Hydrodynamic effects on supersaturated solutions
The interplay between precipitation, absorption and bioavailability of supersaturated solutions in the gastrointestinal-tract

Linda Gabrielsson
Abstract

Along with development of high throughput screening, the number of drug candidates with poor solubility in water and/or poor permeable abilities has increased. This is a problem when oral drug delivery is the single most common route of administration since the bioavailability of poorly soluble and/or poorly permeable drugs is low. These drugs belong to the BCD class II. Often drug candidates with these properties are deselected in the fast screening methods because it is predicted that it will show low bioavailability. But the assays for testing or predicting these abilities are primitive and the computerised methods are not sufficiently developed. An increased sensitivity in the design of drug testing assays where the objective to mimic the human gastrointestinal tract could alter the outcomes for several deselected drugs. Hank's balanced salt solution is somewhat of a standard solution for testing drug solubility and it does not mimic the gastrointestinal tract, although it will be used in this master thesis since there are several different interesting parameters that can be evaluated on this subject.

This master thesis wants to explore the fundamentals of the concept of supersaturation as a method for evading problems with poor solubility.

Supersaturation is defined as the phenomenon when molecules are in solution at concentrations above their thermodynamic solubility equilibrium. By achieving this state with different solid-state forms of drugs, the concentration gradient can be affected by the high concentration and will force more drug solution over the wall of the small intestine by mechanisms of passive transport. Since this is an unstable state for the solution, problems with precipitation are likely to be seen. The mechanisms of precipitation are not fully understood with supersaturated solutions, especially when in the presence of biological membranes and other physical factors like stirring.

The aim of the master thesis is thus to conduct an assay for testing how and if supersaturation could be a method for increasing the bioavailability of drugs belonging to BCD class II and if it is affected by different rates of stirring. The new drug ezetimibe will be our model substance.

The Caco-2 cell monolayer is a well-known model for predicting drug permeability in vitro. It is very similar to the human small intestine; the epithelial cells form very tight junctions, which makes it suitable for studying passive diffusion. Caco-2-cells were cultured in flasks and then seeded onto permeable membrane inserts. Experiments with Caco-2 cells were performed in these Corning Costar plates with inserts. Dissolution tests were performed in eppendorf-tubes. In both experiments an orbital shake in a heating cabinet was used. The dissolution media used was HBSS. Results showed that no supersaturation was achieved during the timeframe of our experiments. The amorphous ezetimibe had in fact lower permeability and lower solubility than the crystalline ezetimibe both in the solubility tests and the caco-2-experiments. There is no sign of better dissolution with higher stirring rates. It is likely that most of the scattered data and questionable results is a consequence of the problems with the model substance in both crystalline and amorphous forms.

In conclusion a fairly good assay for performing drug permeability studies with Caco-2 cells in controlled temperature and with different stirring rates has been designed. Due to problems with the drug substance the results collected in this project are a subject of caution. No supersaturation could be seen in either solid-state form of ezetimibe. The amorphous ezetimibe had the lowest solubility in all experiments. A higher stirring rate seems to affect the dissolution of crystalline ezetimibe negatively. The substance choice should have been made more carefully and a more well-known and well-studied substance should have been chosen.

Caco-2 cell monolayer; drug absorption; supersaturated solution, BCD Class II drug.
1. Introduction

1.1 Drug development

The development of drugs has not always been as fast and elaborate as it is today. Only 20 years ago the discovery of new drugs was more of a random chance event. Potential drugs could be found manually in compounds tried for other purposes or diseases than the final current use (1). As an example, the discovery of propulsion inhibitors like loperamide resulted from the development of synthetic opioids (1,2). At the moment drugs are generally developed starting from a drug target involved in a specific process in the human body. The target can for instance be a receptor of which the chemical structure is investigated for clues for finding a possible substrate. The potential drug molecule or “the lead compound” is then adapted to suit the environment in the human body as much as possible. There are a lot of factors that have to be considered when designing a drug molecule which affect the bioavailability of the drug. Examples are the molecule weight and lipophilicity of the drugs (1).

Large parts of the screening process are currently performed in computer models and the process of testing for the suitable properties can be preformed in automated or highly efficient laboratory methods e.g. by robotics. This enables a very fast screening for potential drugs that was never before possible. These methods of high throughput screening has been evolved throughout the last 15 years (3) and it has increased the number of hydrophobic drug candidates so that today 40% of the newly discovered drugs are poorly water-soluble, and/or exhibiting poor permeation (4). In the quest of developing drugs with high biological receptor activity, the absorption, distribution, metabolism and excretion (ADME) is neglected. ADME is currently problematic to predict since the complete picture of the interplay of the processes involved in ADME is not fully understood whilst it is translated into computer models (in silico) (5).

When a lead compound is found in the search for new drug candidates, the compound has to undergo numerous tests to evaluate if its properties are suitable for drug development. This project is based on the proposed theory that since these tests are the moment where a lot of innovative oral drug candidates are rejected for their bad performance, the need for valid results are crucial. It is possible that a drug candidate can be rejected by false indications if the test assay is not simulating in vivo human conditions to a sufficient degree. Drug tests tend to be very simple in its design and the computer models are not yet sophisticated enough. But there are several on going projects for better prediction with computerized models (6–8). The problem is as mentioned that the interplay between the underlying factors and their significance is not fully understood, and this complicates the designing of assays, both computerised and not.

Solubility tests are currently performed by studying drug dissolution in a relatively simple buffer solution. The experiments of this study are performed in Hank's balanced salt solution, which belong to this group. As always, the requirements are for the evaluating methods to be efficient and this is the reason for the wide use of this setup. Recent studies have suggested that it can be problematic to use such simple medias since they do not simulate the environment in the gastro intestinal tract, which specific properties may alter the outcomes. Tests with other dissolution medias are currently evaluated (9,10). Factors affecting the dissolution of a drug can be pH, ionic strength, volume available for dissolution, bile salts, surfactants e.g. lipids, all of which can differ depending on dissolution media at hand (11). But since it is the most widely used setup, and because these possible factors will not be the subject of this project, the Hank's balanced salt solution will be used in our experiments.
The BCD classification system is a renowned tool for classification of biopharmaceuticals regarding permeability and solubility. The BCD class II includes drugs that have a good permeability but poor solubility, which means that their oral absorption is dependent upon dissolution (4). These properties will result in reduced or absent bioavailability for oral formulations of the drug candidates. Since oral delivery of drugs is the single most predominant route of administration, the need to overcome these problems is huge (12,7).

Ezetimibe is the sole member of a new class of lipid-altering agents. It exerts its effect by inhibiting the adsorption of cholesterol but its mechanism of action is not known. It has been found to be located mainly on the tips of the intestinal villi. It belongs to the BCD class II and will be used as model drug in this project (13). As mentioned above, pH is a factor affecting dissolution. Ezetimibe is a weak base and would hence benefit from a dissolution media more comparable to the lower pH of the gastrointestinal tract, Hank’s Balanced Salt solution (HBSS) and the drug solution used in these experiments will be adjusted to pH 6 instead of 7.4. pH 6 was chosen in hope to increase solubility whilst mimicking the environment of the small intestine where the drug is in solution and being adsorbed.

1.2 Caco-2-cell monolayers

Permeability, metabolism and transport tests are preferably performed with a biological model line-up such as the one consisting of Caco-2-cells. Caco-2-cells have been used since the late 1980’s and are a human colon epithelial cancer cell line that mimics the human small intestine in a satisfactory manner. They undergo spontaneous differentiation and grow in a monolayer with tight junctions that are somewhat tighter than the tight junctions of human small intestine (14–16). They display all the routes of drug absorption shown below in figure 1.

The great advantages of cell-based models over other available assays are that it is easily maintained, easily modified, reliable, enterocyte-like, well characterized, and only requires small amounts of compounds. Also, the method is less expensive than animal assays. Caco 2 cells are cultured for approximately 21 days before they exhibit the right features. One of the most important features of Caco-2 cell monolayers is the
in vivo-like transcellular permeability. They are cultured in flasks and then put onto permeable supports in which form they constitutes a drug transport model shown in figure 2 below.

![Figure 2. The Caco-2-cell monolayer as a transport model. (a) Apical side and (b) basolateral side. Revised with inspiration from (15).](image)

A disadvantage with Caco-2 cell monolayers compared to some other biological models is that it does not express all the important metabolic enzymes of the CYP450-system, which are involved in drug metabolism. The lack of CYP3A4 could be especially problematic, since it is involved in the first passage metabolism of many compounds. But if it is required, there are methods to increase in expression of CYP3A4 to satisfactory levels compared with in vivo (14,15). In this project this will not be of matter since the permeability route of study will be passive transport and this route does not engage the CYP450-system.

Several techniques for characterizing the properties of the Caco-2-cell-monolayer and comparing it to the human intestine have been carried out. Among them the mRNA expression profiles of Caco-2-cells and human intestinal cells have been compared with correlating results (14,15). However, it must be remembered that Caco-2-cells are not human intestine cells; they are immortalized cultured cells that despite comparative tests possibly can display unknown differences compared to the in vivo situation which has impact on experiment results. Although the mRNA profiles have been compared, the actual protein expression was not determined and compared.

In this project the transport of a model drug under different stirring rates and in different types of solutions (thermodynamically stable and supersaturated) will be studied. As mentioned above, the transcellular permeability of Caco-2-cell monolayers and that of the human intestine are quantitatively comparable and the paracellular transport is fairly comparable in Caco-2 and in the human intestine. Further the first pass metabolism will not be studied, and the properties of active transport are not relevant when studying the effect of supersaturated solutions since they presumably engage only passive transport. This altogether means that it will be suitable to use Caco-2-cellmonolayers as permeability barriers for these experiments.

A standard setup for testing the membrane properties is to conduct tests with metoprolol, atenolol and propanolol. This gives an opportunity to obtain a relationship between fraction absorbed in humans and drug permeability. The tests are conducted in the same manner as the experiments for this project; by placing drug solution apically and collecting samples basolaterally at given time points. These three compounds are common beta-blockers whose permeability are widely studied in Caco-
2-cells and have become somewhat of a standard trio for Caco-2 permeability studies. They represent three different classes of permeability: low=atenolol, medium=metoprolol, high=propranolol, and because their correlations are known it is possible to compare results of the cultured caco-2-cells with the literature to ensure coherence and thereby proper function of the monolayer. The widespread use of these as model drugs of permeability studies, and the possibility to compare results with other laboratories makes it suitable to also use them in this project. Caco-2-cells cultured form the same cell-line as the one used in this project, has been subjected to these tests.

14C-mannitol can further determine the integrity of the paracellular transport over the cell monolayer. Because of its inability to be transported in other ways than paracellular, a defect in the cell monolayer will be readily detected.

As mentioned the Caco-2-cellmonolayers may be sensitive to stirring and this is why using mannitol is a good way to evaluate the integrity associated with experiments with different stirring rates. Carbon-14 is a radioactive label used as a marker in verifying transport assays. The common carbon isotope (12C) in one position in the mannitol molecule is substituted with the 14C-isotope, which makes it detectable with a scintillation counter (14,17).

1.3 Supersaturation

When solubility problems are not possible to overcome only by the means of chemical modification, the alternative is to develop drug formulations to reach the intended goal. Several formulation methods are proposed to circumvent the solubility problem. For instance: physical modifications like reduction of particle size to nano formulations, the use of polymorphs, surfactants and use of carriers such as amorphous solid dispersions (12).

One interesting approach is the use of supersaturated solutions of solid-state forms in the gastrointestinal environment to increase intestinal absorption. The use of supersaturated solutions has proven to improve the bioavailability in several attempts. The term supersaturation is referring to the phenomena when molecules are in solution at concentrations above their thermodynamic solubility equilibrium. An example of a supersaturated solution versus a “normal” thermodynamically stable solution is illustrated in figure 3. The theory is that the concentration gradient will be affected by the high saturation and hence more solution passes through the wall of the small intestine by passive transport mechanisms. A greater extent of supersaturation is achieved with particles in amorphous form than with the corresponding crystalline form due to its kinetic solubility (18–20).

![Figure 3. Concentration/time-profiles for supersaturated solution (red line) compared to thermodynamically stable solution (black line). Revised with inspiration from (18).](image-url)
Supersaturation is a thermodynamically unstable situation. The supersaturated solutions are at high risk of precipitating into the more ordered crystalline solid form, which requires lower energy. The influence of different factors on the supersaturated state is not fully understood. Some clues have been revealed as to it has been seen that precipitation will have a higher tendency to take place if the dissolution occurs too fast and it will also occur faster the higher the saturation. Precipitation can be prevented with the aid of excipients or with formulation of controlled-release formulations, but again, the total interplay is not understood (18,19).

It has become evident that the sink conditions, which are presently used for dissolution assays, are not suitable for studying supersaturated solutions. Sink conditions is when a drug is dissolved in a solvent-containing chamber that is much larger than needed for its complete dissolution. This setup allows for studies of dissolution rate without physiological factors. Sink conditions can for instance mask the effect of any precipitation-inhibitor, since precipitation may not occur in sink conditions (19). It is hereby proposed that dissolution in a restricted volume (non-sink conditions) would be more consistent with in vivo environment in the gastrointestinal tract, and therefore, it seems unsuitable to constantly evaluate any drug performance in sink conditions, regardless of supersaturation or not.

Other factors than the dissolution volume are defined to affect the duration of supersaturation in the gastrointestinal tract; motility, gastric emptying and bile salts (20).

1.4 Thermodynamics and hydrodynamics

Precipitation is influencing the bioavailability by affecting the rate and extent of drug absorption. When a molecule is precipitated from a supersaturated solution, it is transformed to its crystalline form, removed from the solution and hence does not contribute to the supersaturation effect on the concentration gradient. This in turn leads to decreased absorption and thus decreased bioavailability (20). Further it can be imagined that when molecules are absorbed over the membrane, the absorption rate decreases since the saturation decreases (19). This due to the decreased influence on the concentration gradient by the decreasing number of drug molecules in the small intestine.

The precipitation from a supersaturated solution is depending on the processes of nucleation and crystal growth. As previously stated, an increasing degree of supersaturation increases the risk of nucleation and crystallization, which leads to faster precipitation. Nucleation can be described as the formation of a solid phase in the supersaturated solution in an attempt to separate the solute from the solution and thereby reducing the total Gibbs free energy, which is very high in supersaturated solutions (4). Crystallization is described as the further growth of the nuclei, resulting in precipitation. It is important to point out that these processes occur simultaneously (18,21).

It is known that stirring is a factor that affects dissolution, and it will be the subject of our experiments and constitute the hydrodynamic effects on the drug solutions. At present, the USP paddle method is the standard method for stirring in dissolution tests of drugs. When there are biological models like Caco-2-cellmonolayers present in the setup, these paddles are not suitable. The use of an orbital shaker is more suitable since it engages an outer force instead of an in solution mixing that would damage the permeability model. A substantial degree of stirring is taking place in the stomach and gastrointestinal tract, which are the locations of dissolution of oral drugs. This in turn gives rise to questions about the importance of mode of stirring in a
dissolution assay, questions that have not been raised to any greater extents as far as can be found in the literature.

These facts have given the idea for this project to discuss around a concept called “Give the molecule a choice” (illustrated in figure 4), which basically means that a particle in a supersaturated solution has a number of different options to choose from when choosing what to do next. It can return to its prior undissolved state, be absorbed over the small intestine, start to aggregate and precipitate or stay in solution. The interplay between factors affecting these options is believed to hold the key for the understanding and use of supersaturated drug formulations and thereby circumventing the solubility problems of many new drug candidates.

![Figure 4. Different options for the molecule in solution. (1) Return to drug particle (remain in amorphous state) (2) Precipitate as crystalline material (non-amorphous). (3) Stay in solution. (4) Absorption. Figure by author with inspiration from the supervisor.](image)

In summary the effect of hydrodynamics is important and it has impact on several steps in the process described above, which are the permeability, the state of dissolved molecules in the supersaturated solution and the properties of particles present in solution. Among these processes the hydrodynamic impact on permeability is relatively well studied. The processes that lack further insight on the impact of hydrodynamics are the behaviour of supersaturated solutions and the particle properties.
1.5 Aim of the master thesis

The aim of this master thesis is to study and design an assay consisting of supersaturated drug solutions with crystalline or amorphous particles at different stirring rates. This will be achieved by the following subsidiary aims, which is to study the effect of different stirring rates on:

- The permeability of the model drug in thermodynamically stable drug solutions (this is well-studied, see introduction 1.4).
- The stability of supersaturated solutions (This is not thoroughly understood see introduction 1.4 and method 2.2).
- The dissolution rate and solubility of amorphous particles in supersaturated state. (This is not well-studied, see introduction 1.4)

The model substance selected for this project is the lipid altering agent ezetimibe.

2. Material and method

2.1 Growing caco-2-cells in culture media

Caco-2-cells were grown in Dulbecco’s modified eagle medium with DMEM high glucose 4,5 g glucose/L, non-essential amino acids, 10% bovine calf serum and PEST (penicillin/streptomycin). When ready, phosphate buffered saline (PBS) with pH 7,4 was used for washing and trypsin enzyme solution for the release of cells from the culturing flask. The cells were then placed on Coring Costar permeable membranes at a seeding density of 500,000 cells/cm². Medium change occurred every other day on both culturing flasks and membranes. Experiments were always performed on cells that had received fresh medium 4-18 hours earlier.

2.2 Cell environment during experiments and characterization of Caco-2-cells as a functional membrane

All experiments were performed in Hank's balanced salt solution (HBSS) and 25 mmol HEPES buffering agent at pH 7.4. It was used for washing the Caco-2-cells prior to experiments at the apical side, and as dissolution media at the basolateral side during experiments. Apical drug solutions all had pH 6.

Transepithelial electrical resistance (TEER) provided a measurement of the electrical resistance through the Caco-2-cellmonolayer. In practice, a positive and a negative electrode were placed on either side of the biological membrane. The TEER measurements can be correlated to the barrier properties of Caco-2-cellmonolayers; high resistance correlates to decreased paracellular permeability. TEER measurements were made prior to the start of experiments. The relevance of TEER measurements lies in obtaining information regarding if the barrier properties are normal or comparable to other laboratories. The apparatus used was Endohm. It is very user friendly and it has a minimal distance between the electrodes, which ensures that the resistance
measure is of the monolayer resistance, and not the resistance of the surrounding liquid.

As described in the introduction, the 14C-mannitol method is a good measurement of the integrity of the paracellular transport over the cell monolayer. Tests with beta-blockers are also valuable for testing membrane properties. But because of the mishaps with cells and analysis method, time became an issue and it was not possible to perform this test. It would have been used in order to ensure that the experiments were performed on cells with functioning parallel transport. Although, deficiencies in the integrity of the Caco-2 cell monolayers will be visible in the results, it is always better to be sure of this in advance to avoid wasting time.

### 2.3 Main experiments

A standard USP-paddle bath is commonly used in drug dissolution and solubility experiments but was not used in this project. The reason for this is that the paddle method would damage the Caco-2-cell monolayers, an orbital shaker was used instead. A small-scale setup for solubility and dissolution rate tests was used similar to the one developed by Bergström et al. (8).

Ezetimibe was prepared in Luleå by another research group. All experiments involved ezetimibe in different concentrations dissolved in water and/or HBSS. Due to the electrostatic nature of the crystalline ezetimibe, the solution process was very difficult. It was not possible to acquire a homogenous suspension at any concentration in HBSS or water. Ezetimibe formed at its best a milky coating at the liquid surface. This coating adhered to everything it came in contact with, e.g. pipette tips, the pH electrode etc. This property makes it severely difficult to predict if the generated concentrations are in fact correct.

In ethanol the substance was completely endlessly soluble but it is not suitable to use ethanol at high concentrations with Caco-2 cell monolayers. At the first addition of water or HBSS to the ethanol-ezetimibe solutions precipitation occurred instantaneously. Further, the use of pure ethanol as solvent for an oral administration would not be suitable for obvious reasons.

All experiments are conducted to compare the effects of supersaturation, the presence of a permeability barrier and the stirring rate. Different concentrations of ezetimibe were used to compare ezetimibe at its normal solubility equilibrium to its potential supersaturated state. Amorphous ezetimibe was compared to crystalline in the potential of reaching supersaturation. Tests with and without Caco-2 cells as an absorption site were performed to evaluate the concept of “give the molecule a choice” in the sense of exploring what happens to the supersaturated solution when molecules are taken away from the solution and absorbs through the permeability barrier. All these properties are individually evaluated at different stirring rates to estimate the impact of stirring in supersaturated solutions compared to “normal” equilibrated solutions. A summary of all performed experiments can be seen in table 1 and 2.
Table 1. Summary of performed dissolution and solubility tests.

<table>
<thead>
<tr>
<th>Drug solution (concentration, phase)</th>
<th>pH drug solution and HBSS</th>
<th>Stirring rate (rpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>111.5 mg/L crystalline</td>
<td>7.4</td>
<td>100</td>
</tr>
<tr>
<td>111.5 mg/L crystalline</td>
<td>7.4</td>
<td>500</td>
</tr>
<tr>
<td>111.5 mg/L crystalline</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>111.5 mg/L crystalline</td>
<td>6</td>
<td>500</td>
</tr>
<tr>
<td>500 mg/L crystalline</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>500 mg/L crystalline</td>
<td>6</td>
<td>500</td>
</tr>
<tr>
<td>500 mg/L amorphous</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>500 mg/L amorphous</td>
<td>6</td>
<td>500</td>
</tr>
</tbody>
</table>

Table 2. Summary of performed experiments with Caco-2-cells.

<table>
<thead>
<tr>
<th>Drug solution (concentration, phase)</th>
<th>pH drug solution</th>
<th>Stirring rate (rpm)</th>
<th>Number of filters</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mg/L crystalline (2.3.1)</td>
<td>6</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>5 mg/L crystalline (2.3.1)</td>
<td>6</td>
<td>500</td>
<td>4</td>
</tr>
<tr>
<td>500 mg/L crystalline (2.3.4)</td>
<td>6</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>500 mg/L crystalline (2.3.4)</td>
<td>6</td>
<td>500</td>
<td>4</td>
</tr>
<tr>
<td>500 mg/L amorphous (2.3.4)</td>
<td>6</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>500 mg/L amorphous (2.3.4)</td>
<td>6</td>
<td>500</td>
<td>4</td>
</tr>
<tr>
<td>40 mg/L (standard dose 10 mg in 250 mL HBSS) resembling physiological conditions (2.3.4)</td>
<td>1-6 (pH switch)</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>40 mg/L (standard dose 10 mg in 250 mL HBSS) resembling physiological conditions (2.3.4)</td>
<td>1-6 (pH switch)</td>
<td>500</td>
<td>4</td>
</tr>
</tbody>
</table>

2.3.1 Evaluation of hydrodynamic effects in thermodynamically stable drug solution in presence of Caco-2-cell monolayers

These reference tests performed on thermodynamically stable solutions were used as reference for section 2.3.4, where stirring rate was to be evaluated in presence of Caco-2-cellmonolayers and in supersaturated drug solution.

HBSS pH 7.4 and crystalline drug solution comparable to the maximum solubility of ezetimibe in water (5mg/L) at pH 6 was pre-heated to 37°C in a heating cabinet. In an empty Corning Costar plate, 1.2 mL preheated HBSS was placed in the first two rows of wells. Then 4 Caco-2-cell monolayer grown filters were removed from their culturing wells and the apical media was poured off. Then they were transferred to the first row of HBSS-containing wells and 0.5 mL HBSS was added apically. The plates
were then incubated for 10 minutes in the heating cabinet. After 10 minutes, the filters were released from their apical HBSS and relocated to the next row of HBSS containing wells, whereupon 0.5 mL drug solution was placed on the apical side. Initial concentration sample $C_{start}$ was taken directly from the apical in a quantity of 100μl and then the intended drug solution volume of 0.4 mL remained. The plate was then placed on an orbital shaker at a rate of 100 rpm. At time intervals 5, 10, 15, 20, 25, 30, 60, 90, 120 minutes the plate was temporarily removed from the orbital shaker and from the basolateral side, samples of 0.6 mL were taken and replaced with 0.6 mL 37°C HBSS. At 120 minutes, a 100μL apical sample ($C_{end}$) was taken and the samples were placed in a refrigerator. These samples where not centrifuged. The same procedure was repeated at 500 rpm.

2.3.2 Evaluation of hydrodynamic effects on a supersaturated solution.

5 mg of crystalline ezetimibe was weighed in an Eppendorf test tube and suspended in 1.5 mL distilled water with the aid of a vortex mixer until it was visually considered to be as homogenous as possible at this high concentration. The undissolved particles formed something resembling a milky thin film in both plastic tubes and glass tubes. From this tube, 50μL was placed in 24 Eppendorf tubes, which at the start of the experiment were filled with 1450μL HBSS each.

The sample time intervals of these experiments were 5, 10, 15, 30, 45, 60, 90 and 120 minutes and there were three equal sets made simultaneously, thereby 24 tubes. Because of tight time intervals the samples at 5 and 10 minutes were carried out separate from the other first, and then the remaining samples were done. Accordingly, 1450μL of HBSS with pH 6 (or pH 7.4 initially) was placed in the six tubes corresponding to 5 and 10 minutes and the tubes were placed on an orbital shaker placed in a heat cabinet. The first samples were taken at 5 minutes and than spun for 5 minutes in a table centrifuge at 100 rpm. From the centrifuged sample, 1 mL was removed and placed in a new tube that was placed in a refrigerator. The same procedure was then repeated for the next time intervals. No visible pellet was noticed in any of the samples.

2.3.3 Evaluation of hydrodynamic effects on particle dissolution and solubility on a supersaturated solution.

These experiments were performed in the same manner as described above with the exception of pure amorphous phase ezetimibe was used instead of crystalline. The excess of particles in the solution was achieved with a concentration of 500mg/L as before and thus non-sink conditions were applied for this and the next experiment. The added amount of excess particles would preferably be determined based on the result of the previous sections. But since the LCMS apparatus broke down at the time planned for analysis, the entire range of experiments had to be performed prior to analysis.

2.3.4 Evaluation of hydrodynamic effects on supersaturated systems in the presence of Caco-2-cell monolayers. (Evaluation of above headings...)

In this section all the above parameters were combined in the presence of caco-2-cell monolayers. That is the effect of stirring in supersaturated solutions with the presence
of Caco-2-cell monolayers. The same procedure as in 2.3.1 was used except for the pH switch that was conducted as follows:

5 mg ezetimibe (amorphous or crystalline) was weighed and placed in a 150 mL beaker. HBSS was pre-warmed to 37°C in a heat cabinet. 10 minutes prior to experiment start, the caco-2-cells were put in the heat cabinet for pre-incubation as in 2.3.2. 125 mL heated HBSS and a magnet stirrer was added to the beaker. pH was lowered to 1.17 through addition of 3 mL 5M HCl and the drug solution was mixed for 30 minutes with the aim of achieving the highest possible concentration. pH was then altered by adding 2.5 mL 5M NaOH and additional drops of 1M NaOH to reach 6.0. At this point, a timer was started, measuring the time passed from the pH switch until the drug solution was placed on the apical side of the Caco-2-cell-monolayers. The time passed was about 5 minutes. The same procedure as described in 2.3.1 was then carried out.

### 2.3.5 Sample analysis

The samples were analysed with an HPLC-UV method. The wavelength was 232nM and a C18 column was used, with mobile phase AcN; MQ (52:48) and 20 mM formic acid with a flow rate of 1 ml per minute.

### 3. Results

#### 3.1 Selected samples

Nearly 500 samples were taken but not all were analyzed due to time constraints. The following samples (table 3 and 4) were selected to give an adequate picture of the effects of supersaturation and the presence of a permeability barrier (Caco-2 cell monolayers).

*Table 3. Selected solubility tests marked with *.

<table>
<thead>
<tr>
<th>Drug phase</th>
<th>solution (concentration, pH drug solution and HBSS)</th>
<th>Stirring rate (rpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>111.5 mg/L crystalline</td>
<td>7.4</td>
<td>100</td>
</tr>
<tr>
<td>111.5 mg/L crystalline</td>
<td>7.4</td>
<td>500</td>
</tr>
<tr>
<td>111.5 mg/L crystalline</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>111.5 mg/L crystalline</td>
<td>6</td>
<td>500</td>
</tr>
<tr>
<td>500 mg/L crystalline*</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>500 mg/L crystalline*</td>
<td>6</td>
<td>500</td>
</tr>
<tr>
<td>500 mg/L amorphous</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>500 mg/L amorphous*</td>
<td>6</td>
<td>500</td>
</tr>
</tbody>
</table>
Table 4. Selected Caco-2-experiments marked with *.

<table>
<thead>
<tr>
<th>Drug solution (concentration, phase)</th>
<th>pH drug solution</th>
<th>Stirring rate (rpm)</th>
<th>Number of filters</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mg/L crystalline (2.3.1)</td>
<td>6</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>5 mg/L crystalline (2.3.1)*</td>
<td>6</td>
<td>500</td>
<td>4</td>
</tr>
<tr>
<td>500 mg/L crystalline (2.3.4)</td>
<td>6</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>500 mg/L crystalline (2.3.4)*</td>
<td>6</td>
<td>500</td>
<td>4</td>
</tr>
<tr>
<td>500 mg/L amorphous (2.3.4)</td>
<td>6</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>500 mg/L amorphous (2.3.4)*</td>
<td>6</td>
<td>500</td>
<td>4</td>
</tr>
<tr>
<td>40 mg/L (standard dose 10 mg in 250 mL HBSS) resembling physiological conditions (2.3.4)</td>
<td>1-6 (pH switch)</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>40 mg/L (standard dose 10 mg in 250 mL HBSS) resembling physiological conditions (2.3.4)</td>
<td>1-6 (pH switch)</td>
<td>500</td>
<td>4</td>
</tr>
</tbody>
</table>

3.2 Graphs of solubility tests and Caco-2 experiments

All solubility tests were performed in series of three. Figures 5-8 show the results from solubility tests while figures 9-12 show experiments done with Caco-2 cells. Figures 8 and 12 represents combined data for easier overview. For the solubility tests, it is notable that the amorphous ezetimibe has the lowest solubility compared to both stirring rates with crystalline ezetimibe. There is also a difference between the crystalline rates where the lower rate has experienced larger permeation.

For the Caco-2 experiments, it is noticeable that the amorphous ezetimibe showed the lowest amount collected basolaterally. The ezetimibe solution with a concentration of 500 mg/L had the highest cumulative amount collected as expected, but there were no big differences between the 500mg/L and the 5mg/L solutions (see figured 9-12).
Figure 5. Solubility test crystalline ezetimibe 500 mg/L, 100 rpm.

Figure 6. Solubility test crystalline ezetimibe 500 mg/L, 500 rpm.
Figure 7. Solubility test amorphous ezetimibe 500 mg/L, 500 rpm.

Figure 8. Solubility tests ezetimibe - average/comparison.
Figure 9. Caco-2 experiments with amorphous ezetimibe 500 mg/L, 500 rpm.

Figure 10. Caco-2 experiments with 500 mg/L crystalline ezetimibe at 500 rpm.
Figure 11. Caco-2 experiments with 5mg/L crystalline ezetimibe at 500 rpm.

Figure 12. Average caco-2 experiments with ezetimibe
4. Discussion

4.1 Method discussion

Since this project was a pilot study, naturally one of the secondary objectives was to explore the methods for tests with supersaturated BCD class II drug solutions. A lot can be learned from this experience.

4.1.1 Solubility tests

The crystalline drug substance delivered was as mentioned extremely difficult to work with. It was a very light, static powder, which adhered to most surfaces: pH electrode, pipette tips and the surface of the solvent. It was not possible to get a homogenous suspension, at best a grainy, milky film formed after a long time use of the vortex. Both glass and plastic tubes was tried but it did not seem to adhere specifically to plastic, as some hydrophobic substances tend to do. Therefore the choice fell on eppendorf tubes since they are easy to handle. Because of the time limitations of this project, it was not possible to adjust the resulted data for potential adherence losses in the dilution process. The delivered amorphous form of ezetimibe was quite the opposite, it consisted of larger, hard crystal-like bits.

Another complexity created by the properties of ezetimibe was that the lowest weighable amount at our laboratory is 5 mg, which means that the ezetimibe was dissolved and then diluted in many steps before use. Also in this procedure a trial and error approach was conducted. Therefore, ezetimibe solutions for solubility tests were initially made by dispersing 5mg in 1,5 mL deionized water which was then diluted (hence the 111 mg/L concentration in the initial solubility tests). Later it was determined that 500 mg/L was a more reliable concentration for excess particles. The solutions with 5mg/L were prepared in the same manner. Since the dilution steps where numerous with a substance that adhered to these many surfaces, it was definitely adding to the uncertainty about the concentrations.

Ethanol was tried as solvent and it completely dissolved ezetimibe far beyond its saturation limit in water, but mainly because of its inappropriateness in Caco-2 assays, the decision fell on water. Ethanol has a toxic effect on Caco-2 cells and thus the integrity of the monolayer cannot be guaranteed.

The solubility test samples were centrifuged to get rid of excess particles, but it would have been more consistent with pharmaceutical protocols if they were also filtered through a 0,2 μm sterile filter, although this would have been positive for the static light substance, it could lead to further problems with adherence for such lipophilic substances.

All experiments were performed in pH 6 (except for the pH-switch), which was adjusted in the diluted drug solution used for experiments. As previously mentioned, there were extreme problems with drug adherence to the pH-electrode, particularly with the extremely saturated solutions. The decision of adjusting pH arose after initial dissolution tests, pH 6 was used in all drug solutions since a lower pH value is predicted to increase the solubility of basic compounds and to mimic the physiological pH of the small intestine. A positive note is that the solutions and samples are stable for at least 10 weeks when refrigerated, although a new drug solution was made prior to each experiment (both Caco-2-experiments and dissolution tests) because it was considered to generate more accurate results when there were large problems with the drug solutions.
4.1.2 Caco-2-experiments

It was somewhat tricky to perform, but overall the Caco-2 experiments went smoothly. The ezetimibe solutions adhered to pipette tips, pH-electrode and accumulated at the surface of the solvent as previously described, but it was possible to fetch the plate and collect samples at a relatively fast speed despite their difficult location in the heating cabinet. The heating cabinet gave good temperature control in both solubility tests and Caco-2 experiments.

On a possible negative note is that the inserts were probably moving inside the wells as they were shaking on the orbital shaker. This was noted since there is an opening angle that is best for collecting samples. When the insert had been set in this specific angle, some inserts had randomly moved with this opening pointing at another direction. This could result in altered stirring rate and pattern if the inserts are not following the movements of the orbital shaker to a sufficient degree. Minimum spillage was noted by wetting of the plastic gloves at the point of sample collecting for some Caco-2 experiments (e.g. crystalline 5 mg/L at 15 minutes) with 500 rpm on orbital shake and it was not possible to know if this liquid originated from the apical or basolateral side of the inserts. This could of course also affect the results in a negative way if the concentrations are altered by loss of drug solution apically or dissolution media HBSS basolaterally.

These samples were not centrifuged nor filtrated which would have been preferable, for comparison with other tests, especially for the comparability between the start concentrations in the Caco-2 experiments and the concentrations in dissolution tests.

4.1.3 Sample analysis

For the aspects of the analysis it would also have been better to choose a well-known drug substance. It was hard enough to fit the sample analysis with either LC-MS or HPLC within the time frame for this project, but because of the rather new untried substance it took time to establish a protocol.

4.2 Discussion of results

Because of the time constrains, not all tests performed were analysed for this project (selected samples listed in table 3 and 4). For the solubility tests, the crystalline solutions of 500mg/L at both rpm were chosen along with the high speed rpm for the amorphous solutions for a possible comparison in dissolution between solid state forms and stirring rates. For the Caco-2 experiments, only the high speed stirring rates were chosen for both solid-state forms and both concentrations of crystalline ezetimibe. These choices were hopefully going to give us clues about how these parameters affect each other in supersaturated solutions with the presence of a permeability barrier.

The actual results for all experiments are very scattered. This might be a consequence of the handling problems with ezetimibe. This is why (unfortunately) these results are not as good as a hint as we had hoped for.
4.2.1 Hydrodynamics and the effect on thermodynamically stable drug solutions and drug supersaturated solutions

Hydrodynamics in this context can be narrowed down to the process of stirring. It is known that stirring affects the unstirred water layer around particles in a thermodynamically stable drug solution (22). But little is known about its impact on supersaturated solutions. It is known that precipitation of supersaturated solutions can occur quicker if it is dissolved too fast or if the saturating is too high (18). Our prediction was that a higher stirring rate on a supersaturated solution might increase the solubility of the supersaturated solution (see figure 5).

Unfortunately, our goal with achieving supersaturated solutions with ezetimibe at 500 mg/L was a failure with both solid state forms. No supersaturation can be seen in the resulting graphs (see figures 1-8 above) and the concentration never reaches above 0.2 mg/L on average for dissolution tests, or above 1.6 mg/L (cumulatively collected) on average for the Caco-2-experiments. Compared to the literature value of 5mg/L in water this is not even close to that and ezetimibe might have required more time to dissolve to reach its literature value. The rather immediate dilution process cannot have been helpful if that is the case.

Although it is possible that supersaturation occurred before the first time interval of 5 minutes, the use of any shorter time spans would be difficult. 2-3 minutes might be possible with our setup but the intensity of the experiments will then reach a higher ground, which would result in decreased control. Nevertheless it is necessary to be able to hold a supersaturated state for longer than seconds or minutes if it is supposed to use for experiments or in the prolongation as a drug in any form.

In the comparison between average solubility tests of crystalline ezetimibe (figure 4) at both 100 and 500 rpm it is noted that the opposite of what was expected has occurred; a slight tendency toward better solubility is seen with the lower stirring rate. Furthermore, the amorphous ezetimibe, which was expected to have better solubility, did on the contrary have the lowest solubility.

Figure 5. Possible impact of stirring on supersaturated solutions. Suggestively both red lines represent supersaturated solutions exposed to different stirring rates. Will higher stirring rate result in the bright red line? Figure by author inspired from supervisor.
4.2.2 Hydrodynamic effects on thermodynamically stable drug solutions and supersaturated drug solutions with the presence of Caco-2 cell monolayers

It is known that stirring affects permeability. By varying stirring rate it is possible to determine the contribution of hydrodynamics on permeability. For drugs with high cellular permeability, the thickness of the unstirred water layer on top of the caco-2-cell monolayers will have impact on the total permeability of the system. This is described in the following formula:

$$\frac{1}{P_{\text{tot}}} = \frac{1}{P_{\text{uw}}} + \frac{1}{P_{\text{caco2}}}$$

Equation 1. Equation derived from Eq.8 (22).

If the stirring is decreased, the unstirred water layer will thicken and thereby also the permeability decreases for these drugs. Changes in concentration over time can be used to calculate the permeability coefficient.

The average results seen in figure 8 shows that the crystalline ezetimibe with excess particles (500 mg/L) has a somewhat higher concentration continuously, compared to the thermodynamically stable (5 mg/L) solution. Thus, a difference of about 0.1 mg/L it is not particularly thrilling. Figure 8 shows, as in figure 4, amorphous ezetimibe as the least soluble compound. For the Caco-2-experiments a start and end concentration sample of the apical drug solution was collected. It was noticeable that the $C_{\text{end}}$ samples held larger concentrations than the $C_{\text{start}}$ with the amorphous ezetimibe solutions (data not shown in report). But the problem with this data is that these samples were not centrifuged and thus might be subjected to variations due to excess particles remaining in the samples. The opposite would have been expected and a possible explanation is that the hard “crystals” of the amorphous ezetimibe might have prolonged the dissolution process.

The addition of an excess of particles allowed for studies of the contribution of particle surface on the stability of the supersaturated solution at different stirring rates. But since no supersaturation occurred it is not possible to say anything about precipitation besides that it is possible that it already had precipitated as crystals at the first time interval in our experiments. Still there is a distinguishable difference in sample concentration between the original crystal form and the (at least initially) amorphous form, which speaks against it and rather points to the dissolution problems.

Overall, the results collected in this project are a subject to caution. Both because of the problems with the handling of ezetimibe, and that the results are not what we expected according to the literature. The substance choice was most unfortunate. Another drug from the BCD class II with known and well-studied properties ought to give more reliable (and foremost comparable) results, for example the calcium channel blocker felodipine.
Conclusion

A fairly good assay for performing drug permeability studies with Caco-2 cells in controlled temperature and with different stirring rates has been designed. Due to problems with the drug substance the results collected in this project are a subject to caution. No supersaturation could be seen in either solid-state form of ezetimibe. The amorphous ezetimibe had the lowest solubility in all experiments. A higher stirring rate seems to affect the dissolution of crystalline ezetimibe negatively. The substance choice should have been made more carefully and a more well-known and well-studied substance should have been chosen.

Acknowledgements

Many thanks to my supervisor Staffan Tavelin who enthusiastically designed the framework for this project and acted as support when the cells decided to kick the bucket.

Thanks to the entire department of pharmacology at Umeå University for your kindness, help, support and amusing lunch-hours. Also thanks to professor Sitaram Velaga and colleagues at Luleå University of technology for donating the model drug and for experience exchange.

Thanks to my fellow students Birgitta and Jasmin for letting me unload and making me laugh.

And last but not least thanks to my partner Carl who always supports and encourages me.
References


