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Intestinal mucus is capable of stabilizing supersaturation of poorly water-soluble drugs

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Abstract

The utilization of polymers to stabilize drug supersaturation and enhance oral drug absorption has recently garnered considerable interest. The potential role of intestinal mucus in stabilizing drug supersaturation, however, has not been previously explored. The ability for intestinal mucus to stabilize drug supersaturation and delay drug precipitation is potentially useful in enhancing the absorption of orally dosed compounds from drug delivery systems that generate supersaturation within the gastrointestinal tract (e.g., solid dispersions, lipid-based drug delivery systems). This work aims to evaluate the precipitation-delaying abilities of intestinal mucus using carvedilol (CVDL) and piroxicam (PXM) as model drugs. In supersaturation-precipitation (S-P) experiments, CVDL and PXM supersaturation were induced in test media (0, 0.1, 0.2, 0.4 % w/v mucin and 8 % w/v native pig intestinal mucus (PIM)) via the solvent-shift method at supersaturation ratios (SSR) of 5 and 6, respectively. Time to drug precipitation was assessed using ion-selective electrodes and HPLC. The S-P experiments showed that increasing mucin concentration led to increasingly delayed CVDL precipitation, while PXM precipitation was prevented at all mucin concentrations studied. The ability of mucus-stabilized CVDL supersaturation to translate into enhanced CVDL absorption was evaluated in transport experiments using mucus-producing (90% Caco-2:10% HT29-MTX-E12 co-cultures) vs. non-mucus-producing intestinal monolayers (100% Caco-2 cultures). The absorption enhancement of CVDL (SSR = 5 relative to SSR = 1) was higher across mucus-producing than non-mucus-producing intestinal monolayers. This work demonstrates for the first time the potential for intestinal mucus to delay the precipitation and enhance the absorption of poorly water-soluble compounds, suggesting that drug supersaturation can be stabilized in close proximity to the absorptive site, thereby presenting a possible novel approach for targeted supersaturating drug delivery systems.

Graphical abstract

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Keywords

intestinal mucus; mucin; supersaturation; polymeric precipitation inhibitors; poorly water-soluble drugs; ion-selective electrodes

1. Introduction

The potential for drug supersaturation in the gastrointestinal (GI) tract to enhance drug absorption has recently garnered considerable interest^{1,2}. In the context of oral drug delivery, supersaturation refers to the transient generation and maintenance of drug concentrations above the equilibrium drug solubility in GI fluids. Supersaturation increases the thermodynamic activity of solubilized drugs, and can significantly increase oral drug absorption especially in the case of poorly water-soluble drugs (PWS), where free aqueous concentration is often low and limiting to absorption. Oral drug delivery systems that generate drug supersaturation in the GI tract include solid dispersions and lipid-based drug delivery systems; where the former typically aims to generate drug supersaturation upon dissolution from dosage forms, and the latter generate drug supersaturation during endogenous processing of formulation lipids such as dosage form dispersion³, digestion^{4–6} and absorption⁷. Since supersaturated systems are inherently at risk of precipitation which compromises absorption, precipitation inhibitors (usually polymers) such as hydroxypropyl methylcellulose (HPMC) and polyvinylpyrrolidone (PVP) have been employed to stabilize drug supersaturation in the GI tract and maximize the duration over which drug is present at high concentrations⁸. The mechanisms of action of such polymers are not fully understood, but proposed to include: altered surface tension and increased solution viscosity to in turn decrease the rate of nucleation, prevention of nucleation through interactions with the drug, prevention of crystal growth following direct adsorption onto developing nuclei, and reduction of supersaturation by increasing drug solubility⁸. Considering the proposed mechanisms of actions of these polymers, it is possible that intestinal mucus, which consists of ~ 95% water, 0.5–5% glycoproteins and lipids, 0.5–1% mineral salts and 1% free proteins⁹, and is immediately adjacent to the absorptive epithelium, may also stabilize drug supersaturation leading to enhanced drug absorption (for example, through reducing the rate of nucleation and/or crystal growth due to mucus' higher viscosity, and/or ability to directly interact with drug molecules)^{10,11}.

This potential role of intestinal mucus in stabilizing drug supersaturation has not been previously explored. Indeed, mucus is often considered as a potential barrier to drug delivery^{12,13}. Mucus is synthesized by underlying cells and ultimately sloughs into the intestinal lumen with a reported turnover rate of 4–6 h^{14,15} such that there is an effective velocity of the mucus layer away from the underlying epithelium¹², potentially hindering drug absorption. Further, drugs and drug carriers can undergo interactions with mucins, including hydrophobic and electrostatic interactions, that can slow diffusive transport

through the mucus layer^{13,16}. The mesh-like network formed by interacting mucins has a nominal pore size of approximately 100 nm, allowing the mucus gel to also act as a physical barrier to microcarrier systems. However, drugs and drug carriers are still able to penetrate the mucus layer, and indeed all orally delivered drugs must penetrate mucus in order to be absorbed. Thus, mechanisms by which mucus may impact overall drug absorption are of interest.

The work described in this manuscript aims to evaluate the supersaturation-stabilizing abilities of intestinal mucus, by monitoring changes in free drug concentration upon induction of supersaturation, in the absence and presence of mucin (the major constituent of mucus) and native pig intestinal mucus (PIM), using carvedilol (CVDL) and piroxicam (PXM) as model drugs. The ability of mucus-stabilized drug supersaturation to translate into enhanced drug absorption was evaluated in CVDL transport experiments using mucus-producing vs. non-mucus-producing intestinal monolayers.

2. Materials and methods

2.1 Materials

Carvedilol (CVDL), piroxicam (PXM), potassium phosphate monobasic (KH_2PO_4), potassium phosphate dibasic (K_2HPO_4), sodium azide, mucin from porcine stomach (Type III), albumin from porcine serum, ammonium dihydrogenphosphate ($\text{NH}_4\text{H}_2\text{PO}_4$), 4-morpholineethanesulfonic acid (MES) hydrate, bovine serum albumin (BSA), linoleic acid (LNA), L- α -phosphatidylcholine (PC, from dried egg yolk), cholesterol, TWEEN[®] 80 and antibiotic antimycotic solution 100x were obtained from Sigma-Aldrich, MO. Eagle's minimum essential medium (EMEM), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and dimethylsulfoxide (DMSO) were from ATCC, VA. Hank's Balanced Salt Solution (HBSS) and HEPES, L-glutamine and MEM non-essential amino acids 100x were from Gibco[®], CA. Acetonitrile (HPLC grade), methanol (HPLC grade), ethanol, glacial acetic acid, and sodium hydroxide pellets (NaOH) were from Fisher Scientific, MA. Ultrapure water (18 M Ω) was obtained from an in-house Picopure[®] 2 system (Hydro[®] Service and Supplies, NJ).

2.2 Calibration of ion-selective electrodes (ISE)

Ion-selective electrodes (ISE) (Octens, Belgium) were employed to monitor and quantify the real-time changes in CVDL concentration in test media. ISE have previously been successfully utilized as *in situ* probes to accurately determine real-time drug concentrations in numerous varied media¹⁷, including complex media such as milk¹⁸ and simulated gastric fluids¹⁸. Prior to experiments, the electrodes were conditioned in base buffer (10 mM phosphate buffer, to pH 6.50) containing 50 $\mu\text{g/mL}$ drug at 37 °C for 7 days, conditions previously determined to sufficiently condition the ISE to CVDL (data not shown). Calibration curves were generated at the beginning of each experiment, and were constructed by stepwise addition ($5 \times 20 \mu\text{L}$ additions) of a 10 mg/mL CVDL in methanol standard solution into a glass vial containing 20 mL test medium, achieving an end concentration of 50 $\mu\text{g/mL}$.

2.3 Native mucus collection and preparation

Porcine intestines (Research 87, MA) were obtained from a local abattoir within 2 h of slaughter. Native mucus was scraped with a spatula from pig jejunum and stored at -80°C until use, as previously described¹⁹. Mucus collected from porcine intestine was used as is (i.e. not purified or processed) and represents a complex mixture of proteins, glycoproteins, lipids, electrolytes, cellular debris, and water^{20,21}. Porcine mucin structure, molecular weight, and composition are similar to those of human mucin^{22,23}.

2.4 Supersaturation-precipitation (S-P) experiments

In S-P experiments, drug supersaturation was induced in test media via the solvent shift method, and time to drug precipitation was assessed using ISE (CVDL) and HPLC (CVDL and PXM). HPLC analysis was conducted to confirm ISE's ability to measure free CVDL concentration in the test media. For CVDL and PXM studies, test media included 0, 0.1, 0.2, 0.4 %w/v mucin and 8 %w/v native pig intestinal mucus (PIM) in 10 mM phosphate buffer (pH 6.50). The concentration of mucin investigated was based on a previously reported artificial mucus model, where a solution of 0.4 %w/v mucin was determined to correspond to 8 %w/v native mucus²⁴. Prior to use, all test media other than 8% w/v native PIM were filtered (5.0 μm nylon filter, Fisher Scientific, MA) to remove particulates. 8% w/v native PIM could not be filtered due to rapid clogging of the filters. 20 mL test media were added to glass vials containing stirrers (0.079 in. by 0.25 in.), stirring at 500 rpm, 37°C . Prior to S-P experiments analyzed via ISE, preconditioned ISE and reference electrodes were immersed into test media and equilibrated for ~ 30 min, after which calibration curves were generated as described in Section 2.2. To commence S-P experiments, 400 μL of an appropriate drug stock solution (CVDL in methanol, PXM in DMSO) was spiked into test media such that a supersaturation ratio (SSR, calculated according to Equation 1) of 5 and 6 were achieved for CVDL and PXM, respectively. Real-time changes in CVDL concentration in test media were measured using ISE up to 24 h post-spiking (voltage data was collected every 0.034 min). Samples were taken at different time points ($t=0.5, 5, 10, 20, 40, 60, 120, 180, 240, 300, 360, 1260, 1320, 1440$ min) for analysis of drug concentration by HPLC. The SSRs were selected based on desirable supersaturation-precipitation profiles achieved in base buffer during preliminary experiments, where intended degree of drug supersaturation was maintained for at least 5 min, and drug precipitation occurred relatively quickly (within 1 h for CVDL and 5 h for PXM), allowing potential precipitation-delaying effects of additives to be sufficiently captured within the experimental timeframe. Final solvent concentration in test media was 2.5 %v/v.

$$\text{Supersaturation ratio (SSR)} = \frac{\text{Drug concentration in medium}}{\text{Equilibrium solubility of drug in medium}} \quad \text{Equation 1}$$

2.5 Equilibrium solubility determination

The equilibrium solubility (C_s) of CVDL and PXM in test media was determined via HPLC. To prepare mucin/native PIM-containing media, the appropriate mass of the ingredients were weighed in a glass beaker, made up to volume with base buffer, and stirred overnight at

37 °C. The final pH of all test media was adjusted to 6.50 ± 0.02 with NaOH. To determine C_s of the drugs in buffer containing 0, 0.1, 0.2, 0.4 %w/v mucin, excess solid drug was added to 1 mL formulation in glass vials. Vials were briefly vortexed, incubated at 37 °C, and samples taken every 24 h over a period of 72 h. During sampling, vials were centrifuged (4,000 xg, 5 min, 37 °C), 100 μ L of supernatant sampled, and vials re-vortexed. To determine C_s of the drugs in PIM suspension, excess solid drug was added to 3 mL formulation in glass vials and stirred at 500 rpm, 37 °C. 100 μ L samples were taken every 12 h over a period of 48 h. The samples were immediately centrifuged (4,000 xg, 2 min, 37 °C), and 50 μ L of the supernatant sampled. C_s was defined when drug concentrations in consecutive samples varied by $\leq 5\%$, and was determined on three separate occasions.

2.6 Caco-2 & HT29-MTX-E12 cell culture

Caco-2 and HT29-MTX-E12 cells were purchased from ATCC (VA) and Public Health England (UK), respectively. The cells were maintained in complete medium (Caco-2 medium: EMEM with 20% FBS and 1% antibiotic antimycotic solution (100x); HT29-MTX-E12 medium: DMEM with 10% FBS, 1% antibiotic antimycotic solution (100x), 1% non-essential amino acids, and 2 mM glutamine), in Corning™ 25 cm² polystyrene culture flasks in an incubator at 37 °C with 5% CO₂ (Forma Series II, Thermo Scientific, MA). Cells (either 100% Caco-2 or 90% Caco-2:10% HT29-MTX-E12 co-cultures) were seeded at a density of 50,000 cells/cm² (seeding volume 1.5 mL) on 23.1 mm polyethylene terephthalate (PET) culture inserts (0.4 μ m pore size; Falcon™, MA). Following seeding, apical and basolateral media (1.5 mL and 2.5 mL, respectively; Caco-2 medium for 100% Caco-2; HT29-MTX-E12 medium for co-cultures) were replaced 3 times a week. Passage numbers 50–60 and 60–70 for Caco-2 and HT29-MTX-E12, respectively, were used for the experiments described in this article.

2.7 CVDL transport experiments

CVDL was dosed to mature (21 days) Caco-2 and co-culture monolayers at a concentration corresponding to SSR = 1 and SSR = 5. The degree of drug flux enhancement in both monolayer types (SSR = 5 relative to SSR = 1) was compared to evaluate the impact of a mucus layer (present on the surface of co-culture monolayers, absent on the surface of Caco-2 monolayers) on supersaturation stabilization and subsequent flux enhancement. Monolayers were rinsed thrice with apical and basolateral buffer, consisting of HBSS (to pH 6.50 ± 0.02 with 20 mM MES) and 1% BSA in HBSS (to pH 7.40 ± 0.02 with 20 mM HEPES), respectively. Monolayers were then equilibrated with apical buffer at 37 °C for 30 min. After the equilibration period, reference trans-epithelial electrical resistance (TEER) was measured using an EVOM meter with STX2 electrode (World Precision Instruments, FL), and transport experiments initiated by the replacement of apical buffer with CVDL-containing buffer in the apical compartment. To prepare CVDL-containing buffer (SSR of 1 or 5), 100 μ L of an appropriate CVDL in ethanol standard solution was spiked into 10 mL buffer, stirred for 1 min (500 rpm, 37 °C) and immediately dosed to monolayers. Cells were placed on an orbital shaker (55 rpm; Orbi-Shaker™ CO₂, Benchmark Scientific, NJ) at 37 °C and 5% CO₂ throughout the experiment. The apical and basolateral volumes used for the experiments were 1 mL and 2 mL, respectively. 0.2 mL samples were taken from the basolateral compartment at 15, 30, 45, 60, 75, 90 min, and replenished with equal volume of

fresh basolateral buffer. Initial and final samples (100 μ L) were taken from the apical compartment, centrifuged (4,000 $\times g$, 2 min, 37 $^{\circ}$ C) immediately, and the supernatants assayed for CVDL content via HPLC. At the end of the experiment, a final TEER value was measured. TEER values were expressed as % relative to reference.

2.8 Sample preparation and HPLC assay conditions for CVDL and PXM

Samples from test media were first diluted 1:4 v/v with acetonitrile, centrifuged (4,000 $\times g$, 2 min, 37 $^{\circ}$ C), and the supernatant further diluted by a minimum of 4-fold with respective mobile phases prior to injection into the HPLC. Samples from basolateral compartment in transport experiments were diluted 1:4 v/v with acetonitrile, followed by centrifugation (4,000 $\times g$, 2 min, 37 $^{\circ}$ C), and the supernatant injected directly into the HPLC. The HPLC-UV-system (Agilent, CA) used to assay CVDL and PXM content consisted of an Agilent 1260 Infinity Quaternary Pump, a 1260 Infinity Multiple Wavelength Detector VL, a 1260 Infinity High Performance Autosampler and an Agilent OpenLAB chromatography data system (version A.02.01). The injection volume for all samples was 50 μ L. CVDL was eluted by a ZORBAX ODS 5 μ m, 4.6 $\times$ 150 mm column using 80% v/v Acetonitrile:20% v/v 10 mM $\text{NH}_4\text{H}_2\text{PO}_4$ as mobile phase (flow rate 1 mL/min). PXM was eluted by a ProntoSIL Phenyl column using 50% v/v Acetonitrile:50% v/v 0.85% glacial acetic acid as mobile phase (flow rate 1 mL/min). UV detection was set at 242 nm for CVDL and 360 nm for PXM. The retention times for CVDL and PXM were 4.0 min and 6.0 min, respectively. The standard curve range for both CVDL and PXM was 0.1–100 μ g/mL.

2.9 Quantitative analysis

For ISE analysis, the conversion of the measured potential to drug concentration was carried out using an in-house Potential-to-Concentration software (LabVIEW, Version 6.1, National Instruments). The ISE system measured the electrochemical potential of the solution every 0.034 min. C_{max} was defined as the maximum concentration measured throughout the monitoring period. Time to precipitation (T_{ss}) was defined as the duration post-spiking over which drug concentration was maintained at 95% C_{max} . Absorptive flux enhancement in CVDL transport experiments was calculated according to Equation 2.

$$\text{Absorptive flux enhancement} = \frac{\text{Average flux in SSR} = 5 \text{ experiment}}{\text{Average flux in SSR} = 1 \text{ experiment}} \quad \text{Equation 2}$$

3. Results and Discussion

3.1 Mucin and native PIM delayed/prevented drug precipitation in S-P experiments. Increasing mucin concentration led to increasingly delayed CVDL precipitation from supersaturated solutions, while PXM precipitation was prevented at all mucin concentrations studied.

The equilibrium solubilities of CVDL and PXM in test media are shown in Figure 1. CVDL solubility in buffer did not change with the addition of mucin, while the addition of mucin appeared to increase PXM solubility to an equal extent regardless of mucin concentration.

The addition of 8 %w/v PIM significantly increased the solubilities of both CVDL and PXM, potentially due to the presence of lipids and proteins in native mucus that increase the solubilization capacity for the drugs.

CVDL S-P profiles were analyzed by ISE and HPLC (Figure 2A and Figure 2B, respectively). Since ISE measurements were collected ~ every 2 sec, T_{ss} (defined in Section 2.9) for each test medium was calculated from data in Figure 2A, and presented in Figure 3. Following induction of CVDL supersaturation ($SSR = 5$) in test media, T_{ss} significantly increased with increasing mucin concentration, up to 0.2 %w/v mucin (Figure 3). 0.4 %w/v mucin and 8 %w/v PIM also significantly delayed CVDL precipitation (CVDL concentration remained above a SSR of 3 throughout the monitoring period - (Figure 2A)), however, since CVDL precipitation was not completely prevented and CVDL concentration progressively declined from the start of the experiment, their T_{ss} values (defined as the duration over which drug concentration was maintained at 95% C_{max}) were much lower than that for 0.2 %w/v mucin. The data suggest that 0.2 %w/v mucin was sufficient to prevent CVDL precipitation, and that mucin may be the main component responsible for the supersaturation-stabilizing abilities of native mucus. The concentration of mucin in 0.4 %w/v mucin and 8 %w/v PIM were comparable (assuming that native mucus contains 5% glycoproteins) as suggested by a previously reported artificial mucus model²⁴. It is noted that 8 %w/v PIM was used unfiltered, such that particulates that were present may have provided sites for CVDL nucleation, thereby reducing its efficacy in stabilizing CVDL precipitation. A slower onset and rate of CVDL precipitation (reflected by slopes that were less steep during the precipitation phase) were also observed in 0.1–0.4 %w/v mucin and PIM-containing buffer relative to those in buffer alone (Figure 2A). HPLC analysis indicated that CVDL at $SSR = 5$ rapidly precipitated from buffer solution after 10 min, and the addition of mucin and PIM slowed the onset and rate of CVDL precipitation (Figure 2B) in a comparable manner to Figure 2A, thus confirming the validity of ISE as a tool to measure free drug concentration in the test media.

PXM S-P profiles were analyzed by HPLC only, and are shown in Figure 4. Following induction of PXM supersaturation ($SSR = 6$) in buffer alone, PXM started to precipitate after 5 h and reached a SSR of ~4 at 24 h. In the presence of mucin (regardless of concentration) and PIM, PXM concentration was maintained at SSR of 6 throughout the monitoring period.

Taken together, the data suggest that, in the concentration range tested, native PIM, in particular its major component mucin, was able to stabilize drug supersaturation in a concentration-dependent manner for CVDL and concentration-independent manner for PXM. The mechanism(s) by which mucin stabilizes drug supersaturation could not be definitively determined in these studies, however properties of intestinal mucus such as high viscosity^{25,26} and ability to directly interact with charged compounds^{10,27} (CVDL and PXM are expected to be ionized at pH 6.50) were potential contributors to the observed precipitation-delaying/obliterating effects. Polymers that increase the viscosity of bulk solutions, including HPMC and other non-ionic cellulose derivatives, have widely been shown to delay the precipitation of basic/acidic drugs⁸. Viscosity-mediated delays in precipitation are believed to be due to the slower rate of drug diffusion, which in turn decreases intermolecular collision frequency (required for nucleation) and the rate of drug

transfer from bulk solution to the nuclei or growing crystal surface⁸. Mucus contains high molecular weight glycoproteins (up to 20 MDa), and increases bulk solution viscosity by forming interconnected networks of polymer chains^{28,29}. Anionic polymers such as poly(styrene sulfonic acid) have been shown to more effectively delay the precipitation of weakly basic drugs, while cationic polymers such as polydiallyldimethylammonium chloride show the opposite trend of having superior capacity to delay the precipitation of weakly acidic drugs³⁰. These observations speak to the potential for electrostatic interactions between drug and polymer to stabilize supersaturated drug and delay precipitation^{30,31}. Positively-charged CVDL may interact with negatively-charged mucin sugars, while negative-charged PXM may interact with regions of the positively-charged mucin protein backbone¹⁶. Alternatively, either molecule could undergo hydrophobic interactions with portions of the mucin backbone. Closer examination of the S-P profiles (in the presence of mucin) of CVDL reveal a progressive decrease in drug concentration that is indicative of nucleation and crystal growth (Figure 2) – a feature that was not evident in the PXM S-P profiles in the presence of mucin (PXM concentration remained constant throughout the test duration) (Figure 4). This difference could indicate that the mechanisms through which mucin stabilizes supersaturation are drug-specific – with slowing of crystal growth being likely in the case of CVDL (supported by mucin's lack of effect on CVDL solubility in Figure 1); and the formation of mucin–drug interactions in solution being likely in the case of PXM (supported by mucin's effect on PXM solubility in Figure 1).

3.2 The absorption enhancement of CVDL when dosed in supersaturated solutions was higher across mucus-producing than non-mucus-producing intestinal monolayers

CVDL was dosed at SSRs of 1 and 5 to both mucus-producing (Caco-2:HT29-MTX-E12 co-culture) and non-mucus-producing (Caco-2) intestinal monolayers, to assess the impact of a mucus layer on supersaturation stabilization and subsequent flux enhancement. CVDL appearance in the basolateral compartment in transport experiments, when dosed in buffer containing CVDL at SSRs of 1 and 5, is shown in Figure 5. The absorptive flux enhancement (defined in Section 2.9) for CVDL at each sampling point across Caco-2 and co-culture monolayers is shown in Figure 6. Flux enhancement was greater at all time points for mucus-producing co-culture monolayers, when compared to non-mucus-producing Caco-2 monolayers (Figure 6). Since flux enhancement calculations were performed using the same monolayer type (thus eliminating differences in CVDL flux across different monolayer types), the difference in flux enhancement between the mucus-producing and non-mucus-producing monolayers was likely due to different degrees of supersaturation in the apical compartment *in situ*, even though CVDL was dosed at the same SSR to both monolayer types initially. The data therefore suggest that the presence of a mucus layer immediately adjacent to small intestinal monolayers helped stabilize CVDL supersaturation and led to enhanced CVDL absorption.

The integrity of the monolayers following exposure to buffer containing CVDL at SSRs of 1 and 5 for the duration of the transport experiment, as indicated by TEER measurements, is shown in Figure 7. Both Caco-2 and co-culture monolayers tolerated CVDL at a SSR of 1 well, where the TEER of monolayers remained at >80% starting value over a 100 min incubation (Figure 7). When CVDL was dosed at a SSR of 5 however, the TEER of both

types of monolayers were reduced to ~60% starting value, indicating that high concentrations of CVDL had a slight toxic effect on the monolayers. Even though CVDL appeared to compromise the integrity of the monolayers slightly at a SSR of 5, flux enhancement comparisons (i.e., flux at SSR = 5 relative to flux at SSR = 1) were still performed as the comparisons were made within each monolayer type, and the integrity of both Caco-2 and co-culture monolayers appeared to be compromised to a similar extent (Figure 7).

The difference in flux enhancement across the two monolayer types was not as prominent as may be expected from S-P experiments, where ISE measurements indicated that supersaturated CVDL precipitated within 19 ± 4 min (average $\pm$ SEM; $n=3$) min in the absence of mucus, but remained supersaturated for 24 h in the presence of 8 %w/v native PIM (Figure 2A). A possible explanation for this is the additional transport barrier to drug absorption that is effected by the mucus layer in the co-cultures³². Many authors have suggested that the transport of poorly water-soluble compounds across an “unstirred water layer” adjacent to the epithelium is rate-limiting to absorption^{33,34} (the unstirred water layer is coincident with, and indistinguishable from, the mucus layer^{35–37}). In the case of the mucus-producing monolayers, it is expected that CVDL diffusion across the unstirred water layer is slower than in the non-mucus-producing monolayers, decreasing positive effects of mucus on absorption due to stabilization of supersaturation (when dosed with a SSR of 5 vs. 1). The relative amounts of drug and mucin/mucus in the two experimental systems also likely played a role in the muted absorption enhancement. A relatively thin layer of mucus exists on Caco-2:HT29-MTX-E12 co-cultures ($\sim 3\text{--}6$ m³⁸). Assuming a relatively uniform layer that is 5 μm thick, the mucus layer in these cultures has a volume of 0.00209 mL and contains approximately 0.1045 mg mucin (assuming the reported 5 %w/v of mucins in native mucus). In contrast, in S-P experiments, there is 20–80 mg of mucin in 20 mL base buffer. Thus, the ratio of drug to mucin mass in the transport and S-P experiments were 2.20 and 0.05–0.23, respectively, suggesting that the mucus layer in the transport experiments had a reduced capacity to stabilize CVDL supersaturation due to a much higher drug:mucin mass ratio (~ 10 -fold higher than in the S-P experiments). The native mucus layer in the intestine varies in thickness from approximately 10 to 750 μm ^{39,40} and thus may provide a greater capacity for maintaining supersaturation, depending on local drug concentration and fluid volume. Additional factors that may impact the significance of the mucus layer could include the presence of an absorption sink in transport experiments and differences in the mucus type and form between S-P and transport experiments. The presence of an absorption sink has been previously reported to lower drug SSR and delay the onset and rate of drug precipitation^{41,42}. Likewise, in our transport experiments, the SSR of CVDL in the apical compartment may be lowered by the presence of an absorptive monolayer, therefore CVDL supersaturation may have been maintained longer in the apical compartment of the Caco-2 monolayers (when compared to the S-P experiments), attenuating the expected difference in CVDL flux enhancement when compared to the co-culture monolayers. Lastly, the type and form of mucus in the S-P vs. transport experiments were different, in that (porcine) mucus was mixed into the buffer in the S-P experiments, whereas (human) mucus was present as a discrete and continuous layer immediately adjacent to the cell monolayer in the transport experiments. Mucus that is secreted by HT29-MTX cell lines has been well-characterized in

the literature, and consists mainly of MUC5AC and MUC1, as well as MUC2, MUC3A, and MUC5B mucins^{43–46}. While the main secreted gel-forming mucin produced by HT29-MTX cultures is MUC5AC, the major gel-forming mucin in the small intestine is MUC2. Thus, while HT29-MTX represents a commonly utilized model of human intestinal epithelium that produces mucins as well as other components of mucus important in mucosal immunity, such as trefoil factor family peptides⁴⁷, a shortcoming of this model is this difference in predominant gel-forming mucin type.

Taken together, data from the transport experiments indicate that the absorption of supersaturated CVDL was greater across Caco-2:HT29-MTX-E12 co-culture monolayers than Caco-2 monolayers, suggesting that the presence of a mucus layer immediately adjacent to intestinal absorptive cells helped stabilize CVDL supersaturation leading to enhanced absorption. Indeed, the generation of drug supersaturation at the small intestinal mucus layer has recently been suggested as an intrinsic mechanism of action for lipid-based formulations⁷, and the data presented here suggest that the ability of mucus to stabilize supersaturation may well be complementary to this mechanism. These findings motivate the exploration of supersaturable formulations targeting the mucus layer, for example by the inclusion of mucoadhesive and/or pH-responsive components. As noted above, the mucus layer is coincident with an acidic ‘unstirred water layer’^{35–37} directly adjacent to the absorptive epithelia. The pH of the unstirred water layer is documented to be 5.3–6.2⁷, as opposed to 6.5 in the bulk lumen, and thus enteric coated formulations designed to release drug in response to a decrease in pH⁴⁸ could be used to target the mucus layer. Further, these findings provide significant insight into the mechanisms of enhanced drug absorption from formulations that result in drug supersaturation, and could be useful in understanding and predicting the impact of such drug delivery systems on pharmacokinetic profiles.

4. Conclusion

This study demonstrates for the first time the potential for intestinal mucus to delay the precipitation of two model poorly water-soluble drugs, suggesting that drug supersaturation can be stabilized in close proximity to the absorptive site. As only two model drugs were tested to date, the ability of mucus to stabilize supersaturation of compounds with broad physical and chemical properties needs to be analyzed to assess the generalizability of this phenomenon. Consideration of the potential impact of mucin on stabilization of supersaturation may be important in the interpretation of *in vivo* performance of drug delivery systems resulting in a supersaturated state in the GI tract. Further, this ability for small intestinal mucus to stabilize drug supersaturation may present a novel approach for targeted supersaturating drug delivery systems to enhance drug absorption. This study also demonstrates the value of using ISE for real-time continuous monitoring of drug concentration in complex media without additional sample processing.

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Highlights:

- The potential for intestinal mucus to stabilize drug supersaturation was evaluated
- Mucin and porcine mucus delayed the precipitation of carvedilol (CVDL) and piroxicam
- Supersaturated CVDL was absorbed to a greater extent in mucus-producing monolayers

Figure 1. Equilibrium solubility (37 C) values of CVDL and PXM in test media. Data represent mean SEM of n = 3 determinations. * denotes significant difference relative to buffer (p < 0.05).

Figure 2.

(A) Real-time changes in CVDL concentration measured by ISE following supersaturation induction (SSR = 5) in 0, 0.1, 0.2, 0.4 %w/v mucin and 8 %w/v PIM. Data was filtered using Microsoft® Excel prior to plotting (1st and every 100th data point thereafter were plotted). Data represent average $\pm$ SEM (n = 3). (B) CVDL concentration measured by HPLC following supersaturation induction (SSR = 5) in 0, 0.1, 0.2, 0.4 %w/v mucin and 8 %w/v PIM. Data represent average $\pm$ SEM (n = 3). [NB: Color should be used for this figure in print]

Figure 3.

T_{ss} of CVDL following supersaturation induction (SSR = 5) in buffer with and without mucin/PIM. For 0.2 % w/v mucin, 0.4 % w/v mucin, and 8 % w/v native PIM (denoted ^), although CVDL concentration did decrease to < 95% C_{max} (thus enabling quantification of T_{ss}), marked precipitation did not occur as evident in Figure 2A. Data represent average ± SEM (n=3). * denotes significant difference relative to buffer (p < 0.05).

Figure 4.
PXM concentration measured by HPLC following supersaturation induction (SSR = 6) in 0, 0.1, 0.2, 0.4 % w/v mucin and 8 % w/v PIM. Data represent average $\pm$ SEM (n = 3). **[NB: Color should be used for this figure in print]**

Figure 5. Cumulative CVDL appearing in the basolateral compartment with respect to time, following dosing of buffer containing CVDL at SSR =1 and SSR = 5, to (A) Caco-2 and (B) co-culture monolayers. Data represent average $\pm$ SEM (n=3).

Figure 6:
CVDL absorptive flux enhancement (SSR=5 relative to SSR=1) across Caco-2 and co-culture monolayers at each sampling point. Flux enhancement derived from average data in Figure 5.

Figure 7. Trans-epithelial electrical resistance (TEER) of Caco-2 and co-culture (Co-C) monolayers, expressed as percent relative to reference (i.e., $t = 0$), following a 100-min exposure to buffer containing CVDL at a SSR of 1 and 5 in the apical compartment during transport experiments.