Increasing Solubility of Dexamethasone by Complexation with Beta and Gamma Cyclodextrins

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6TH SEMESTER, BACHELOR PROJECT
SPRING 2017
ROSKILDE UNIVERSITY

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Abstract

The solubility of dexamethasone (DMS) upon adding β- and/or γ- cyclodextrin (CD), was measured by the absorbance at 250 nm and was determined with HPLC. The concentrations of DMS were plotted against the amount of added cyclodextrin to yield a Phase-Solubility Diagram. Three sets of samples were prepared, one for each type of cyclodextrin individually, and one where both types were added. The first two were used to find the concentration of DMS in solution at different amounts of added CD, and yielded B-type Phase-Solubility Diagrams. The βCD complexes had a higher solubility seen by a concentration of dissolved DMS plateau value of 8.4 mM, whereas the γCD data had a plateau value of 7.4 mM. The third experiment was conducted to determine how the concentration of DMS was affected when adding multiple types of CD. The slope of the increase in solubility showed an additive effect, on solubility, from the combined cyclodextrins, however the results indicate that there is a synergistic effect on the maximum solubility of DMS.

Introduction

Limited solubility, limits the efficacy of drugs and is therefore a vital part of drug discovery and development. Drug discovery and development is the field of study relating to drug design, in which each potential drug molecule goes through a stringent set of tests, to eliminate all but one which succeeds to be a drug suitable for the market. Solubility is one of the key properties of a successful drug. A drug must be soluble enough in the gastrointestinal system in order for it to enter the blood and be distributed to its site of action. If a drug is not soluble enough, then there won't be enough that enters the bloodstream to exert the desired effect. Dexamethasone is a drug on the market, yet it is poorly soluble, which might lower its bioavailability. Bioavailability is the percentage of the administered drug that reaches the bloodstream. This study aims to be an investigation of whether, and to what extent, β-and γ- cyclodextrins increase the solubility of dexamethasone. The solubility is measured by HPLC and the results are analysed with Phase-Solubility Diagrams.

Cyclodextrins can be used to increase the solubility of drugs by the formation of inclusion complexes, as cyclodextrins are hydrophilic. The cyclodextrin encapsulates the drug, which increases the total amount of drug in solution. The total amount of drug that can be in solution is then the solubility of the drug itself, plus the solubility of the inclusion complex. The size of the cyclodextrin determines what drugs it can form inclusion complexes with. We use β- and γ- cyclodextrin as we expected them to be able to form inclusion complexes with dexamethasone, as this was observed by a previous group at RUC (Bartoš, Hansen, Pedersen, Larsen, & Lund, 2016).

Phase-Solubility Diagrams show the total concentration of one compound on the y-axis against the added amount of another compound on the x-axis. In this project, the y-axis shows the total amount of dexamethasone in solution against the added amount of cyclodextrin on the x-axis. The Phase-Solubility Diagram gives a visual representation on how the amount of dexamethasone in solution is influenced by the amount of cyclodextrin added. The diagram yields information about the solubility limit of dexamethasone and the complexes. It is also used to indicate how many dexamethasone and cyclodextrin molecules are found in each complex, and how stable the complexes are. This information can be used to find the optimal amount of added cyclodextrin and why the concentration drops when high amounts of cyclodextrin is added. To supplement the Phase-Solubility Diagram, gravimetric analysis was performed on the samples, to find how the cyclodextrin was distributed between the precipitate and solution, which yields additional information about the complexes.

The calculations in this project rely heavily on two types of chemical equilibria. The first is solubility equilibria and the second is stability equilibria. Solubility equilibria is used to describe the interaction between a compound in precipitate and the compound dissolved in solution. The solubility interaction has the useful property that the concentration of the compound in solution is constant if the compound is also in precipitate, independent of the amount of precipitate. This property is used to explain some of the behaviours observed in the Phase-Solubility Diagrams. In this project, the stability equilibria represent how quickly the complexes are formed compared to how quickly they disassociate. This is a measure of how favourable the complexation reaction is.

We conduct three experiments, for which the samples were created by mixing a constant mass of dexamethasone, with different masses of cyclodextrin, and a millilitre of water. The samples were mixed for at least a week, to let them equilibrate. The first two experiments use the two types of cyclodextrins individually to determine how they influenced the solubility of dexamethasone. In the third experiment,

both types of cyclodextrins were used to find the combined impact on the solubility of dexamethasone. The possible impacts are antagonistic, additive, or synergistic. Additive means that it is equal to the sum of the individual effects, synergistic means that it exceeds the sum of the individual effects, and antagonistic means that it is less than the sum of the individual effects.

Problem Formulation

How is the solubility of dexamethasone influenced by utilizing β and γ cyclodextrins? Does the utilization of both cyclodextrins simultaneously result in a non-additive effect on the solubility of the drug?

Hypothesis

- The solubility of dexamethasone should increase due to complexation with cyclodextrins.
- The increase in solubility of dexamethasone with the addition of cyclodextrin should follow a B-type phase solubility, due to an expected limited solubility of the complexes.
- How the amount of dexamethasone in solution changes, will most likely differ depending on the type of cyclodextrin added. We expect a difference due to the distinct physicochemical properties of each cyclodextrin, yielding different intramolecular interactions with the drug.
- The use of both types of cyclodextrins simultaneously may change the solubility of dexamethasone more or less than the additive effect of the two cyclodextrins. Three possible effects for the combination of the two cyclodextrins are: additive, synergistic or antagonistic.

Theory

Drug Discovery & Development

Drug discovery and development is a field of research involving the process of designing new drugs, through which a vast number of potential drug compounds are eliminated, while passing each stage of validation as a drug, from laboratory tests (pre-clinical trials) to human testing (clinical trials). In the end, one drug which is able to withstand each stage is considered authorized to be sold on the market. The later the stage in which a potential drug compound gets rejected, the higher the expense and the more time-consuming. Thus, it is crucial for researchers to perfect the pre-clinical trials to save time and money. The motivation of researchers in drug discovery typically falls under two categories: to stop or reverse the effects of a disease based on new insights of the disease process, as well as taking an existing drug with side effects and modifying the functional groups to hinder the toxic influence on the human body (The Drug Development Process, 2017). During pre-clinical trials, researchers aim to form a successful drug by designing molecules with a good balance of key properties, in order to produce a drug with high efficacy and safety, while maintaining a low cost. Researchers alter potential drug compounds with the aim of including a sufficient amount of the following vital characteristics of a successful drug: potency, safety, absorption, metabolic stability and solubility. One of the first pre-clinical tests done in the laboratory is on solubility, which is the focus of this study.

Solubility as a Determinant of Absorption, Bioavailability and Half-life

Solubility is defined by the maximum quantity of solute that can dissolve in a certain amount of solvent at a specified temperature and pressure. Bioavailability is denoted as the percentage of the administered drug that enters (is absorbed) into the bloodstream, which is therefore able to exert an effect. In order for a drug to enter the bloodstream it must first be soluble in the gastrointestinal system. Thus, bioavailability often goes hand-in-hand with solubility. Furthermore, non-polar compounds are more likely to interact with the main metabolizing enzyme CYP450. The solubility of dexamethasone is rather low at 0.050 mg/ml (Dexamethasone, 2016), its bioavailability is 78% (Brophy, McCafferty, & Tyrer, 1983), and half-life is 34-54 h. Half-life is the rate in which a drug is eliminated from the blood; thus, the solubility of a drug also influences its half-life. If the solubility of a drug is high, more drug is dissolved in the gastrointestinal system, and thus transferred to the bloodstream and absorbed into tissues or eliminated from the plasma, thereby, increasing its half-life. In this study, we aim to establish that adding cyclodextrins, increases the solubility of dexamethasone from 0.050 mg/ml. This could raise its bioavailability to over 78% and expand its half-life.

Problems with Solubility

For orally administered drugs, solubility is a key rate-limiting parameter which hinders the desired concentration in systemic circulation. If the solubility of a drug is too low, it would not dissolve in the fluids of the body on the way to the target cell, such as the fluid of the lumen (i.e., inside space of the intestine), interstitial fluid (i.e., liquid between cells and blood), so that it can enter the bloodstream. This leads to an insufficient amount of active drug in systemic circulation, which lowers the amount present at the target cell to exert its effect (i.e., drug efficacy). Since high bioavailability is a favourable characteristic of an active drug, increasing the solubility of the drug and thereby raising the bioavailability would make for an overall improved drug (Savjani, Gajjar, & Savjani, 2012).

Pathway of Drug: DMPK and ADME

An oral drug or tablet goes through the following pathway in the body: tablet enters the gut, drug in tablet enters the gut, drug is absorbed into the blood, then distributed into the tissues, and interacts with the receptor on a target cell. Two acronyms consisting of these crucial traits of successful drugs, used widely in drug discovery and development, are drug metabolism and pharmacokinetics (DMPK), in addition to, absorption, distribution, metabolism and excretion (ADME).

DM or drug metabolism, refers to how the body breaks down molecules, and PK or pharmacokinetics, denotes the total processes of drug disposition. ADME consists of four important characteristics of drug discovery which are absorption, the movement of the drug from the site of administration to the site of measurement; thus, it usually refers to the absorption from the gut to the bloodstream. Distribution, the reversible transfer of drug between the site of measurement and other sites within the body, which is typically the movement of drug from the blood into the tissues. Metabolism, that is the loss of drug from the body by biochemical conversion which usually occurs in the gut or liver. Finally, elimination, which is the loss of drug from the site of measurement within the body, and excretion is an irreversible elimination or loss of drug from the body.

In vivo vs. *in vitro* Effectivity of Drug

As a common idea, any research carried out *in vitro* does not entirely represent the realistic outcome *in vivo.* In this study, Phase-Solubility Diagrams are used to determine whether, and if so, to what extent the solubility of dexamethasone in the gut is increased by complexation with individual and combined cyclodextrins, and thus, indicate an *in vitro* solubility, and absorption in the blood. However, there is no guarantee that the discovered solubility of the drug *in vitro* will be the same *in vivo,* in the human body. Even if this study is able to reveal that the solubility of dexamethasone increased in complexation with cyclodextrins, this does not indicate the overall effectiveness and potency of dexamethasone. This is because, the exact way the drug behaves in the body, through absorption, metabolism, distribution and excretion (i.e. DMPK), are not considered. This is especially true in this research, since the drug is dissolved in deionized water, which does not represent the following fluids in the body, in which the drug passes: saliva and the lumen, before reaching the bloodstream.

Chemical Equilibria

Chemical equilibrium is a state where the concentration of the components of a chemical reaction does not change with time. Equation 1 shows a chemical reaction which will be used as an example.

$$a \cdot [A] + b \cdot [B] \underset{r_2}{\overset{r_1}{\rightleftharpoons}} c \cdot [C] + d \cdot [D]$$

Equation 1

In this example A and B react to form C and D. The double arrow shows that C and D also react to form A and B. The rates of reaction (r_1, r_2) from one side of the equation to the other, is proportional to the concentration of the reactants. If there is a high concentration of A and B then r_1 is large, causing C and D to be formed faster than if the concentrations of A and B were lower. Likewise, a high concentration of C and D gives a larger r_2, increasing the rate at which A and B is formed. If A and B are reacting faster than C and D $(r_1 > r_2)$, then the concentrations of A and B will decrease and the concentrations of C and D will increase. The changes in concentration will cause r_1 to decrease and r_2 to increase. At some point $r_1 = r_2$ and the concentrations stop changing. The reactions are still taking place but the speed at which

A and B reacts to form C and D, is equal to the rate at which C and D react to form A and B. The concentrations at which this occurs is the chemical equilibria and is described by Equation 2

$$K = \frac{[C]^c \cdot [D]^d}{[A]^a \cdot [B]^b}$$

Equation 2

K is called the equilibrium constant and only varies as a function of temperature. That means that if K is known at a given temperature, the concentration of any of the components can be calculated, if the concentration of the others are known. By using Equation 1 and Equation 2, the equilibrium concentrations can be calculated, if K and the starting concentrations are known.

There are many types of chemical equilibria, but we will focus on two types. The first is solubility equilibria and the second is stability equilibria.

Note that the terms r_1 and r_2 represent the rate of reaction in each direction respectively, and K represents an equilibrium constant. They are unique for each equation unless otherwise stated. For example, K is a constant but can have different values and units for each equation. A specific example of this is that the K in Equation 1 has a different value than the K in Equation 4.

Solubility Equilibria

Solubility equilibria describe the relationship between the rate of dissolution (r_1) of the precipitate (C_s) and the rate of precipitation (r_2) of the solute (C_{aq}). A reaction of this type is shown in Equation 3.

$$C_s \underset{r_2}{\overset{r_1}{\rightleftharpoons}} C_{aq}$$

Equation 3

Solubility equilibria are especially interesting when the amount of C added to the system exceeds the solubility limit of C. Consider a container with a solvent. The compound C is added in excess so that the solvent is saturated with C and the remaining C precipitates. The solubility constant, or just solubility is defined by Equation 4. C_0 is the intrinsic solubility of C, it is the concentration of C when the solvent is saturated with C.

$$K = \frac{[C_{aq}]}{1} = C_0$$

Equation 4

In this scenario, all additional C added to the system precipitates. r_1 is dependent on the concentration of C_s, however C_s is a precipitate and therefore the concentration isn't defined and does not increase when the amount of precipitate increases. When r_1 doesn't increase, the $[C_{aq}]$ doesn't increase, and therefore r_2 doesn't increase. This property means that the value of the denominator in Equation 4 is constant, when there is sufficient C to form precipitate. As the value is constant, it can be transformed to 1 and K becomes equal to the solubility limit of the compound.

This property, exhibited by a saturated system, can be used in experimental setups to keep the concentration of a compound in solution constant even if some is used in a reaction. This lowers the number of variables, and the effects of the concentration of other compounds is more easily determined.

Stability Equilibria

As the name suggests, stability equilibria describe how stable a compound or complex is. It compares the formation/complexation rate (r_1) and the disassociation rate (r_2). A simple reaction of this type is shown in Equation 5.

$$A + B \underset{r_2}{\overset{r_1}{\rightleftharpoons}} C$$

Equation 5

A stability constant or the disassociation constant can be found using Equation 2 to form Equation 6. The two constants are the inverse of each other. The stability constant represents how A and B react to form C, and the disassociation constant represents how C disassociates into A and B.

$$K_{stability} = \frac{[C]}{[A] \cdot [B]} \vee K_{disassociation} = \frac{[A] \cdot [B]}{[C]}$$

Equation 6

The larger the stability constant is, the more stable the compound/complex is. A large disassociation constant, means the compound/complex quickly disassociates. In this project, the stability constant is used. Disassociation constant is mentioned so that the reader is aware of the relation between the two representations.

Inclusion Complexes

An inclusion complex is formed when one chemical compound (host) has a cavity, which is occupied by a second chemical compound (guest). The two compounds do not form covalent bonds and a complex is therefore not a molecule. In this project cyclodextrin is used as the host to increase the amount of guest molecules in solution.

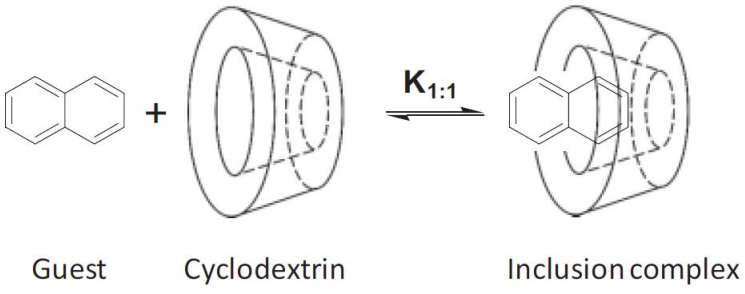

Figure 1. Formation of inclusion complex. *The guest compound binds inside cyclodextrin, forming an inclusion complex. The complex displayed in this figure is a 1:1.*

Equation 7 is the general formula for complexation. It shows m guest (G) molecules associating with n host (H) molecules to form an inclusion complex. In Figure 1 one host and one guest form a complex, which is equivalent to $m = n = 1$ in Equation 7.

$$m \cdot G + n \cdot H \underset{r_2}{\overset{r_1}{\rightleftharpoons}} G_m{:}H_n$$

Equation 7

The stability constant for the general complexation reaction is given by Equation 8.

$$K_{m:n} = \frac{[G_m{:}H_n]}{[G]^m \cdot [H]^n}$$

Equation 8

In some cases, more than one type of complex is formed with each its own stoichiometric ratio. The stoichiometric ratio is a measure of how many guest molecules are present per host molecule in the complex and is equal to $\frac{m}{n}$. It is also possible that more than one complex has the same ratio, for example, a complex with $m = 1, n = 2$ and a complex with $m = 2, n = 4$ would have the same ratio, the calculations done in this project cannot distinguish between complexes with the same ratio. Each type of complex has its own stability constant $K_{m:n}$.

Dexamethasone

Description

Dexamethasone (DMS) is a drug which belongs to the class of organic compounds called 21-hydroxysteroids (i.e., steroids carrying a hydroxyl group at the 21-position of the steroid backbone). The chemical formula is C22H29FO5 (see Figure 2). The indication of the drug when orally administered is to treat allergic responses and bronchial asthma (Dexamethasone, 2016). An orally active drug must be water soluble to reach the intestines, be absorbed into the plasma. Simultaneously, it must have lipophilic properties to allow its penetration into the phospholipid bilayer of intestinal cells, reach the blood, and enter target cells in order to exert its effect. Dexamethasone has a lipophilicity (logP) of 1.68, which is less than 5, required according to Lipinski's Rule of 5, and is thus sufficient to enter cells (Leeson, 2012).

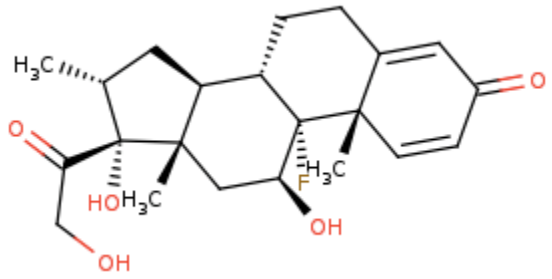

Figure 2. Dexamethasone structure. *The drug is composed of three six-carbon rings, in which one is bound to an oxygen atom. The six-carbon ring furthest from the one attached to the carbonyl oxygen, is bound to a five-carbon ring and an ethane group which is attached to an oxygen atom as well as a hydroxyl group. Obtained from protein data bank (rcsb.org).*

Mechanism of Action

Dexamethasone, a glucocorticoid agonist, crosses cell membranes and binds to cytoplasmic glucocorticoid receptors. This drug-receptor complex acts as a transcription factor and binds to DNA segments (glucocorticoid response elements), which modifies transcription, and thus, protein synthesis – resulting in the following: inhibition of leukocyte infiltration at the inflammation site, interference in the function of inflammatory response mediators, and suppression of humoral immune responses – which overall

causes a reduction in edema or scar tissue. The anti-inflammatory effects of dexamethasone are thought to occur due to the control of the biosynthesis of potent mediators of inflammation such as prostaglandins and leukotrienes, by phospholipase A2 inhibitory proteins (i.e., lipocortins) (Dexamethasone, 2016).

DMS has targets such as the following enzymes: derivatives of Cytochrome P450, aromatase, steroid 17-alpha-hydroxylase/17,20 lyase, mitochondrial cholesterol side-chain cleavage enzyme. Also, it is an agonist of targets which include the following: glucocorticoid receptor, nuclear receptor subfamily 0 group B member 1, annexin A1 and nitric oxide synthase (Dexamethasone, 2016).

Cyclodextrins

Cyclodextrins (CD) are rings consisting of linked glucopyranose units. Common cyclodextrins include αCD, βCD, and γCD, containing 6, 7, and 8 glucopyranose units respectively. The cyclic shape of CD makes it more resistant to hydrolysis than linear sugars (starch) and allows for guest molecules to reside inside the cavity of the ring, forming inclusion complexes (Figure 1). The number of glucopyranose units determines the size of the ring, which in turn determines what size of molecules the CD can form inclusion complexes with. Cyclodextrins with more than 8 units are possible but are of limited interest for creating inclusion complexes with drugs.

Derivatives of the cyclodextrins can be made by substituting the hydroxy groups with other functional groups. Each glucopyranose unit has three hydroxy groups that can be substituted. Substitutions can increase aqueous solubility and/or increase the ability to form drug/CD complexes. The number of hydroxy groups that have been substituted are often given as a percentage of the total number of hydroxy groups, or as the number of substituents per glucopyranose unit. The first gives a number between 0 and 1, 0 being that no substitutions are present and 1 representing that all the groups are substituted. The second common way of representing the number of substituents has a value between 0 and 3, as there are 3 groups that can be substituted per unit. 0 means no substituents are present and 3 means that all groups are substituted.

βCD, and γCD were chosen as the host molecules for DMS in this project as αCD was considered too small. The cyclodextrins used did not have any hydroxy groups substituted. When working with substituted CD then there will be many distinct CD types in each sample, this is because the substitutions are random. The average number of substitutions is known but the exact number and location of each group varies. By choosing CD without any substitution, it is easier to determine the cause of any observed behaviour as there is only one or two host molecules present (βCD and/or γCD).

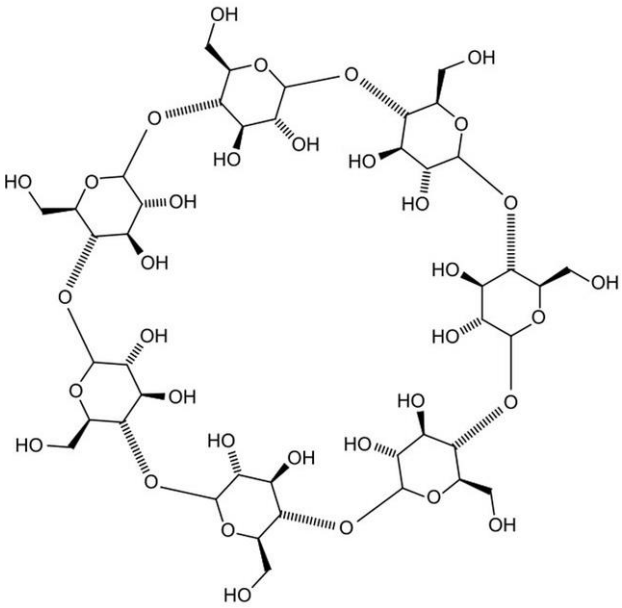

Figure 3. β-Cyclodextrin structure. *Seven heterocyclic compounds composed of five carbons and an oxygen, as well as three hydroxyl groups branching out of each heterocyclic compound, all arranged in a cyclic structure, bound by an oxygen in between each.*

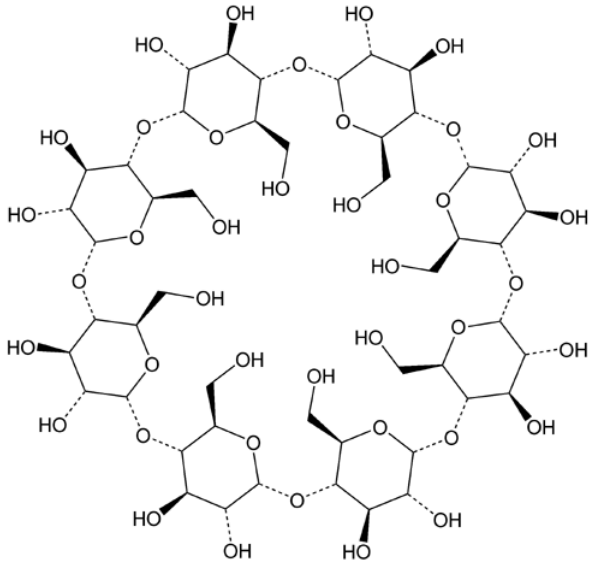

Figure 4. γ-Cyclodextrin structure. *Eight heterocyclic compounds composed of five carbons and an oxygen, as well as three hydroxyl groups branching out of each heterocyclic compound, all arranged in a cyclic structure, bound by an oxygen in between each.*

Phase Solubility Diagrams

The focus of the project is to increase the amount of dissolved DMS (guest(G)). To do this CD (host(H)) is added so that inclusion complexes are formed. This section starts by representing the equilibria that are used to explain Phase-Solubility Diagrams, before explaining the diagram itself.

Equation 9 shows the total amount of guest in solution, when there is excess guest in the form of precipitate. It is the sum of the intrinsic solubility of G (G_0), and the amount of the guest found in the dissolved complexes. G_{tot} is then all G in solution, including that found in dissolved complexes.

$$G_{tot} = G_0 + m_1 \cdot \left[G_{m_1} : H_{n_1}\right] + \cdots + m_i \cdot \left[G_{m_i} : H_{n_i}\right]$$

Equation 9

At low concentrations, it is assumed that only one complex is formed and it has the ratio $1:1$ giving the reaction and equilibria shown in Equation 10. Other complexes may be present at low concentrations but in very small quantities and are therefore not regarded.

$$[G] + [H] \underset{r_2}{\overset{r_1}{\rightleftharpoons}} [G:H] \quad K_{1:1} = \frac{[G:H]}{[G] \cdot [H]}$$

Equation 10

The equations describing the complexation was described in the section Inclusion Complexes. There are three different compounds, each of which can be in equilibrium with precipitate, should the amount exceed the solubility limit. These equilibria are shown in Equation 11

$$G_s \underset{r_2}{\overset{r_1}{\rightleftharpoons}} G_{aq} \qquad K_G = \left[G_{aq}\right] = G_0$$
$$H_s \underset{r_2}{\overset{r_1}{\rightleftharpoons}} H_{aq} \qquad K_H = \left[H_{aq}\right] = H_0$$
$$G:H \underset{r_2}{\overset{r_1}{\rightleftharpoons}} G:H_{aq} \quad K_{G_m:H_n} = \left[G:H_{aq}\right] = G:H_0$$

Equation 11

A Phase-Solubility Diagram shows the amount of one compound in solution against the amount added of another compound. In this project, the y-axis shows the total amount DMS (mM) in solution and the x-axis is the apparent concentration of CD (mM). Here apparent concentration is the number of grams added divided by the solvent volume, the actual concentration can be smaller as some of the CD can be bound in the complexes and precipitate.

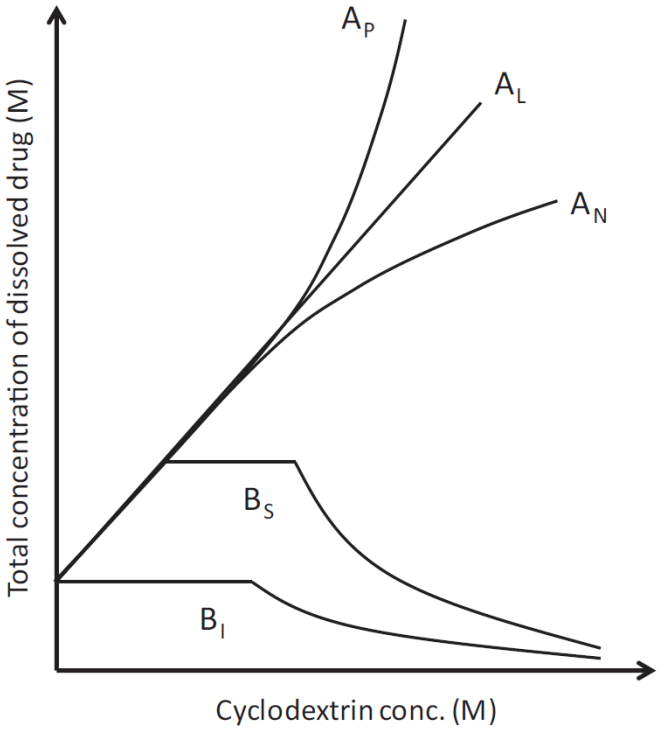

Figure 5. Different types of Phase-Solubility Diagrams. *There are three A-Types and two B-Type Phase-Solubility Diagrams. The A-Type typically grows linearly, exponentially or falls slightly after increasing. The B-Type consists of three segments, a growth, stationary and descending segment.*
Drug Delivery Strategies for Poorly Water-Soluble Drugs, Dennis Douroumis & Alfred Fahr, 2013, Chapter 3, pg. 74, Figure 3.3

The Phase-Solubility Diagrams can be split into two types, called A-type and B-type, and can be used to calculate stoichiometry and stability constants of the complexes. The difference in the types is that, A-type Phase-Solubility Diagrams exhibit a continuous growth in the total amount of drug in solution when adding CD, whereas B-type Phase-Solubility Diagrams have a limit to how much CD can increase the total amount of drug in solution (see **Figure 5**). This limit is normally explained by the complexes having a limited solubility. If additional complexes are formed once the limit is reached, they precipitate and the total amount of drug in solution doesn't increase. A-type phase-solubility diagrams are commonly observed when using CD with substituted hydroxy groups. B-type phase-solubility diagrams are common when using unsubstituted CD (Douroumis & Fahr, 2013) which should increase the solubility of the complexes, and partly due to that a sample with substituted CD will form many distinct complexes that each have their own solubility limit. Unsubstituted CD only has one distinct type of CD, limiting the number of distinct complexes. limiting the number of distinct complexes. limiting the number of distinct complexes. limiting the number of distinct complexes.

In dilute solutions 1:1 complexes are the most common and the slope of the A-type and the initial slope of the B-type Phase-Solubility Diagrams, can be used to calculate the stability constant for the 1:1 complex using Equation 12. G_0 is solubility limit of the guest (DMS) and is called the intrinsic solubility of the guest and can be seen on the Phase-Solubility Diagram as it is equal to the y intercept.

$$K_{1:1} = \frac{\text{Slope}}{G_0 \cdot (1 - \text{Slope})}$$

Equation 12

In dilute solutions, the total concentration of guest in solution, for A-type and the initial slope of the B-type Phase-Solubility Diagrams is given by Equation 13, if the guest is found in the precipitate, as the concentration of guest will be at the solubility (see Solubility Equilibria).

$$G_{tot} = G_0 + [G{:}H] = G_0 + K_{1:1} \cdot G_0 \cdot [H]$$

Equation 13

The concentration of the complex was expanded in Equation 13 by using Equation 10. Note that Equation 13 is not able to represent the total amount of guest in solution, from the beginning of the plateau of the B-type Phase-Solubility diagrams.

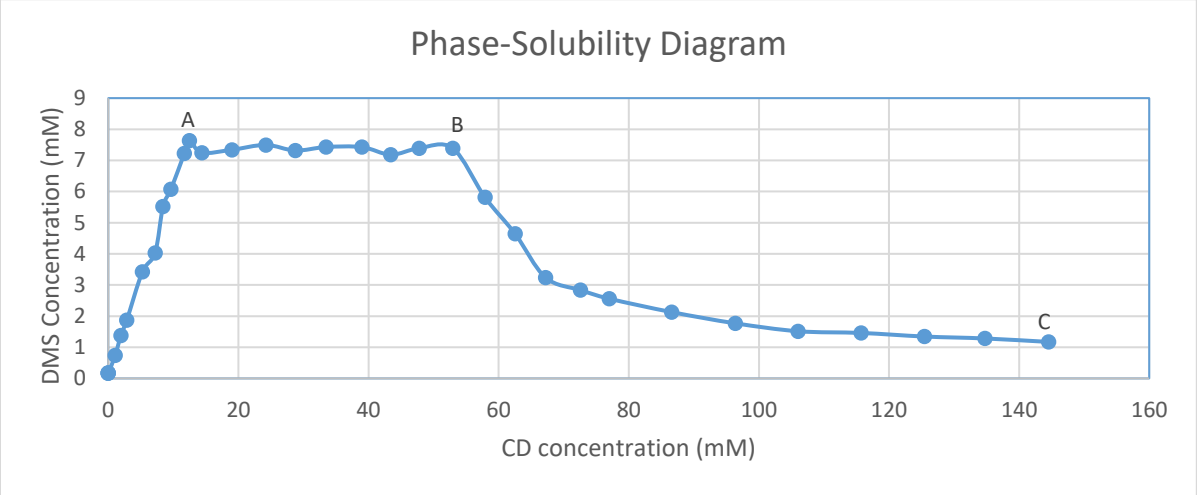

Figure 6. Phase-Solubility Diagram. *The figure shows the concentration of DMS in solution against the amount of γCD added.*

A B-type Phase-Solubility Diagram is shown for the γCD experiment in **Figure 6**. The initial slope used to calculate the stability constant is the points before point A. The plateau seen between points A and B can be explained by a limited solubility of the complexes. The decrease between points B and C is due to depletion of free drug in the precipitate, allowing the concentration of free drug in solution to decrease. Assuming only 1:1 complexes are formed then the total amount of drug in solution should decrease by the intrinsic solubility of the drug. The total concentration of drug in solution is the concentration of free drug plus the concentration of the complex.

$$G_{tot} = [G] + [G{:}H]$$

Equation 14

If the concentration of free drug goes to zero, then the concentration of the complex is the only contribution to the total concentration of dissolved drug.

The decrease in the total concentration of drug from points B to C in **Figure 6**, is much greater than the intrinsic solubility. This can be due to there being more than one type of complex formed or due to non-

inclusion complexes and aggregates being formed. Complexes and aggregates with more than one DMS and/or more than one CD, become more likely the higher the concentration of the two compounds are. This means that the number of larger complexes compared to the total number of complexes, increases proportionally to the amount of CD added.

The stoichiometric ratio between DMS and CD in the complexes can be calculated by using the plateau between points A and B. This is done by assuming, that during the plateau, precipitated drug is replaced by precipitated complex. We also assume that at point A no complex has precipitated and that at point B no free drug is left in the precipitate. By comparing the amount of drug converted between points A and B with the amount of CD added in the same section you get the stoichiometric ratio (Equation 17).

The number of moles of DMS that has complexed during the plateau is then equal to the number of moles added to the samples (DMS_{add}) minus the total number of moles of DMS in solution at point A $\left(c(DMS_A)\right)$.

$$DMS_{add} - c(DMS_A)$$

Equation 15

The number of moles of CD that has complexed is equal to the number of moles of CD added at point B $\left(CD_{add_B}\right)$ minus the amount added at point A $\left(CD_{add_A}\right)$.

$$CD_{add_B} - CD_{add_A}$$

Equation 16

Combining the two gives Equation 17.

$$\frac{DMS}{CD} = \frac{DMS_{add} - c(DMS_A)}{CD_{add_B} - CD_{add_A}}$$

Equation 17

HPLC

High Performance Liquid Chromatography (HPLC) was employed in this study to obtain the absorbance of the samples. A calibration curve, is used to determine the relationship between absorbance and concentration. Using the Lambert-Beer's Law, the amount of UV absorption can be correlated to the concentration of DMS, expressed in the equation: $A = \varepsilon \cdot l \cdot c$, where ε is the molar absorptivity, l is the length of light passing the cuvette which is proportional to the length of the cuvette, and c is the concentration of the analyte (Clark & Gunawardena, 2017). This allows for the determination of DMS concentration in solution of each sample, which is the experimental variable in this research. It is a highly improved column chromatography, which separates, identifies and quantifies components in a sample mixture. Unlike column chromatography, where gravity drives the separation, HPLC is operated by a high pressure of up to 400 atmospheres to move the sample and mobile phase solvent (which is methanol and water in this experiment) through a column (stationary phase) in order to identify, quantify, and separate substances in a sample based on hydrophobicity or hydrophilicity, much more quickly than column chromatography. If the column is hydrophobic, as in this experiment, the sample leaves the hydrophobic stationary phase in the order of increasing hydrophobicity. This is because hydrophobic substances will stick to nonpolar silica, causing polar substances to exit first. Thus, the complexes are separated in the

column, as CD is more polar than DMS, and CD exits the column first and DMS exits later. The separation makes it possible to measure the total concentration of DMS, without interference by complexes. If the HPLC column is hydrophilic, the sample exits the hydrophilic stationary phase, in the order of increasing hydrophilicity. The absorbance is measured by a detector and the information is sent to a computer to be analysed, where it is converted into a chromatogram. The chromatogram can be used to analyse the samples from the milli absorbance units (mAu), and retention time (i.e., the time between injection and detection of sample) of the peaks. The software displays the area under each peak which is proportional to the concentration of the specific substance, due to the established relationship between the two variables on the calibration curve. (Waters: The Science of What's Possible, 2017)

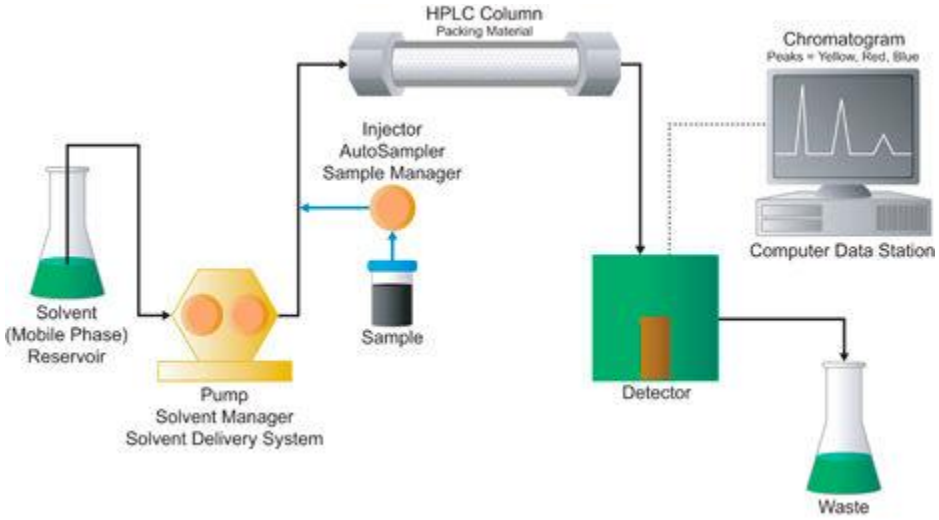

Figure 5. HPLC flowchart displaying each component of the system in the order the sample flows through them. *The mobile phase is passed through to the pump, and upon the stimulus of a high pressure, the sample is added through an injector. The mobile phase and sample then enter the stationary phase when it reaches the HPLC column, where the sample is separated, and subsequently, reaches the detector. The detector then sends the signals to a software on a computer, which converts it into a chromatogram. The chromatogram displays retention time on the x-axis and milli absorbance unit (mAu) on the y-axis. The rest of the mobile phase exits the system as waste (Waters, 2017).*

Methods

This chapter contains the experimental procedure and a section describing some of the choices that were made when designing the experiments.

Protocol

Materials

- 2 ml Eppendorf tubes
- Shaking table (IKA ® KS 4000 ic control)
- HPLC
- HPLC vials
- Scale
- Pipette 1000 µl, 20-100 µl
- 50 ml volumetric flasks
- Filters with 0.2 µm pore size
- Vacuum oven

Reagents

- β-cyclodextrin:
  SIGMA-ALDRICH. Purity: ≥97%;
  Product Id: C4767-100G; Lot #: MKBX4220V
- γ-cyclodextrin:
  SIGMA-ALDRICH. Produced by Wacker Chemie AG, Burghausen, Germany.
  Purity: ≥98%;
  Product Id: W779547-1KG; Lot #: STBF2563V
- Dexamethasone (DMS):
  ACRÖS ORGANICS. Purity: 96%, Code: 230300050, Lot: A036
- Demineralized water
- 99.85% Methanol
- 96% Ethanol

Procedure

Calibration Standards Preparation

Standards with known concentrations of DMS are run through the HPLC to establish the relation between absorbance and concentration of DMS. We want a range of concentrations that can be approximated by a linear relationship between concentration and absorbance. This makes it possible to determine the concentration of DMS in the samples by measuring their absorbance.

1. Prepare two 1 mg/ml stock solutions (Stock A and Stock B) by adding 50 mg $DMS$to a 50 ml volumetric flask and filling with 96% ethanol. Calculate the exact concentration. The concentration will be 2.548 mM if exactly 50 mg $DMS$is added.
2. Dilute the stock solutions 5 and 10 times respectively, by adding 10 ml of Stock A to a 50 ml volumetric flask, and adding 5 ml of Stock B to another 50 ml volumetric flask. Both flasks are filled to 50 ml with demineralized water. Calculate the exact concentration of the work solution it should be about 0.5 mM DMS for Work A and 0.25 mM Work B.

3. Seven calibration standards are prepared from each Work solution, containing 1%, 2%, 3%, 25%, 50%, 75% and 100% of the Work solution respectively. The calibration standards are called A1-7 and B1-7.
   B1 has the lowest concentration $1\% \cdot 0.25\ mM\ =\ 0.0025\ mM$, and A7 has the highest concentration $100\% \cdot 0.5\ mM\ =\ 0.5\ mM$. The exact concentration is calculated.
   A1-3 and B1-3 are prepared in volumetric flasks and 1 ml is transferred to a HPLC vial. A4-7 and B4-7 are prepared directly in the HPLC vials. Each HPLC vial should contain 1 ml solution.
   E.g. A2 is prepared by adding 1000 µl of Work A into a 50 ml volumetric flask and then filled to the mark with demineralized water, after mixing 1 ml is transferred to a HPLC vial. B4 is made by adding 250 µl Work B and 750 µl demineralized water directly into a HPLC vial and mixed.
4. The standards are analysed by HPLC (see HPLC section of Methods).

Preparation of DMS and Cyclodextrin Samples

Different masses of cyclodextrin are added to vials containing a fixed mass of DMS and water. After a week, the supernatants from the vials are measured by HPLC to obtain the absorbance, which tells us the total concentration of DMS in solution.

1. 33 Eppendorf tubes are marked and weighed for each of the individual CD experiments, and 28 Eppendorf tubes are marked and weighed for the combined CD experiment.
2. 13.74 mg of DMS are added to each Eppendorf tube. The exact weight of DMS in each sample is recorded. 13.74 mg of DMS dissolved in 1 ml of solvent is a concentration. This concentration was chosen based on the solubility of γ-cyclodextrin and data previously obtained by another student group (Bartoš, Hansen, Pedersen, Larsen, & Lund, 2016). The amount of 35 mM DMS.
3. The tubes containing DMS are weighed and the weight is recorded.
4. Cyclodextrin is added to the samples.
   a. For each
   b. individual CD experiment, 3 samples are prepared without adding any CD.
      In each of the remaining 30 samples from each experiment, between 1 mM to 150 mM CD is added. The amount added we added can be seen in Appendix II, and the reasons for the specific concentrations is given in the next section. How the number of mg needed to add is calculated, is shown in Example Calculations in the next chapter.
   c. For the combined CD experiment, 1 sample is prepared without adding any CD. 3 samples are prepared with only γCD and 3 samples are prepared with only βCD. 21 samples are prepared with both types of CD. How much we added is explained in the next section.
5. The tubes are weighed again and the weight is recorded.
6. 1 ml water is added to all samples.
7. The tubes are vortexed, sonicated and placed on the shaking table. They are shaken at 400 rpm and 25 degrees for at least one week.
8. The tubes are centrifuged and the supernatant is removed and filtered using a 2 ml syringe (BD Plastipak) and a filter (Phenex RC Membrane with a pore size of 0.2 µm, 4 mm syringe filter), and put in a new marked Eppendorf tube.
9. The remaining precipitate is weighed with the vials and the weight is recorded. The precipitate in the vials, is then dried in a vacuum oven. The samples are dried overnight at 50℃ and 20 mbar. The tubes are weighed again after drying and the weight is recorded.

10. The supernatants are diluted directly in the HPLC vials. The samples from the γCD experiment are diluted 20x, the samples form the βCD are diluted 40x, and the samples from the combined CD experiment are diluted 50x.
11. The dilutions are analysed by HPLC (see HPLC section of Methods).

HPLC

The HPLC is prepared, using necessary materials and adjusting the settings, for injection of the samples. The results are collected with ChemStation.

1. A methanol and demineralized water, mobile phase is prepared in the ratio 7:3, v/v.
2. 1.5 ml of the mobile phase is added to a HPLC vial. This vial will be used to rinse the injector needle between samples.
3. The samples are loaded into the HPLC tray.
4. The Agilent 1100 Series HPLC is turned on for a couple of hours before running the samples, to allow the pressure of the mobile phase to stabilize and to let the lamp in the detector warm up.
5. The settings are adjusted to the following: a flow of 0.8 ml/min, 1 injection of 20 µl per sample, and absorbance is measured for 250 nm. The temperature of the C18 column from phenomenex is set to 30℃. We had a pressure of 115 bar but this is dependent on instrument, column, and flow rate. The absorbance is measured for 8 minutes after each injection.
6. The graph is analysed with Agilent ChemStation to find the area under the absorbance curve with units of mAU*s. (for example see Appendix III)

Experimental Design

This section is split into calibration, Phase-Solubility Samples, and HPLC. These subsections contain the design choices made for the respective protocol sections.

Calibration

Being unfamiliar with the laboratory where we worked, we decided to make two independent stock solutions, to make the calibration standards from. We consider them independent as the two stocks were made in each their own volumetric flask, by adding a determined mass of DMS powder to each flask individually and then filling the flasks to the mark with ethanol. Ethanol was used for the stock as DMS has a low solubility in water. We diluted the two stocks with demineralised water to make the working solutions. They were diluted differently so that a larger range of concentrations could be covered by the calibration standards, without risking increasing errors by serial dilution. Demineralised water was used for all further dilutions of the calibration solutions, as our samples were prepared with demineralised water as the solvent. We wanted the solvent in the calibration standards to be similar to the solvent in our samples, in case the solvent influenced the absorbance or retention time in the HPLC. Seven standards were prepared from each work solution so that if either set of standards gave any obvious deviations, then the other set could be used. The seven calibration standards were prepared in the same way for both working solutions. Should there be a consistent error in the preparation of the calibration standards, then any deviations should have the same pattern in both sets of standards. This could be used to help determine the cause of any observed deviation. The weakest standards were prepared in volumetric flasks, due to the high dilution factor. The high dilution factor meant that the concentration was more sensitive to the accuracy of the pipettes than the standards with a lower dilution factor.

Phase-Solubility Samples

The samples were weighed multiple times during preparation, this was partly done as a precaution to help us explain any deviations we observed, and partly to let us do additional gravimetric analysis to supplement the absorbance data from the HPLC. The change in mass can be used to calculate how many mg has been dissolved. The gravimetric analysis is shown in the next chapter.

In each of the individual CD experiments, 3 samples without any CD were prepared and measured, to determine the intrinsic solubility of DMS. From the paper from a previous group (Bartoš, Hansen, Pedersen, Larsen, & Lund, 2016), we approximated the concentrations, where the different segments of the Phase-Solubility Diagrams would be, when adding an apparent concentration of 35 mM DMS to the samples. Using this information, we chose how much CD to add to each sample so that were 10 samples for each predicted segment. This gave us a total of 33 samples for each individual CD experiment.

The combined CD experiment was performed because, we wanted to determine if there was a non-additive effect on the solubility of DMS when both types of CD are added. We expected that a non-additive effect would affect how much DMS was dissolved per added amount of CD. Therefore, the amount of CD added to each sample was based on the growth segment of the individual CD Phase-Solubility Diagrams, as it was here we expected to see a difference, as these concentrations were known to increase the total concentration of DMS in solution. In some of the samples we added more CD, so that the concentration of added CD was expected to be in the stationary segment. This was a precaution in case we misjudged what concentration the growth segment ends at. The amount of CD to add to the combined CD samples was calculated as a percentage, of the concentration of added CD at the end of the growth phase, from the initial CD data, for βCD we approximated this value to 20 mM and for γCD we approximated to 10 mM. A table with percentages from 0% to 140% with increments of 20, along each axis gave us an 8x8 table of samples. An axis for each type of CD. From the 64 samples, 28 were chosen. A table showing which samples were used for the combined CD experiment can be found in Appendix I. 1 sample was prepared without any CD, which was expected to have the same concentration as the 6 samples without CD from the other two experiments, as they were prepared the same way. It was included so that if the concentrations weren't the same then we knew something was wrong. 3 samples were prepared for each type of CD, containing only that type of CD. These samples were used to compare the combined CD experiment to the individual CD experiments. Ideally, the concentration of these combined CD samples, plotted against the apparent concentration of CD, should give a slope that is identical to the slope of the individual CD experiments' growth segment. Large deviations in the slope indicate an experimental error or low reproducibility. The remaining samples were chosen so that the samples had different ratios of βCD and γCD. If there is a non-additive effect in the growth phase, then the ratio should influence the magnitude of the effect. Knowing how the ratio influences the total concentration of dissolved DMS could yield information about what is causing any observed non-additive effect.

The samples were shaken at 25 degrees, as we wanted to be sure that the temperature was constant. At a lower temperature, we risked that the room became warmer than the temperature we set the shaking table to, leading to the temperature not being held constant, as the table can only heat the samples and not cool them. A higher temperature brought the risk of the supernatant forming precipitate after it had been extracted from the vials. The samples were shaken for at least a week as we wanted to be sure the samples had reached equilibrium, waiting even longer could have been beneficial, but we were also

limited by time. The supernatant was filtered as particulates can have a negative effect on the accuracy of the HPLC. The temperature and pressure at which the precipitate was dried, was determined so that the samples would be completely dry, without a high risk of chemical reactions due to increased temperature.

The dilution factors for the supernatant from the individual CD experiments were determined using the previous group's Phase-Solubility Diagrams. The samples without cyclodextrin were not diluted for these experiments as they were expected to have low enough concentrations. The dilutions should have concentrations of dissolved DMS between 0.0025 mM and 0.5 mM, as this would yield an absorbance between 52.7 mAU*s and 9847.4 mAU*s on the HPLC, and therefore be within our calibration range (Appendix IV). For the γCD experiment, the data of the previous group showed that the concentration of DMS never exceeded 6 mM. Diluting 20x would give a maximum concentration of 0.3 mM which is within our range. As we observed a slightly higher concentration of dissolved DMS in our γCD data than the previous group and the highest concentration of dissolved DMS was about 10 mM for the previous group's βCD experiment, we decided to use a 40x dilution for the βCD samples. The dilution factor for the combined CD experiment was determined from the results of the first two experiments. The highest concentration we should observe, should be the sum of the heights of the stationary segments from the initial CD experiments, giving $8.2 + 7.4 = 15.6\,mM$. The numbers can be found in the next chapter. Diluting 50x puts the concentration within our calibration range. We decided to dilute the sample without CD as well, as the dilution should still lie within the calibration range.

HPLC

The choice of mobile phase, injection volume, flow rate, column temperature, which wavelength absorbance was measured at, and how long the absorbance was measured for after injection, were all chosen by our supervisor Christian Schönbeck. The wavelength that is measured is where DMS has a good absorbance. The mobile phase and column were chosen based on DMS being very non-polar. This means that a non-polar column slows down DMS and CD passes through quickly, effectively separating the two so that the concentration of DMS can be measured directly, without worrying about if the complex affecting the absorbance. The mobile phase needs to be partly non-polar, or DMS will not leave the column.

Results/Analysis

This section contains our results and how they were analysed. We include the calculations that were applied to our results. The chapter is split into 4 parts:

1. Calibration data
2. How the calculations were performed on the experimental data
3. Experimental data from the first two experiments, one for each individual CD
4. Experimental data for the experiment with combined CD

Calibration Data

Knowledge of the relation between absorbance and concentration is vital because the HPLC yields an absorbance, and the concentration of DMS in solution (mM) is necessary to plot a Phase-Solubility Diagram. This was achieved with a calibration curve. Our aim in carrying out the calibration is to find a range where a linear correlation, with a R^2 value close to 1, is observed between the absorbance and concentration. An absorbance (mAU*s) that lies within a range with this behaviour can be directly assigned to a concentration. Thus, the absorbance obtained from the HPLC within this interval, would most likely correlate accurately to the concentration of DMS.

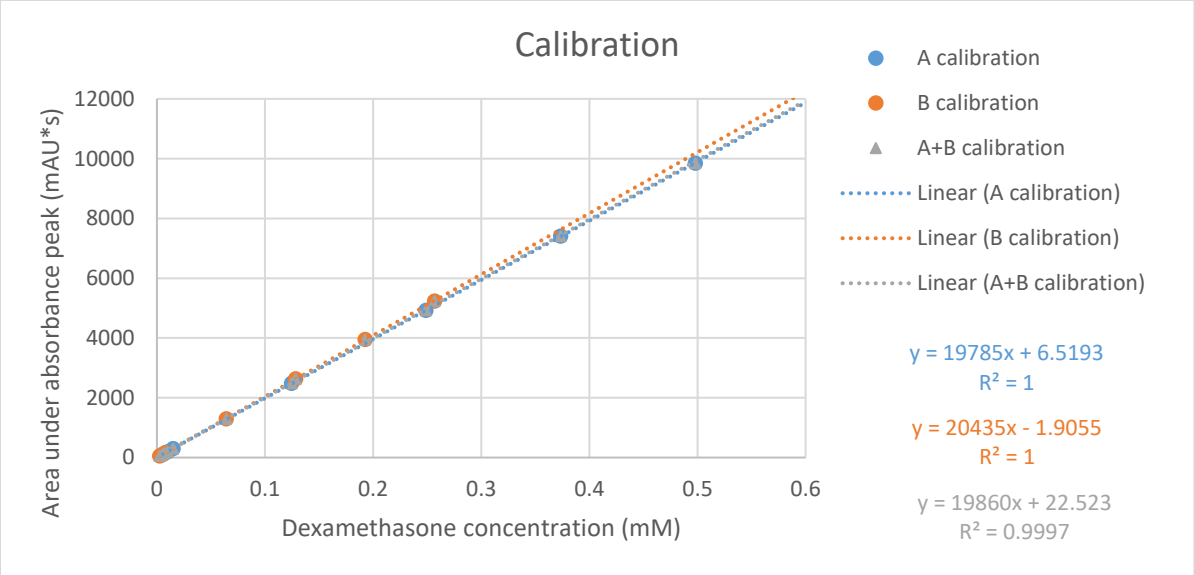

Figure 7. Calibration curves for dilution A, B and the average of A and B. *Dilution A is in blue, dilution B is in orange and the average of A and B is displayed in grey. The variables are the following: dexamethasone concentration (mM) on the x-axis and area under absorbance peak (mAU*s) on the y-axis. The linear equations and R^2 values for the calibration of dilution A, B and the average of A and B are shown in their respective colours on the right hand side. The specific values used can be seen in Appendix IV.*

The two sets of calibration standards, A and B, both have correlation coefficients R^2 not significantly different to 1, which means there is a strong correlation between absorbance (mAU*s) and DMS concentration (mM). The R^2 values also indicate that there are only minor experimental deviations, due to factors such as pipetting and instrumental error. However, the two datasets do not coincide as shown by the difference in slope (See **Figure 7**). Calibration standards A and B were made and determined within minutes of each other so factors such as temperature, humidity and HPLC conditions are unlikely the cause of the difference observed. The difference is most likely due to gravimetric errors rather than

pipetting errors. The standards were made by pipetting x ml stock and y ml of water into vials. If the volumes pipetted to make the standards deviated, then the R^2 value would be smaller, as the concentration of the calibration standards would deviate from the expected. As the difference in slope is not likely due to pipetting errors from preparing the standards, it can be concluded that the stocks from which the standards were produced, are the likely source of error. The concentration of the stock was determined from the weight of the DMS added and the volume of the volumetric flask. As standards A1-3 and B1-3 also were diluted by use of volumetric flasks and that their responses were accurate, the gravimetric uncertainty from weighing the DMS is the most likely reason for the difference between the datasets. The minor difference in slope indicates that the uncertainty is small. It is also possible that the purity of the DMS powder was not homogeneous in the container, causing the calculated concentration to deviate, however gravimetric uncertainty is still more probable.

We use both sets of data points for our calibration curve as they are very similar and there is no indication as to one being more correct. The combined data has a R^2 of 0.997. Even though it is a smaller coefficient, we consider it to be a better representation than either set alone, as it incorporates the slight variance of the gravimetric measurements.

Example Calculations

This section contains all the constants and calculations employed while analysing the data. There are two tables: the first displays the constants used in the calculations, and the second contains descriptions and any calculations carried out. The second table is separated into rows representing those in the results tables (see Appendix II). The calculations shown are the same as those used on the data.

Constants	Value	Abbreviation	Notes
Gamma CD g/mol	1297.12	γCD_{Mw}	$\frac{g}{mol} = \frac{mg}{mmol}$
Beta CD g/mol	1134.98	βCD_{Mw}	$\frac{g}{mol} = \frac{mg}{mmol}$
DMS g/mol	392.46	DMS_{Mw}	
Sample Volume ml	1	V_s	
Dilution factor	20, 40, 50	D_F	Each experiment had its own D_F
Calibration Intercept mAU*s	22.52	C_{Inter}	
Calibration Slope mAU*s/mM	19859.65	C_{Slope}	
Impurities mg	0.7499	DMS_{imp}	This is the average weight loss of the vials without added CD. The weight loss is determined, using the weights of the vials with all the components, and the weight of the vials with the precipitate after the supernatant was removed and subsequently dried. The first weight, minus the second weight, minus the weight of added water, minus the amount of mg of DMS measured in solution, is the calculated amount of impurities in the DMS. The average of the impurities is then calculated.

Name	Description/calculation
Chosen [CD] (mM) CD_{chosen}	Each vial had a chosen concentration. Our choices are described in the method chapter in the section Experimental Design.
Required CD (mg) CD_{req}	The amount of CD needed to give the desired concentration. $$CD_{req} = CD_{chosen} * CD_{Mw} * \frac{V_s}{1000}$$
Actual CD (mg) CD_{add}	The amount of CD that was added to the sample.
Actual DMS (mg) DMS_{add}	The amount of DMS that was added to the sample.
Water added (mg) V_{add}	The amount of water added by pipette. It was calculated by weighing the vial with water and subtracting the weight before water was added. This was measured as a precaution. It is assumed that 1000 µg of water is equal to 1 ml.
HPLC output (mAU*s) A_s	The output is determined with ChemStation in mAU*s and written into Excel. An example of a HPLC report, made by ChemStation can be seen in Appendix II.
Relative Absorbance (%) R_{absorb}	4 calibration standards were measured alongside the samples in the HPLC for each experiment. The average difference in the original response (A_{co}) and the response gained alongside the samples (A_{cs}), was used to compensate for day to day variations. $$R_{absorb} = \frac{\frac{A_{cs1}}{A_{co1}} + \frac{A_{cs2}}{A_{co2}} + \frac{A_{cs3}}{A_{co3}} + \frac{A_{cs4}}{A_{co4}}}{4}$$
Adjusted output (mAU*s) A_{rs}	The value that is given when HPLC output is adjusted for day to day variations. $$A_{rs} = \frac{A_s}{R_{absorb}}$$
Calculated [DMS] (mM) $c(DMS)$	The total concentration of dissolved DMS in the samples is calculated from the adjusted output, the calibration data, and the dilution factor. This calculation has two steps. First the A_{rs} is put into the trendline equation for the calibration standards. $$y = a * x + b$$ $$A_{rs} = C_{Slope} * c(DMS_{HPLC}) + C_{Inter}$$ And then rearranged to isolate the total concentration of DMS in the HPLC vials. $$c(DMS_{HPLC}) = \frac{A_{rs} - C_{Inter}}{C_{Slope}}$$ The second step is to multiply by the dilution factor to find the original concentration in the sample. $$c(DMS) = c(DMS_{HPLC}) \cdot D_F$$
Calculated [CD] (mM) $c(CD)$	The apparent concentration of CD in the sample. $$c(CD) = \frac{CD_{add}}{CD_{Mw}} \cdot \frac{1}{V_{add} \cdot 10^{-6}}$$

Calculated [CD]aq (mM) $[CD]_{aq}$	Each vial was weighed multiple times throughout the project which allowed us to conduct gravimetrical analyses, to determine how the CD was distributed between solution and precipitate. This was calculated in multiple steps. The first step was to calculate the weight difference between the weight of the added water (V_{add}), and the weight lost after removing the supernatant and then drying. Vial_{before} is the weight of the vials before the supernatant is removed and Vial_{after} is weight of the vial with dried precipitate. The weight difference represents how much mass was dissolved in the water. $$W_{dif} = V_{add} - (\text{Vial}_{before} - \text{Vial}_{after})$$ The next step was to determine how much of the weight loss was due to dissolved DMS. This is determined using the calculated concentration of DMS and the volume of the solvent. $$DMS_{dis} = c(DMS) \cdot DMS_{Mw} \cdot V_{add} \cdot 10^{-6}$$ The third step is to calculate how many mg of CD was in the solution. This is done by assuming that the weight loss, that isn't explained by dissolved DMS and DMS_{imp}, is due to dissolved CD. The result of this step is shown in the table in Appendix II. $$CD_{aq} = -W_{dif} - DMS_{dis} - DMS_{imp}$$ The last step is to calculate the concentration. This value is plotted in **Figure 8** and **Figure 9**. $$[CD]_{aq} = \frac{CD_{aq}}{CD_{Mw}} \cdot \frac{1}{V_{add} \cdot 10^{-6}}$$
Calculated [CD]s (mM) $[CD]_s$	The amount of CD in the precipitate is calculated by taking the weight of CD added to the sample minus the weight in solution. Appendix II shows this result in the table. $$CD_s = CD_{add} - CD_{aq}$$ To make it easier to read the figures, the amount of CD in the precipitate was also calculated as a concentration. This value is used in the figures. $$[CD]_s = c(CD) - [CD]_{aq}$$

Individual CD

This section is divided into five subsections: Overview of the Phase-Solubility Diagram and distribution of CD, intrinsic solubility, growth segment, stationary segment and descending segment. It is organized this way to focus on specific parts of the Phase-Solubility Diagram for both types of CD simultaneously, as each part was analysed in the same way for both types of CD. This categorization also makes it easier to compare the two Phase-Solubility Diagrams.

Phase-Solubility Diagram and CD Distribution

This subsection includes general descriptions of the Phase-Solubility Diagrams plotted from our data, and how CD was distributed between precipitate and solution, in our samples. The figures are compared with the theoretical B-Type Phase-Solubility Diagram and deviations are discussed. Correlations between the distribution of CD and the Phase-Solubility Diagrams are also noted.

Beta

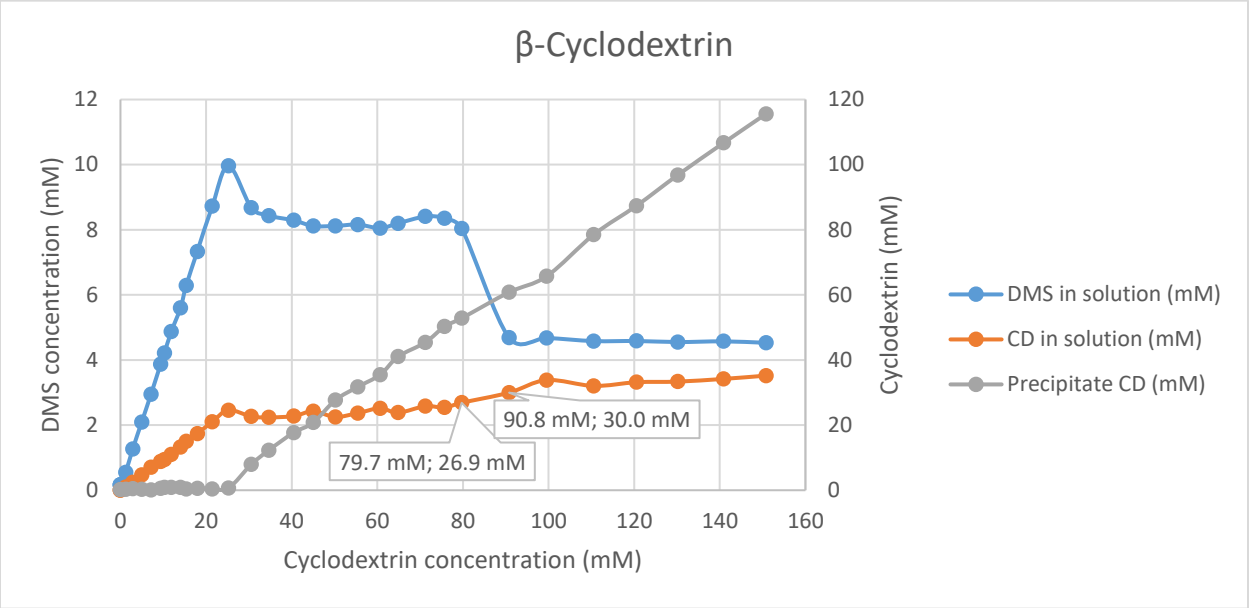

Figure 8. βCD Phase-Solubility Diagram and distribution of βCD in solution and precipitate. *The blue line displays "Calculated DMS" vs "Calculated [CD]" giving a Phase-Solubility Diagram. The orange line exhibits the "Calculated CDs" vs "Calculated [CD]" and the grey line displays "Calculated CDaq" vs "Calculated [CD]" (See Appendix II).*

There are two immediately apparent deviations between the data and the expected B-Type Phase-Solubility Diagram. The first one is the high concentration of dissolved DMS at around 25 mM of CD added, before the plateau. If the data had followed the theory, then the concentration of dissolved DMS would have increased until it reached the plateau. Instead, what is observed is that the concentration of dissolved DMS overshoots before decreasing to the plateau. The reason for this spike could be due to oversaturation of complex. Oversaturation is when there is more solute in the solvent than the intrinsic solubility, if this is the case, then the samples had not reached equilibrium. Oversaturation could happen if the complexes are forming and dissociating before precipitation can occur. The second deviation is the sudden decrease seen at the end of the plateau at 80 mM of added CD, after which the concentration of dissolved DMS stays relatively constant. The concentration of dissolved DMS is expected to gradually approach a stable value, rather than the abrupt drop observed. One reason could be that the fall only appears to be abrupt due to an absence of samples between 80 and 90 mM of added CD. Another reason could be due to the solubility of beta cyclodextrin which is 18.4 mg/ml (Douroumis & Fahr, 2013). This solubility is between 15-20 mM CD, meaning more beta cyclodextrin than is soluble was added to the majority of samples. The calculations indicate that the concentration of dissolved CD exceeds the solubility limit of pure beta cyclodextrin, at two plateaus at the same intervals of added CD, as the two plateaus of the Phase-Solubility Diagram. This could indicate that the change in plateau value is due to the depletion of free DMS in precipitate. To show this, the following reactions are assumed to apply:

$$DMS + \beta CD \rightleftharpoons DMS{:}\,\beta CD$$
$$DMS{:}\,\beta CD + (n-1)\cdot \beta CD \rightleftharpoons DMS{:}\,\beta CD_n$$

Equation 18

With the equilibria:

$$K_1 = \frac{[DMS{:}\,\beta CD]}{[DMS] \cdot [\beta CD]}$$

Equation 19

$$K_2 = \frac{[DMS{:}\,\beta CD_n]}{[DMS{:}\,\beta CD] \cdot [\beta CD]^{n-1}}$$

Equation 20

Two complexes are assumed to form in the system: $DMS{:}\,\beta CD$ and $DMS{:}\,\beta CD_n$. The difference is that one contains one βCD and the other one contains more than one. Initially, the concentration of dissolved DMS is constant; therefore, as $[\beta CD]$ increases, K_1causes $[DMS{:}\,\beta CD]$ to rise. This in turn causes $[DMS{:}\,\beta CD_n]$ to increase due to K_2. This continues until the first plateau, where the solubility limit of $DMS{:}\,\beta CD$ is reached. At this point, the precipitate only has free DMS and DMS_{tot} is the sum of the intrinsic solubility of the drug plus the intrinsic solubility of $DMS{:}\,\beta CD$ plus the $[DMS{:}\,\beta CD_n]$.

$$DMS_{tot} = DMS_0 + DMS{:}\,\beta CD_0 + [DMS{:}\,\beta CD_n] = DMS_0 + DMS{:}\,\beta CD_0 + K_1 \cdot K_2 \cdot DMS_0 \cdot [\beta CD]^n$$

Equation 21

During the first plateau, the βCD_{tot} is constant even though the amount of added βCD increases. There is excess DMS in the precipitate, so the $[DMS]$ is constant due to solubility equilibria. For K_1 to be constant, the $[\beta CD]$ and the $[DMS{:}\,\beta CD]$ need to be constant. Alternatively, the $[\beta CD]$ and the $[DMS{:}\,\beta CD]$ increases simultaneously but this does not fit with gravimetric data, and we therefore exclude this possibility. For the $[\beta CD]$ to stay the same it must either precipitate or form $DMS{:}\,\beta CD$. Similarly, the $[DMS{:}\,\beta CD]$ stays the same by the complex precipitating or reacting to form $DMS{:}\,\beta CD_n$. The $[DMS{:}\,\beta CD_n]$ must also stay the same due to K_2 so if the complex is formed it must precipitate.

Since free βCD has a higher solubility than the complexes, adding additional βCD will form complexes that precipitate when DMS is available rather than stay as free βCD in solution, so the solubility limit of βCD is not reached during the first plateau. Therefore, during this plateau DMS in the precipitate is replaced by the complexes.

The drop between the plateaus can be explained if the DMS in the precipitate is depleted at this point. Since up to this point, DMS in precipitate was replenishing the $[DMS]$, now that DMS in precipitate has been depleted, the $[DMS]$ can decrease. To satisfy Equation 19 either the $[\beta CD]$ must increase and/or the $[DMS{:}\,\beta CD]$ must decrease to compensate for the decrease in the $[DMS]$. Even though βCD_{tot} does increase, it is not sufficient to compensate for the $[DMS]$, that is presumably approaching 0; therefore, the $[DMS{:}\,\beta CD]$ must be the experimental variable that is decreasing. If the $[DMS{:}\,\beta CD]$ is decreasing, then $DMS{:}\,\beta CD$ cannot be present in the precipitate. Therefore, the drop in DMS_{tot} can be explained by the depletion of the $[DMS]$ and the $[DMS{:}\,\beta CD]$ in the system. DMS_{tot} is then equal to the solubility of $DMS{:}\,\beta CD_n$, from this point and onwards (see Equation 22).

$$DMS_{tot} = DMS{:}\,\beta CD_{n_0}$$

Equation 22

Given that the system can be explained by two complexes, the following can be calculated:

- The solubility limit of $DMS{:}\,\beta CD$
- The value of n in $DMS{:}\,\beta CD_n$

The solubility limit of $DMS{:}\,\beta CD$ can be calculated from the values of the plateaus on the Phase-Solubility Diagram. The upper plateau is the solubility of DMS, $DMS{:}\,\beta CD$ and the $[DMS{:}\,\beta CD_n]$. The $[DMS{:}\,\beta CD_n]$ is likely to be close to the intrinsic solubility of $DMS{:}\,\beta CD_n$ and is assumed to be equal. The lower plateau is the solubility limit of $DMS{:}\,\beta CD_n$. The solubility of DMS is significantly smaller than that of the two types of complexes, so it is disregarded. Therefore, the solubility of $DMS{:}\,\beta CD$ is equal to the value of the upper plateau minus the value of the lower plateau (see Equation 23)

$$DMS_{totPlat1} \approx DMS{:}\,\beta CD_0 + DMS{:}\,\beta CD_{n_0} \wedge$$
$$DMS_{totPlat2} = DMS{:}\,\beta CD_{n_0} \Longrightarrow$$
$$DMS{:}\,\beta CD_0 \approx\ DMS_{totPlat1} - DMS_{totPlat2} = 8.2 - 4.5 = 3.7\ mM$$

Equation 23

The solubility limit of $DMS{:}\,\beta CD$ is therefore 3.7 mM.

By comparing DMS_{tot} with βCD_{tot} at the lower plateau, a rough estimate of n can be calculated. DMS_{tot} will be the amount of DMS in $DMS{:}\,\beta CD_n$, and βCD_{tot} will be the amount of βCD in the complex plus the solubility of βCD. With these numbers, then the number of βCD in the complex compared to the number of DMS in the complex can be calculated (see Equation 24).

$$\beta CD_{complex} \approx 35 - 17 = 18\ mM$$
$$DMS_{complex} \approx 4.5\ mM \Longrightarrow$$
$$n = \frac{18\ mM}{4.5\ mM} = 4$$

Equation 24

This result indicates that there are 4 times as many βCD than DMS in the large complex.

Gamma

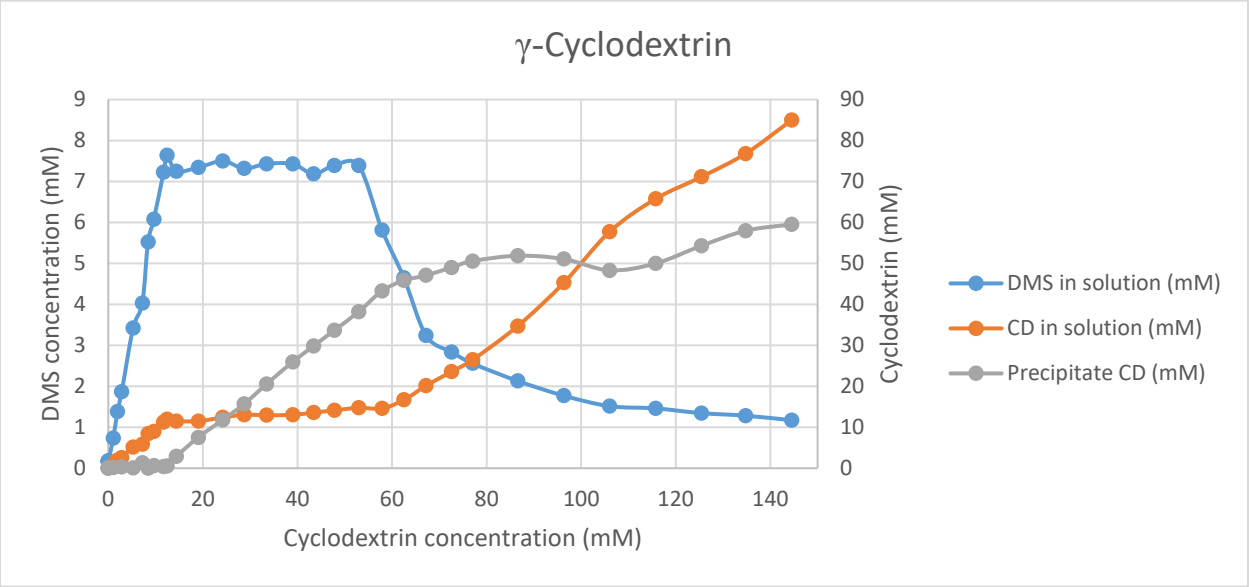

Figure 9. γCD Phase-Solubility Diagram and distribution of γCD in solution and precipitate. *The blue line displays "Calculated [DMS]" vs "Calculated [CD]" giving a Phase-Solubility Diagram. The orange line exhibits the "Calculated [CD]s" vs "Calculated [CD]" and the grey line displays "Calculated [CD]aq" vs "Calculated [CD]" (see Appendix II)*

There is one immediately apparent deviation between the data and the expected B-Type Phase-Solubility Diagram. This deviation is the drastic amount that the total concentration of DMS decreases after the plateau. The decrease is much larger than what can be theoretically explained if $DMS{:}\,\gamma CD$ is the only complex present. This seems to indicate that there is at least one additional type of complex formed. We will consider all possible additional complexes as one complex, namely, $DMS{:}\,\gamma CD_n$. n represents the average number of γCD in the additional complexes.

This gives the following reactions:

$$DMS + \gamma CD \rightleftharpoons DMS{:}\,\gamma CD$$
$$DMS{:}\,\gamma CD + (n-1)\cdot \gamma CD \rightleftharpoons DMS{:}\,\gamma CD_n$$

Equation 25

With the equilibria:

$$K_1 = \frac{[DMS{:}\,\gamma CD]}{[DMS]\cdot[\gamma CD]}$$

Equation 26

$$K_2 = \frac{[DMS{:}\,\gamma CD_n]}{[DMS{:}\,\gamma CD]\cdot[\gamma CD]^{n-1}}$$

Equation 27

Initially, the concentration of dissolved DMS is constant; therefore, as the $[\gamma CD]$ increases, K_1 causes the $[DMS{:}\,\gamma CD]$ to rise. This in turn causes the $[DMS{:}\,\gamma CD_n]$ to increase due to K_2. This continues until the

plateau, where the solubility limit of $DMS{:}\,\gamma CD$ is reached. At this point, the precipitate only has free DMS, and DMS_{tot} is the sum of the solubilities of DMS, $DMS{:}\,\gamma CD$ and the $[DMS{:}\,\gamma CD_n]$.

$$DMS_{tot} = DMS_0 + DMS{:}\,\gamma CD_0 + [DMS{:}\,\gamma CD_n] = DMS_0 + DMS{:}\,\gamma CD_0 + K_1 \cdot K_2 \cdot DMS_0 \cdot [\gamma CD]^n$$

Equation 28

During the plateau, the γCD_{tot} is constant even though the amount of added γCD increases. There is excess DMS in the precipitate, so the $[DMS]$ is constant due to solubility equilibria. For K_1 to be constant, the $[\gamma CD]$ and the $[DMS{:}\,\gamma CD]$ need to be constant. For the $[\gamma CD]$ to stay the same it must either precipitate or form $DMS{:}\,\gamma CD$. Similarly, the $[DMS{:}\,\gamma CD]$ stays the same by the complex precipitating or reacting to form $DMS{:}\,\gamma CD_n$. Since $DMS{:}\,\gamma CD_n$ has reached its solubility limit, the $[DMS{:}\,\gamma CD_n]$ must stay the same due to K_2, so if additional complex is formed, it must precipitate.

The drop after the plateau can be explained if the DMS in the precipitate is depleted at this point. When the DMS in precipitate has been depleted, the $[DMS]$ can decrease. To satisfy K_1, the $[\gamma CD]$ must increase and/or the $[DMS{:}\,\gamma CD]$ must decrease to compensate for a decrease in the $[DMS]$. γCD_{tot} increases as seen in the gravimetric data (see **Figure 9**). Given as $DMS{:}\,\gamma CD$ is assumed to have reached its solubility limit, the increase in γCD_{tot} must be due to the $[\gamma CD]$. It is possible that $DMS{:}\,\gamma CD_n$ was not quite at its solubility limit, and therefore the $[DMS{:}\,\gamma CD_n]$ might have a small contribution. According to K_2, an increase in the $[\gamma CD]$ will increase the amount of the $DMS{:}\,\gamma CD_n$ being formed, of which the majority will precipitate, since it is close to its solubility limit. Therefore, DMS_{tot} will approach the intrinsic solubility of $DMS{:}\,\gamma CD_n$.

$$DMS_{tot} = DMS{:}\,\gamma CD_{n_0}$$

Equation 29

The solubility of $DMS{:}\,\gamma CD$ can be calculated from the value of the plateau, and the value DMS_{tot} approaches, on the Phase-Solubility Diagram. The plateau is the solubility of DMS, $DMS{:}\,\gamma CD$ and the $[DMS{:}\,\gamma CD_n]$. The $[DMS{:}\,\gamma CD_n]$ is likely to be close to the intrinsic solubility and is assumed to be equal. DMS_{tot} approaches a stable value that is assumed to be the solubility of $DMS{:}\,\gamma CD_n$. The solubility of DMS is significantly smaller than that of the two types of complexes, so it is disregarded. Therefore, the solubility of $DMS{:}\,\gamma CD$ is equal to the value of the plateau minus the value that the total concentration of dissolved DMS approaches (see Equation 30).

$$DMS_{totPlat} \approx DMS{:}\,\gamma CD_0 + DMS{:}\,\gamma CD_{n_0} \wedge$$
$$DMS_{totApproach} = DMS{:}\,\gamma CD_{n_0} \Longrightarrow$$
$$DMS{:}\,\gamma CD_0 \approx\ DMS_{totPlat} - DMS_{totApproach} = 7.4 - 1.1 = 6.3\ mM$$

Equation 30

The solubility of $DMS{:}\,\gamma CD$ is therefore 6.3 mM.

To calculate n, a sample saturated with γCD is necessary. Since γCD_{tot} does not reach a plateau after the drop in $[DMS]$, then the samples are not saturated with γCD. This is due to the high intrinsic solubility of γCD. The reason a saturated sample is needed is because, n is calculated from γCD_{tot} of a saturated sample.

Intrinsic Solubility

For each of the two individual CD experiments, three samples were prepared without CD. These samples were used to determine the intrinsic solubility of DMS. This value is needed for many of the calculations.

Beta

The following table shows the data from the βCD samples without CD.

Name	Initial 1	Initial 2	Initial 3
DMS added (mg)	13.81	13.65	13.82
*Milli Absorbance units*s*	3412.38	3454.7	3411.89
Concentration (mM)	0.1644	0.1665	0.1644

Table 1. Intrinsic Solubility of DMS from Beta CD data. *Concentration is calculated using the calibration data.*

The average concentration of DMS from the three samples was calculated to $0.1651\ mM$, and used as the intrinsic solubility.

The standard deviation of the concentration of DMS is $1.19 \cdot 10^{-3}\ mM$.

Gamma

The table below displays data from the γCD samples without CD.

Name	Initial 1	Initial 2	Initial 3
DMS added (mg)	13.52	13.65	13.78
*Milli Absorbance units*s*	3629.85	3562.20	3402.18
Concentration (mM)	0.1774	0.1741	0.1662

Table 2. Intrinsic Solubility of DMS from Gamma CD data. *Concentration is calculated using the calibration data.*

We use the average to get a DMS_0 of $0.1726\ mM$.

The standard deviation of the concentration of DMS is $5.75 \cdot 10^{-3}\ mM$. This value is higher than the standard deviation of the βCD samples. The Initial 3 value is significantly closer to the βCD samples than Initial 1 and 2. We were not able to determine the cause of this.

Growth Segment

In this section, the first segment of our B-Type Phase-Solubility Diagrams produced from the βCD and γCD data, are analysed. This segment can be used to determine the stability constant from the slope of the line.

Beta

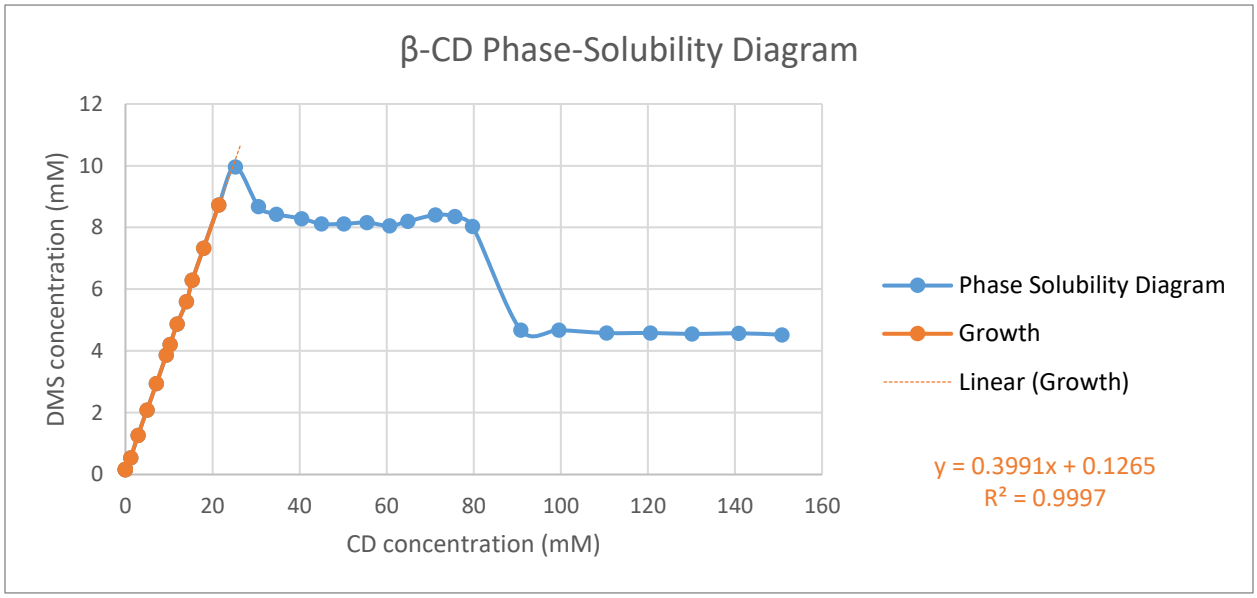

Figure 10. βCD Phase-Solubility Diagram. *The βCD Phase-Solubility Diagram is displayed including the ascending βCD concentration (mM) on the x-axis, and DMS concentration (mM) on the y-axis. The growth segment is highlighted in orange.*

The growth segment is the data points starting from the intrinsic solubility samples and ending at the start of the plateau. The stability constant for the $\beta CD{:}\, DMS$ complex was calculated using the slope of the trendline of the growth segment and the intrinsic solubility previously determined. The peak point around 25 mM βCD, was disregarded, as it is above the plateau.

$$\text{Slope} = 0.3991\ \frac{mM_{DMS}}{mM_{\beta CD}}$$

$$DMS_0 = 1.651\ mM$$

The stability constant for the $\beta CD{:}\, DMS$ complex is given by Equation 31.

$$K_{1:1} = \frac{0.3991 \dfrac{mM_{DMS}}{mM_{\beta CD}}}{1.651\ mM_{DMS}\left(1 - 0.3991 \dfrac{mM_{DMS}}{mM_{\beta CD}}\right)} = 0.4023\ mM_{DMS}^{-1}$$

Equation 31

Gamma

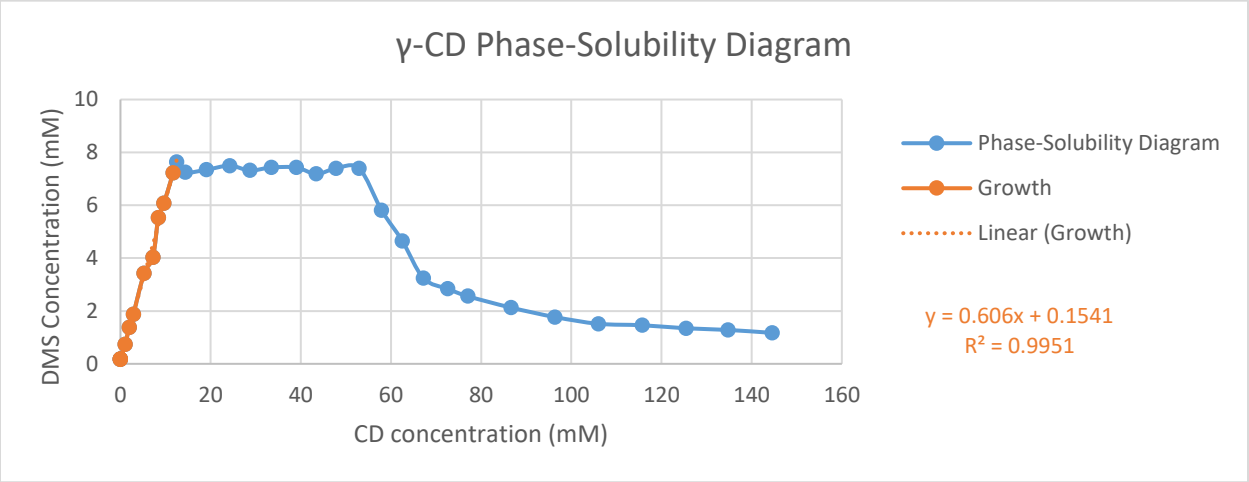

Figure 11. γ-CD Phase-Solubility Diagram. *The γCD Phase-Solubility Diagram is shown, consisting of the ascending γCD concentration (mM) on the x-axis and DMS concentration (mM) on the y-axis. The growth segment is highlighted in orange.*

The growth segment is the data points starting from the intrinsic solubility samples and ending at the start of the plateau. The stability constant for the $\gamma CD{:}\,DMS$ complex was calculated using the slope of the trendline of the growth segment and the intrinsic solubility previously determined.

$$\text{Slope} = 0.606\frac{mM_{DMS}}{mM_{\beta CD}}$$
$$DMS_0 = 1.726\ mM$$

The stability constant for the $\gamma CD{:}\,DMS$ complex is given by Equation 32.

$$K_{1:1} = \frac{0.606\ \frac{mM_{DMS}}{mM_{\beta CD}}}{1.726\ mM_{DMS}\left(1 - 0.606\ \frac{mM_{DMS}}{mM_{\beta CD}}\right)} = 0.891\ mM_{DMS}^{-1}$$

Equation 32

Stationary Segment

The second segment of the B-Type Phase-Solubility Diagram is the plateau, between the growth and the descending segments. This section can be used to determine the stoichiometric ratio of the precipitate, as well as the maximum value of DMS_{tot}.

Beta

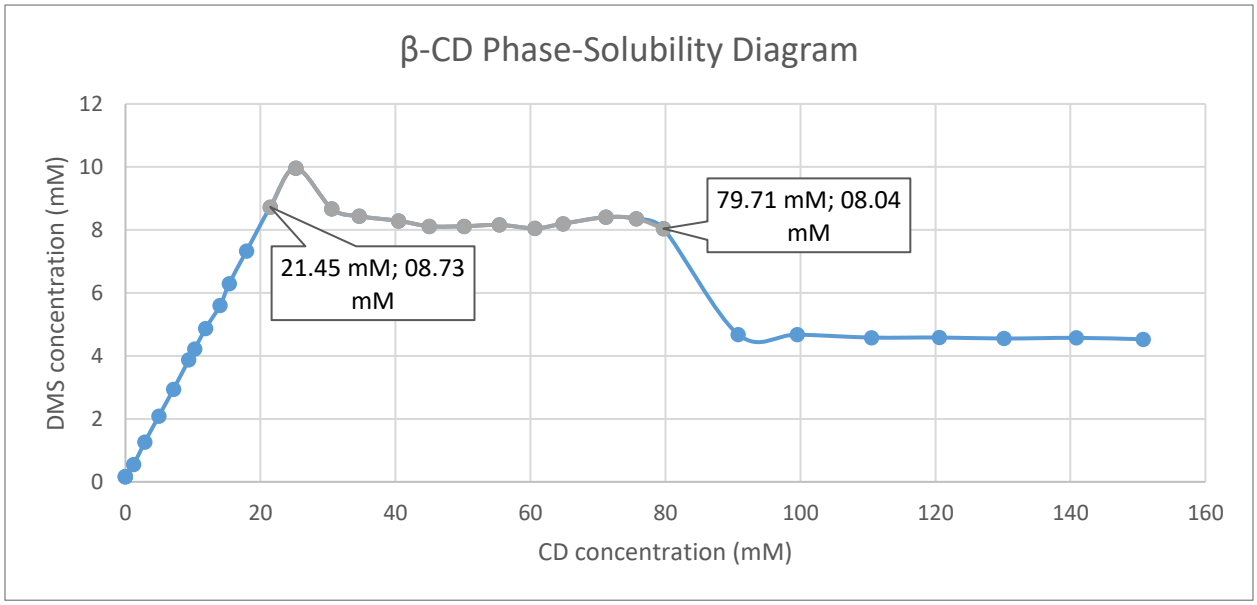

Figure 12. βCD Phase-Solubility Diagram. The βCD Phase-Solubility Diagram is displayed, consisting of ascending βCD concentrations (mM) on the x-axis and DMS concentration (mM) on the y-axis. The stationary segment is highlighted in grey. The values at point A are 21.4547 mM βCD and 8.7269 mM DMS, and the values at point B are 79.7058 mM βCD and 8.0414 mM DMS.

The stationary segment of βCD exhibits a relatively stable plateau, disregarding the second point. The plateau indicates that the highest amount of DMS that can be dissolved including the complexation with βCD is approximately 8.2 mM.

To calculate the stoichiometric ratio between DMS and βCD, the following values were used:

$$DMS_{add} = 35\ mM$$
$$c(DMS_A) = 8.73\ mM$$
$$\beta CD_{add_A} = 21.45\ mM$$
$$\beta CD_{add_B} = 79.7\ mM$$

Using the following equation, the ratio of the complex is calculated. More information about this equation can be found in the theory chapter in the section "Phase Solubility Diagrams".

$$\frac{DMS}{\beta CD} = \frac{35\ mM - 8.73\ mM}{79.7\ mM - 21.45\ mM} = \frac{26.27\ mM}{58.25\ mM} \approx \frac{1}{2}$$

Equation 33

The stoichiometric ratio is about $1{:}\,2$ DMS to βCD.

Gamma

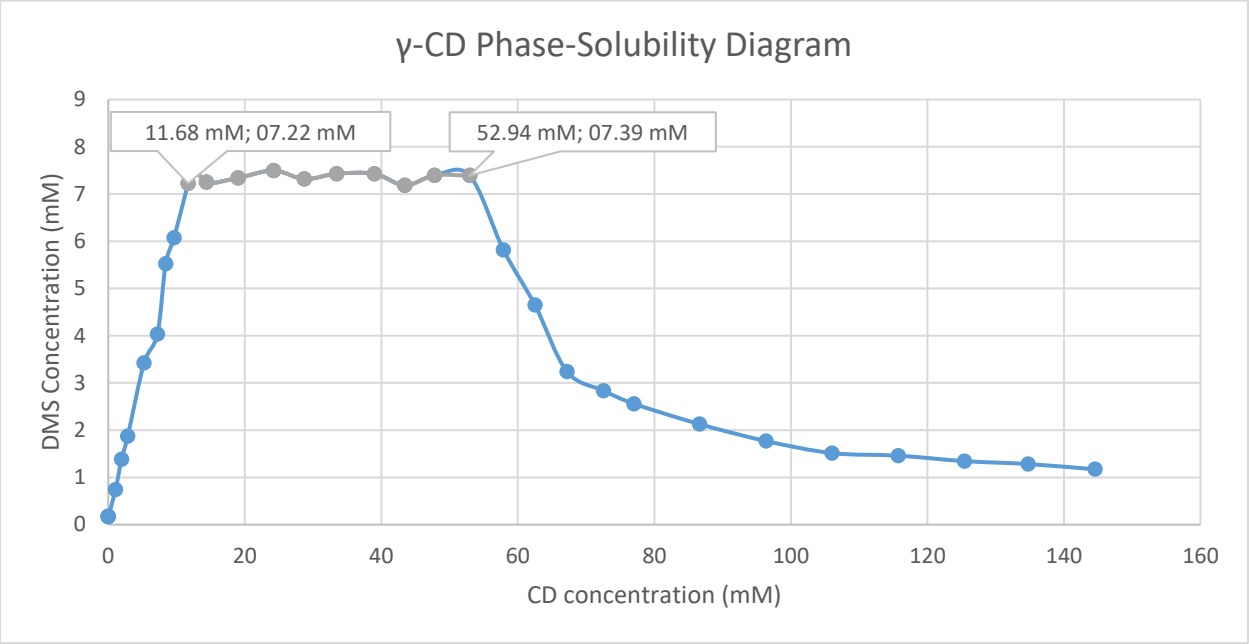

Figure 13. γCD Phase-Solubility Diagram. *This consists of increasing γCD concentrations (mM) on the x-axis and DMS concentration (mM) on the y-axis. The stationary segment is highlighted in grey. The values at point A are 11.6831 mM γCD and 7.2235 mM DMS, and the values at point B are 52.9403 mM γCD and 7.3911 mM DMS.*

The stationary segment of γCD exhibits a stable plateau. The plateau indicates that the highest amount of DMS that can be dissolved including the complexation with γCD is approximately 7.4 mM.

To calculate the stoichiometric ratio between DMS and γCD, the following values were used:

$$DMS_{add} = 35\ mM$$
$$c(DMS_A) = 8.73\ mM$$
$$\gamma CD_{add_A} = 21.45\ mM$$
$$\gamma CD_{add_B} = 79.7\ mM$$

Using the following equation, the ratio of the complex is calculated. More information about this equation can be found in the theory chapter in the section "Phase Solubility Diagrams".

$$\frac{DMS}{\gamma CD} = \frac{35\ mM - 7.22\ mM}{52.9\ mM - 11.68\ mM} = \frac{27.78\ mM}{41.22\ mM} \approx \frac{2}{3}$$

Equation 34

The stoichiometric ratio is about $2{:}\,3$ DMS to γCD.

Descending Segment

The last segment is the remaining data points after the plateau. This section provides information about the concentration of DMS when large concentrations of CD is added, additionally, this segment indicates the concentration of added CD at which DMS in precipitate has been depleted.

Beta

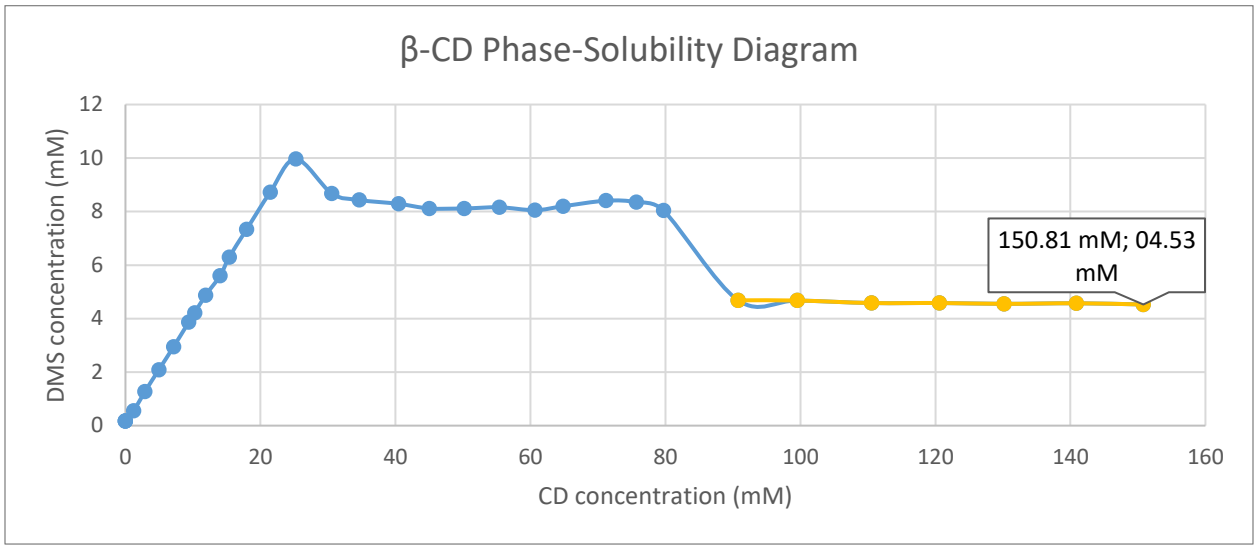

Figure 14. βCD Phase-Solubility Diagram. *This contains increasing βCD concentrations (mM) on the x-axis and DMS concentrations (mM) on the y-axis. The descending segment is highlighted in yellow. The values at the end-point of the descending segment is 150.8061 mM β-CD, 4.5276 mM DMS.*

The concentration of dissolved DMS approaches 4.5 mM when the precipitated DMS is depleted. Precipitated DMS was depleted at 80 mM of added βCD.

Gamma

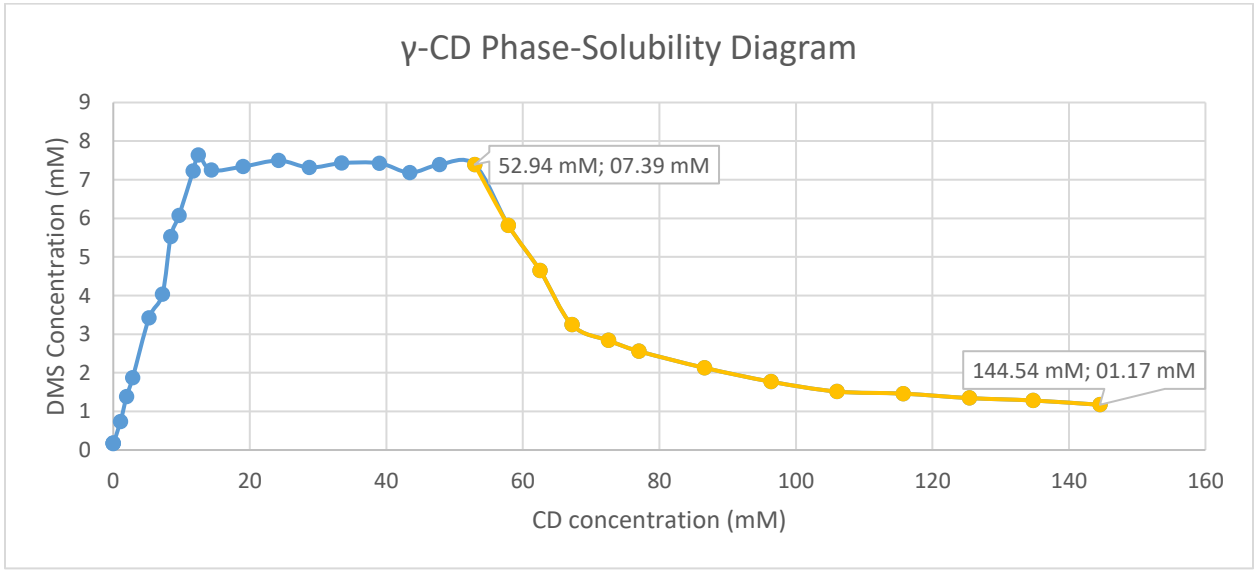

Figure 15. γCD Phase-Solubility Diagram. *This consists of ascending γCD concentrations (mM) on the x-axis and DMS concentrations (mM) on the y-axis. The values at the end-point of the descending segment is 144.5403 mM γ-CD, 1.1716 mM DMS.*

The concentration of dissolved DMS approaches 1 mM when the precipitated DMS is depleted. Precipitated DMS was depleted at 53 mM of added γCD.

Combined CD

In this experiment, one of the aims was to determine whether there was a non-additive effect on the solubility of DMS when γCD and βCD were used simultaneously. The samples were prepared using various ratios of CD so that if any non-additive effects were present, it was possible to determine whether the ratio influenced the magnitude of the effect. The samples contained concentrations of CD that correspond to the concentrations of the growth segment and the start of the plateau from the individual CD experiments. The distribution of CD was determined gravimetrically.

This chapter is divided into three parts. The first part covers the general description of the data, and shows the concentrations of dissolved DMS and CD distribution against the apparent concentration of added CD. The second section focuses on whether the data exhibited a non-additive effect. This is done by comparing the expected DMS concentration with the observed DMS concentration. The third section compares this experiment with the individual CD experiments. Specifically, the increase in DMS concentration was compared for each type of CD in the two sets of experiments. The outcome is expected to be similar; thus, the focus of this section is to determine the accuracy and reproducibility of the data.

DMS Concentration and CD Distribution

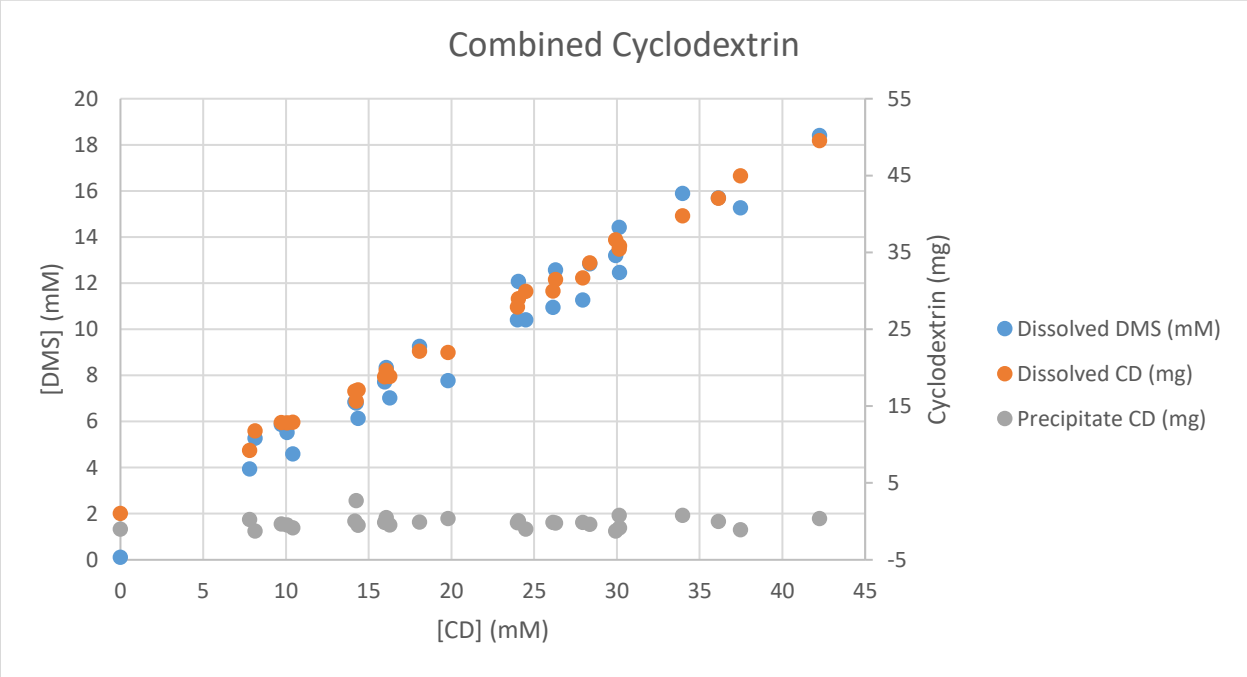

Figure 16. Growth segment of combined cyclodextrin shown as dissolved DMS, and dissolved and precipitated CD. *[CD] (mM) is on the x-axis and [DMS] (mM) is plotted on the y-axis. Dissolved DMS (mM) is shown in blue, dissolved CD (mg) is exhibited in orange and finally, precipitated CD (mg) is displayed in grey.*

Figure 16 shows the concentration of DMS plotted against the total concentration of added CD. It also shows the distribution of CD, in precipitate and solution, against added CD. Note that the 0 value on the two y-axes are shifted from each other.

It is immediately apparent that there is a linear trend to the concentration of dissolved DMS; however, there are deviations from the trend line. These deviations are covered in more detail in the next section. Furthermore, it is apparent that all the CD seems to dissolve, shown by the mass of CD precipitate that is

close to zero, for all samples. In addition, the fact that the value of precipitated CD is frequently below 0 is most likely due to systematic errors, such as absorbed water in CD, impurities and/or instrument inaccuracies. Moreover, the observation that all CD was dissolved fits the expectation, as the amount added was primarily in the range where it dissolved in the individual CD experiments. Additionally, the data point for precipitated CD at 14 mM added CD, is the only data point that is noticeably above 0 mg CD. This data point is from a sample that contains a high amount of γCD, and no βCD, so the precipitation is expected, since samples in the previous experiment with this amount of γCD had precipitated γCD also.

Predicted vs Measured [DMS]

To verify whether or not an additive effect on the DMS concentration occurred, when combining βCD and γCD, the measured concentrations of dissolved DMS was plotted against predicted DMS concentrations. The predicted DMS concentration was calculated by multiplying the concentration of each type of CD with the slope of their respective growth segment, determined from the previous experiments. The slope represents how much the concentration of DMS increases per mM of added CD. As the slope is different for each type of CD, this approach should decrease the variance observed in **Figure 16**. The two values were then added together with the average of the intercepts of the growth segments to give the predicted concentration, in the following equation:

$$[DMS]_{pred} = \text{Slope}_{\beta} \cdot [\beta CD] + \text{Slope}_{\gamma} \cdot [\gamma CD] + \frac{\text{Intercept}_{\beta} + \text{Intercept}_{\gamma}}{2}$$

Equation 35

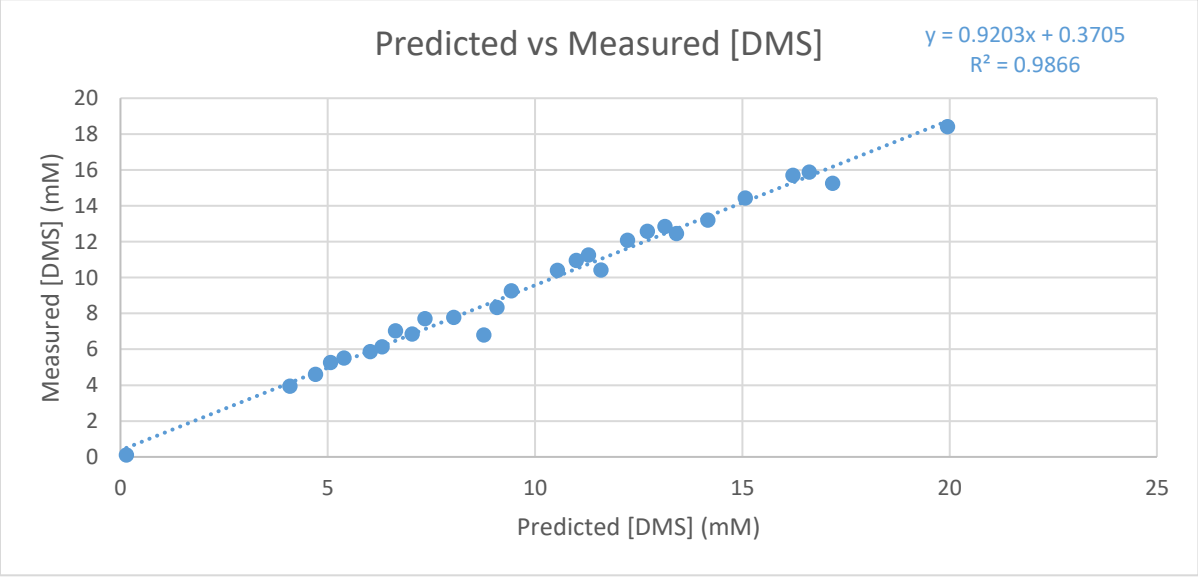

Figure 17. Predicted versus measured dissolved DMS concentrations in solution. *Predicted dissolved [DMS] (mM) is plotted on the x-axis and measured dissolved [DMS] is on the y-axis. The R^2 value is 0.9866 and the equation of the line is y = 0.9203x + 0.2414.*

As shown in **Figure 17**, the variance is much lower after the data has been transformed. If the effect is additive then the slope is expected to be 1, with an R^2 value close to 1. Since the slope is 0.9203, which is approximately 8% lower than 1. This seems to indicate an antagonistic effect; however, given the variety of the composition of the samples, a large variance would be expected. There is however a small variance indicated by the high R^2 value of 0.98663, which contradicts the idea that there is an antagonistic effect.

Therefore, it is likely that there is a systematic error of some form. A possible systematic error could be water content and impurities in the CD that were not taken into account in the calculations.

There are two points which noticeably deviate from the trend line. The first one is approximately 9 mM predicted $[DMS]$ and the other is at 17 mM. These two points have high concentrations of added CD which might explain the deviations, since some of the CD could have precipitated and therefore not contribute to the $[DMS]$. This theory is supported by the previous figure (see **Figure 16**) for the point at 9 mM predicted $[DMS]$, as this point is the same sample that had noticeable precipitate.

Even though there is no conclusive evidence that there is an additive or a non-additive effect, the data point with the highest value, which is 18.3 mM dissolved $[DMS]$, warrants additional research. The concentration of dissolved DMS at this point exceeds the sum of the maximum dissolved $[DMS]$ from the individual CD experiments. The maximum values from the individual CD experiments are 8.2 and 7.4 for βCD and γCD samples, respectively, the sum of which is 15.6 mM $[DMS]$. 18.3 mM is notably greater than 15.6 mM, which could indicate a synergistic effect at higher $[DMS]$ concentrations, that increases the plateau value.

Comparing Results

The growth segments of the individual CD experiments were compared to the specific data points from the combined CD experiment where only one type of CD was added (see Appendix I). The two should approximate to the same line; thus, large deviations could indicate measurement errors.

Beta

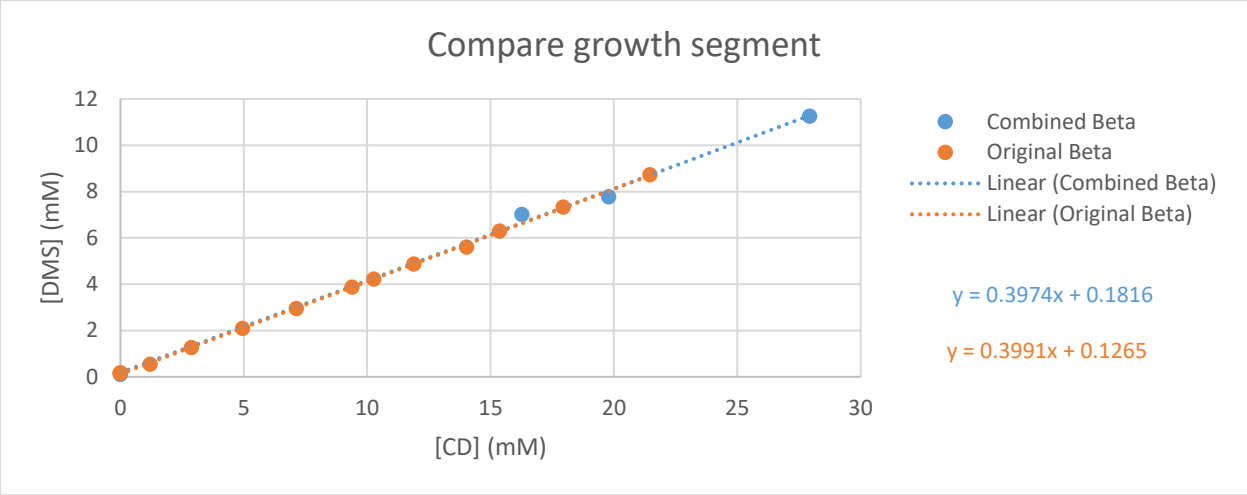

Figure 18. Growth segment from βCD experiment and data points without γCD from combined CD experiment compared. *[CD] (mM) is plotted on the x-axis and [DMS] (mM) is on the y-axis. Original βCD is exhibited in orange, with a linear equation of y = 0.3991x + 0.1265. Combined βCD is displayed in blue, including a linear equation of y = 0.3974x + 0.1816.*

The trend lines of both datasets are almost identical. The slope of the combined beta is 0.3974 and the slope of the original beta is 0.3991, which are exceedingly close. The powerful correlation between the two datasets, even though the experiments were carried out independently, indicates that the experiments are reproducible.

Gamma

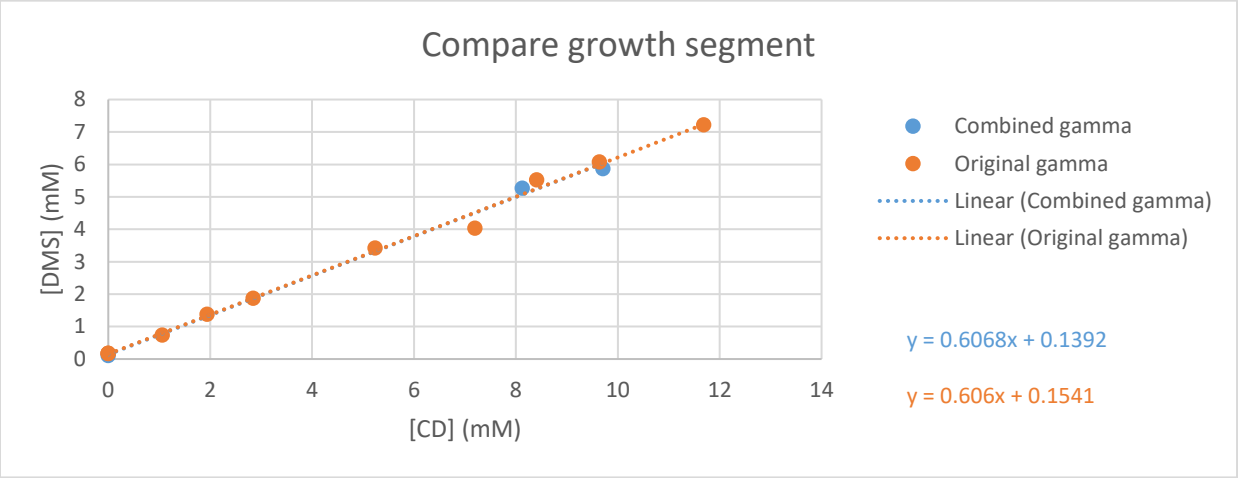

Figure 19. Growth segment from γCD experiment and data points without βCD from combined CD experiment compared. *[CD] (mM) is plotted on the x-axis and [DMS] (mM) is on the y-axis. Original γCD is exhibited in orange, with a linear equation of y = 0.606x + 0.1541. Combined γCD is displayed in blue, including a linear equation of y = 0.6068x + 0.1392.*

The trend lines of both datasets are almost identical. The slope of the combined gamma is 0.6068 and the slope of the original beta is 0.606, which are exceedingly close. The powerful correlation between the two datasets, even though the experiments were carried out independently, indicates that the experiments are reproducible.

Discussion

The analysis section contains the discussions about each individual graph. The remaining discussion can be found here. The discussion is split into three parts. The first part is the discussion concerning the phase solubility diagrams that were the result of the two individual CD experiments. The two diagrams are compared to each other and the theory. The second part is the discussion of experimental errors and some of the experiments that could be used to verify our results. The third part covers the potential application of our results and our recommendations for future research.

Phase-Solubility Diagram

We will start by comparing the individual segments of the diagrams and then compare how the CD was distributed between precipitate and solute in each diagram. The slope of the growth segment is used to determine the stability constant of the 1:1 complexes. By comparing them we can determine which complex is the most stable. The stability constant for the βCD experiment is 0.4032 mM^{-1} (Equation 31) and 0.891 mM^{-1} (Equation 32) for the γCD experiment. The value of the stability constant for γCD is more than twice as large. This indicates that the γCD complexes are more stable than the βCD complexes. The difference could be due to the larger cavity in the γCD, making it easier for the drug to enter and form a complex. The larger stability constant does not necessarily mean that the γCD complex stays as a complex for a longer time, it only indicates that the rate that complexes are formed compared to the rate at which they disassociate, is larger for the γCD complex (see Equation 6).

The spike observed at the beginning of the stationary segment on the βCD Phase-Solubility Diagram, is also present to a smaller degree on the γCD Phase-Solubility Diagram. The explanation for the peak in the βCD diagram, given in the analysis, is oversaturation. This explanation is also valid for the γCD diagram. To test this explanation, samples with the same concentration of added CD as the peak, given different amounts of time to react, can be used. If the peak is due to oversaturation, then the height of the peak should decrease as a function of time. The fact that the peak is smaller for the γCD could indicate that the samples were closer to equilibrium. The reason that the peak is found at the beginning of the plateau could be due to the types of complexes present at this point. During the growth segment 1:1 complexes are the most prominent. As the concentration of dissolved DMS approaches the stationary segment, the proportion of larger complexes increases. At the intersection of the growth segment and the stationary segment, there is no complex in precipitate. Adding CD at this point will create more complexes, if the new complexes dissociate before they can precipitate then the concentration of DMS in solution will be higher, as the DMS that participated in the complexes is replenished by the precipitate. Given enough time the new complexes will precipitate. Larger amounts of complex will increase the speed at which precipitation takes place, which is why the rest of the stationary segment equilibrates faster.

The height of the stationary segment gives the maximum amount of DMS that can be dissolved at equilibrium, by use of each type of CD. The values of the stationary segments are 8.2 for βCD and 7.4 for γCD. This shows that more DMS can be dissolved by use of βCD than γCD. This is not intuitive given as γCD has a higher intrinsic solubility (Douroumis & Fahr, 2013) and stability constant than βCD. The higher value for βCD indicates that the intrinsic solubilities of the beta complexes are higher, despite βCD having a lower intrinsic solubility than γCD. The length of the stationary phase differs between the two types of CD. The stationary phase for βCD is 58.25 which is longer than 41.22 for γCD, this indicates that γCD forms smaller complexes than βCD, so that less γCD is needed to deplete DMS in the precipitate.

The diagram for γCD has a much slower decent than the diagram for βCD. This could have a relation to the size of the complexes being formed. Due to the nature of stability equilibria, large complexes are more strongly influenced by changes in concentration than smaller complexes. Equation 36 will be used to exemplify this.

$$K = \frac{[DMS{:}\, CD_n]}{[DMS] \cdot [CD]^n}$$

Equation 36

The larger n is, the more a small change in the concentration in CD will affect the concentration of DMS and/or the complex. Therefore, the larger βCD complex will cause the concentration of DMS to drop more than a smaller γCD complex will when the same amount of CD is added. The values that the Phase-Solubility Diagrams approach is 4.5 mM and 1 mM for βCD and γCD respectively. These values represent the concentration the dissolved DMS approaches after the stationary segment when additional CD is added. The values are significantly lower than the stationary segment minus the intrinsic solubility of DMS, as the theory would predict if only 1:1 complexes are formed. This supports the hypothesis that additional types of complexes are formed. We hypothesise that the values that the diagrams approach are equal to the intrinsic solubilities of the complex with the highest number of CD. For βCD we determined in the analysis that this complex has 4 CD for each DMS. The largest complex for γCD was not determined. Assuming the hypothesis is true, the solubility of the largest βCD complex is greater than that of the largest γCD complex.

The behaviour of the distribution of the CD is the same for both types of CD during the growth segment and stationary segment. In the growth segment, all CD dissolves, then during the stationary phase all added CD precipitates. The precipitated CD is assumed to be in a complex. Then during the descending segment the behaviour differs for the two types of CD. The βCD in solution has a sudden small increase and then stays relatively constant, while the majority of the βCD precipitates. The γCD in solution increases for all the remaining samples, while the change in the amount γCD precipitate initially drops before increasing again after 100 mM added CD. Presumably the amount of γCD in solution will increase until it reaches the intrinsic solubility of γCD plus the intrinsic solubility of the largest γCD complex. The plateaus seen for CD in solution for both types of CD have a small slope, this could be due to impurities in the CD as the concentration is calculated from the change in weight. If there are impurities with a higher solubility than the CD, then the precipitate will lose weight due to the impurities dissolving, resulting in a higher calculated concentration.

Experimental Errors and Verification

First possible errors in our experiment will be discussed, followed by a description of some additional analyses that could be used to verify our results. A likely error that could influence our calculations is that the water content of the CD was not determined, this means that the calculated concentrations are likely to be too high in our results. This error is consistent for all samples so this will not affect the behaviours seen in our data. The impurities in the chemical compounds were likewise not accounted for. The affect the impurities have on the data will likely be proportional to the amount of compound added, so it might have an influence on the observed behaviours.

During our γCD experiment, the fan in the HPLC had to be replaced, this meant that the HPLC had to be interrupted and disassembled. This could change experimental conditions such as pressure. Additionally,

it was observed that the HPLC needed time, before the pressure equilibrated and the light intensity in the spectrophotometer stabilised. The instrument did not automatically wait until reaching an equilibrated state, therefore if the samples were run shortly after the machine was turned on, then the response might be incorrect for the first few samples. The laboratory scale was inconsistent when determining the weight of our samples, the weight could change by up to a milligram when waiting before registering the result. The amount of time waited for each measurement varied, so this could mean the registered masses were incorrect, however our results generally seem accurate so this inconsistency is likely to be small.

To help determine the stoichiometry of the complexes, the dried precipitate could be analysed by crystallography and/or NMR (Nuclear Magnetic Resonance), as other researchers for an example have carried out the structure determination of protein-ligand complexes (Ziarek, Peterson, Lytle, & Volkman, 2011). The information determined from these techniques could be used to supplement or even verify our results.

ITC (Isothermal Titration Calorimetry) can be used to determine the stability constant of the complexes. ITC is usually performed at low concentrations of the compounds so it can give a very accurate determination of the stability constant for the 1:1 complexes. The determination of the stability constant by ITC, can be used to verify the stability constant derived from the slope of the growth segments. The stability constant was determined with ITC by the previous group (Bartoš, Hansen, Pedersen, Larsen, & Lund, 2016). We planned to do ITC ourselves but were prevented due to time constraints.

Applications and Future Directions

This section addresses our ideas on how our results can be used and what experiments would be a natural extension of this project.

Our results can be used to find the optimal ratio of DMS and CD, so that the concentration of DMS is optimised. Our research also shows that utilising multiple types of CD can be used to further increase the solubility of drugs, than if only one type of CD is used. Our results indicate that the maximum concentration of dissolved DMS, when using both types of CD, exceeds the sum of the maximum concentrations when using the two types of CD individually. We only have one data point with sufficient CD to show this synergistic effect. We recommend additional research on combining the two types of CD to increase the solubility of DMS, selecting concentrations of the two CD types that exceed their respective growth segments. Additionally, measuring samples containing higher amounts of individual CD, can be used to determine if the concentration of dissolved DMS does stabilise as part of the descending segment or if there is any further changes in concentration. This information can be used to support or refute our hypothesis that the concentration of DMS in solution approaches the intrinsic solubility of the complex with the most CD.

Conclusion

The solubility of dexamethasone increases, when γCD and/or βCD is added. The maximum amount of DMS that can be dissolved, is 8.2 mM for βCD, which is higher than 7.4 mM for γCD. The Phase-Solubility Diagrams follow a B-type, when utilising either of the two types of CD used in this project, this indicates that the complexes have a limited solubility. This result was expected as the previous group's data also exhibited a B-type (Bartoš, Hansen, Pedersen, Larsen, & Lund, 2016). The differences observed in the Phase-Solubility Diagrams indicate that βCD forms larger complexes than γCD and that the βCD complexes are more soluble, even though βCD is less soluble than γCD. The slope of the growth segments indicate that the 1:1 $DMS{:}\,\gamma CD$ complex is more stable than the $DMS{:}\,\beta CD$ complex. The combined impact of CDs on the concentration of dissolved DMS is additive, seen by the linear correlation between the expected and measured dissolved DMS concentrations. However, the concentrations continued to increase and reached a value, exceeding the sum of the maximum concentrations of dissolved DMS observed using individual CD types, which can indicate a synergistic effect on the maximum solubility. As only one sample had sufficient added CD to show this synergistic effect, further research is necessary to determine whether this effect is synergistic on the maximum solubility of DMS.

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Appendix I

Gamma|Beta %	0	20	40	60	80	100	120	140
0								
20								
40								
60								
80								
100								
120								
140								

Table 3. Table displaying the percentage content of each type of CD in samples of combined CD experiment. *Blue cells represent samples of combined CD experiment. To test the reproducibility of data, dissolved DMS in individual and combined CD experiments were compared, shown in red, purple and orange. Red cells represent samples used in comparing βCD on dissolved DMS, in individual and combined CD experiments, and thus contains 0% γCD, or only contains βCD. Purple cells represent samples used in comparing γCD on dissolved DMS, in individual and combined CD experiments, and so contains 0% βCD, or only contains γCD. The orange cell is the content of CD which is 0%, and therefore this represents the D_0 or intrinsic solubility of dexamethasone used in the analysis.*

Appendix II

Gamma

Gamma sample name	S0 g1	S0 g2	S0 g3	g1	g2	g3	g4	g9	g6	g7	g8
Chosen [CD] (mM)	0	0	0	1	2	3	5	7	9	10	12
Required CD (mg)	0.00	0.00	0.00	1.30	2.59	3.89	6.49	9.08	11.67	12.97	15.57
Actual CD (mg)	0	0	0	1.36	2.61	3.84	7.06	9.7	11.34	13	15.74
Actual DMS (mg)	13.52	13.65	13.78	13.74	13.76	13.75	13.64	13.73	13.81	13.74	13.65
Water added (mg)	1020.6	1042.2	1042.6	993.3	1038.9	1041.1	1039.5	1039.1	1039.7	1039.9	1038.6
HPLC output (mAU*s)	3629.9	3562.2	3402.2	773.0	1426.3	1924.3	3498.8	4120.7	5638.5	6197.5	7365.0
Relative Absorbance (%)	102.36	102.36	102.36	102.36	102.36	102.36	102.36	102.36	102.36	102.36	102.36
Adjusted output (mAU*s)	3546.2	3480.1	3323.8	755.2	1393.4	1880.0	3418.1	4025.7	5508.6	6054.7	7195.4
Calculated [DMS] (mM)	0.177	0.174	0.166	0.738	1.381	1.871	3.420	4.031	5.525	6.075	7.224
Calculated [CD] (mM)	0.00	0.00	0.00	1.06	1.94	2.84	5.24	7.20	8.41	9.64	11.68
Calculated [CD]aq (mg)	0.11	-0.08	-0.03	1.12	2.62	3.47	6.94	7.89	11.33	12.14	15.20
Calculated [CD]s (mg)	-0.11	0.08	0.03	0.24	-0.01	0.37	0.12	1.81	0.01	0.86	0.54

Gamma sample name	g5	g12	g11	g10	g13	g14	g15	g16	g17	g18	g19
Chosen [CD] (mM)	13	15	20	25	30	35	40	45	50	55	60
Required CD (mg)	16.86	19.46	25.94	32.43	38.91	45.40	51.88	58.37	64.86	71.34	77.83
Actual CD (mg)	16.78	19.35	25.66	32.64	38.83	45.2	52.61	58.63	64.43	71.39	78.1
Actual DMS (mg)	13.98	13.83	13.79	13.76	13.73	13.67	13.72	13.71	13.8	13.74	13.75
Water added (mg)	1038.9	1037.4	1039.8	1038.8	1041.8	1041.1	1039.9	1040.9	1039.5	1039.6	1039.9
HPLC output (mAU*s)	7784.3	7389.6	7485.6	7643.2	7459.8	7575.0	7572.3	7325.1	7537.6	7535.4	5932.5
Relative Absorbance (%)	102.36	102.36	102.36	102.36	102.36	102.36	102.36	102.36	102.36	102.36	102.36
Adjusted output (mAU*s)	7605.0	7219.3	7313.2	7467.1	7288.0	7400.5	7397.9	7156.3	7364.0	7361.8	5795.8
Calculated [DMS] (mM)	7.636	7.248	7.342	7.497	7.317	7.430	7.427	7.184	7.393	7.391	5.814
Calculated [CD] (mM)	12.45	14.38	19.03	24.22	28.74	33.47	39.00	43.42	47.79	52.94	57.90
Calculated [CD]aq (mg)	16.10	15.46	15.50	16.71	17.64	17.46	17.59	18.34	19.03	19.89	19.71
Calculated [CD]s (mg)	0.68	3.89	10.16	15.93	21.19	27.74	35.02	40.29	45.40	51.50	58.39

Gamma sample name	g20	g21	g22	g23	g24	g25	g26	g27	g28	g29	g30
Chosen [CD] (mM)	65	70	75	80	90	100	110	120	130	140	150
Required CD (mg)	84.31	90.80	97.28	103.77	116.74	129.71	142.68	155.65	168.63	181.60	194.57
Actual CD (mg)	84.3	90.69	97.87	103.8	116.8	130	142.7	155.8	168.9	181.7	194.9
Actual DMS (mg)	13.74	13.73	13.68	13.7	13.75	13.69	13.67	13.71	13.62	13.79	13.72
Water added (mg)	1039.4	1040.5	1039.7	1039.3	1039.5	1040.4	1037.8	1037.8	1038.1	1039.1	1039.7
HPLC output (mAU*s)	4731.3	3309.2	2897.8	2615.8	2180.5	1816.7	1558.3	1502.6	1385.8	1322.6	1210.4
Relative Absorbance (%)	102.06	102.06	102.06	102.06	102.06	102.06	102.06	102.06	102.06	102.06	102.06
Adjusted output (mAU*s)	4635.8	3242.4	2839.4	2563.1	2136.5	1780.0	1526.8	1472.3	1357.8	1295.9	1185.9
Calculated [DMS] (mM)	4.646	3.243	2.837	2.558	2.129	1.770	1.515	1.460	1.345	1.282	1.172
Calculated [CD] (mM)	62.53	67.19	72.57	77.03	86.59	96.36	106.02	115.73	125.43	134.77	144.54
Calculated [CD]aq (mg)	22.49	27.17	31.77	35.68	46.81	61.14	77.69	88.50	95.78	103.51	114.63
Calculated [CD]s (mg)	61.81	63.52	66.10	68.16	69.95	68.90	65.03	67.29	73.12	78.15	80.29

*Table 4 Masses of γCD and DMS, calculations and HPLC output data for γCD samples. "Chosen [CD]" is the concentration (mM) of CD that was chosen for that sample. "Required CD" is the mass (mg) of CD necessary to weigh out in order to reach the concentration in "Chosen CD". "Actual CD" is the mass (mg) of CD that was actually weighed. "Actual DMS" is the mass of DMS that was actually weighed. "Water added" is the weight of water that was pipetted into each sample to achieve the respective dilutions. "HPLC output" is the absorbance (mAU*s) measured in the HPLC for each sample. "Relative Absorbance" is the difference in percentage between the absorbance (mAU*s) originally measured from a calibration standard versus the absorbance of a standard measured with the γCD samples. "Adjusted output" is the adjusted absorbance (mAU*s) of the γCD samples or "HPLC output" according to the HPLC output of the standard. "Calculated [DMS]" and "Calculated [CD]" are the concentrations (mM) of DMS and CD and are calculated from the "Adjusted output" and "Actual CD" respectively. "Calculated [CD]aq" is the mass (mg) of CD in solution and "Calculated [CD]s" is the mass (mg) of solid CD or CD in the precipitate. [CD]aq and [CD]s were calculated gravimetrically.*

Beta

Beta sample name	S0 b1	S0 b2	S0 b3	b1	b2	b3	b4	b5	b6	b7	b8
Chosen [CD] (mM)	0	0	0	1	3	5	7	9	10	12	14
Required CD (mg)	0.00	0.00	0.00	1.13	3.40	5.67	7.94	10.21	11.35	13.62	15.89
Actual CD (mg)	0	0	0	1.37	3.26	5.62	8.08	10.64	11.65	13.47	15.82
Actual DMS (mg)	13.81	13.65	13.82	13.64	13.76	13.72	13.69	13.69	13.79	13.73	13.84
Water added (mg)	999.4	999.8	999.6	1000.4	1000.6	1000.5	998.8	999.2	1000.2	998.7	993.5
HPLC output (mAU*s)	3412.4	3454.7	3411.9	306.2	676.1	1099.6	1541.2	2018.2	2196.6	2535.6	2910.2
Relative Absorbance (%)	103.79	103.79	103.79	103.79	103.79	103.79	103.79	103.79	103.79	103.79	103.79
Adjusted output (mAU*s)	3287.7	3328.5	3287.3	295.0	651.4	1059.4	1484.9	1944.4	2116.3	2442.9	2803.9
Calculated [DMS] (mM)	0.164	0.166	0.164	0.549	1.267	2.088	2.945	3.871	4.217	4.875	5.602
Calculated [CD] (mM)	0.00	0.00	0.00	1.21	2.87	4.95	7.13	9.38	10.26	11.88	14.03
Calculated [CD]aq (mg)	-0.26	-0.40	-0.54	0.70	2.32	4.91	7.60	9.58	10.30	12.08	14.50
Calculated [CD]s (mg)	0.26	0.40	0.54	0.67	0.94	0.71	0.48	1.06	1.35	1.39	1.32

Beta sample name	b9	b10	b11	b12	b13	b14	b15	b16	b17	b18	b19
Chosen [CD] (mM)	15	18	20	25	30	35	40	45	50	55	60
Required CD (mg)	17.02	20.43	22.70	28.37	34.05	39.72	45.40	51.07	56.75	62.42	68.10
Actual CD (mg)	17.42	20.33	24.32	28.59	34.63	39.2	45.78	51.13	56.82	62.7	68.5
Actual DMS (mg)	13.79	13.75	13.67	13.67	13.76	13.83	13.68	13.71	13.79	13.75	13.74
Water added (mg)	999.0	998.1	998.7	998.4	997.9	997.4	997.0	1000.5	998.0	996.8	994.8
HPLC output (mAU*s)	3265.8	3802.0	4520.5	5159.7	4494.2	4369.7	4294.4	4206.2	4206.2	4228.2	4172.6
Relative Absorbance (%)	103.79	103.79	103.79	103.79	103.79	103.79	103.79	103.79	103.79	103.79	103.79
Adjusted output (mAU*s)	3146.5	3663.1	4355.3	4971.2	4330.1	4210.1	4137.5	4052.6	4052.6	4073.7	4020.2
Calculated [DMS] (mM)	6.292	7.333	8.727	9.967	8.676	8.434	8.288	8.117	8.117	8.160	8.052
Calculated [CD] (mM)	15.36	17.95	21.45	25.23	30.58	34.63	40.46	45.03	50.16	55.42	60.67
Calculated [CD]aq (mg)	16.63	19.28	23.46	27.46	25.24	24.91	25.36	27.14	25.10	26.40	28.03
Calculated [CD]s (mg)	0.79	1.05	0.86	1.13	9.39	14.29	20.42	23.99	31.72	36.30	40.47

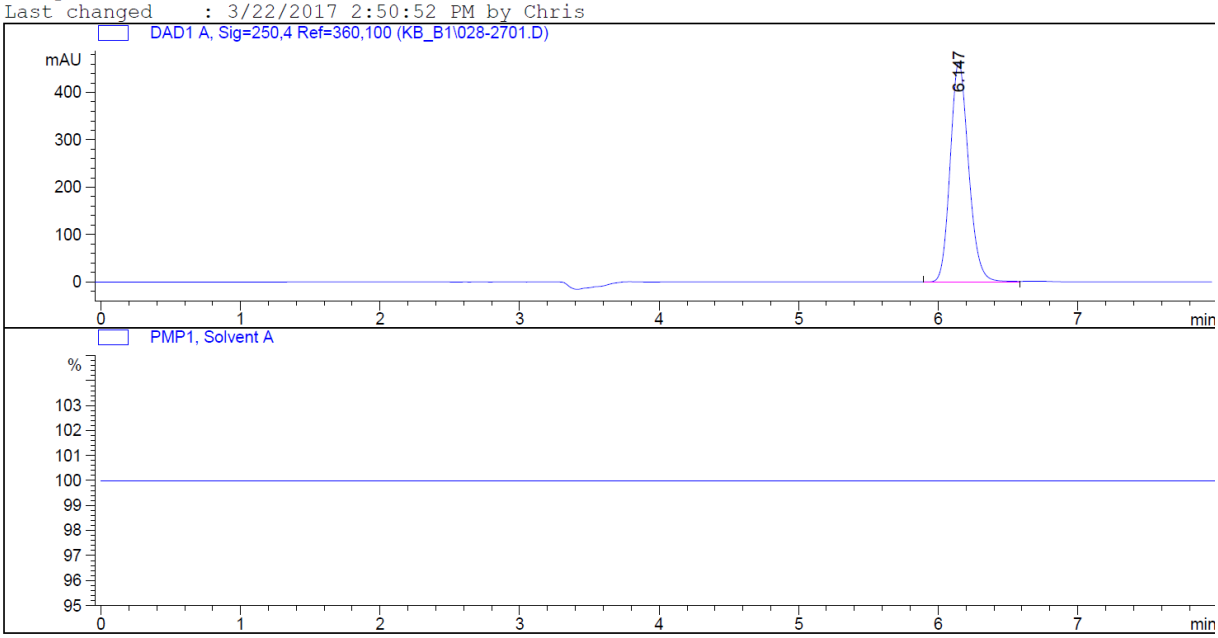
Beta sample name	b20	b21	b22	b23	b24	b25	b26	b27	b28	b29	b30
Chosen [CD] (mM)	65	70	75	80	90	100	110	120	130	140	150
Required CD (mg)	73.77	79.45	85.12	90.80	102.15	113.50	124.85	136.20	147.55	158.90	170.25
Actual CD (mg)	73.29	80.51	85.61	90.08	102.7	112.6	125	136.4	147.3	159.2	170.5
Actual DMS (mg)	13.82	13.69	13.75	13.67	13.61	13.77	13.72	13.71	13.74	13.81	13.82
Water added (mg)	996.0	996.5	996.3	995.8	996.6	996.8	996.4	996.4	996.7	995.7	996.3
HPLC output (mAU*s)	4248.1	4355.2	4328.0	4167.2	2436.6	2433.0	2384.5	2385.4	2369.0	2379.9	2356.5
Relative Absorbance (%)	103.79	103.79	103.79	103.79	103.79	103.79	103.79	103.79	103.79	103.79	103.79
Adjusted output (mAU*s)	4092.9	4196.1	4169.9	4015.0	2347.6	2344.1	2297.4	2298.2	2282.4	2293.0	2270.4
Calculated [DMS] (mM)	8.198	8.406	8.353	8.041	4.683	4.676	4.582	4.584	4.552	4.573	4.528
Calculated [CD] (mM)	64.83	71.18	75.71	79.71	90.78	99.54	110.53	120.57	130.16	140.88	150.81
Calculated [CD]aq (mg)	26.52	28.78	28.31	29.96	33.50	37.87	35.84	37.11	37.34	38.24	39.40
Calculated [CD]s (mg)	46.77	51.73	57.30	60.12	69.18	74.75	89.15	99.24	109.91	120.97	131.12

*Table 5. Masses of βCD and DMS, calculations and HPLC output data for βCD samples. "Chosen [CD]" is the concentration (mM) of CD that was chosen for that sample. "Required CD" is the mass (mg) of CD necessary to weigh out in order to reach the concentration in "Chosen [CD]". "Actual CD" is the mass (mg) of CD that was actually weighed. "Actual DMS" is the mass of DMS that was actually weighed. "Water added" is the weight of water that was pipetted into each sample to achieve the respective dilutions. "HPLC output" is the absorbance (mAU*s) measured in the HPLC for each sample. "Relative Absorbance" is the difference in percentage between the absorbance (mAU*s) originally measured from a calibration standard versus the absorbance of a standard measured with the βCD samples. "Adjusted output" is the adjusted absorbance (mAU*s) of the βCD samples or "HPLC output" according to the HPLC output of the standard. "Calculated DMS" and "Calculated CD" are the concentrations (mM) of DMS and CD and are calculated from the "Adjusted output" and "Actual CD" respectively. "Calculated [CD]aq" is the mass (mg) of CD in solution and "Calculated [CD]s" is the mass (mg) of solid CD or CD in the precipitate. [CD]aq and [CD]s were calculated gravimetrically.*

Appendix III

```
Data File C:\CHEM32\1\DATA\KB_B1\028-2701.D
 Sample Name: 20

    ========================================================================
    Acq. Operator    : Chris                          Seq. Line :  27
    Acq. Instrument  : Instrument 1                    Location : Vial 28
    Injection Date   : 3/31/2017 5:51:09 PM                 Inj :   1
                                                     Inj Volume : 20 µl
    Acq. Method      : C:\CHEM32\1\METHODS\CDGROUP.M
    Last changed     : 3/31/2017 1:23:29 PM by Chris
    Analysis Method  : C:\CHEM32\1\METHODS\CDGROUP.M
    Last changed     : 3/22/2017 2:50:52 PM by Chris
```




```
    ========================================================================
                              Area Percent Report
    ========================================================================

    Sorted By             :      Signal
    Multiplier            :      1.0000
    Dilution              :      1.0000
    Use Multiplier & Dilution Factor with ISTDs


    Signal 1: DAD1 A, Sig=250,4 Ref=360,100

    Peak RetTime Type  Width     Area      Height     Area
      #   [min]        [min]   [mAU*s]     [mAU]       %
    ----|-------|----|-------|----------|----------|--------|
       1   6.147 BV    0.1408 4248.05322  463.94046 100.0000

    Totals :                   4248.05322  463.94046


    ========================================================================
                              *** End of Report ***
```

Figure 20. Example of HPLC report

Appendix IV

	A1	A2	A3	A4	A5	A6	A7
Percent Work	1%	2%	3%	25%	50%	75%	100%
Concentration	0.00498 mM	0.00996 mM	0.01494 mM	0.12447 mM	0.24894 mM	0.37341 mM	0.49789 mM
Retention time	6.912	6.171	6.169	6.169	6.17	6.17	6.169
Area	99.98 mAU*s	196.41 mAU*s	298.58 mAU*s	2488.32 mAU*s	4929.49 mAU*s	7403.08 mAU*s	9847.40 mAU*s
	B1	B2	B3	B4	B5	B6	B7
Percent Work	1%	2%	3%	25%	50%	75%	100%
Concentration	0.00257 mM	0.00514 mM	0.00771 mM	0.06421 mM	0.12842 mM	0.19263 mM	0.25684 mM
Retention time	6.176	6.509	6.175	6.178	6.177	6.173	6.17
Area	52.70 mAU*s	102.57 mAU*s	157.14 mAU*s	1291.39 mAU*s	2634.00 mAU*s	3955.09 mAU*s	5230.00 mAU*s

*Table 6. Absorbance data of the calibration standards obtained from the HPLC. Shown are the 1%, 2%, 3%, 25%, 50%, 75% and 100% percentage dilutions of the Work solutions diluted from stock solutions A and B, respectively are shown on the top row. The measured absorbance (mAU*s) corresponding to the concentration (mM) for each standard are displayed, along with the retention time.*

Appendix V: Extended Abstract

Increasing the Solubility of Dexamethasone by Complexation with β- and γCD

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Roskilde University

Overview

Several properties are sought for in a drug during the design, in the scientific field of drug discovery. A successful drug must possess a solubility in an ideal range specific to each drug, as it determines the way the drug should behave in the body to exert its effect to its full potential. This project aims to increase the solubility of a drug with relatively low solubility, dexamethasone (DMS), by complexation with β- and γ-cyclodextrin individually, and in combination, to find how the solubility increase. Three experiments were carried out in the same fashion, by mixing dexamethasone with βCD and γCD in water for a week – individually in two separate experiments, and with combined β- and γCD in the final experiment – until they were assumed to have reached solubility equilibria. Samples were weighed before water was added and after the supernatants were extracted, to find the amount of CD in precipitate, and therefore, in solution. Supernatants of samples were put into the HPLC and the absorbance obtained was correlated to concentration of dexamethasone in solution, using a calibration curve. The data were plotted in phase-solubility diagrams which indicated the presence of more than two types of $DMS{:}\,CD$ complexes. The phase-solubility diagrams for the three experiments yielded the following results: βCD samples could increase the solution of DMS than γCD samples, the highest concentrations were 8.2 mM and 7.4 mM, respectively. The stoichiometric ratio of the precipitate for $DMS{:}\,\beta CD$ was 1:2, and 2:3 for $DMS{:}\,\gamma CD$. Finally, the combined β- and γCD samples yielded a dissolved dexamethasone concentration of 18.3 mM, which is larger than the sum of the individual CD results which is 15.6 mM, most likely indicating a synergistic effect.

Keywords

Additive: the effect of combined substance gives the sum of the individual effects.
Antagonistic: the effect of the combined substance gives an effect which is lower than the sum of the individual effects.
Synergistic: the effect of the combined substance gives an effect that is greater than the sum of the individual effects.
Cyclodextrin (CD): a cyclic carbohydrate chain used to increase the solubility of dexamethasone.
Dexamethasone (DMS): the drug with low solubility which the aim is to increase using cyclodextrin.
Solubility equilibrium: the point where no more solute can dissolve in a solvent.
Oversaturation: the point when the concentration of solute exceeds that of the solvent.

Introduction

Drug discovery and development is a field of study regarding the process of designing a drug, from identifying chemical entities, carrying out laboratory and human tests on them, until all but one drug is eliminated that is most promising and thus suitable for being sold on the market. Several properties are sought for in a drug. The property that will be focused on in this study, is solubility. It is a key property a successful drug must possess, in an optimal range specific to each drug, as it is a crucial determinant of the way the drug behaves in the body. If a drug is too soluble, thus, too polar, the drug will have trouble being absorbed through cells, and lower the amount of drug at the target, since hydrophobicity is a

necessary characteristic of molecules to passively transport through cells. This naturally hinders the drug's pharmacological effect. On the other hand, if a drug is not soluble enough, hence, if it is non-polar, the drug would not be able to dissolve, and thus its presence in solution would be insufficient. Therefore, it would not be in its active form to exert its effect to its full potential. This project aims to increase the solubility of a low-soluble drug, dexamethasone, by complexation with a cyclic carbohydrate chain called cyclodextrin. Two types of cyclodextrins, β- and γ-cyclodextrins, are utilized alone and in combination, to investigate the possible effect of both, which we hypothesize may be an additive, antagonistic or a synergistic effect. Three experiments were carried out using the same method. Different masses of CD in increasing increments were added to samples containing a fixed mass of DMS and water, for the first two experiments. The samples in the third experiment consisted of a fixed mass of DMS and water, as well as both types of CD added in varying percentages. All of the samples, in the three experiments, were weighed before water was added and after supernatants were extracted, in order to calculate the amount of CD in precipitate and therefore solution, knowing the amount of CD added in each vial. After having mixed them for a week, they were assumed to have reached equilibrium. The supernatants were extracted and processed by the HPLC to obtain absorbance, which was correlated to the concentration of DMS in solution for the samples. For the samples in each experiment, the dissolved DMS concentrations were plotted against increasingly added CD concentrations to form phase-solubility diagrams. Phase-solubility diagrams plotted from the data in each of the three experiments were analysed to find, among other information, the maximum concentration of dissolved dexamethasone. Solubility and stability equilibria were also used to analyse the data. Solubility equilibria describes the interaction between a compound in precipitate and the compound dissolved in solution, and stability equilibria represent how quickly the complexes are formed compared to how quickly they disassociate. This tells us how favourable the complexation reaction is, which among other information, provides understanding on the presence of $DMS{:}\,CD$ and $DMS{:}\,CD_n$ complexes in different segments of the diagram, which contributes to knowledge on the amount of DMS in solution.

Methods

Calibration Standards Preparation

By running standards with known concentrations of DMS through the HPLC, a linear relationship between absorbance and concentration of DMS can be established; thus, the absorbance obtained from the HPLC can be approximated to the concentration of DMS in solution for plotting the phase-solubility diagrams.

1. Prepare two 1 mg/ml DMS stock solutions (Stock A and Stock B) to achieve a concentration of 2.548 mM of DMS. Dilute stock solutions to create work solutions of 0.5 mM DMS for Work A and 0.25 mM for Work B made up to 50 ml demineralized water.
2. Prepare seven calibration standards from the two Work solutions: 1%, 2%, 3%, 25%, 50%, 75% and 100%, respectively. Prepare the concentrations of standards A1-7 and B1-7, B1 being the lowest ($0.0025\ mM$) and A7 being the highest ($0.5\ mM$), and transfer to HPLC vials.

Preparation of DMS and Cyclodextrin Samples

Add increasing increments of cyclodextrin to vials with a fixed mass of DMS and water. After a week, measure the absorbance of supernatants in vials measured by HPLC, giving the total concentration of DMS in solution.

1. Add 13.74 mg of DMS to each Eppendorf tube, 33 tubes for the first two individual CD experiments and 28 for the third combined CD experiment. The concentration of 35 mM DMS was chosen

based on data previously obtained (Bartoš, Hansen, Pedersen, Larsen, & Lund, 2016). Weigh DMS tubes and record.

2. Add CD to the samples.
    a. For each individual CD experiment, prepare 3 samples without adding any CD. 1 mM to 150 mM CD is added to the remaining 30 samples.
    b. In the third experiment, to observe if the ratio between the types of CD influences the solubility, varying amounts of CD were calculated based on the B-type Phase-Solubility Diagrams, and added.
3. Weigh tubes, add 1 mL of water, vortex, sonicate and shake at 400 rpm and 25 degrees for a minimum of one week to reach equilibrium. Centrifuge tubes, extract supernatant using a 2 ml syringe and a filter, and put in a new Eppendorf tube.
4. Weigh precipitate in tubes and dry in a vacuum oven overnight. Weigh tubes after drying.
5. Dilute samples for first two experiments in HPLC vials, excluding samples without CD, using dilution factors taken from previous student group (Bartoš, Hansen, Pedersen, Larsen, & Lund, 2016), the concentrations must lie within the calibration range.
6. Analyse samples in the HPLC by preparing reagents and adjusting the settings.

Discussion/Conclusion

The data from the individual CD experiments generally follows the B-Type Phase-Solubility Diagram (see Fig. 1 and Fig. 2). Two complexes are assumed to form in the system: $DMS{:}\,CD$ and $DMS{:}\,CD_n$. The difference is that one contains one CD and the other one contains more than one. Initially, the concentration of dissolved DMS increases linearly, until reaching a plateau, due to the solubility limit of the $DMS{:}\,CD$ complex being reached. At this point, the total concentration of dissolved DMS is the sum of the solubility limit of DMS plus the solubility limit of $DMS{:}\,CD$ plus the concentration $DMS{:}\,CD_n$.

During the plateau, the total concentration of DMS is not increasing, due to the limited solubility of the complexes. Increasing the amount of added CD causes more complexes to be formed with the DMS in solution. The DMS in solution is replenished by the DMS in precipitate and the extra complexes precipitate. Essentially the DMS in precipitate is replaced by complex during the plateau.

In the descending segment, the DMS in precipitate is depleted. Increasing the amount of added CD causes all the DMS and complexes in solution to become the larger $DMS{:}\,CD_n$. The total amount of DMS in solution then approaches the solubility limit of $DMS{:}\,CD_n$.

The plateau indicates the maximum concentration of dissolved DMS that is possible by adding CD. In the individual βCD and γCD experiments, the start of the plateau has as a spike in concentration, which could be due to oversaturation, and was therefore not used to determine the maximum concentration of dissolved DMS (see Fig. 1 and Fig. 2). The maximum concentration of dissolved DMS was 8.2 mM for βCD and 7.4 mM for γCD. βCD increased the solubility of DMS more than γCD. The precipitation formed during the plateau was used to calculate the ratio of DMS and CD in the complexes. The ratio between $DMS{:}\,\beta CD$ is 1:2 and $DMS{:}\,\gamma CD$ is 2:3.

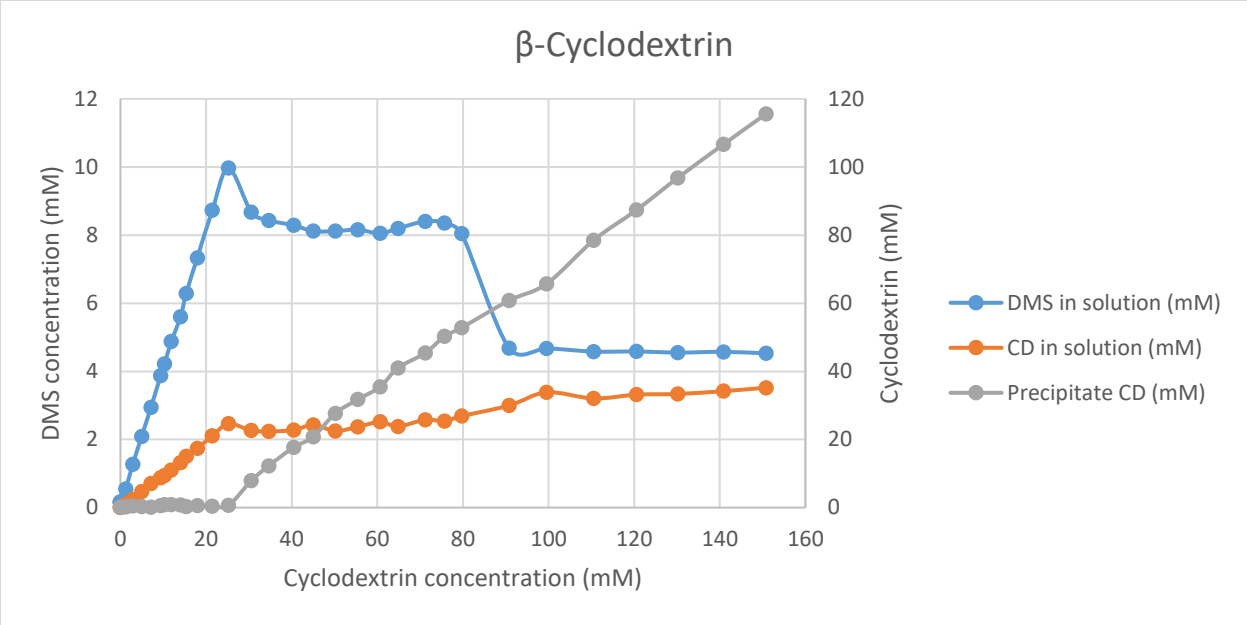

Figure 1. βCD Phase-Solubility Diagram and distribution of βCD in solution and precipitate. *The blue line displays "Calculated DMS" vs "Calculated [CD]" giving a Phase-Solubility Diagram. The orange line exhibits the "Calculated CDs" vs "Calculated [CD]" and the grey line displays "Calculated CDaq" vs "Calculated [CD]".*

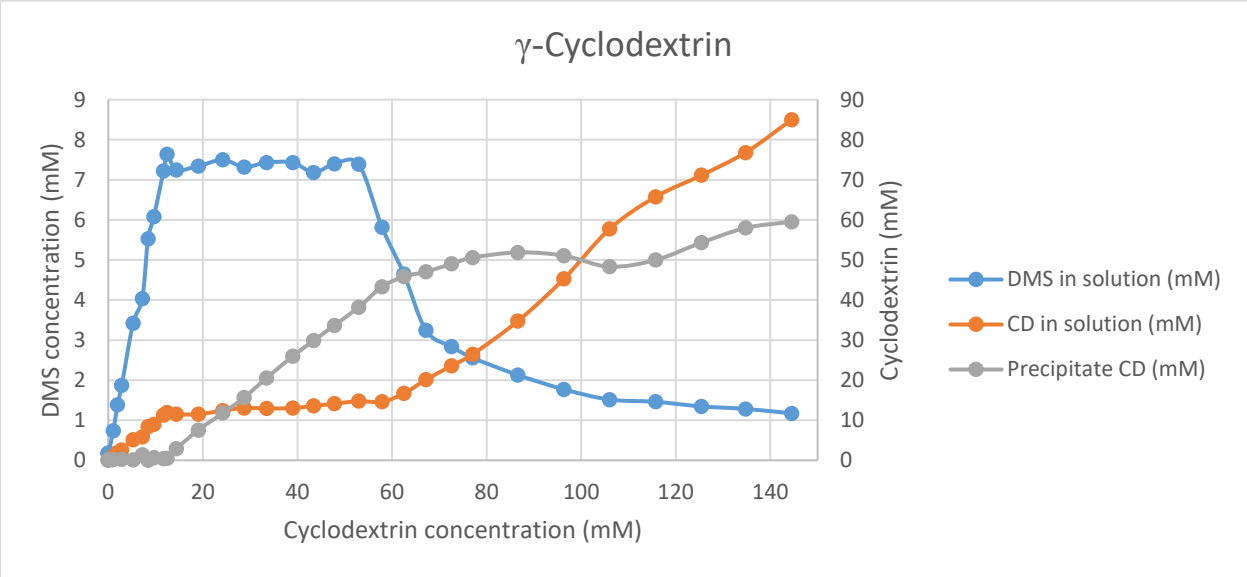

Figure 2. γCD Phase-Solubility Diagram and γCD in solution and as precipitate. *The blue line displays "Calculated [DMS]" vs "Calculated [CD]" giving a Phase-Solubility Diagram. The orange line exhibits the "Calculated [CD]s" vs "Calculated [CD]" and the grey line displays "Calculated [CD]aq" vs "Calculated [CD]"*

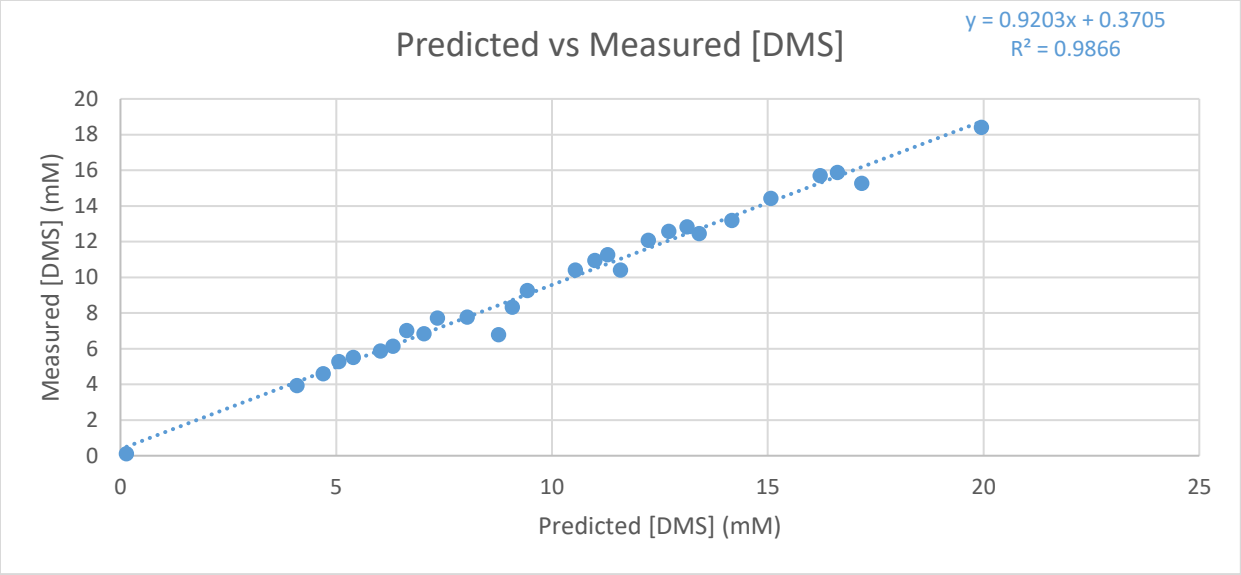

Figure 3. Predicted versus measured dissolved DMS concentrations in solution. *Predicted dissolved [DMS] (mM) is plotted on the x-axis and measured dissolved [DMS] is on the y-axis. The R2 value is 0.9866 and the equation of the line is y = 0.9203x + 0.2414.*

To verify if the increase in concentration of dissolved DMS in the combined CD experiment, was additive, then the predicted and measured concentrations of DMS were plotted against each other. The predicted concentration of DMS was calculated by multiplying the concentration of each type of CD with the slope of their respective growth segments, determined from the previous two individual CD experiments. The slope represents how much the concentration of DMS increases per mM of added CD. The plot should have a slope of 1 if there is an additive effect. The slope is 0.9203 which is lower than 1, and therefore indicates an antagonistic effect, however an antagonistic or synergistic effect should of given a lot of variance, so the difference in slope is probably a systematic error. The data point with the highest value, at 18.3 mM dissolved $[DMS]$, exceeds the sum of the maximum dissolved $[DMS]$ from the individual CD experiments which is 15.6. This indicates a synergistic effect on the maximum solubility. Since there is only one point that can show this synergistic effect, further research is necessary, using higher concentrations of CD, to conclude whether combining both types of CD, results in a synergistic effect on the maximum concentration of DMS.

The results from this study can be applied in a number of ways. Firstly, to find the optimal ratio of DMS and CD, so that the concentration of DMS is optimized. Secondly, utilizing multiple types of CD can be used to further increase the solubility of drugs, compared to using only a single type of CD. The stoichiometry of the complexes could be verified by employing crystallography and/or NMR to analyse the dried precipitate. The stability of the complexes could be verified by ITC.