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FLUTICASONE PROPIONATE/GAMMA - CYCLODEXTRIN COMPLEXES FOR PULMONARY DELIVERY

KOMPLEKSI FLUTIKAZONPROPIONATA Z GAMA CIKLODEKSTRINI ZA DOSTAVLJANJA V PLJUČA

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I, Urška Leben, student of pharmacy at the University of Ljubljana, Faculty of Pharmacy performed the thesis research work within Erasmus mobility exchange program at the University of Lisbon, Faculty of Pharmacy, at the department of Pharmaceutical Technology. Host supervisor was Prof. Dr. Helena Maria Cabral Marques, home supervisor was Prof. Dr. Julijana Kristl. Preparation, characterisation and formulation of inclusion complexes along with Twin Impinger analysis were made at the Faculty of Pharmacy, University of Lisbon. The SEM images were made at Kemijski inštitut in Ljubljana, dissolution tests were performed at the Faculty of Pharmacy, University of Ljubljana.

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Statement

I declare that I made this thesis research work under the supervision of Prof. Dr. Julijana Kristl and Prof. Dr. Helena Maria Cabral Marques.

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Abstract

Fluticasone proprionate (FP) is a corticosteroid drug used as anti-inflammatory agent in asthma, chronic obstructive pulmonary disease and allergic rhinitis. It is a highly lipophilic drug with poor water solubility.

This study aimed to prepare and characterise inclusion complexes of FP with the most suitable cyclodextrin (CD) in order to meet the augmented lung deposition and water solubility compared to formulations of free FP. In the research HP-β-CD and γ-CD were used. Using methods of molecular modelling and phase-solubility studies, γ-cyclodextrin resulted to be the most appropriate choice for inclusion complex formation with FP. Kneading methods were not efficient for FP/γ-CD formation, therefore precipitation method from water was used. Inclusion was tested with IR spectroscopy and inspection of FP solubility differences between free drug, physical mixture (FP and γ-CD) and FP/γ-CD. In the IR analysis the characterictic absorption bands were eliminated on complex spectrum. UV analysis of FP solubility showed 20-fold higher absorbance compared to free FP. SEM images were taken for size and morphology observation of the FP, γ-CD, physical mixture of pure components and FP/γ-CD particles. Particle size distribution spectrum of FP/γ-CD was obtained by Aerosizer® Aerodynamic performaces were carried out on Twin Impinger comparing the deposition of FP/γ-CD and free FP in formulation with α-lactose monohydrate. Capsules were manually filled, so that each capsule contained 40 ± 1 mg or 20 ± 1 mg of powder mixture. The inhaling device Rotahaler® was used for discharging the powder from the gelatine or HPMC capsules at a flow rate of 60 ± 5 l/min. Respirable fraction and emitted dose were evaluated. Formulations with FP/γ-CD inclusion complexes did not exhibit better aerosol performances compared to free FP, except complex formulation of 20 mg in HPMC capsules resulted in a slightly improved performance (higher respirable and emitted fraction). On the other hand, dissolution test demonstrated already in 15 minutes the 27-fold increase in water solubility of FP/γ-CD compared to free FP. Regarding FP dissolution, the use of γ-CD is beneficial for inclusion complex formation.

Key words: fluticasone propionate, γ-cyclodextrin, pulmonary delivery, Twin Impinger, deposition, dissolution

Povzetek

Flutikazon propionat (FP) je kortikosteroidna učinkovina s protivnetnim delovanjem, ki se uporablja pri zdravljenju astme, kronično obstruktivne pljučne bolezni in alergijskega rinitisa. Učinkovina je lipofilna ter izredno slabo topna v vodi.

Cilji diplomskega dela so bili priprava in karakterizacija inkluzijskih kompleksov flutikazonpropionata z najustreznejšim ciklodekstrinom, da bi izboljšali nalaganje v pljučih in povečali topnost v vodi v primerjavi s prosto učinkovino. V proučevanje smo vključili hidroksipropil-β-CD (HP-β-CD) in gama-CD (γ-CD). Z molekularnim modeliranjem in določitvijo fazne topnostni smo ugotovili, da je γ-CD najustreznejši za kompleksiranje FP. Metode priprave kompleksov z gnetenjem se niso izkazale za uspešne pri kompleksiranju z γ-CD, zato smo uporabili metodo precipitacije iz vode. Uspešnost vključevanja smo preverili z IR in opazovanjem razlike v topnosti (v vodi) med prosto učinkovino, fizikalno zmesjo ter kompleksi. Na IR spektru kompleksov v primerjavi s samo učinkovino so izginili karakteristični vrhovi flutikazonpropionata, medtem ko se je pri preskusu raztapljanja v vodi topnost učinkovine v kompleksirani obliki povečala 20-krat. Analizo velikosti in morfologije delcev FP, γ-CD, fizikalne zmesi obeh komponent ter FP/γ-CD kompleksov smo določili na SEM posnetkih, spekter distribucije velikosti delcev FP/γ-CD kompleksov pa na aparaturi Aerosizer®. Aerodinamične značilnosti smo preverjali na Twin Impingerju, kjer smo primerjali nalaganje formulacij s kompleksi oz. s prosto učinkovino v kombinaciji z α-laktozo monohidratom. Kapsule smo polnili ročno, tako da je vsaka kapsula vsebovala 40 ±1 mg oz. 20 ± 1 mg praška. Pri analizi s Twin Impingerjem smo uporabili inhalator Rotahaler®, v katerega smo vstavili želatinsko oz. HPMC kapsulo z ustrezno formulacijo pri pretoku zraka 60 ± 5 l/min in dobili podatke za inhalabilno in izločeno frakcijo. Formulacije z FP/γ-CD inkluzijskimi kompleksi se niso izkazale za učinkovitejše glede na aerodinamične značilnosti v primerjavi s formulacijami, ki so vsebovale samo učinkovino. Nekoliko izboljšano obnašanje je bilo izkazano le pri formulaciji FP/γ-CD v HMPC kapsulah z 20 mg vsebine praška (višja inhalabilna in izločena frakcija). Test raztapljanja pa je pokazal 27-krat višjo topnost kompleksov v primerjavi s prosto učinkovino že v 15-ih minunah. Z ozirom na izboljšano topnost lahko sklepamo, da je uporaba γ-ciklodekstrinov koristna za tvorbo inkluzijskih kompleksov s flutikazonpropionatom.

Ključne besede: flutikazon propionat, γ-ciklodekstrin, dostavljanje v pljuča, Twin Impinger, depozicija, raztapljanje

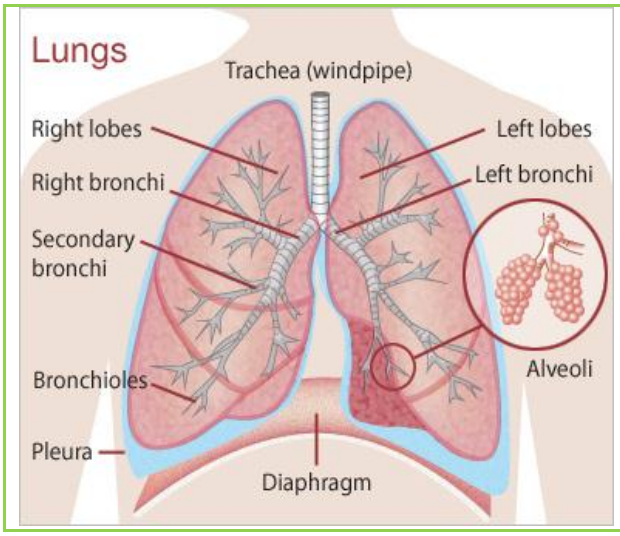
List of abbreviations

AT I cells	alveolar type I cells
GIT	gastrointestinal tract
MDI	metered dose inhaler
FP	fluticasone propionate
DMSO	dimethyl sulfoxide
DMF	dimethylformamide
COPD	chronic obstructive pulmonary disease
FP	fluticasone propionate
IL	interleukin
NF-κB	nuclear factor kappa B
CYP	cytochrome P
CD	cyclodextrin
D	drug
K_c	stability constant
K_d	dissociation constant
NMR	nuclear magnetic resonance
pKa	acid dissociation constant
HPLC	high-performance liquid chromatography
GRAS	generally recognized as safe
FK224	cyclopeptide substance P antagonist
UV	ultraviolet
IR	infrared
SEM	Scanning Electron Microscopy
TI	Twin Impinger
RC	regenerated cellulose
EtOH	ethanol

1 Introduction

1.1 Pulmonary route for drug delivery

The lungs offer a large surface for absorption when the drug is administrated in gaseous, aerosol mist or ultrafine solid particle form. Inhalation therapy has a long history in medical treatment with origins which date back 4000 years especially in India, where people smoked the leaves of *Atropa belladonna* plant to supress cough. Smoking of tobacco products is one of the most common examples of pulmonary delivery through history (1,2).



Conducting zone	Respiratory zone
• Nose • Pharynx • Larynx • Trachea • Bronchi • Bronchioles • Terminal bronchioles	• Respiratory bronchioles • Alveolar ducts • Alveolar sacs

Figure 1: Anatomic scheme of lungs (adopted from (9))

In the last decades pulmonary delivery has emerged as a promising possibility for the non-invasive local and systemic drug delivery. Commonly used pharmacologic aerosols for respiratory diseases are bronchodilators, anti-inflammatory agents such as steroids and antibiotics. The respiratory system is functionally divided into a conducting and a respiratory zone (see *Figure 1*). The conducting zone is considered to be a conduit between the external environment and the respiratory zone, thus it does not contribute to the gas exchange. The respiratory region, mainly alveoli, of the lung provides a total surface area of 80-100 m^2 (adult). The alveolus is the principle site of gas exchanges as well as of the drug absorption. In alveolus regions significant decrease in the velocity of air flow takes place as a result of

greatly increased cross-sectional area between the terminal bronchiole and the alveolar sac. Lung contains only small volumes of liquid which is consequently distributed as a thin layer measured between 21 and 200 nm on alveolar surface covering air-blood barrier (3–8).

1.1.1 Advantages of using pulmonary delivery for respiratory diseases

Lungs provide an excellent surface area with a very good blood supply and a minimum physical barrier area between airspace and bloodstream. By inhalation a high drug concentration is delivered to the target organ reducing systemic exposure and therefore producing lower adverse effects. The epithelium of the alveolus consists mainly of flat AT I cells which are in intimate contact (in the most pulmonary regions only between 0.1 μm and 0.2 μm) with blood vessels. Thus it appears that after deep lung inhalation (placing the drug into alveolus) the drug can be rapidly absorbed. Moreover, drugs administered via lungs avoid first pass liver metabolism, they meet low intrinsic enzymatic activity and are not exposed to GIT conditions (e.g. low pH level in the stomach, catabolic enzymes ...), which gives the possibility of accomplishing a higher bioavailability of some drugs. As a result smaller doses are needed to achieve a similar therapeutic effect, for example oral salbutamol 2-4 mg is therapeutically equivalent to 100-200 μg by MDI (5,10,11).

1.1.2 Disadvantages and challenges of using pulmonary delivery for respiratory diseases

Development of optimal drug formulation and delivery devices to produce an aerosol with desirable mass and size often encounters problems. For that reason it requires a lot of research and expenses. The technology is clearly more challenging than in the case of pills development. Therapeutic effect might be affected by the site of deposition in the respiratory tract and the delivered dose to that site. Barriers to the uniformity of drug delivery are also airway geometry, humidity, clearance mechanisms and presence of various lung diseases (4,11). The use of different devices for pulmonary delivery demands certain skills of the patient and an adequate breathing manoeuvre to accomplish suitable deposition (6).

1.2 Inhaled corticosteroids (ICS)

Inhaled glucocorticosteroids were introduced in 1972. After 40 years they are still the most frequent choice for the first-line treatment in asthma. They are also indicated for chronic obstructive pulmonary disease and intranasal therapy for allergic rhinitis. They are anti-inflammatory drugs with remarkably better therapeutic index when compared to oral corticosteroids due to their topical application. For that reason they have almost entirely replaced oral corticosteroids which show major adverse effects such as hypothalamic-pituitary-adrenal-axis suppression, osteoporosis, growth retardation in children, cataract and glaucoma (12–14).

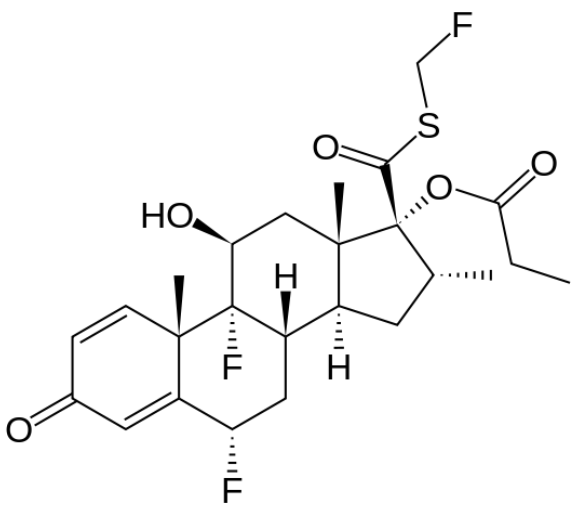
The ideal corticosteroid for use by inhalation is desired to have high efficacy in the airways together with minimum systemic effects within its dose range. In order to accomplish this, the compound should have a high intrinsic topical activity (anti-inflammatory potency) combined with low oral systemic bioavailability (15). Moreover, the therapeutic index is also function of pharmacokinetic parameters of a drug. Improved parameters such as high clearance rate, high volume of distribution, high level of protein binding as well as short elimination half-life and a long terminal half-life should have augmented the therapeutic index as a result of increased receptor binding affinity, slower absorption from the lung after inhalation and rapid systemic clearance. Equally important for ICS's efficacy is the high pulmonary deposition which results in greater pulmonary anti-inflammatory activity (16).

Besides the side effects of ICS that occur mostly due to their systemic absorption, they can complicate the treatment because they can cause candidiasis in oropharynx and dysphonia. Both local side effects are significantly dependent on the daily doses of ICS (17).

1.3 Fluticasone propionate

FP is an androstane synthetic steroid of glucocorticoid family used as an anti-inflammatory agent, specifically designed to have topical activity and high first-pass metabolism, thereby minimising the risk of associated systemic adverse effects. It is a highly lipophilic drug (log P = 3.46) (22) passing readily through the cell membrane and binding with the glucocorticoid receptor (21,22). The result of binding to the glucocorticoid receptor is a conformational change in the receptor, followed by dissociation from its chaperone proteins

and translocation to the cell nucleus. Dimerization of ligand-receptor occurs and binds to a specific DNA sequence known as the glucocorticoid response element (GRE). Consequently it increases the expression of anti-inflammatory molecules. Genes that are recognized to be upregulated by glucocorticoids and being involved in resolution of inflammation are lipocortin I and p11/calpactin binding protein. They both play part in suppressing release of arachidonic acid (23). On the other hand, binding to the negative GRE inhibits expression of pro-inflammatory cytokines such as IL-4 and IL-5, which are important in the allergic immune response, participating in immunoglobulin E (IgE) production, mast-cell activation and mediator release, and eosinophil differentiation. The action of glucocorticoids can also take place in cytoplasm by inhibiting cytoplasmic pro-inflammatory transcription factors, such as activator protein-1 and nucleus factor kappa B (NF-κB) (24).



The physico-chemical properties of FP	
State (18)	Solid
Colour (18)	White
Average molecular weight (18)	500.571
Melting point	272-273 °C
Solubility (19)	Ins. in water, freely soluble in DMSO, DMF, slightly soluble in methanol, ethanol
log*P* (22)	3.46
Exp. water solubility (18)	0.51 mg/L (insoluble)

Figure 2: Chemical structure of FP (20) and physicochemical properties

In asthma patients it is recommended to administrate 50-1000 µg of FP twice daily depending on disease severity and patient's age (25). FP is highly selective for the GR with <0.001 of the relative potency for human androgen, estrogen and mineralocorticoid receptors. The half-life of the steroid-receptor complex for FP is >10 hours, therefore it has a high lung retention which is also the consequence of the drug's high degree of lipophilicity (26). As 10-20 % of the inhaled dose is deposited in the lung, the rest is deposited on the oropharynx and can be

swallowed, it is therefore available for systemic absorption (24). Once systemically absorbed, FP undergoes a rapid first-pass metabolism by the cytochrome P-450 isoenzyme CYP3A4 to an inactive metabolite 17-carboxylic acid derivative (27). Since total blood clearance is similar to hepatic blood flow, oral bioavailability is reported to be around 1% (24,28). The study was done to mimic swallowed fraction of inhaled FP. 21 healthy male volunteers were enrolled in a study and received oral FP of 0.1 mg, 1 mg or 10 mg, or oral placebo twice daily according to a randomised double-blind crossover design. Unchanged plasma FP was detected only in 10 mg oral FP group, proving the oral bioavailability to be around 1% (29).

A randomised cross-over study comparing pharmacokinetics and systemic effects of inhaled FP in patients with asthma and healthy volunteers showed that the systemic availability of inhaled FP was more than halved in asthma patients when compared with healthy controls (12).

1.4 Cyclodextrins

Cyclodextrins (CDs) are cyclic oligosaccharides obtained by the enzymatic degradation of starch. The most common naturally occurring CDs are composed of six (α-CD), seven (β-CD) and eight (γ-CD) glucose residues, respectively (30).

They were first described as ‘cellulosine’ by a French scientist A.Villiers in 1891 when he obtained 3 g of crystalline material from 1000 g of starch digest of *Bacillus amylobacter* probably contaminated with *Bacillus macerans*. The substance seemed to be resistant to acid hydrolysis and did not show reducing properties. Soon after that an Austrian microbiologist F. Schardinger in 1903 managed to isolate α-CD and β-CD, whereas γ-CD was discovered by Freundenberg and Cramer later, i.e. in 1935. By the mid of 1980s the existence of large CDs was also proven; on the other hand, CDs with less than 6 units cannot be prepared due to steric reasons (31,32). The main role in research and application of CDs is their ability to selectively form inclusion complexes with other molecules, ions or even radicals (33).

1.4.1 Chemical structures and properties of natural occurring cyclodextrins

Cyclodextrins are cyclic oligosaccharides consisting of glucopyranose units linked by α-(1,4) bonds. Natural CDs are the primary product of starch degradation by cyclodextrin glucosyltransferase enzyme (CGTase), where chain splitting undergoes an intramolecular reaction without the participation of a water molecule. However, starch by enzymatic degradation usually results in the production of glucose, maltose, maltotriose, etc. (dextrins) which is a real hydrolytic process, as the origin product reacts with one molecule of water when splitting the glycosidic linkage. The three-dimensional structure of a CD molecule forms a shallow truncated cone with a hydrophobic centre cavity and a hydrophilic exterior (34).

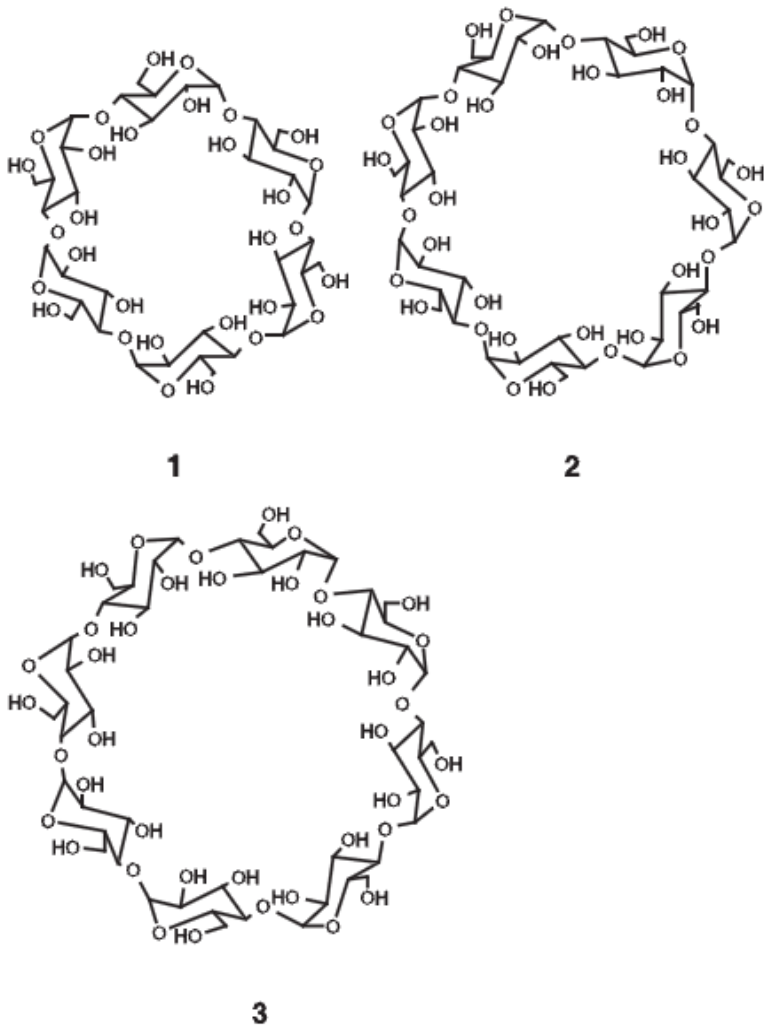


Figure 3: Structures of α-CD (1), β-CD (2), γ-CD (3), adopted from (33)

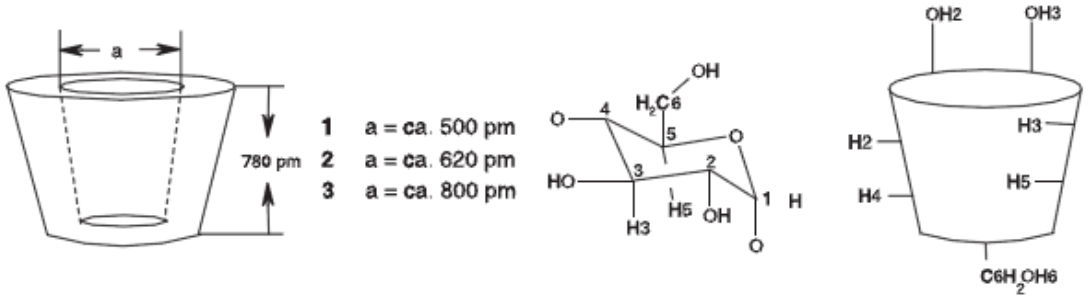


Figure 4: Schematic view of the 3D structure, atom numbering of glucopyranoside ring, average orientation of the most important atoms and OH groups (adopted from (33))

Because of the C1 conformation of the glucopyranose units, all secondary OH groups are situated on one of the two edges of the ring, and the entire primary on the other. The result of the conformation is the hydrophilic nature of the surface of the CD molecule. The lipophilic character of CD's cavity is a result of H3 and H5 protons lining the cavity and lone pairs of glycosidic oxygen atoms lying in a plane. The non-bonding electron pairs of the glycosidic oxygen bridges are directed towards the inside of the cavity, producing a high electron density and lending it some Lewis-base character (35).

The C2-OH group of one unit can form a hydrogen bond with the C3-OH group of the neighbour unit. Because of these hydrogen bonds the CD molecule is a rigid structure. The lowest solubility of β-CD can be explained by intramolecular H-bonds. The hydrogen belt is incomplete in α-CD molecule, as one of the units is in a distorted position which is why only 4 of 6 H-bonds can be established. The γ-CD is the most soluble of the three due to its non-coplanar structure, which is more flexible. Free rotation of the primary OH-groups will reduce the effective diameter of the cavity on the side on which they occur (35).

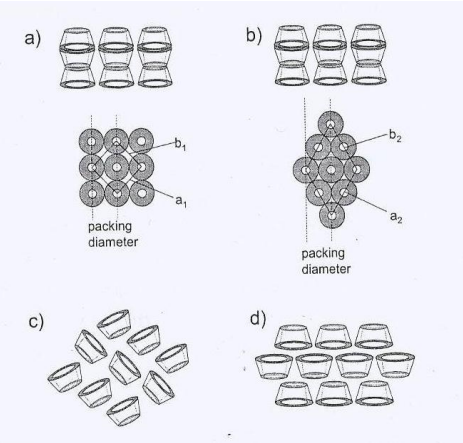
The macrocycle ring of CDs is distorted from its ideal structure with a n-fold symmetry to compensate the steric effects between glucopyranose units and distortion of the glycosidic linkage. Upon complex formation CDs can change their macrocyclic structure and adjust the structure of the cavity to accommodate the guest molecule. The shape of the macrocyclic ring is well explained by using a polygon consisting of glycosidic O4 atoms which are nearly coplanar. The radius of the polygon, the distance from the centre of polygon to O4 atom, is a good measure to obtain the size of macrocycle (33).

Table 1: Characteristics of α-CD, β-CD, γ-CD (adopted from (35))

	α-CD	β-CD	γ-CD
No. of glucose units	6	7	8
Molecular weight	972	1135	1297
Solubility in water [g/100 mL] at 25°C	14.5	1.85	23.2
Cavity diameter [Å]	4.7-5.3	6.0-6.5	7.5-8.3
Crystal water (%)	10.2	13.2-14.5	8.13-17.7
Water molecules in cavity (33)	6	11	17

Cyclodextrins crystallized from water have their cavities filled with water molecules; some are integral parts of the crystal structure (crystal water). Inclusion occurs with the substitution of included water in the cavity by the guest molecule. Crystal structure of CD turns to amorphous state by eliminating the water (dehydration) (35).

Cyclodextrins crystallise in two main types of crystal packing (see *Figure 5*): these are channel structures and cage structures, depending on the CD and guest molecule (31).



CDs can change their crystal structure due to the complexation. For instance, γ-CD when uncomplexed typically forms a cage herringbone type of crystal, whereas in complex forms the columnar channel type packing, which is either tetragonal or hexagonal, determined by the packing of the γ-CD molecules in the respective two-dimensional unit cells (34).

Figure 5: Crystal structures of CDs: a) tetragonal columnar channel-type, b) hexagonal columnar channel-type, c) cage herringbone-type and d) cage brick-wall-type structures (adopted from (34))

1.4.2 Modified cyclodextrins

Various CD derivatives have been developed by the chemical substitution of the primary or secondary hydroxyl groups of natural CDs in order to alter their limited aqueous solubility, complexing properties or parenteral safety (34).

Random modifications of hydroxyl groups to hydroxylpropyl, sulfopropyl, carboxymethyl or silyl functions can be easily achieved. Hydroxyl groups have nucleophilic properties, consequently electrophilic attack on these positions is the initial reaction (33). It was discovered that substitution of any of the hydroxyl groups, even by hydrophobic moieties such as methoxy functions, resulted in a dramatic increase in their aqueous solubility. The main reason for the solubility enhancement in the alkyl derivatives is that chemical manipulation transforms the crystalline α-, β- and γ-CD into amorphous mixture of isomeric derivatives (30).

1.4.3 Production of cyclodextrins (solvent, non-solvent process)

The production of CDs consists of several phases. Normally they are prepared by enzymatic degradation of starch (usually of corn or potato) with CTGase at 30-90 °C. The main steps of preparation include:

- Cultivation of a microorganism that is able to produce CTGase enzyme, such as *Bacillus macerans, Bacillus megaterium, Bacillus subtilis, Bacilus firmus, Bacillus circulans, Klebsiella pneumonia, Klebsiella oxytoca*
- Separation and purification of the enzyme
- Pre-hydrolysis of starch with amylases
- Enzymatic conversion of starch
- Inactivation of the enzyme by heat treatment
- Separation of the CDs from the conversion mixture (mixture of α-, β-, γ-CDc along with series of acyclic dextrins)
- Purification and crystallization of CDs from water

Separation of the conversion mixture can be achieved by the addition of an appropriate solvent or using a solvent-free method which is more desirable (36).

1.4.3.1 Solvent process

To improve the yield of production and to alter the ratio of different CDs obtained, complexing agents can be added. Mostly they form insoluble complexes with CDs, while the rest of the CDs and acyclic dextrins remain in the mother liquid. The complex can be easily separated from the liquid conversion. For cyclodextrin isolation the complex is dissociated by steam distillation or extraction of the complexing agent with an organic solvent (36). The choice of an appropriate enzyme and complexing agent determines the selectivity of CD formation and recovery. The yield of production can be improved also by using specific CTGase enzymes (37).

1.4.3.2 Non-solvent process

The non-solvent process is used when CDs are intended to be used for food or pharmaceutical purposes. The reaction mixture is composed only of water, starch and enzyme. The final crystallization of CDs is the most challenging step in this process. Production costs are usually higher than in the solvent process (36). β-CD can be easily purified by crystallisation steps due to its low water solubility. Purification of α- and γ-CD was achieved via complex and expensive chromatography with low yields and a range of by-products. However, more economical processes have recently been published, which also may have some potential environmental advantages (37).

1.4.4 Inclusion complexes

The term ″inclusion complex″ was presented in 1950 by Schlenk. Among all other names it seems to be the most appropriate one, considering there is no covalent bond established between the host and the guest molecule and, moreover, the dissociation-association equilibrium in a solution is one of the most characteristic features of the host-guest association (35). The ability to form inclusion complexes is CD's main applicable character. In these complexes a guest molecule is captured within the cavity of CD (host molecule); in particular, it can be described as a dimensional fit between host cavity and guest molecule. Consequently, geometrical factors rather than chemical are crucial in determining the kind of

guest will penetrate into the CD's cavity. While the height of CD cavity among all three types of CDs is the same, the number of glucopyranose units determines the internal diameter of the cavity and its volume. Based on these dimensions, α-CD can typically complex low molecular weight molecules or compounds with aliphatic side chains, β-CD will complex aromatics and heterocycles and γ-CD can accommodate larger molecules such as macrocycles and steroids (31,35).

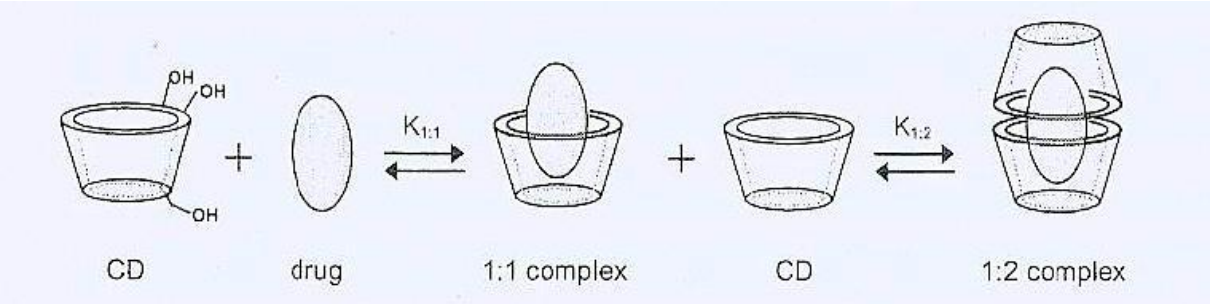


Figure 6: Drug/CD complex formation scheme (adopted from (34))

The main driving force of complex formation is the release of enthalpy-rich water molecules from the cavity, displacing them with more hydrophobic guest molecule present in the solution. Apolar-apolar association is formed which consequently decreases the ring strain of CD. The result is the establishment of a more stable/lower energy state. Thus only molecules which are less polar than water molecules can be complexed by CDs (31).

To sum up, the energetically favourable interactions that help shift an equilibrium to form the inclusion complex are:

- The displacement of polar water molecules from the apolar CD cavity
- The increased number of hydrogen bonds formed as the displaced water returns to the larger pool
- A reduction of the repulsive interactions between the hydrophobic guest and the aqueous environment
- An increase in the hydrophobic interactions as the guest inserts itself into the apolar CD cavity

While the initial equilibrium to form the complex is very rapid (often within minutes), the final equilibrium can take much longer to be reached. Once inside the CD cavity, the guest

molecule makes conformational adjustments to take maximum advantage of the weak van der Waals forces that exist (31). The guest molecules are usually orientated in the cavity in such position as to attain the maximum contact between the hydrophobic part of the guest and the apolar CD cavity. The hydrophilic outside remains as much as possible at the outer face of the complex to ensure the maximum contact with both the solvent and the hydroxyl groups of the host. It is also possible to form inclusion complexes with molecules significantly larger that the cavity. In this case only certain groups or side chains penetrate into the CD cavity (35).

The dissociation of the inclusion complex is a relatively fast procedure usually driven by a large increase in the number of water molecules in the surrounding environment. The resulting concentration gradient shifts the equilibrium in *Eq 1* (see *Table 2*) to the left. In the highly dilute and dynamic system like the body, the guest has difficulty finding another CD to reform the complex and is left free in the solution (31).

1.4.4.1 The association-dissociation equilibrium

Complexation of the drug to CD occurs through a non-covalent interaction between the molecule and the CD cavity. This is a dynamic process whereby the drug molecule continuously associates and dissociates itself from the host CD. Measurement of association or stability constant (K_c) or the dissociation constant (K_d) of the drug-CD complexes are very important because this is an index of changes in physicochemical properties of a compound upon inclusion. Determining the K-values is usually based on titrating the changes in the physicochemical properties of the guest molecule and then analysing the concentration dependencies. Additive properties of the drug or guest molecule that can be studied in this way are aqueous solubility (phase-solubility relationship), chemical reactivity, molar absorptivity and other spectroscopic properties, NMR chemical shifts, pKa values and HPLC retention times among others.

Assuming a 1:1 complexation, the spontaneous process of interaction can be described by the following *Eq 1* in the equilibrium where D is uncomplexed guest molecule, CD is uncomplexed cyclodextrin and D*CD is the complex between drug and cyclodextrin. The stability constant is better written as $K_{m:n}$ to indicate the stoichiometric ratio of the complex (see *Eq 4*).

Table 2: Equations describing inclusion complex formation

1:1 stoichiometry		m:n stoichiometry	
$D + CD \leftrightarrow D * CD$	***Eq 1***	$\underset{[a-mx]}{mCD} + \underset{[b-nx]}{nD} \overset{K_{m:n}}{\rightleftarrows} \underset{[x]}{CD_m * D_n}$	***Eq 2***
$K_c = \frac{[D*CD]}{[D]*[CD]}\left(M^{-1}\right)$	***Eq 3***	$K_{m:n} = \frac{[x]}{[a-mx]^m [b-nx]^n}$	***Eq 4***
$K_{diss} = \frac{1}{K_c}$	***Eq 5***	$K_{diss} = \frac{1}{K_{m:n}}$	***Eq 6***

K-values are trustworthy only when a temperature and pH for ionisable guests are also given. With an increasing temperature K_c decreases significantly as the complex dissociates. Constants can be well determined by the linear part of phase-solubility diagram as presented by Higuchi and Connors (31,35,38).

1.4.4.2 Phase-solubility studies

The knowledge of phase-solubility behaviour provides the information on their relative stoichiometric amounts and indicates their complexation efficiency (i.e. ratio between complexed and uncomplexed CD) in the solution, while it also represents a useful tool for determining K-values (34). Phase–solubility analysis involves an examination of the effect of a solubilizer, i.e. cyclodextrin or ligand on the drug being solubilized, i.e. the substrate. It was introduced by Higuchi and Connors in 1965 (61) and later reviewed by Connors (38).

Practically, into several vials of constant aqueous volume containing successively larger concentrations of CD excess of poorly soluble drug is added. Vials are agitated at constant temperature until the equilibrium is established. The excess of drug provides as high thermodynamic activity of the drug as possible. The aliquots of suspensions from vials are filtered and the total drug concentration (Dt) is examined with an appropriate analytical procedure (UV spectroscopy, HPLC…). The phase-solubility profile is later on studied on the effect of CD on the apparent solubility of the drug. Profiles fall into two major groups: A and B-type (39).

According to solubility isotherm of inclusion complexes, a potential guest in a heterogeneous system consisting of CD, water and the guest at equilibrium may change its solubility. This change may be an increase, either monotonously or to a certain limit, or it may even decrease. This correlation between guest solubility and CD concentration is illustrated in *Figure 7*. If only a dissolved complex is formed between D and CD, the phase solubility isotherm is of type A. When all formed complexes are of 1.order (that is D*CD, D_2CD,…,D_nCD) a diagram A_L is achieved. A_P type is seen with complexes that form reaction of order higher than 1 (that is D^*CD_2, D^*CD_3 ,…, D^*CD_n). A_N type profiles have several explanations of negative deviation from the linearity (i.e. CD is proportionally less effective at higher concentrations). It can indicate bulk changes imparted to the solvent by solubilizer at various concentrations (solubilizer is acting as a chaotrope or kosmotrope or is altering the bulk properties of the media by changing its viscosity, surface tension or conductivity) or self-association of the solubilizer at high concentrations.

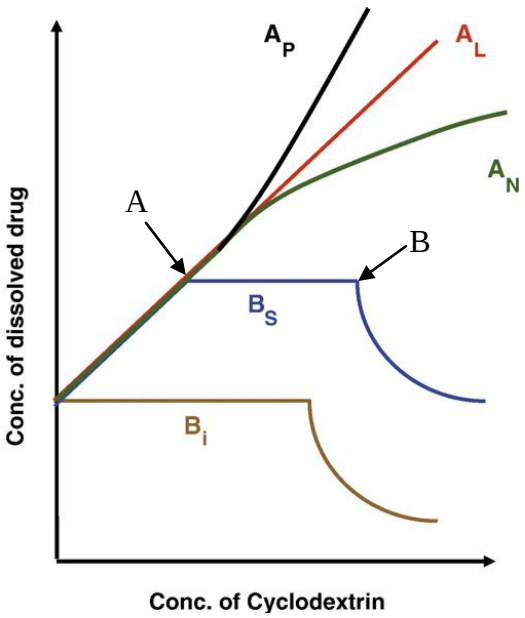


Figure 7: Solubility isotherm types (adopted from (38))

K-value in A-profile type can be derived in many ways. The equilibrium constant for a complexation of interest is given by *Eq 7*.

$$K_{1:1} = \frac{slope}{S_0(1-slope)} \qquad \textit{Eq 7}$$

B-type profiles are seen when formation of complexes has limited aqueous solubility and are normally observed with naturally occurring CDs, especially β-CD. B_S-type isotherm is obtained when as CD concentration increases, a soluble complex forms which increases the total solubility of the drug; following at a certain point in this solubility process, the maximum solubility of the drug is achieved. The latter is described as the sum of S_o (drug intrinsic solubility) plus any drug solubilized in the form of CD complexes. The addition of CD results in the formation of additional complexes which precipitates but as long as there is still solid drug available in the solution, which is seen as plateau.

At the point when no solid drug remains, any further increase in the CD concentration results in no further increase in solubility, but in the precipitation of the microcrystalline complex (B_S type isotherm). Reaching point B means that all solid guest molecules have been converted to a less soluble inclusion complexes; therefore, on adding even more CD to the system, the association-dissociation equilibrium will be shifted to association and the solubility asymptotically approximates to the inherent solubility limit of the complex (D_C). K-value for the B_S-type profile can be estimated to be similar than for the A_L-type from ascending part of the isotherm. The stoichiometry of the inclusion compound for B-type can be deduced by analysing the length of the plateau region, AB, according to the following equation:

$$\frac{[\text{guest}]}{[\text{CD}]} = \frac{(\text{total guest added to system}) - (\text{guest in solution at point A})}{[\text{CD}]_t \text{ corresponding to plateau region}} \qquad \textit{Eq 8}$$

In the case where the formed complex is insoluble (type B_I) the solubility of the guest remains unaltered until all guest molecules are converted into insoluble complexes; thereafter the concentration of dissolved guest begins to decrease (35,38).

1.4.4.3 Drug release from the CD complex

In the past with first CD-containing formulations, it was believed the drug availability would be impeded by the slow release of the drug from the CD cavity. However, later on it was proven that the kinetics of inclusion complex formation and dissociation between the drug and CD is very fast. The rates for formation/dissociation were measured to be very close to diffusion controlled limits with the complexes being continuously formed and broken down. Complexation of poor aqueous solubility drugs is shown to enhance the bioavailability at the hydrated surface (30,39).

Different mechanisms play an important role in drug release from the drug-CD complex. Dilution is one of the most important processes to explain drug release, particularly when releasing a drug from weak drug/CD complexes, and also a significant contributor for strongly bound drugs. Other mechanisms that play a significant role in drug release are competitive displacement, plasma and tissue binding, drug uptake into tissue, CD elimination, change in ionic state and temperature. The relative contribution of these factors is however dependent on the route of administration (dilution effects), volume of distribution of drug and CD (dilution effects), binding strength and concentrations of D and CD (dilution effects), binding constant and concentration of competing agent (competitive displacement) and

association constant and protein concentration (protein binding) (40). The published studies regarding drug release from CD complex in pulmonary delivery are rather limited. Lung has a very thin aqueous layer covering alveolus, thus dissolution and/or drug release from the complex might be limited by this finite fluid volume (8,40).

A study by Yang *et al.* (41) explored the *in vivo* pharmacokinetics in mice of itraconazole/HP-β-CD after pulmonary administration. The nebulized droplets containing itraconazole/HP-β-CD coalesced into the thin layer of lung fluid and demonstrated significantly higher itraconazole concentration compared to the equilibrium solubility of itraconazole alone. The major driving force for drug release from the dynamic inclusion complexes was estimated to be simple dilution (41).

An investigation by Cabral Marques was made studying salbutamol/HP-β-CD complexes in order to slow the pulmonary absorption. Since the absorption was not significantly prolonged, Cabral Marques suggested the low solubility constant of salbutamol/HP-β-CD (60-70 M^{-1}) is the reason for unaltered absorption (39). Furthermore, Wall *et al.* noticed a similar problem with rolipram and testosterone, which is why the authors assumed that the reason for rapid dissociation of the drug from HP-β-CD which may be occurring *in vivo* is the potential competition of these drugs for the CD by endogenous molecules such as cholesterol (39).

1.4.4.4 Complexation techniques

Complexation may be performed under several conditions, like in a homogeneous solution, or in a suspension, under pressure or by simple mixing of the compounds or by melting together the potential guest with CD. For each pair drug/CD a special procedure has to be developed to give the most suitable outcome (35).

1.4.4.4.1 Co-precipitation

CD is dissolved in water and the guest is added while stirring the CD solution. This is the technique most widely used in laboratories. The initial CD concentration must not be too high (i.e. for β-CD 20 % max) since the solubility is rising with higher temperature that is often applied during the process. If the sufficiently high concentration is chosen, the solubility of D/CD will be exceeded as the complexation reaction proceeds or as cooling is applied. The precipitate can be collected by decanting, centrifugation or filtration (31,35). Scale-up is the most difficult step in the production as the limited solubility requires large volumes of water.

Consequently, tank capacity, time and energy for heating and cooling may become important cost factors (31).

1.4.4.4.2 Slurry complexation

In the so called slurry method, the CD and the drug are not dissolved, but only finely suspended in water and stirred at ambient temperature (35). CD can be added to water as 50-60 % solids and stirred. The drug to be encapsulated can be either dissolved in an appropriate solvent or added directly to the well stirred CD suspension. As the CD complex saturates the water phase, the complex will crystalline or precipitate out of the aqueous phase. Favoured circumstance with this method is a reduced amount of water required meaning that a smaller size of reactor is needed in the scale-up (31).

1.4.4.4.3 Paste complexation – 'kneading method'

It is a variation method of slurry complexation, where even less water is combined to CD and the calculated amount of drug is added directly without using any solvent. This process can be done using a mortar and a pestle in a laboratory scale or kneader on larger scale. The time required for the complexation to be efficient is dependent on the drug molecule. The driving force for the kneading method lies in the energetically less favoured initial water/CD complex compared to D/CD complex. Finally the water is removed from paste-like product by drying in an air stream (31,35).

1.4.4.4.4 Damp mixing and heating

This method uses no or very little amount of water, which can range from the hydration of CD and added drug up to 20-25 % water on a dry basis. The mixture of the drug and CD is well mixed and placed into a sealed container which is then heated to about 100 °C. The contents are then removed and dried. The temperature applied must not reach the thermal degradation of CD that is about 180 °C (31).

1.4.4.4.5 Extrusion

CD, drug and water can be premixed as added to the extruder. This technique has an advantage of being a continuous system using very little water (31).

1.4.4.4.6 Dry mixing

Sometimes the drug can be complexed simply by mixing together the drug and CD. This is the most applicable method for oils or liquid guests (31).

1.4.5 Toxicological evaluations for pulmonary delivery

Polar components like CDs cannot readily penetrate biological membranes when administrated *per os* due to their bulkiness and hydrophilicity. On the other hand, there is evidence about the systemic absorption after pulmonary administration. In 1991 Cabral Marques *et al.* studied the bioavailability of intratracheally administered β-, DM-β-, HP-β- and reported them to be as high as 66, 74 and 80 % in rabbits, respectively (42).

In rats by parenteral administration of LD_{50} doses, signs of intoxication are seen as nephrotoxicity, in particular alterations in the vacuolar apparatus of the proximal tubules, accompanied by cytoplasmic vacuolation, cell disintegration and amorphous mineralization (35).

In vitro tests were performed on bronchial Calu-3 cells and primary cultured alveolar cell models using fluorescein isothiocyanate (FITC)-labeled dextrans of different molecular weight. With both models pores of 6 nm were observed concluding that macromolecules with radius < 5μm corresponding to dextrans of size 4-40 kDa can be transported across bronchial and alveolar epithelia *in vitro* by paracellular diffusion (43).

In vitro study by Matilainen *et al.* (42) showed that the pulmonary safety of CD is concentration-dependent and related to the CD used. γ-CD seems to be the safest of the natural CDs according to the research. Even at high concentrations of 50 mM it caused less than half Calu-3 cells deaths compared to α-CD that led all cells to die. A 50 mM solution of β-CD could not be prepared due to its low solubility although at concentrations >5 mM a decrease in cell viability was observed. HP-α-CD and HP-β-CD demonstrate similar toxicity profiles than γ-CD. The latter study also finds γ-CD to be the least cytotoxic at local level for pulmonary delivery, meaning its ability to solubilize and remove components from the cell membranes is the poorest (42).

CDs after inhalation can cause pulmonary responses such as changes in epithelial integrity or number of inflammatory cells (44). Overall, the long term safety of inhaled CDs still needs to

be clarified as until now there have been only a few studies investigating the acute pulmonary administration of CDs (34).

γ-CD is recognized as GRAS due to acute, subchronic and chronic oral and parenteral studies. Even at dietary levels up to 20 % in rats no significant adverse effects were noticed. A human tolerance study clarified that a single dose of 8 g γ-CD provokes no more gastrointestinal side-effects than the ingestion of 8 g maltodextrin (45).

1.4.6 CDs as pharmaceutical excipients in pulmonary delivery

Inclusion complexes of CDs with various drugs for pulmonary application have been studied to improve pulmonary absorption and bioavailability of drugs. In the *Table 3*, some examples of inclusion complexes for inhalation are presented together with the augmentation of aerodynamic behaviour.

Table 3: In vitro aerodynamic behaviour of inclusion complexes for pulmonary application in DPIs, presented as emitted dose (ED) and fine particle fraction (FPF); a-physical mixture of drug and CD, b-formulation without bulk carrier, c-lactose as a carrier in the formulation, d-dose = 4mg, e-dose = 10 mg

Drug/CD in DPI	ED (% of nominal dose)		FPF (% of ED)		Reference
	With CD	Without CD	With CD	Without CD	
Beclomethasone/HP-β-CD	n.a.	n.a.	39.5, 33.1[a]	9.3	(34) Leite Pinto, Cabral Marques
Cyclosporine A/HP-α-CD	59[b], 64[c]	106	33.0[b], 34.6[c]	19.8	(46)
FK244/β-CD	70.2[d], 67.4[e]	n.a.	47.2[d], 45.2[e]	n.a.	(47)
Lipid-polycation-pDNA vector/DM-β-CD	84.4	90.4	48.2	15.6	(48)

1.5 Lung deposition

Drug delivery to the lung may be aggravated by the fact that nature developed extensive means to keep particulate matter from depositing there. For example, oropharynx has a 90° bend that encourages impaction and deposition of the particles at the back of the throat during inhalation. Further on, the continuous branching and narrowing of the airway paths makes it

difficult for the particles to navigate without depositing in areas where suboptimal pharmacological effect might be produced. High humidity and mucociliary clearance mechanisms further inhibit the progress of inhaled drug particles in these regions (49).

The important *in vivo* parameters when considering the performance of inhaled aerosol are the total dose that reaches the patient and the deposition pattern of the inhaled dose in the airways. Deposition patterns in the human airways are controlled by three major factors: airway geometry, the aerodynamic particle size distribution of the inhaled aerosol and the inhalation flow rate at which it is inhaled. The first of them is patient characteristic whereas the latter two can both be characteristic of a delivery device/formulation. Usually it is the aerodynamic particle size that is surrogate for deposition and can be determined in laboratory tests (50).

The aerodynamic diameter (d_{aer}) of the particle has been used as a simple indicator to provide information about particles capability of reaching the lower parts of lungs. The following equation describes the aerodynamic parameters, which are size, shape and density of a particle. The equation is applicable only for particles at the Stokes flow regime of Re<0,1.

$$d_{aer} = \sqrt{\frac{\rho}{\rho_0 \chi}} * d \qquad \textbf{Eq 9}$$

ρ is the particle density, ρ_0 is the unit density (1 g/cm^3), χ is the dynamic shape factor, which is defined as the ratio of the drag force on a particle to a drag force on the particle volume-equivalent sphere at the same velocity (51), and d is the geometric diameter of the particle. Hence, the aerodynamic diameter can be reduced by one or more of the following manipulations:

- Decreasing the volume-equivalent particle diameter (d)
- Reducing the particle density (ρ)
- Increasing the particle dynamic shape factor (χ)

In general, the aerodynamic diameter should be between 1-5 μm if the aerosol is intended for deep lung inhalation. In addition to the particle size, many other physicochemical properties are known to affect the interparticulate interactions and the overall performance of inhalation

powders, such as particle shape, surface texture and energy, velocity, charge, hygroscopicity, interparticulate contact area and ambient humidity (51).

Deposition dose can be estimated as shown in the following equation:

Deposited dose = mass concentration [mg/m^3] * minute ventilation [m^3/min]*exposure time [min]*deposited fraction (52) ***Eq 10***

Mechanisms which are significant for removing aerosol from the site of deposition include mucociliary clearance, mostly important in the upper airways, phagocytosis in alveolar region, lymphatic clearance or absorption through the pulmonary epithelium (34).

1.5.1 Physical mechanisms causing deposition of aerosols

There are five basic mechanisms affecting the deposition of aerosol: inertial impaction, sedimentation, Brownian diffusion, interception and electrostatic precipitation. Inertial impaction results as a particles tendency to move in a straight direction instead of following the gas streamlines. Thus, deposition may occur on the obstacle downstream the trajectory of the particle (for example oropharynx). This mechanism is very important for describing impaction of particles larger than 10 μm. Sedimentation of the dispersed phase takes place when particles move under force of gravity, especially in the particle range 0.5–5 μm. Diffusion is a significant deposition mechanism for very small particles (< 0.5 μm) as the particles are too small to deposit due to sedimentation. It can often happen that particles are exhaled prior to deposition. Interception takes place when particles are moving with the gas stream but a distal part of the particle is already touching a solid or liquid surface. It is of greater importance for fibres rather than spherical particles. Electrostatic precipitation is quite rare but it may be significant when a charged particle can induce a charge of opposite sign in the walls of the airways and consequently becomes electrostatically attached to it (5).

1.6 Dry powder inhalers

The classification of inhaled drug delivery systems falls into 3 categories: pressurized metered-dose inhalers (pMDIs), dry powder inhalers (DPIs) and nebulizers, each one with its unique advantages and weaknesses. These categories are based on physical states of dispersed-phase and continuous medium. Nebulizers are particularly different from the other

two, since not only the drug is dissolved or suspended in a polar medium usually water but also the aerosol is delivered continuously over an extended period of time. pMDIs contain a drug solution or suspension mixed with a propellant, such as hydrofluoroalkenes (53).

DPIs are the newest type of aerosol delivery device and are significantly increasing on the market due to environmental reasons, inhaler performance, product stability and their ease of use. The DPIs are filled with a drug powder with or without a drug carrier. Force of inhalation is needed to release the dose from a pierced blister or capsule (pre-metered type) or bulk powder (reservoir-type inhalers) (34). The energy from the patient's inhalation deaggregates the powder into smaller particles. A fact that DPIs are activated by the patient's conscious inspiratory airflow frequently resulted in a better lung delivery compared to pMDIs, since DPIs require little coordination of actuation and inhalation (53). In general DPIs are more patient friendly, very portable, quick to use and easier to teach the patients about their adequate use. There are some limitations for usage especially for young children under 5 years, since higher inspiratory flow and the cognitive ability are necessary to operate a DPI. In addition, attention has to be put on patient's adequate lung volume to inhale the drug into the lower airways (54). Regulatory bodies are very strict on pharmaceutical and manufacturing standards for DPIs, as it is very challenging to demonstrate the device reliability in terms of delivered dose uniformity (55).

1.6.1 Formulation approaches

Performance of DPIs is highly dependent on the particulate characteristics like geometric and aerodynamic particle size distribution, particle shape and powder dispersion characteristics (56). Before drug can be deposited in the lung, drug particles have to undergo a flow, fluidization and deaggragation, meaning that the particles must leave a DPI and they have to be deaggregated from each other and other components in the formulation (57).

Formulation typically consists of micronized drug blended with a carrier and their size distributions are optimized during early formulation. The drug can be micronized either by milling or as a result of the particle processing method (e.g. spray drying, spray-freeze drying, supercritical fluid methods, emulsion-based techniques). Commonly α-lactose monohydrate is used as a carrier in conventional dry powder formulations with the intention to increase the formulation bulk, improve the flow properties of the powder, facilitate the drug release from the inhaler and promote the proper aerosolization for the deep lung deposition (34,53,58).

The geometric particle size distribution plays an important role for the uniformity of dose and emitted dose. Interparticulate forces that bind particles in bulk powder have to be overcome to achieve an efficient dispersion of DPI aerosols. Mostly, in respiratory size range the dominating forces are van der Waal forces, which will increase in parallel with the geometric particle size. Between small and irregularly shaped particles the agglomeration occurs naturally due to cohesive forces. For that reason the carrier is added so that cohesive forces are broken and replaced by weaker adhesion between the carrier and drug particles. Enhanced performance of inhalation powders in DPIs can be achieved with formulation manipulations, for example, generating highly porous particles with large geometric diameters but small aerodynamic diameters. Cyclodextrins may also improve aerosol performance as the drug particles are in a monomolecular dispersion after inclusion. Another approach is to provide efficient energy for adequate aerosol dispersion from the device itself (58,59).

1.6.2 Types of DPIs

DPIs can be classified into 3 categories:

- First generation (breath activated single dose units from capsules, like Spinhaler® and Rotahaler®)
- Second generation (multi dose DPIs, the dose is measured from reservoirs and multi-dose units premetered into blisters, disks, dimples, tubes and strip by the manufacturer)
- Third generation (active devices, which employ compressed gas or motor driven impellers or use electronic vibration to disperse drug from the formulation) (60)

Each of these devices uses a different method for aerosolizing the powder. Spinhaler® was presented already in 1971. As it is a unit-dose inhaler, a capsule is inserted in a rotor holder inside the inhalation tube for each dose. The device pierces the capsule and the rapid inspiratory flow of the patient causes the rotor to spin and vibrate, therefore the powder is released into the airstream. Aerolizer® uses a similar mechanism. Rotahaler® is also a unit-dose device in which the capsule is opened and powder released during high air velocity and turbulence in the chamber. Turbohaler® is a multi-dose device containing bulk powder. With each twist of the device fresh powder is prepared in the path of the inspired air. Unlike Turbohaler®, Easyhaler® is less dependent on patient's inspiratory flow leading to higher dose

uniformity. Deagglomeration enhancement with both latter devices is achieved by directing the powder into a narrow mouthpiece, thus creating a high turbulence shear. Diskhaler® is a premetered multi-dose device. It uses a circular disk that contains 4-8 powder doses in the blister. For each application an aluminium blister is pierced and its contents released into the dosing chamber. Blisters are usually sufficient for 1-2 days and then have to be replaced with a new one. Because of this new inhalers are being developed with more complicated mechanisms which can provide more doses without replacing the blister (52,58,59).

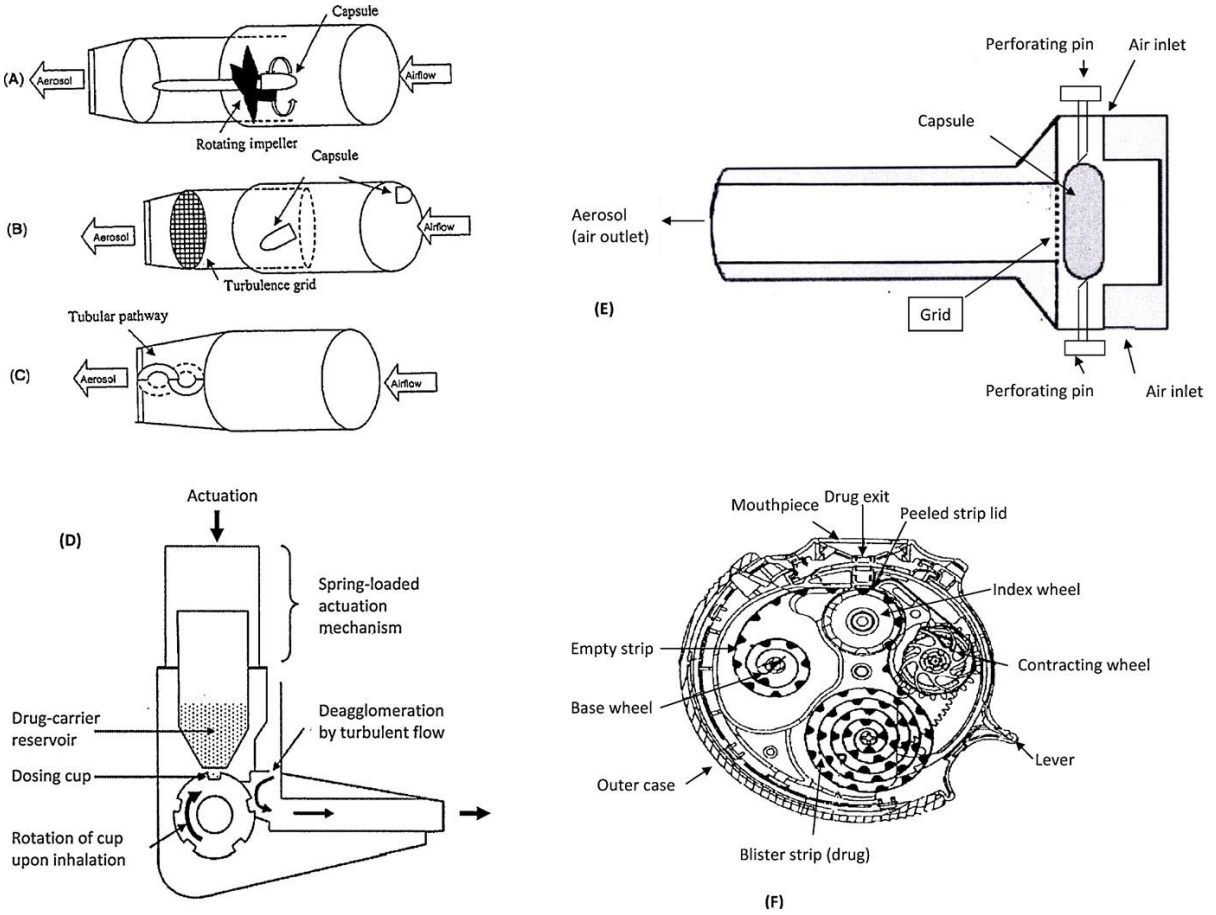


Figure 8: A. Spinhaler®, B. Rotahaler®, C. Turbohaler®, D. Easyhaler®, E. Aerolizer® and F. Diskus® inhaler (adopted from (59))

2 Aim of the study

The aim of this study was to prepare and investigate the use of inclusion complexes between fluticasone propionate, which is a poorly soluble substance, and cyclodextrin (CD) for pulmonary application. Finding the most appropriate cyclodextrin for complex formation was evaluated using molecular modelling and phase-solubility studies. Inclusion formation was tested (IR) and complexes were further on characterized for particle size and morphology (SEM, Aerosizer®). The pulmonary deposition comparing formulation of free drug and complexes was tested on Twin Impinger, where respirable and emitted fractions were evaluated.

The study was carried out with the following questions:

- Is the lung deposition of the drug better when it is loaded in CDs?
- Do inclusion complexes with CDs enhance the solubility of the drug (FP)?

3 Materials and methods

Reagents and materials:

- Fluticasone propionate (Hovione, batch 05ST75.HQ00017)
- HP-β-CD (KLEPTOSE® HP, MS = 0.85, Roquette))
- γ-CD (CAVAMAX® W8 PHARMA, Wacker-Chemie AG)
- 96 % Ethanol (AGA)
- Purified water
- α-lactose monohydrate (Granulac 70, Meggle)
- Gelatine capsules (Capsugel)
- HPMC capsules (Shionogi Qualicaps)

Equipment

- shaking water bath (HAAKE SWB 20)
- Eppendorf® tubes
- mechanic micropipette 1000 μL
- laboratory centrifuge
- 5 mL syringe with needle
- syringe filters Millipore 0.45 μm Ø 25 mm
- syringe filters RC 0.45 μm Ø 25 mm
- UV/VIS spectrophotometer (HITACHI-2000)
- Rotahaler®

- balance (Mettler Toledo AG245, Toledo Germany)
- balance (AG204 delta range model)
- Aerosizer® (LD API TSI)
- centrifuge (Heraeus instruments, Megafuge 1.0R)
- IR spectrometer (Nicolet spectrophotometer, model Impact 400, USA)
- scanning electron microscope (Carl Zeiss, model SUPRA 35 VP)
- carbon tape (Oxon, Oxford Instruments, Abingdon, UK)
- Twin Impinger (Copley Instruments)
- magnetic stirrer (VARIOMAG POLY)
- UV/VIS spectrophotometer (Hawlett Packard, HP 8453)

Methods

3.1 Calibration curve for detecting FP on UV/VIS spectrophotometer

The concentration of FP was detected on UV/VIS spectrophotometer. Standard solutions in H_2O/ethanol 50/50 % (v/v) with known concentrations were prepared. 2.6 mg of FP were weighted into 50 mL flask to prepare the starting solution with the concentration of 0.104 mM. The dilution of starting solution was made into 8 tubes with concentrations as shown in *Table 4*.

Table 4: Dilution protocol for calibration curve

	Dilution protocol (mL 0.104 mM FP solution + mL H_2O/EtOH)	Concentration [M]
1	1+1	5.195×10^{-5}
2	1+2	3.463×10^{-5}
3	1+3	2.597×10^{-5}
4	1+4	2.078×10^{-5}
5	1+5	1.731×10^{-5}
6	1+6	1.484×10^{-5}
7	1+7	1.299×10^{-5}
8	1+8	1.154×10^{-5}

The solutions were analysed on double beam UV spectrophotometer HITACHI U-2000 using two 10 mm quartz cuvettes; one always contained dilution media as a reference and the other was used for samples.

3.2 Phase-solubility test

The solubility studies were carried out on HAAKE SWB 20 shaking water bath according to the method of Higuchi and Connors (61). Experimentally, the drug of interest was added to several tubes in such an amount so that it was always in excess. The drug solubility was tested

in the presence of HP-β-CD and γ-CD. For HP-β-CD concentrations ranging from 0.00-0.04 mM (n=5) and for γ-CD concentrations from 0.000-0.125 mM (n=5) were used. Two basic solutions of HP-β-CD and γ-CD were made (0.04 M and 0.125 M, respectively) and then tubes for the phase solubility test were prepared as shown in *Table 5* and *Table 6* with 2 parallels for both. The test was carried out at the agitation speed of 100 rpm and 25 °C temperature.

Table 5: Phase-solubility test with FP and HP-β- CD

TUBE	FP [mg]	H_2O [mL]	HP-β-CD c=0.04M [mL]	CONC [M]
1	3.0	10	0.0	0.00
2	3.0	7.5	2.5	0.01
3	3.0	5.0	5.0	0.02
4	3.0	2.5	7.5	0.03
5	3.0	0.0	10.0	0.04

Table 6: Phase solubility test with FP and γ-CD

TUBE	FP [mg]	H_2O [mL]	γ-CD c=0.125M [mL]	CONC [M]
1	3.0	10	0.0	0.000
2	3.0	7.5	2.5	0.03125
3	3.0	5.0	5.0	0.0625
4	3.0	2.5	7.5	0.09375
5	3.0	0.0	10.0	0.125

The samples were agitated for 3 days (previously tested that equilibrium had been achieved in that time) at 100 rpm agitation speed and temperature of 25 °C. The samples were taken every day nearly at the same time. They were collected in the Eppendorf® tubes then taken to the centrifuge for 15 min. Supernatant from Eppendorf® tubes was removed to new tubes and centrifuge procedures was repeated again for 15 min. From each sample 1 mL of supernatant was taken with a micropipette and diluted to 10 mL. The solutions were assayed on UV spectrophotometer for the total concentration of FP. Solubility constants were obtained from *Eq 7*.

3.3 Molecular modelling

Molecular docking, a part of molecular modelling, involves finding the most favourable binding modes of a ligand to the target of interest when forming stable complexes.

Molecular modelling was performed by Prof. Dr. Mire Zloh from UCL School of Pharmacy of London. The docking programme GLUE was used, which fits ligands into a set of GRID maps of a target structures. GLUE is able to carry out the various steps needed to obtain one or more docked positions of the ligand into the target in automatic way.

Visualisations and inspection of possible complexes were done in the programme available on-line Discovery Studio 3.1 Visualizer.

3.4 Preparation of FP/γ-CD complexes

3.4.1 Kneading method

3.4.1.1 Ethanol as solvent

Fluticasone propionate (FP) and γ-cyclodextrin (γ-CD) were weighted in a glass mortar in the ratio of 1:1 giving altogether 5 g of mixture. Then 5 mL of 96 % ethanol was added. The drug was slowly incorporated into the paste and trituration was further continued for one hour until the ethanol was completely evaporated and a powder was formed. The product was stored in the desiccator.

3.4.1.2 Water as solvent

8.0 mL of water were added to 2.5 g 1:1 FP/γ-CD mixture (equivalent procedure as above). The excess of water was added to assure more time for a complex formation.

3.4.2 Precipitation from aqueous solution

The excess amount of fluticasone propionate (160 ± 1 mg) was stirred on the magnetic mixer for 2 days with 125 mL of 0.032 M aqueous γ-CD solution in 1.flask and 100 mL of 0.040 M aqueous γ-CD solution in 2.flask. The suspension was filtered (filter paper, Double ring 102 filter papers) to remove undissolved fluticasone propionate. To reach a descending part at 0.09 M γ-CD 75 mL with 16.278 g dissolved γ-CD was added to the first flask and 100 mL of 16.861 g dissolved γ-CD to the second flask. A solution was stirred at room temperature for 1 day and then placed in the fridge at 4 °C for 1 day. The precipitate which was formed during the stirring was collected by centrifugation (Heraeus instruments, Megafuge 1.0R, sealed

rotor, 4 50-mL Falcon tubes; 2 for each flask) at 4 °C first for 1h at 4332×g and then the joined product in 2 tubes for 40 min at 6238×g. The precipitate was kept at -20 °C for 1 day and then freeze-dried for 1 day (Christ Alpha 1-4, condenser = -40 °C, vacuum=0.03 mbar).

3.5 Characterisation of complexes

3.5.1 Proof of inclusion complex formation

3.5.1.1 IR spectroscopy

IR spectra were obtained for each raw material; FP, complexes and for physical mixture (PM) of powders using a KBr disk method and scanned in the range of 4000–450 cm^{-1}. The procedure consisted of placing a sample of the neat powder dispersed in KBr into the sampling cup, smoothing the powder into a thin bed and compressing the powder bed into the holder using a compression gauge. The sample was placed into the light path and the spectrum was obtained.

3.5.1.2 UV/Vis spectroscopy

In 3 parallels 3 different solutions were prepared and analysed on UV/VIS spectrophotometer as shown in *Table 7*. The differences in solubility were observed between free FP, physical mixture and complexes.

Table 7: Scheme of UV analysis

5 mg FP + 5 mL H_2O	5 mg PM + 5 mL H_2O	5 mg complex + 5 mL H_2O
Filtration (Millipore 0.45 μm)		
2 mL filtrate + 2mL ethanol		
UV detection		

3.5.2 Particle size and morphology

3.5.2.1 SEM

Scanning Electron Microscopy can image and analyse bulk specimens. The beam electrons are emitted from the cathode and accelerated by voltage difference towards anode forming a smallest beam cross section (the crossover) near the anode with a diameter of about 10-50 μm. Since this field is too large to produce a sharp image, it is demagnified by the lens system focused on the specimen surface. SEM can produce an electron beam with a smallest spot size of about 1-10 nm carrying an electron-probe current of 10^{-9}-10^{-12} A. Elastic and inelastic scattering are the elementary atomic interaction processes, though the final signal for image formation is not the result of a single scattering process, but of the complete electron diffusion caused by the gradual loss of the electron energy and by lateral spreading caused by multiple elastic large-angle scattering (67–69).

The images of samples were made by Prof. Dr. Odon Planinšek on Scanning Electron Microscope (Carl Zeiss, model SUPRA 35 VP) at Kemijski inštitut in Ljubljana. Powder samples were deposited on double-sided carbon tape of 12 mm diameter. The beam acceleration voltage used was 1,0 kV and magnification 2000x for FP and 1000x for complexes, physical mixture and γ-CD.

3.5.2.2 Twin Impinger

Twin Impinger (Copley Instruments), also known as Apparatus A by European Pharmacopoeia, is designed to simulate the movement and location of deposition of particles in the respiratory tract (70,71). Among others, Apparatus B and C, their use is compulsory for the *in vitro* evaluation of the performance of aerosols. It is a whole glass apparatus that divides particles in two fractions, smaller and larger than 6.4 μm (72). Particles are deposited in various regions of Apparatus corresponding to respiratory tract, depending on their aerodynamic diameter.

Separately, FP and inclusion complexes powders together with α-lactose monohydrate (previously sieved for intended fraction 63-90 μm) were weighted in order to give the dose 0.22 mg and total amount of capsule 20 mg or 40 mg. Gelatine and HPMC capsules were used. The powders were triturated in a glass mortar. After homogenisation the powders were

manually filled into capsules. At all-time the powders were kept in a desiccator. For each analyse on Twin Impinger 5 capsules were used.

The capsules with powder were inserted into the inhaler device (Rotahaler®), which has a mechanism to open the capsule. The device was introduced at the inlet of Twin Impinger (see *Figure 9*, A). An air stream of 60 l/min was allowed to flow through the system by attaching the outlet of the Twin Impinger to a vacuum pump for 5 s. Aerosol passed through a glass bulb (*Figure 9*: U) which was intended to stimulate oropharynx. Then it proceeded into the upper impinge stage (*Figure 9*, M) consisting of a nozzle and a bulb and containing 7 mL (*Figure 9*, D) of solvent (H_2O/ethanol 50/50% v/v). Particles larger than the cut-off point of this stage were collected in the liquid. Smaller particles proceeded to the lower compartment (*Figure 9*, L) which contained 25 mL of solvent. Compartments and device were separately rinsed with fresh solvent and collected into appropriate volumes (device, upper and medium compartment: 25 mL flask, lower compartment: 50 mL flask) and the drug contents were determined by UV at 237.6 nm.

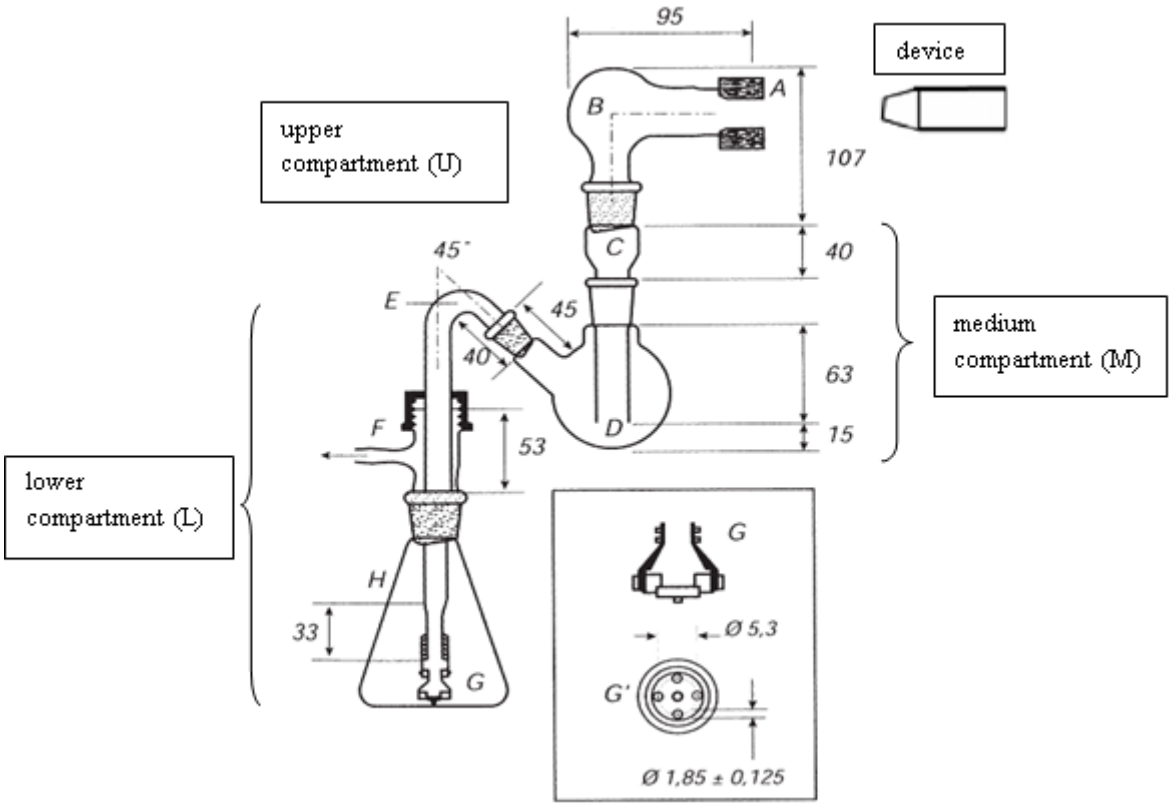


Figure 9: Twin Impinger (Apparatus A)

Emitted and respirable fractions were evaluated.

Emitted fraction = percentage of nominal dose that is discharched to the TI from the device

$$\text{emitted fraction of FP} = \frac{\text{emmited dose of FP (compartment U+M+L)} * 100}{\text{nominal dose}} \qquad \textbf{Eq 11}$$

Where U, M and L refer to upper, medium and lower compartment, respectively.

Respirable fraction = the percentage of nominal dose that reaches the alveolar region of the lung (lower compartment)

$$\text{respirable fraction} = \frac{\text{dose of FP in lower compartment} * 100}{\text{nominal dose}} \qquad \textbf{Eq 12}$$

Nominal dose = the total drug dose in one unit (0.22 mg of FP in 1 capsule)

3.5.2.3 Aerosizer®

The essential part of the apparatus are two laser beams that measure the time taken for the particle to accelerate between the beams and velocity, which is further on converter to aerodynamic diameter by a reference table constructed by manufacturer. The outcome is the spectrum of particle size distribution (66).

The samples for FP/γ-CD complexes were analysed for size distribution on Aerosizer®.

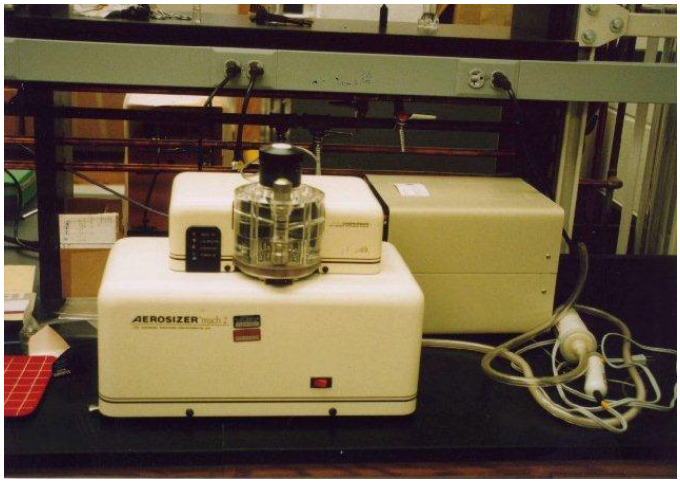


Figure 10: Aerosizer®

3.6 Dissolution test

The basic solution was prepared by weighting 9,98 mg of FP into 1000 mL flask. The solvent was 50/50 % water/ethanol (v/v). 11 dilutions as shown in Table 8 were tested on UV spectrophotometer at 240 nm to obtain a calibration curve.

Table 8: Dilution scheme for calibration curve calculation

	Dilution protocol (mL 0,01994 mM FP solution/flask)	Concentration [M]
1	-	1.994×10^{-5}
2	10/20	1.296×10^{-5}
3	7,5/20	9.970×10^{-6}
4	5/20	7.478×10^{-6}
5	2/20	4.985×10^{-6}
6	1/20	1.994×10^{-6}
7	0,75/20	9.970×10^{-7}
8	0,5/10	7.478×10^{-7}
9	0,5/20	4.985×10^{-7}
10	0,5/50	9.970×10^{-7}
11	0,5/100	1.994×10^{-7}

The dissolution test was performed on a magnetic stirrer VARIOMAG POLY using two 100 mL beakers. Powders of FP and FP/γ-CD were weighted (2.52 mg and 9.56 mg, respectively) in such a way to keep the same amount of drug between free FP and inclusion complexes, predicting 1:1 stoichiometry. The test was carried out in 50 mL of water. Samples of 5 mL were collected every 10 min from 0 - 60 min and every 15 min from 60 – 120 min, filtered through RC 0.45 μm filter and analysed on UV/VIS spectrophotometer. After each sampling the dissolution media was replaced. Concentration was calculated using the equation (see *Eq 13* below) for dissolution test with media replacement.

$$T_1\%\ \text{dissolved} = \left(\left(\left(\left(A_1 * V\right) / A_s\right) * C\right) / W\right) * 100$$

$$T_2\%\ \text{dissolved} = \left(\left(\left(\left(A_2 * V\right) + \left(A_1 * R\right)\right) * k\right) / W\right) * 100$$

$$T_3\%\ \text{dissolved} = \left(\left(\left(\left(A_3 * V\right) + \left(\left(A_1 + A_2\right) * R\right)\right) * k\right) / W\right) * 100$$

$$T_n\%\ \text{dissolved} = \left(\left(\left(\left(A_n * V\right) + \left(\left(A_n - 1 + A_n - 2 + \ldots\right) * R\right)\right) * k\right) / W\right) * 100$$

Eq 13

A_1 = the absorbance of the first sample pull

A_2 = the absorbance of the second sample pull

A_n = the absorbance of the n-sample pull

k = calibration curve coefficient

V = the initial media volume, ml

W = the weight of the sample, mg

R = the volume of media being withdrawn for a single sample pull,

n = the number of the sample pull taken

4 Results and discussion

4.1 Molecular modelling

Molecular modelling showed the ability of the drug to form complexes. FP was tested with 2 cyclodextrins. The purpose of this testing was to prove (along with phase-solubility test) the most promising combination for inclusion formation. From the literature (35) it is known that steroids easily form complexes with γ-CDs.

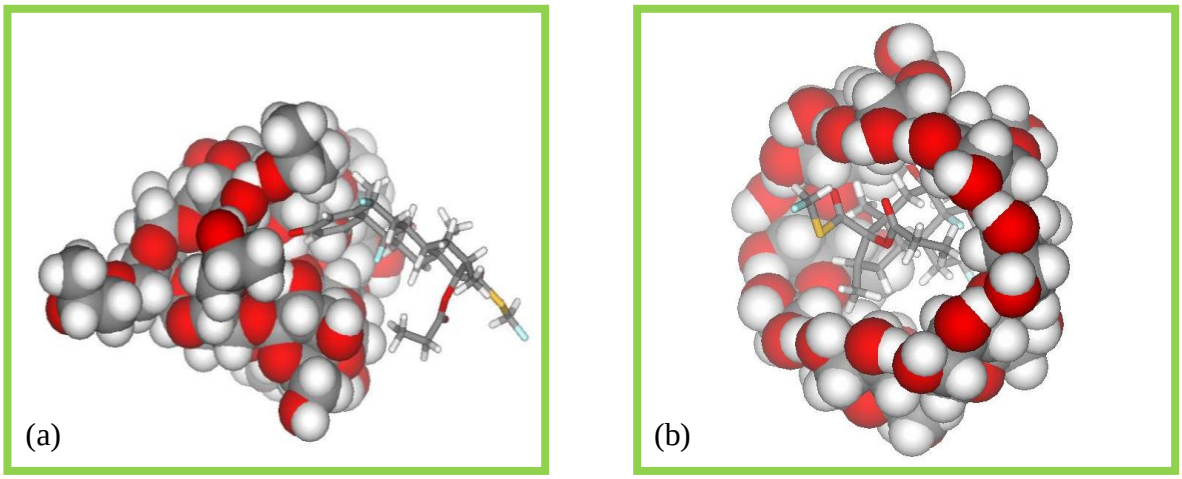


Figure 11: Images of interaction of FP with (a) HP-β-CD, (b) γ-CD

In the case of HP-β-CD, Prof. Dr. Zloh calculated the most stable form when FP is adhered to the CD's surface (see *Figure 11*, image (b)). It is also not very likely the inclusion would occur since the molecule would be entirely exposed to the solvent. γ-CDs can incorporate FP in the cavity, because it has a bigger cleft. Furthermore, with this pair it is also possible that the drug goes through the cleft if there are not enough bonds established. The inspection of the interactions in Discovery Studio 3.1 Visualizer showed the expected H-bonds could be formed between the groups labelled in *Figure 12* and the –OH or -CH_2-OH groups in γ-CD.

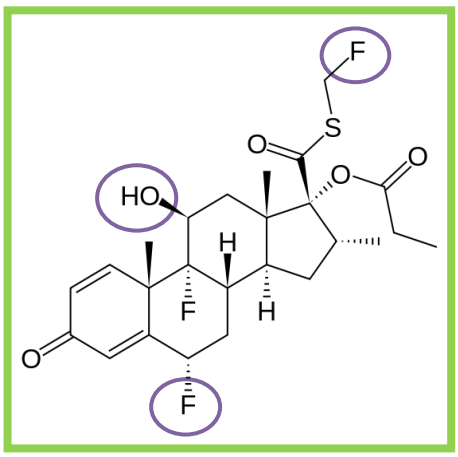


Figure 12: Fluticasone propionate; violet circles for chemical moieties capable of forming H-bonds

4.2 Quantitative determination of FP

A quantitative analysis of fluticasone propionate was carried out using UV/VIS spectrophotometry.

- *Maximum wavelength detection*

For standard scan performance a 0.052 mg/mL solution of FP in 50/50 % (v/v) water/ethanol solution was used. Standard scan spectrum was recorded over a wavelength range 360-200 nm, where according to the literature the maximum absorbance was expected. Spectrum identifies only one peak at 237.6 nm which corresponds to FP.

- *Linearity*

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample. For the establishment of linearity a minimum of 5 different concentrations is recommended. If there is a linear relationship between the absorbance and the concentration, a mathematical equation can be derived.

For the determination of the calibration curve 8 solutions of different FP concentrations were analysed. From the results calibration curve was plotted, see *Figure 13*. Microsoft Excel was used to calculate the linear equation by linear regression using the method of least squares. In addition, the regression coefficient of determination (R^2) was calculated. The coefficient has to be at least 0.99 meaning there are over 99 % of experimental data that correspond to the curve estimated by linear regression.

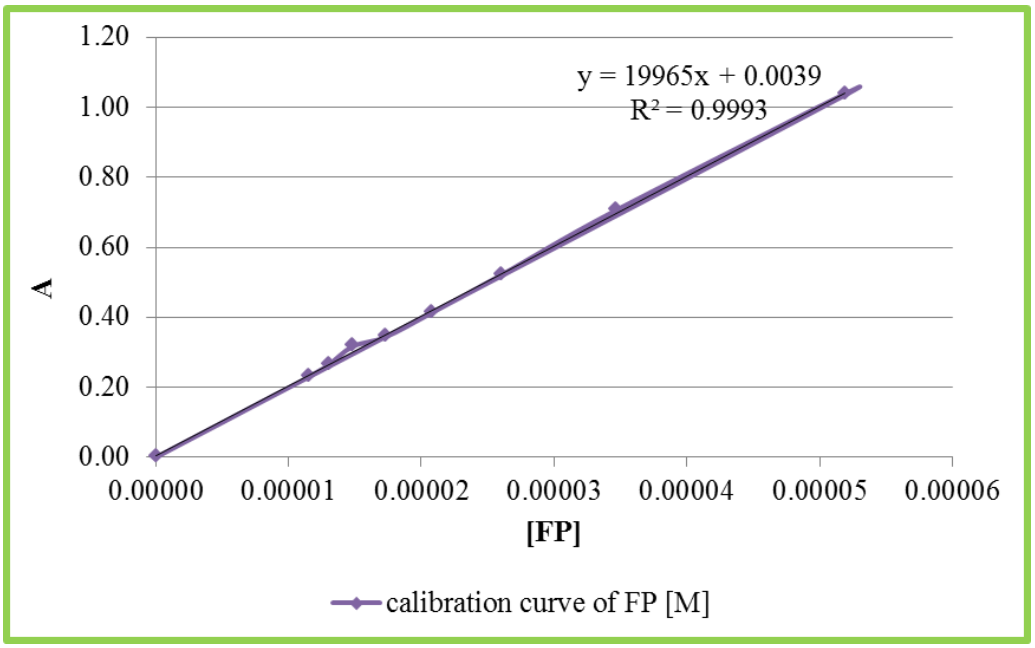


Figure 13: Calibration curve of FP at λ_{max} 237.6 nm, FP concentration is expressed as mg/mL

4.3 Phase-solubility profiles

Two types of cyclodextrins were chosen according to the drug's molecule size compared to the size of CD's cavity as well as regarding the existing literature, where inclusion complexes of steroids were studied.

The presence of a solid drug in these systems is necessary to maximise the thermodynamic activity of the dissolved substrate. Consequently, every day it was visually evaluated whether the solid drug was still present in the testing vials. If not, some more drug was added. In the preliminary 5 days study it was noticed the equilibrium was reached after 3 days with both HP-β-CD and γ-CD. For this reason solutions were examined for 3 days and the samples were pulled out once daily. Prior to each sampling the bath temperature was measured, because the solubility varies with temperature. The bath temperature was set to 25 °C, experimentally it was measured to be 25 ± 1 °C.

4.3.1 FP/HP-β-CD

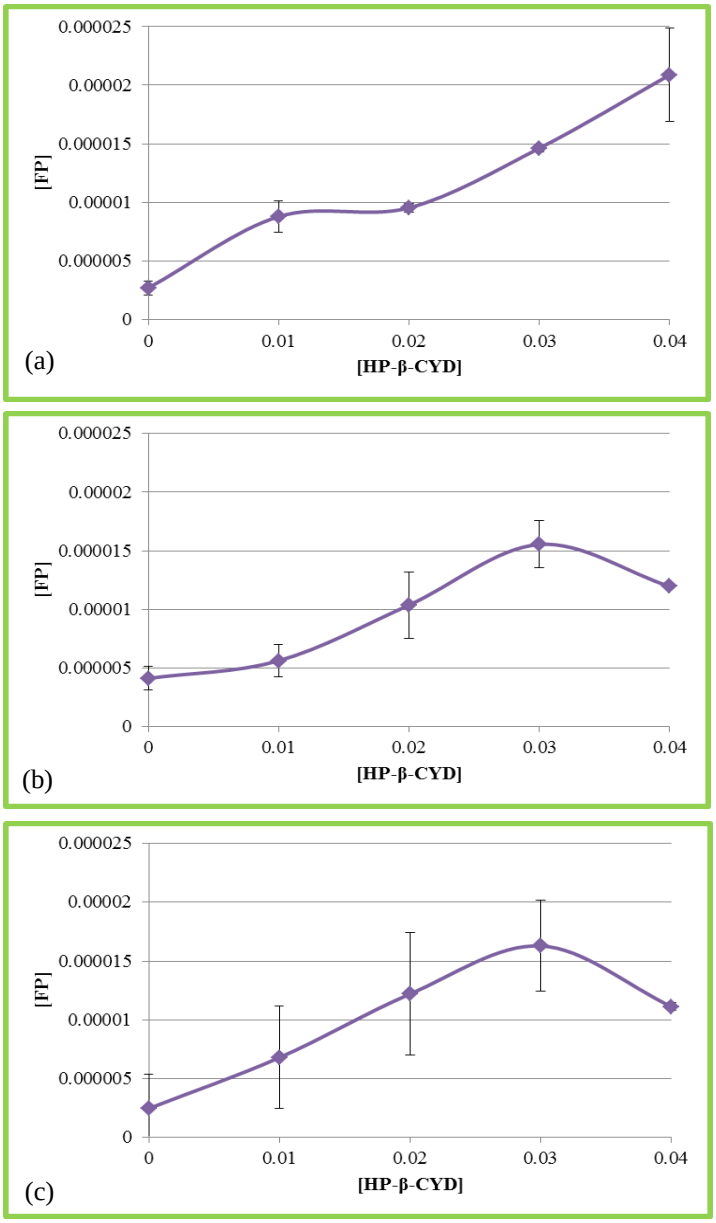


Figure 14: Phase solubility profiles for FP/ HP-β-CD, conc. is expressed as mol/L; (a) – after 24 h, (b) – after 48 h, (c) – after 72 h

In *Figure 14* phase-solubility curves are demonstrated for each day of sampling. On the third day the equilibrium was achieved, consequently the curve (c) represents the phase-solubility type of FP/HP-β-CD interaction. The curve was linear to the 0.03 M HP-β-CD point, afterwards it had negative deviation. From latter observations the curve seemed to be of A_N type. This type is not yet fully understood, however, some explanations are given in 1.4.4.2 (Introduction). The slope of the curve (c) (see *Figure 14*) was calculated from the first 4

points where the curve is linear using linear regression in Microsoft Excel. The constant for the profile was calculated from the *Eq 7*. Intrinsic solubility (S_0) is the solubility of the drug in the absence of CD, so the value can be seen from the ordinate axis on the graph (c) .

Table 9: Data obtained from the phase-solubility test for FP/ HP-β-CD

S_0 (intrinsic solubility)	Slope	Correlation coefficient R^2	$K_{1:1}$
2.44 x 10^{-6}M	4.7 x 10^{-4} M^{-1}	0.9986	192.9 M^{-1}

4.3.2 FP/γ-CD

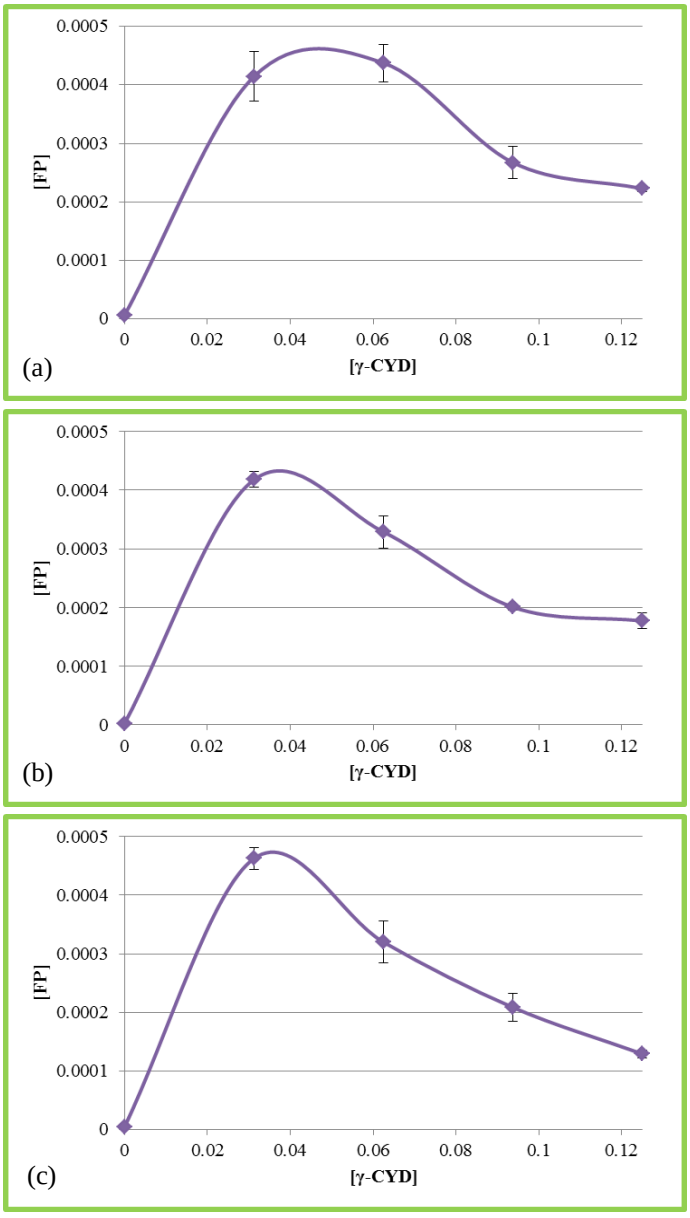


Figure 15: Phase solubility profiles for FP/ γ-CD, conc. is expressed as mol/L; (a) – after 24 h, (b) – after 48 h, (c) – after 72 h

Graph (c) in the *Figure 15* represents the B_S-type behaviour. In the curve (a), showing solubility after 24 h of shaking, the plateau was seen, although there was no significant plateau in the equilibrium (c). It was difficult to predict the plateau, since there were no data points between 0.03125 M and 0.0625 M of γ-CD. The constant $K_{1:1}$ was calculated from the linear part of the graph (c), which was only out of the first two points.

Table 10: Data obtained from the phase-solubility test for FP/γ-CD, a- linear curve was calculated out of the first two points.

S_0 (intrinsic solubility)	Slope	Correlation coefficient R^2	$K_{1:1}$
4.808 x 10^{-6}M	0.015 M^{-1}	1[a]	3093.7 M^{-1}

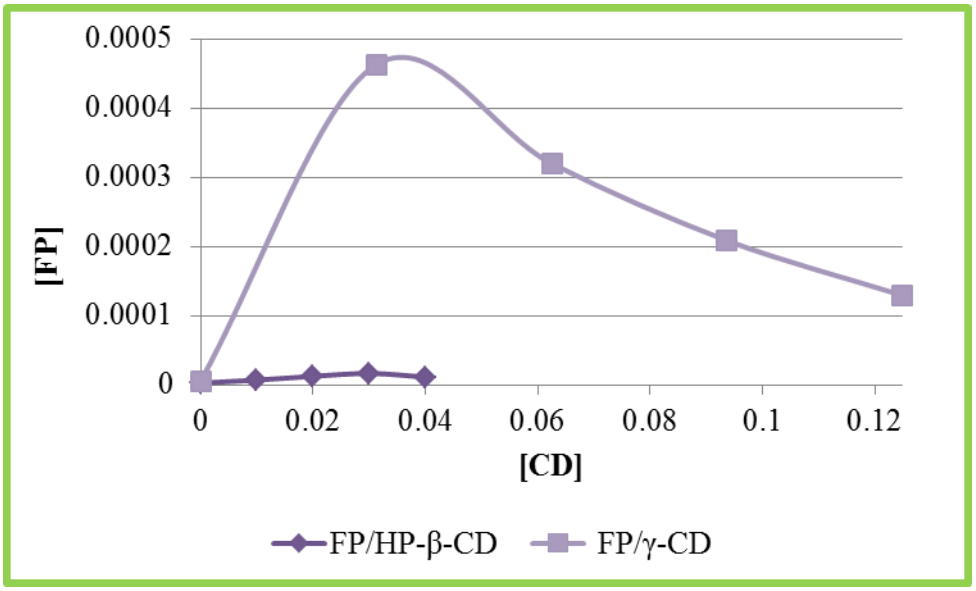


Figure 16: Comparison between equilibrium states of FP/HP-β-CD and FP/γ-CD

From the *Figure 16* it is shown that the solubility of FP in FP/γ-CD was about 10-fold greater than in FP/HP-β-CD. The calculated constant was also much more favourable in the FP/γ-CD pair, which was 3093.7 M^{-1} compared to FP/HP-β-CD, where the constant was 192.9 M^{-1}. Considering the molecular modelling and phase solubility results the complexes of FP/γ-CD were prepared.

4.4 Inclusion formation analyse

For the inclusion formation proving it is recommended to use more than one method. Firstly, IR spectroscopy was used. Spectra were obtained for FP (see *Figure 17*), γ-CD (see *Figure 18*), physical mixture of FP and γ-CD (see *Figure 21*), inclusion complexes FP/γ-CD (see *Figure 21*). If inclusion complexation occurred, the differences in spectra were noticed, i.e. the characteristic absorption bands were not visible or were partly visible because the drug molecule was hidden or partially hidden in CD's cavity.

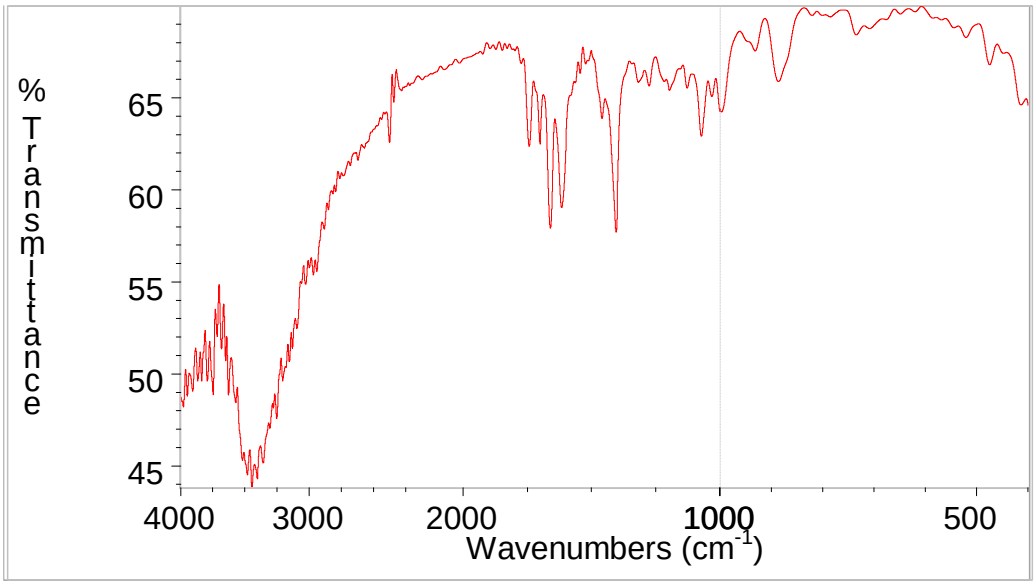


Figure 17: IR spectra of fluticasone propionate

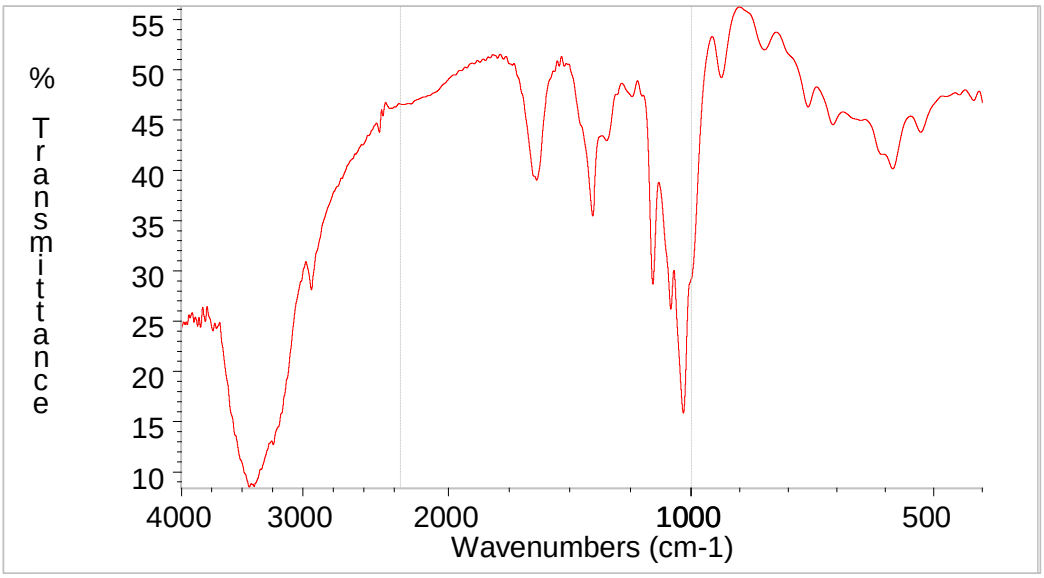


Figure 18: IR spectra of γ-CD

In the area from 1600 cm-^{1} – 1800 cm^{-1} there are the most characteristic peaks for fluticasone propionate since cyclodextrin does not contain functional groups such as C=O, S-C=O, C=C.

4.4.1 Determining complexes prepared by kneading method

Kneading methods were executed using either ethanol in the first case or water in the second case as a solvent. When ethanol was used, the drug was in the dissolved state, whereas with water the CDs were dissolved. The amount of water was calculated to the γ-CD's solubility in water. Since there were no complexes seen in IR spectra using the kneading method with ethanol (see *Figure 19*), water was used as solvent (see *Figure 20*) because it prolonged the kneading time, hence more time was available for the establishment of bonds between FP and γ-CD. *Table 11* shows the comparison of characteristic peaks for both methods. There were no significant differences with the spectra for the free drug (see *Figure 17*). A sharp peak was also seen for γ-CD at 1027 cm^{-1}. The formation of inclusion complexes with kneading methods was not successful.

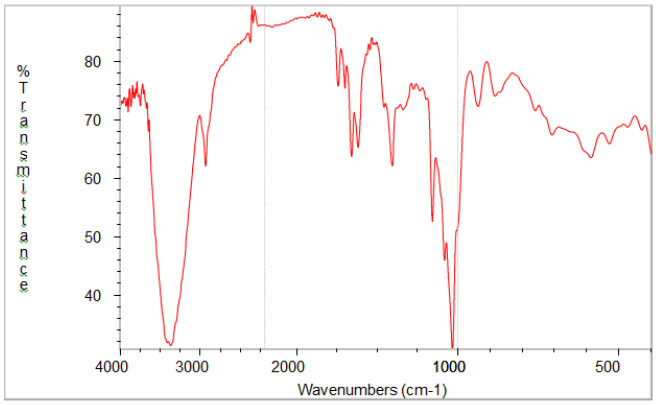


Figure 19: IR spectra of kneading method (ethanol as solvent) for FP/γ-CD complexes

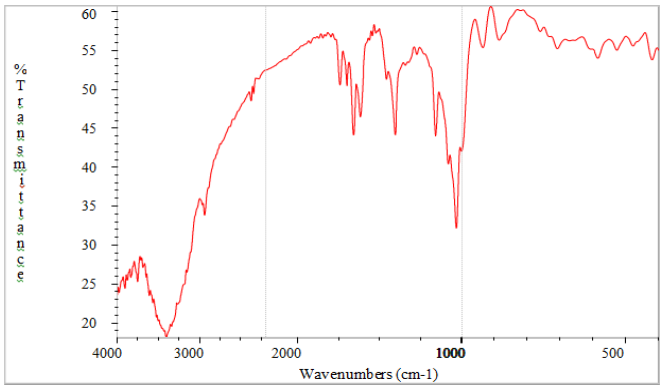


Figure 20: IR spectra of kneading method (water as solvent) for FP/γ-CD complexes

Table 11: Characteristic peaks of FP compared to peaks of two types of kneading methods

Characteristic peaks for FP [cm^{-1}]	Proposed assignment	FP [cm^{-1}]	Kneading with ethanol [cm^{-1}]	Kneading with water [cm^{-1}]
1744	υ (C=O)	1740	1740	1740
1699	υ (S-C=O)	1702	1698	1702
1661	υ (C=O)	1664	1663	1664
1616	υ (C=C)	1619	1607	1619

4.4.2 Determining complexes prepared by precipitation method

An aqueous precipitation method was developed to prepare budesonide/γ-CD complexes. The preparation method, which enabled the production of solid complexes without organic solvent residues, was based on the B_S-type phase-solubility behaviour of budesonide (63). Comparing the phase solubility test graphs and molecular structures of drugs, we used it to produce fluticasone propionate/γ-CD complexes.

The theoretical background of the procedure was adopted from Bs-type behaviour, that is when the complex is more soluble than the free drug, but its solubility limit can be reached within the CD concentration range. Bs-type graph (see *Figure 7*, Introduction) consists of 4 parts:

- *Linear part:* formation of inclusion complexes is higher than precipitation.
- *First plateau:* formation of complexes is equal to precipitation, at this point there is no free drug available.
- *Descending part:* precipitation of inclusion complexes is higher than formation, any further increase in the CD concentration results in no further increase in solubility.
- *Second plateau:* equilibrium between precipitated complexes and unprecipitated complexes is reached (35).

Considering the phase solubility graph (see *Figure 15*, (c)) is lacking a significant plateau, two starting γ-CD concentrations were chosen for the test:

1. flask: before reaching the plateau
2. flask: on the plateau or beyond

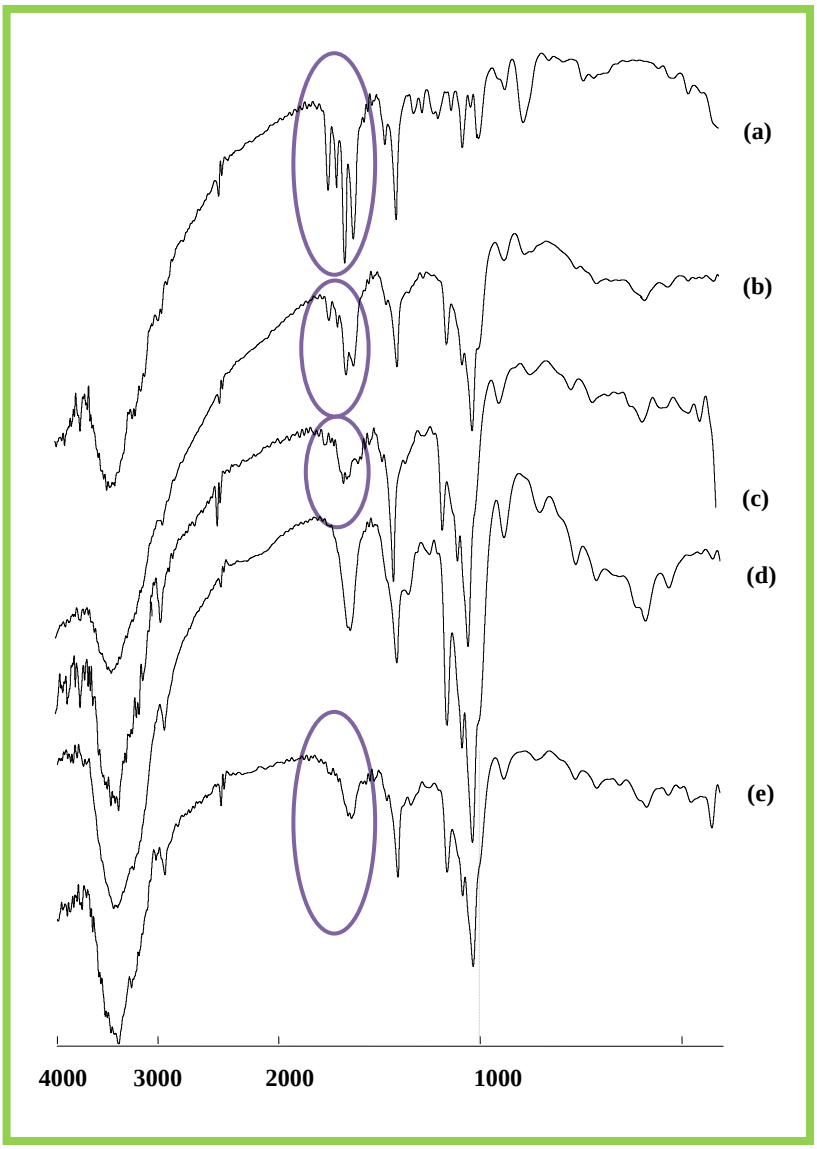


Figure 21: IR spectra, (a) Fluticasone propionate, (b) physical mixture FP/γ-CD, (c) complexes FP/γ-CD flask 1, (d) γ-CD, (e) complexes FP/γ-CD flask 2

IR spectra of complexes (see *Figure 21*, (c), (e)) were changed; the significant peaks for FP described in *Table 11* were absent or augmented. The peaks for thioester and ester carbonyl group (at 1740 cm^{-1} and 1702 cm^{-1}, respectively) were not seen on spectra of complexes, whereas peaks for conjugated C=O and C=C groups were diminished. It was predicted that the circled part of FP molecule (see *Figure 22*) was fully incorporated in the CD's cavity. There were no significant differences in the IR spectra between complexes obtained from flask 1 or 2. The weight of complexes from flask 1 was 93.1 mg and from flask 2 was 103.9 mg. IR spectroscopy is a very feasible method for inclusion proving (65) but it is not the most

reliable one. For this reason another test was done, which observed the solubility differences between free drug, physical mixture and complexes in water using UV/VIS spectroscopy.

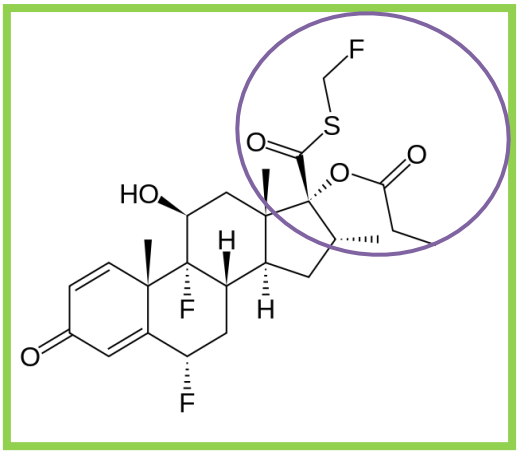


Figure 22: FP molecule; the part in violet circle was predicted to be in the CD's cavity

The UV analysis was designed as shown in *Table 7* (Materials and methods). 2 mL of filtrate was mixed with 2 mL of ethanol and then analysed on UV/VIS spectrophotometer. The test was done in 3 parallels.

Table 12: UV analysis results

	FP	Physical mixture	FP/γ-CD complexes flask 1	FP/γ-CD complexes flask 2
absorbance	0.087	0.177	0.900	0.850

According to UV analysis results there was a significant difference in the solubility between free FP and physical mixture compared to complexes. With two different methods we could predict the inclusion complex formation was successful.

The precipitation from water method was used due to the similarity of the molecules of budesonide and fluticasone propionate. Toropainen *et el.* (34) developed a procedure that used advantages of B_S-type profile properties. For complex formation the concentration of γ-CD

was fixed to be somewhere near the equilibrium of complex formation and precipitation (plateau), whereas for precipitation more γ-CD was added and that led to the decreasing part of the phase-solubility profile. According to Prof. Dr. Zloh's prediction, with molecular modelling the drug could go through γ-CD, which was the reason that the precipitation method was useful because the formation lasted for 48 hours, hence there was more time for bonds establishment and conformations needed for inclusion.

4.5 Deposition of FP in Twin Impinger

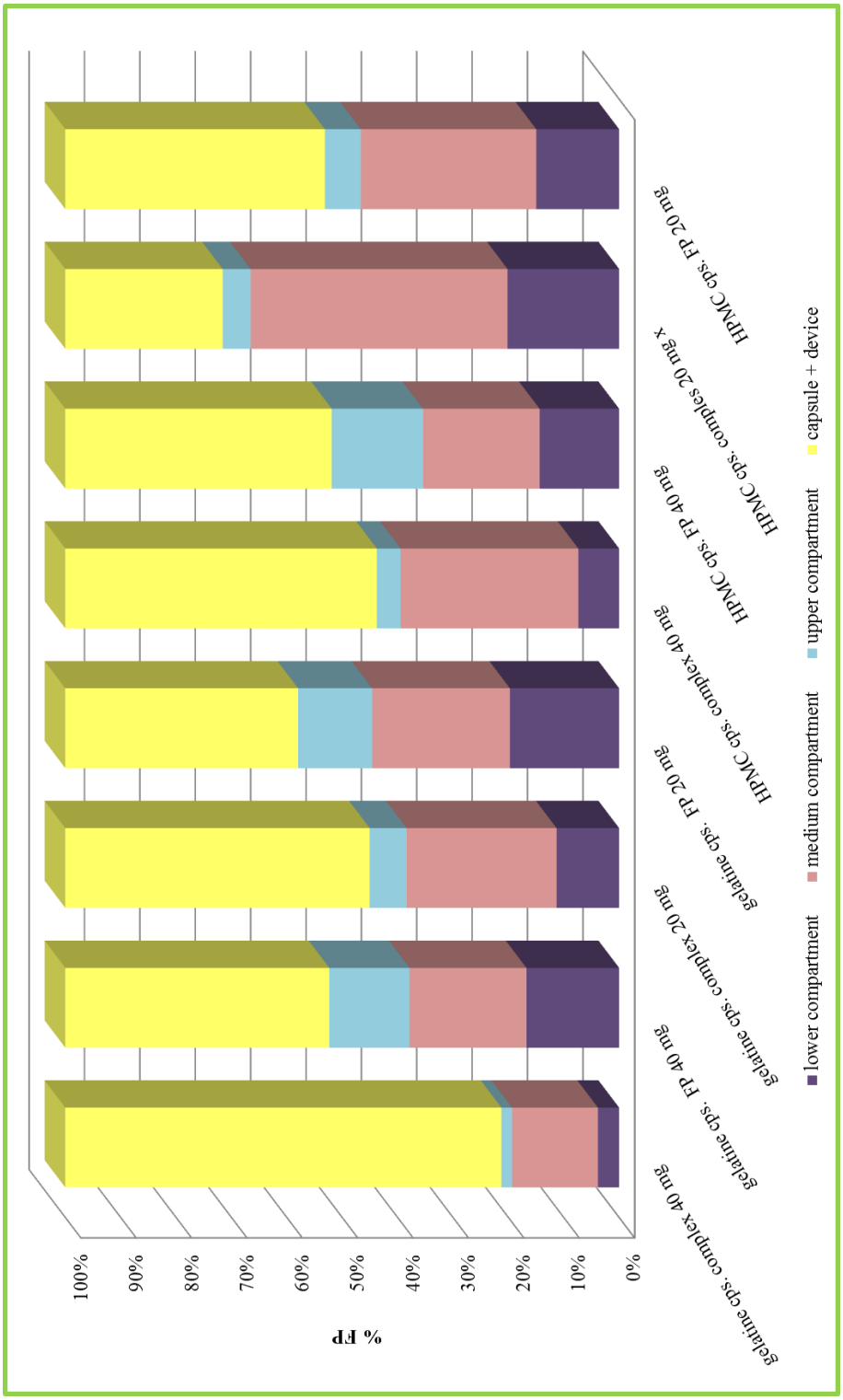


Figure 23: FP distribution in the TI compartments (percentage of nominal dose)

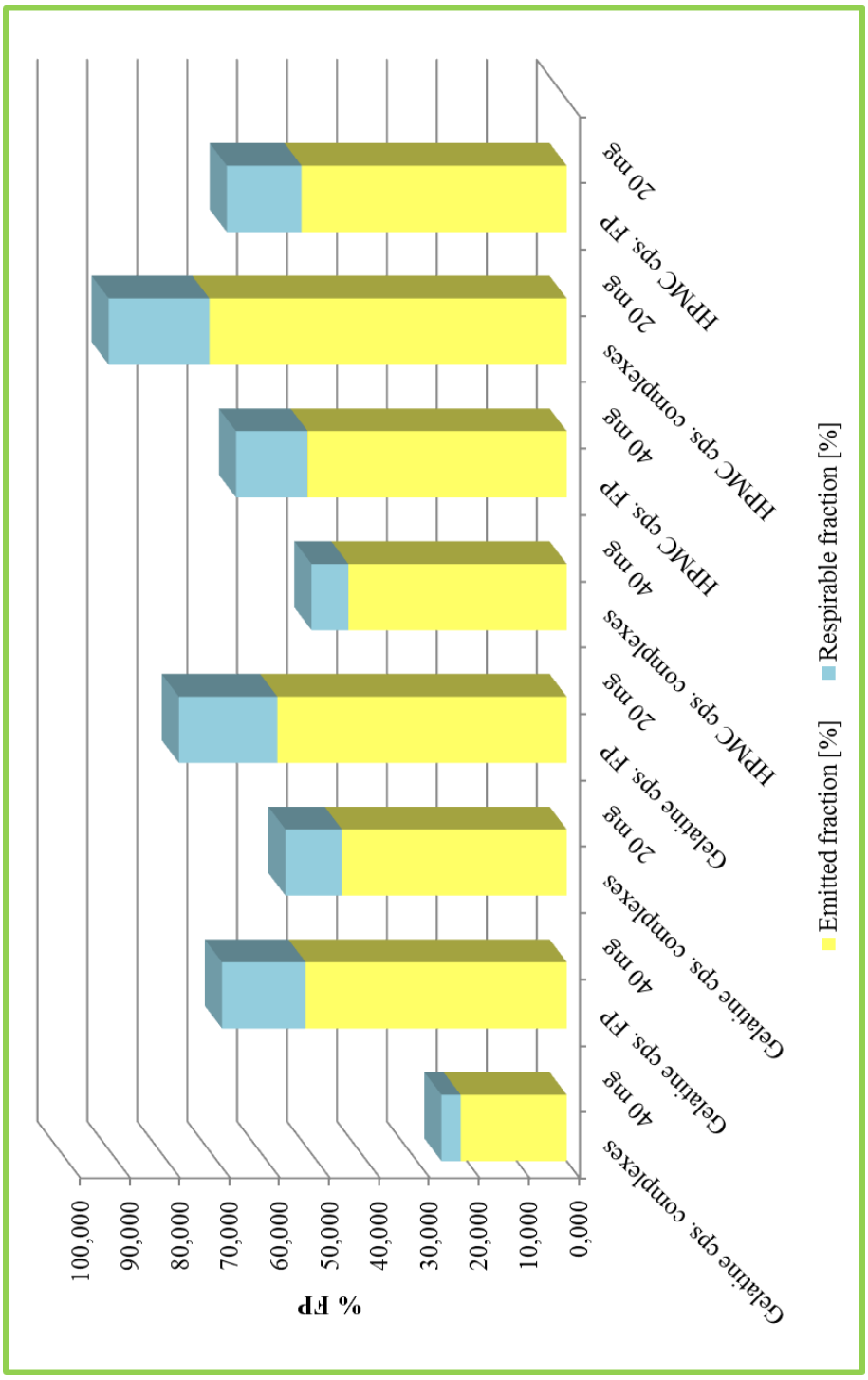


Figure 24: Comparison of emitted dose and respirable fraction (percentage of nominal dose)

Twin Impinger analysis was carried out to find the differences and possible advantages of inclusion complex formation compared to free drug in the formulation with α-lactose monohydrate. The distribution in the TI compartments was compared with the following variables:

- mass of the capsule content (40 mg vs. 20 mg with dose 0.22 mg for both)
- the capsule type (gelatine capsules vs. HMPC capsules)

The test for each combination was carried out with 5 capsules, one after another in order to gain the absorbance in the linear region of the spectrophotometer. Fractions presented in *Figure 23* and *Figure 24* were calculated for 1 capsule.

Gelatine capsules

In both combinations (see *Figure 23*) of formulations with free FP and FP/γ-CD in 40 mg or in 20 mg capsule, the formulation with free FP showed better performance since the fraction of the lower compartment was bigger. The poorest outcome was observed in the 40 mg capsules loaded with FP/γ-CD complex formulation where only 20 % of the drug was emitted from the device (see *Figure 24*). Formulation of the free FP gave similar results for 40 mg and 20 mg capsules. The respirable fraction for FP was 16 % and 20 % in 40 mg and 20 mg capsules, respectively, which was in concordance with the literature (22). Seeing that 40 mg capsules demonstrated worse performance compared to 20 mg, it was assumed that lactose impeded the flow of drug particles. In all combinations emitted fractions were quite low for which gelatine capsules might be the reason. Gelatine capsule shells have 13-16 % water content (76) and due to this gelatine capsules might not be suitable as the moisture affects the flowability of powders. They also have a tendency to adjust its moisture level with the surrounding environment (the relative humidity in Lisbon is quite high). On the other hand, when stored at low humidity conditions they lose its moisture and become brittle. This could be problematic in DPIs since the shear forces could break the capsule into small pieces, hence the capsule pieces could follow the powder flow into the lung.

HPMC capsules

Also with HPMC capsules the complex formulation in 40 mg capsules did not result promising for lung deposition. FP formulations in 40 mg and 20 mg capsules exhibited similar fractions. Despite of that the FP formulation in 20 mg capsules compared to 40 mg

capsules showed smaller upper compartment fraction, which is favourable regarding the candidiase invansion due to corticosteroid deposition in the upper compartment (oropharynx). Slightly better aerodynamic performance gave the complex formulation in 20 mg capsules with the highest emitted fraction (see *Figure 24*); 20 % of respirable fraction and low portion of upper compartment fraction. Above all, the latter combination could be promising for lung deposition and might also be improved if the FP/γ-CD particles would be minimized to a range that is suitable for deep lung inhalation.

4.6 Particle size and morphology analysis

4.6.1 SEM

Scanning Electron Microscopy was used to procure information about the particle size and morphology (*Figure 25*). Pure fluticasone propionate appeared like irregular shaped crystals tending to form aggregates with the size around 10 μm. γ-CD were observed as crystalline particles of various sizes and shapes with a smooth surface. In the physical mixture of FP and γ-CD both types of crystalline particles were seen separately. The image of FP/γ-CD complexes revealed a significant modification in particle morphology. The particles of different shapes and size were observed having possibly an amorphous nature. The pure components could not be differentiated in the image of complexes. To conclude, as previously proven, the interactions occurred between FP and γ-CD. Although not a decisive method to confirm complex formation, it helps to assess existence or absence of pure components in the complex preparation.

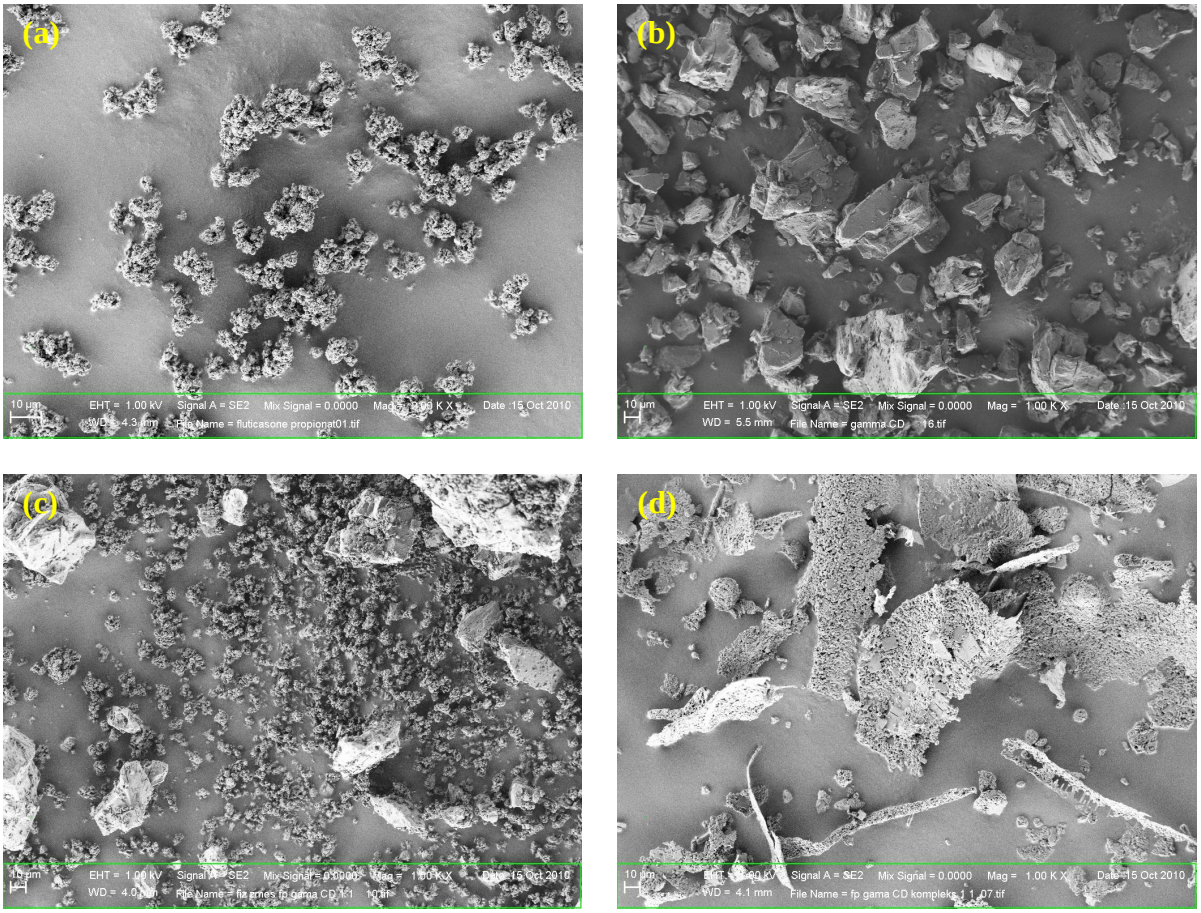


Figure 25: SEM images of (a) – FP, (b) – γ-CD, (c) – physical mixture of FP and γ-CD, (d) – FP/ γ-CD inclusion complexes

4.6.2 Aerosizer®

Aerosizer® is a time-of-flight aerosol spectrometer, by which particle aerodynamic diameter (d_{aer}) of the airborne particles is measured. The particle size determination using Aerosizer® is less time consuming, since the results are obtained in minutes, compared to the Twin Impinger where one analysis takes about half an hour. The particle size distribution spectra differ between powders with different aerodynamic diameters. For example, if the formulation contains the drug (smaller particles) and lactose (larger particles), there are two peaks expected (66).

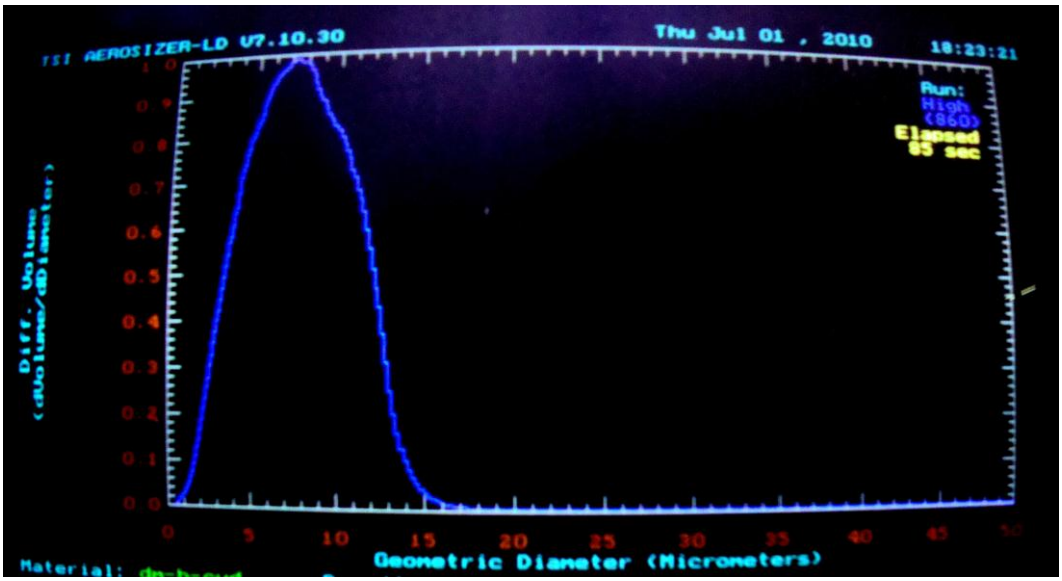


Figure 26: Particle size distribution of inclusion complexes (FP/ γ-CD)

Inclusion complexes FP/γ-CD were analysed for size distribution on Aerosizer®. The distribution was unimodal giving a wide particle range of 0-17 µm, which was in accordance to poor performance on Twin Impinger as well as demonstration of various particles size on SEM images. The obtained particle range is not very appropriate for pulmonary deposition.

4.7 Dissolution test

Dissolution is defined as a process by which a solid substance enters into a solvent to yield a solution and is controlled by the affinity between the solid substance and the solvent. Dissolved drug is necessary for physiologic action therefore dissolution testing is a core performance test in pharmaceutical development and quality control. It is used as a predictive tool to estimate *in vivo* behaviour of a drug product (73–75).

The aerosol particles that are deposited in the lower non-ciliated airways undergo dissolution in the lung fluids. Only dissolved fraction of the dose is available for absorption across alveolar membrane (73). The aim of this dissolution study was to prove the enhancement of dissolution of FP/γ-CD complexes compared to FP alone and not to gain a dissolution rate.

For dissolution test a new calibration curve was obtained (λ_{max} = 240 nm) with coefficient 17601 M^{-1} (R^2 = 0.9989). Percentage of FP was calculated from equation for dissolution test with media replacement described in *Eq 13* (Materials and methods).

FP/γ-CD inclusion complexes exhibited a higher dissolution rate compared to the free drug. The concentration of FP seen on the dissolution curve for FP/γ-CD in *Figure 27* was decreasing from 20.min to 90.min time point. The reasons for decreasing concentration could be adherence of the drug on the needle when sampling, limited FP stability in water and decomposition of complexes leading to exceeding solubility of FP in water. The reason that could be caused with incautious sampling was eliminated by an additional test where samples from 3 different beakers were analysed after 15, 30 and 60 min, respectively. The results demonstrated the decreasing concentration of FP as well. The exact data for fluticasone propionate stability in water was not found in the accessible literature to date. All things considered it was assumed that some complexes got decomposed in the water media. Keeping in mind the complexes were proven only with precipitation method from water that lasted for 2 days, it could be predicted the bonds between FP and γ-CD were not established easily. Therefore with attention to γ-CD's large cavity, which can include 17 water molecules (33), it was assumed the weak bonds in some complexes were broken. The solubility of free FP was then exceeded; as a result the percentage of dissolved FP was decreasing with time.

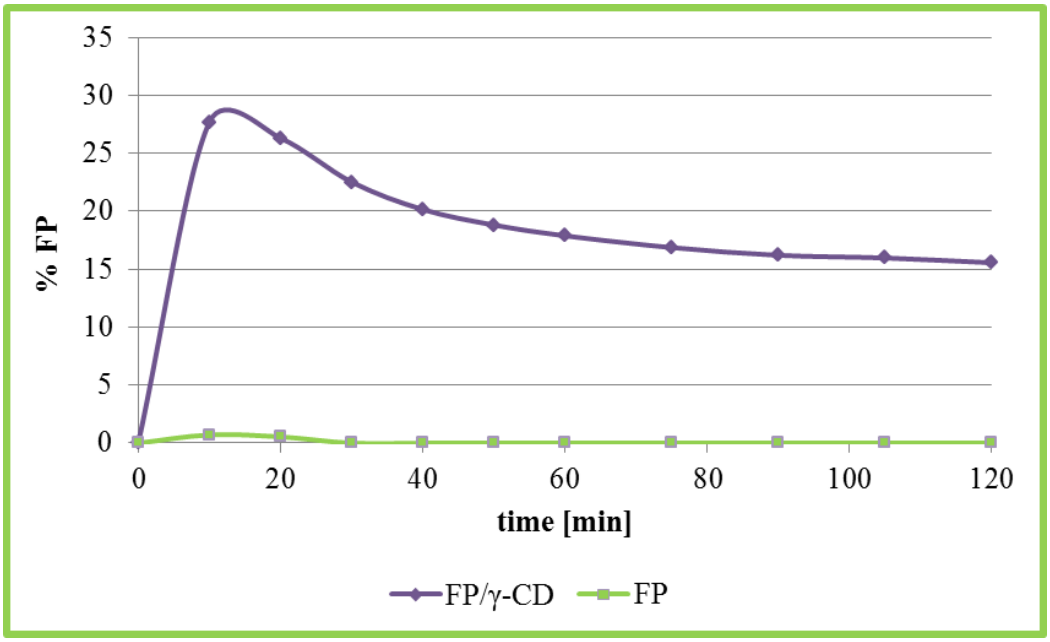


Figure 27: Comparison of dissolution between free drug and FP/γ-CD

5 Conclusions

In my graduate study the drug fluticasone propionate was tested with HP-β- and γ-cyclodextrins aiming for inclusion complexes formation. Firstly, molecular modelling suggested some interactions between FP and HP-β- or γ-cyclodextrin. The phase-solubility studies of HP-β- and γ-cyclodextrin with FP gave significantly higher stability constant for inclusion complex with γ-CD (192.9 M^{-1} and 3093.7 M^{-1}, respectively). Preparation of FP/γ-CD inclusion complexes was carried out with 2 variants of kneading methods (water or ethanol as a solvent) and precipitation from water, which was developed by Toropainen (34). IR spectra and dissolution study proved that complexes were successfully formed only when using precipitation method from water. In IR spectra the characteristic absorption bands of FP were absent and FP solubility was 20-fold higher when complexed compared to solubility of free FP.

FP/γ-CD complexes were further on characterised for particle size and morphology by Scanning Electron Microscopy, Aerosizer® and Twin Impinger that additionally mimics the anatomic structure of lung, thus lung deposition of particles could be predicted. SEM images showed significant differences between FP/γ-CD and pure components. In the FP/γ-CD image particles of pure FP and γ-CD could not be observed, meaning the components underwent some modifications (complex formation). The FP/γ-CD particles were also seen in different shapes and sizes; many particles were larger than 10 μm, which is not appropriate for pulmonary delivery. The observation of SEM images confirmed particle size distribution spectrum obtained by Aerosizer®.

The questions of the graduate study were analysed:

- Is the lung deposition of the drug better when it is loaded in CDs?

The free FP tends to form agglomerates, therefore the particle size exceeds the particle size range suitable for inhalation. The inclusion complexes formation with γ-CD using precipitation method from water did not result in obtaining the smaller particles. Twin Impinger analysis showed that only a complex formulation in HPMC capsule that contained 20 mg of powder exhibited slightly better deposition compared to its free drug formulation pair. It was assumed the preparation method was not suitable to obtain the particles in the

respiratory range or further manipulations of particles would be needed. In order to meet the particle size requirements for pulmonary delivery different particle processing method of preparation should be tested like spray drying, supercritical fluid methods.

- Do inclusion complexes with CDs enhance the solubility of the drug (FP)?

The dissolution analysis showed the solubility of FP/γ-CD inclusion complexes to be 27-fold higher in 15.minute compared to the free FP, although the difference decreased with time. This is advantageous in alveolar region because it is covered only with a thin liquid layer of 21-200 nm. If the particle is 1 μm, only 1/50 to 1/5 of the particle would be immersed in the liquid phase (8). The FP/γ-CD inclusion complexes were expected to be dissolved faster in the relatively small volume of the apical lining fluid, resulting in a higher absorption rate across the cellular epithelial barrier.

To conclude, the use of cyclodextrins is beneficial for the complexation of FP, although the particle size of complexes should be reduced in order to meet requirements for pulmonary delivery. As the FP has 10 h biological half-life, therefore it is retained in the lung, the formulation could be proposed for further work. Combining the micronized free FP and FP/γ-CD inclusion complexes, the complexes would dissolve immediately in the alveolar fluid, which would lead to faster therapeutic effect whereas free FP would be dissolving slowly and that would assure prolonged action.

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