtures with PEG4000, the behavior of which clustered into four generalized groups. In DSC patterns for itraconazole:PEG4000, a distinct $T_g$ was not observed, however, the predicted $T_g$ value was expected commensurate with PEG recrystallization and melting. As samples were heated, distinct crystallization/melting of the PEG4000, followed by distinct crystallization/melting of the itraconazole was clearly observed. This
suggested that solidification resulted in at least some dispersed amorphous itraconazole and polymer. In particular, the enthalpies of recrystallization/melting for the drug were comparable, suggesting that the drug was predominantly amorphous following quenching. Considered in isolation, the DSC data for itraconazole:PEG4000 appear to be consistent with a fully dispersed drug in polymer, however, ambiguity remains as to whether the entire API content was amorphous.

In the second grouping of thermal data, cloperastine:PEG4000, ketoconazole:PEG4000, melatonin:PEG4000, and sulfanilamide:PEG4000, were observed to have a single \( T_g \) indicating persistent amorphous content of the quenched solids. Clear endotherms unambiguously attributable to PEG4000 melting were also observed for each of these solids, none of which were preceded by clear recrystallization, suggesting that the polymer recrystallized during solidification. Following \( T_{m,p} \), recrystallization/melting of the API occurred for each solid, likely because the molten PEG allowed for increased mobility of the API molecules, enabling their crystallization during the temperature ramp. The presence of crystalline API following solidification was more difficult to interpret from the DSC data alone. Ketoconazole:PEG4000 thermograms had only a single \( T_g \) observed per solid, however, these occurred at inconsistent values from sample to sample (Table A.4). Ultimately, they were all skewed towards the \( T_{g,p} \), suggesting that ketoconazole was partially dispersed in PEG4000, resulting in anti-plasticization of \( T_{g,p} \). Additionally, in the melatonin:PEG4000 mixtures, the recrystallization exotherm for the API integrated to a much lower enthalpy relative to the respective melt endotherm, potentially suggesting some crystallization of API during
co-solidification. Taken together, these APIs were classified as partially dispersed in PEG4000.

In the third grouping, solid mixtures that included chlorpropamide, indomethacin, nifedipine, propranolol·HCl, griseofulvin, and quinidine, were observed to have a single $T_g$. As the temperature was increased for these solids, neither crystallization nor melting of the PEG4000 was observed, suggesting that the polymer remained amorphous during solidification, and was inhibited from recrystallization by the very high drug concentration present. Each thermogram also had a distinct recrystallization exotherm prior to the respective melting endotherms for the API. This suggested that these drugs solidified as amorphous solids and only recrystallized as a consequence of heating in the DSC experiments. It should be noted that the API recrystallization observed for chlorpropamide:PEG4000 overlapped with the region where $T_{m,p}$ would normally be observed. Since the DSC signal reports net heat flow, it is entirely possible that melting of polymer that recrystallized during solidification was obscured by the recrystallization of the chlorpropamide. Using only the DSC data, all of the API in this grouping were, therefore, classified as fully dispersed in PEG4000, having no clear evidence of recrystallization during quenching. In the case of chlorpropamide, the ambiguity associated with the overlap exothermic/endothermic signals could easily result in a misclassification of drug:polymer dispersion behavior, highlighting a limitation of DSC interpretation in systems such as these.

Finally, using only the DSC data, terfenedine:PEG4000 was classified as only partially dispersed. These thermograms showed two $T_g$ events at 7.95°C and 61.32°C in 1/3 of the solids, and a single $T_g$ (average value of -30.79°C) in the other two replicate
solids (Figure A.6). Although PEG4000 melting was not observed in these samples, the recrystallization of the API overlapped with the temperature range where PEG melting was expected, potentially obscuring that signal.

![Figure A.6](image)

**Figure A.6.** Overlay of $T_g$ regions in DSC thermograms from top to bottom: Pure terfenadine (in blue), co-solidified 3:1 w/w terfenadine:PEG4000 (in black) and pure PEG4000 (in red). Panel a) a single $T_g$ was measured for the mixture (-28.16°C in this replicate; mean -30.79°C; n=2), which fell intermediate to $T_{g,p}$ and $T_{g,API}$. Panel b) two $T_g$ values were measured (7.96°C and 61.32°C) indicating plasticization and antiplasticization, respectively for $T_{g,API}$ and $T_{g,p}$.

**A.3.6. Combined inferences from physicochemical characterization suite**

A summary of the classifications of dispersion behavior based on each technique in isolation is found in Table A.5, and compared with classifications made by collectively considering all of the characterization data. Of the 15 API:PEG4000 combinations, only 6 resulted in samples whose dispersion behavior was consistently categorized using each of the four methods. That said, owing to limitations described for each individual technique, inferences in isolation of the other techniques were not made with the same confidence relative to those made using all of the pooled data.
Table A.5. Summary of inferences from individual techniques compared with inferences made by collective consideration of all techniques (Combined Inference).

<table>
<thead>
<tr>
<th>Solid Mixture</th>
<th>PXRD</th>
<th>PDF</th>
<th>HSM</th>
<th>DSC</th>
<th>Combined Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorpropamide</td>
<td>Partially dispersed</td>
<td>N/A</td>
<td>Partially dispersed</td>
<td>Fully dispersed</td>
<td>Partially dispersed</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>Fully dispersed</td>
<td>Fully dispersed</td>
<td>Fully dispersed</td>
<td>Fully dispersed</td>
<td>Fully dispersed</td>
</tr>
<tr>
<td>Cloperastine·HCl</td>
<td>Partially dispersed</td>
<td>N/A</td>
<td>Partially dispersed</td>
<td>Partially dispersed</td>
<td>Partially dispersed</td>
</tr>
<tr>
<td>Griseofulvin</td>
<td>Partially dispersed</td>
<td>N/A</td>
<td>Partially dispersed</td>
<td>Fully dispersed</td>
<td>Partially dispersed</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>Fully phase separated</td>
<td>N/A</td>
<td>Fully phase separated</td>
<td>Fully phase separated</td>
<td>Fully phase separated</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>Fully dispersed</td>
<td>Fully dispersed</td>
<td>Partially dispersed</td>
<td>Fully dispersed</td>
<td>Partially dispersed</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>Partially dispersed</td>
<td>N/A</td>
<td>Partially dispersed</td>
<td>Fully dispersed</td>
<td>Partially dispersed</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>Fully dispersed</td>
<td>N/A</td>
<td>Partially dispersed</td>
<td>Partially dispersed</td>
<td>Partially dispersed</td>
</tr>
<tr>
<td>Melatonin</td>
<td>Partially dispersed</td>
<td>N/A</td>
<td>Partially dispersed</td>
<td>Partially dispersed</td>
<td>Partially dispersed</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>Fully dispersed</td>
<td>Fully dispersed</td>
<td>Partially dispersed</td>
<td>Fully dispersed</td>
<td>Partially dispersed</td>
</tr>
<tr>
<td>Propranolol·HCl</td>
<td>Partially dispersed</td>
<td>N/A</td>
<td>Partially dispersed</td>
<td>Fully dispersed</td>
<td>Partially dispersed</td>
</tr>
<tr>
<td>Quinidine</td>
<td>Partially dispersed</td>
<td>N/A</td>
<td>Partially dispersed</td>
<td>Fully dispersed</td>
<td>Partially dispersed</td>
</tr>
<tr>
<td>Sulfanilamide</td>
<td>Partially dispersed</td>
<td>N/A</td>
<td>Partially dispersed</td>
<td>Partially dispersed</td>
<td>Partially dispersed</td>
</tr>
<tr>
<td>Terfenadine</td>
<td>Fully dispersed</td>
<td>Fully dispersed</td>
<td>Partially dispersed</td>
<td>Partially dispersed</td>
<td>Partially dispersed</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>Partially dispersed</td>
<td>N/A</td>
<td>Partially dispersed</td>
<td>Fully phase separated</td>
<td>Partially dispersed</td>
</tr>
</tbody>
</table>
Consider the case of cimetidine, which was classified as fully dispersed in PEG4000 at this composition. The PXRD data suggested a completely amorphous solid (Table A.2), while the PDF analysis suggested that the amorphous phases were intimately dispersed, allowing for interatomic distances unique to a dispersion (Figure A.2). These data were made considerably more compelling, however, by the absence of detectable crystallinity by polarized light microscopy (Figure A.3b), and the presence of a single $T_g$ value comparable to the theoretical $T_g$ and the absence of crystallinity having resulted from solidification cimetidine and PEG4000 (Table A.4, Figure A.5). Similarly, ibuprofen:PEG4000 was unambiguously classified fully phase separated based on complete recrystallization prior to analysis. The PXRD data confirmed that the ibuprofen was entirely crystalline (Figure A.1). The absence of a $T_g$ in the DSC data (Figure A.5), and no observation of any recrystallization in both DSC and HSM further reinforced that no amorphous material was present in any of these samples.

The necessity of using complementary characterization techniques on quenched API:PEG400 mixtures was best illustrated with samples for which inferences based on a single technique were inconsistent (Table A.5). For example, the PXRD data for nifedipine:PEG4000 suggested a completely amorphous solid (Table A.2), supported by PDF analysis, which suggested intimate dispersion of amorphous drug and polymer to form a single phase (Table A.3). The DSC data appeared to also support formation of a fully dispersed solid, evidenced by a single $T_g$, and no detectable crystallinity from either component, formed during co-solidification (Table A.4). Had the microscopy not been performed, these three analyses would have suggested that nifedipine was fully dispersed in PEG4000 at this composition. As shown in Figure A.3j, however, tiny, birefringent
crystalline grains were visible in the polarized light photomicrograph, which comprised a small fraction of the overall sample. This indicated some recrystallization during solidification. Furthermore, the HSM routine showed that some of these crystallites did not melt until $T_{m,API}$, clearly indicating that the nifedipine had recrystallized to some extent. Although this classified the 3:1 solid as partially dispersed, the presence of substantial, persistent amorphous phase suggests that at a lower drug loading a fully dispersed solid may be possible.

Also of note in Table A.5 is the fact that all of the classifications made exclusively using HSM are identical to the final inferences from collective consideration of the data. This could appear to suggest that HSM was superior to the other methods, and potentially the only technique needed to characterize API:PEG4000 solid mixtures. Indeed, polarized light microscopy appears to be able to visualize sample crystallinity too small to be observed in either the PXRD or DSC signals. Like DSC, however, samples subjected to microscopy were limited by the necessity of sub-sampling. Not only are “chips” taken from larger, co-solidified samples for visualization, the field of view at any given time is only a fraction of the chip. While the presence of crystallinity is unambiguous, and the identity of the crystals (API or PEG) can be determined from the observations through the heating cycle, the absence of crystallinity in a microscopy experiment likely needs to be confirmed in many samples to confidently assume that the entire sample contains no crystals.
A.4. Conclusion

A library of 15 API was co-solidified with PEG4000. Characterization of the resulting solids was done using PXRD, PDF analysis, DSC and HSM, and API were classified as either fully dispersed, partially dispersed or fully phase separated in the polymer at a 3:1 weight ratio. The dispersion behavior of only 6/15 of the mixtures was consistently classified using data from each of the techniques independent of the others, reflecting the limits of each method in individually characterizing potential dispersions involving PEG as a carrier. PXRD was limited by the ability to detect diffraction from very small crystallites in an almost entirely amorphous sample. HSM was able to confirm the presence of crystalline API in 4/5 X-ray amorphous patterns, more correctly identifying those API as partially dispersed in PEG4000. The significant amorphous content interpreted from PXRD data complemented by the limited crystallinity observed using HSM suggested that indomethacin, ketoconazole, nifedipine, and terfenedine may be fully dispersable in PEG4000 at a lower concentration. DSC in isolation was limited by potentially overlapping signals from the API and PEG, which made it difficult to distinguish crystallinity formed during solidification vs. recrystallization that occurs exclusively because of the temperature program imposed on the samples by the experiment. Again, HSM was able to confirm the presence of initial drug and polymer crystallites that formed during solidification of the molten mixtures, as well as distinguish events that occurred at experimental temperatures. Overall, the complexity of drug/polymer dispersion behavior, especially in mixtures involving PEG4000, combined with data subtleties that likely need to be interpreted to distinguish between partially and
fully dispersed mixtures, reinforces the need to use complementary techniques in order to ensure thorough characterization.

**A.5. Acknowledgements**

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Bibliography


