## Contents

# บทคัดย่อ

ยาซีลีค็อกซิบเป็นยาต้านอาการอักเสบที่ไม่ใช่สเตียรอยด์ สามารถนำไปใช้ในการรักษาโรคจอประสาทตาเสื่อมและภาวะเบาหวานขึ้นจอตาโดยผ่านการยับยั้งเอนไซม์ COX-2 ที่จอประสาทตา ปัจจุบันมีรูปแบบการบริหารยาเพื่อนาส่งยาเข้าสู่บริเวณส่วนหลังของลูกตา เช่นการบริหารโดยการรับประทาน การฉีดเข้าน้ำวุ้นลูกตา การฉีดเข้าใต้เยื่อบุตา และการหยอดตา ในวิถีการบริหารยาทั้งหมด การบริหารยาโดยการหยอดตาเป็นวิธีที่ทำให้ยาออกฤทธิ์เฉพาะที่ และไม่ทำลายเนื้อเยื่อดวงตา อย่างไรก็ตามเนื่องจากยาซีลีค็อกซิบเป็นยาที่ละลายน้ำได้น้อยจึงเป็นข้อจำกัดในการนำส่งยาเข้าสู่ภายในลูกตา ดังนั้นการศึกษานี้จึงมีวัตถุประสงค์เพื่อพัฒนายาแขวนตะกอนซีลีค็อกซิบในรูปแบบยาหยอดตา ที่มีไซโคลเดกซ์ทรินและพอลิเมอร์เป็นองค์ประกอบเพื่อนำส่งยาไปสู่ส่วนหลังของลูกตา ศึกษาเฟสการละลายของสารประกอบเชิงซ้อน และคุณลักษณะของสารประกอบเชิงซ้อนของยาซีลีค็อกซิบกับไซโคลเดกซ์ทริน 5 ชนิดได้แก่ อัลฟา (alpha-CD) บีต้า (beta-CD) แกมมา (gamma-CD) ไฮดรอกซีโพรพิลบีต้า (HP-beta-CD) และแรนดอมลีเมทิลเลตบีต้า (RM-beta-CD) ไซโคลเดกซ์ทริน และพอลิเมอร์ยึดติดเยื่อเมือก 3 ชนิดได้แก่ ไฮดรอกซีโพรพิลเมทิลเซลลูโลส (HPMC) ไคโตซาน และกรดไฮยาลูโรนิก (HA) จากผลการศึกษาพบว่าไซโคลเดกซ์ทรินชนิด RM-beta-CD มีคุณสมบัติในการละลายยาสูงกว่าไซโคลเดกซ์ทรินชนิดอื่นที่นำมาทดสอบ HPMC มีประสิทธิภาพที่ดีในการเกิดสารประกอบเชิงซ้อนตติยภูมิ ซึ่งสูงกว่าสารประกอบเชิงซ้อนทุติยภูมิซีลีค็อกซิบ/RM-beta-CD ถึง 11 เท่า เมื่อทำการประเมินการเกาะกลุ่มของขนาดอนุภาคสารประกอบเชิงซ้อนตติยภูมิ พบขนาดของอนุภาคอยู่ในช่วง 250 - 350 นาโนเมตร ส่งผลทำให้การละลายของซีลีค็อกซิบสูงขึ้น จากข้อมูลที่ได้รับจากเทคนิค DSC PXRD FTIR และ $^{1}$H-NMR ชี้ให้เห็นว่ามีการเกิดสารประกอบเชิงซ้อนของยาซีลีค็อกซิบและไซโคลเดกซ์ทริน การเตรียมสูตรตำรับยาหยอดตาซีลีค็อกซิบ ได้มีการเตรียมแบบไม่ใช้ความร้อนและการใช้ความร้อนโดยการผ่านคลื่นความถี่สูงที่อุณหภูมิ 70 องศาเซลเซียส เป็นเวลา 1 ชั่วโมง ประเมินคุณลักษณะทางเคมีและกายภาพ คุณสมบัติการยึดติดเยื่อเมือก และการประเมินการซึมผ่านภายนอกร่างกาย ผลการประเมินคุณสมบัติทางเคมีกายภาพของสูตรตำรับ ได้แก่ลักษณะทางกายภาพ ความเป็นกรด-ด่าง ออสโมลาลิตี และความหนืดอยู่ในช่วงที่ยอมรับได้ ขนาดอนุภาคต่ำกว่า 10 ไมโครเมตร บ่งชี้ว่าอาจจะไม่ก่อให้เกิดการระคายเคืองต่อดวงตา สูตรตำรับที่มีพอลิเมอร์ชนิด HA เป็นองค์ประกอบแสดงคุณสมบัติการยึดติดเยื่อเมือกที่ดี ส่วนสูตรตำรับที่ผ่านความร้อนส่วนใหญ่พบว่ามีปริมาณของยาซีลีค็อกซิบที่อยู่ในรูปสารละลายมากขึ้น ส่งผลทาให้ปริมาณยาที่ซึมผ่านเยื่อเลือกผ่านและการซึมผ่านน้ำวุ้นลูกตาจำลองเพิ่มขึ้น โดยเฉพาะสูตรตำรับที่มี RM-beta-CD และ HA ร้อยละ 0.5 โดยน้ำหนักต่อปริมาตร ดังนั้นสูตรตำรับดังกล่าวจึงอาจเป็นสูตรตำรับที่เหมาะสมในรูปแบบยาหยอดตาแขวนตะกอนที่สามารถนำส่งยาซีลีค็อกซิบไปยังบริเวณส่วนหลังของลูกตาในการรักษาโรคจอประสาทตาเสื่อมและภาวะเบาหวานขึ้นจอตาได้

## Abstract

Celecoxib (CCB), a nonsteroidal anti-inflammatory drug (NSAID), could be beneficial in the treatment of age-related macular degeneration (AMD) and diabetic retinopathy (DR) through the inhibition of COX-2 enzyme. Currently, the several methods can deliver drugs to the back of the eye. i.e., oral, intravitreal injection, subconjunctival injection and topical. Among routes of administration, topical eye drop is non-invasive method and local effects. However, due to low aqueous solubility of CCB, it hampers ocular bioavailability. Thus, the aim of this study was to develop topical eye drop suspensions containing cyclodextrin (CD) and polymer delivering to the posterior segment of the eye. CDs, the solubilizer, i.e., alpha-CD, beta-CD, gamma-CD, HP-beta-CD and RM-beta-CD and mucoadhesive polymers i.e., hydroxypropylmethylcellulose (HPMC) chitosan and hyaluronic acid (HA) were used. The phase-solubility profiles and CCB/CD complex characteristics were investigated. RM-beta-CD exhibited the greatest solubilizer among CDs tested. HPMC was the potential polymer to form ternary complex with CCB/RM-beta-CD which gave 11-folds complexation efficiency higher than that of its binary complex. The aggregate size of ternary complexes in solution were found in the range of 250 - 350 nm which could solubilize themselves resulting in increasing CCB solubility. The data obtained from FT-IR, DSC, PXRD and $^{1}$H-NMR indicated that there was interaction of CCB with CD as inclusion complex. The CCB eye drops formulations were prepared by unheated and heating method (sonication at the temperature of 70$^{o}$C for 1 h). The physicochemical and chemical characterizations, mucoadhesive properties and *in-vitro* permeation were determined. The physicochemical properties i.e., appearance, pH, osmolality and viscosity were in acceptable range. The particle sizes below 10 µm indicated possibly no irritation to the eye. The formulation containing HA showed the excellent mucoadhesive properties. The increasing of % CCB content in most cases by heat resulting in higher flux permeation through semipermeable membrane and through simulated artificial vitreous humor, especially the formulation containing RM-beta-CD and HA (0.5% w/v). Therefore, it may be a promising candidate as topical formulation which can deliver CCB to the posterior segment of eye to treat AMD and DR.

## Executive summary

Age-related macular degeneration (AMD) and diabetic retinopathy (DR), retina vessel occlusion and glaucoma are the most common blinding disorders in the aging population. The unique eye anatomy, biochemistry and physiological of it hamper the drug delivery to both retina and the optic nerve. Thus, drug delivery to the posterior segment of the eye is challenging. Currently, the four approaches maybe used to delivery effective doses of drug to the target tissues in the posterior eye are topical, systemic, intraocular and periocular. Although the invasive methods (intraocular and periocular) enable drugs to the posterior segment of the eye, these routes have inherent risks for ocular infections and tissue damage. The development of topically nanoparticles as drug carriers to provide sustained drug release and prolonged therapeutic drug activity are the friendly alternative approaches.

Celecoxib (CCB) is a new non-steroidal anti-inflammatory drug (NSAID) class, able to selectively inhibit COX-2. It has anti-inflammatory, anti-proliferative and/or anti-VEGF activities can be beneficial in the treatment of proliferative diabetic retinopathy, neovascular age-related macular degeneration and some ocular tumors. Both AMD and DR require long-term therapy for their management because they are chronic disorders. CCB has the amide group which is weakly acidic with a pKa value of about 11. For this reason, the aqueous solubility of CCB is 3–7 μg/ml at pH 7. It is a lipophilic compound (log P = 3.5) and has been proven to belong to the class 2 division of the Biopharmaceutical Classification System (BCS Class II), which includes poorly soluble and highly permeable drugs.

Application of cyclodextrins (CDs) by their complexation is the possible to increase aqueous solubility of some lipophilic drugs without changing their molecular structure. CDs are act as true carrier by keeping the hydrophobic drug molecules in solution and delivering them to the surface of the biological membrane, e.g. the ocular barrier, where they partition into the membrane. The relatively lipophilic membrane has low affinity for the hydrophilic nature and large molecular weight of CD and therefore they remain in the aqueous membrane exterior, e.g. the tear fluid. The amount of dissolved drug increases with increasing CD concentration which results in increased drug diffusion to the surface of the barrier.

The project was focused on developing new micro- and nanotechnology platforms to solve problems in ocular drug delivery. They were expected to provide

sustained release and mucoadhesive properties to improve bioavailability. In addition, CCB concentrations would slow down the clearance of the eye due to that self-assembled CD acts as a drug reservoir and reach to the posterior segment of the eye. Thus, the objectives of this present study were follows:

1. To study the effect of cyclodextrin and polymers as ternary complex on the solubility of celecoxib.
2. To develop and characterize of celecoxib microsuspension eye drops containing cyclodextrin.
3. To study the mucoadhesive properties and eye irritation test of the formulation
4. To determine the *in vitro* and *ex-vivo* permeation of celecoxib in the eye drop formulation

The experiments were performed according to research methodology and achieved the objectives of the study. We also have done the special project with the title “Antifungal Activity of Econazole Nitrate/Cyclodextrin Complex: Effect of pH and Formation of Complex Aggregates”

## Introduction

Presently, age-related macular degeneration (AMD) and diabetic retinopathy (DR) are two key prevailing causes of vision impairment. The two disorders combined account for over 60% of cases of blindness in the US (Congdon et al., 2004). AMD is the major cause of central vision loss, typically individual ≥ 65 years of age. The early phase of AMD is characterized by drusen located under the retina pigment epithelium (RPE), and pigment alteration. Two clinically recognized subtypes of AMD are dry and wet AMD. The former represents the severe atrophy of photoreceptors and the underlying RPE and choriocapillaris. The latter accompanies choroidal neovascularization (CNV) in invading the subretinal and sub-RPE space. Patients with neovascular (wet) AMD have a mean age of 70.5 years, compared with 56.8 years for those with dry AMD. It was also found that incidence of disorder was not significantly different from the gender. (Janoria et al., 2007). Some reports showed that the prevalence of AMD in Asian populations was lower than that in Caucasian populations (Jager, Mieler, and Miller, 2008; Yuzawa et al., 1997). Based on the population of Thailand, there were 3% from 10,788 participants diagnosed as having AMD. The mean age was 62.1 (in range 50–98) years old. There were 2.7% and 0.3% participants with early AMD and late AMD, respectively. Of the late AMD, 74.1% were wet AMD and 25.9% were geographic atrophy (Jenchitr et al., 2011).

DR often leads to neovascularization and proliferative retinopathy as well as macular edema. DR is the third leading cause of blindness in the US and the leading cause of new blindness among the age group 20 to 74 years old. Macular edema involves swelling of the macula due to sub-retinal fluid buildup. Macular edema occurs in approximately 10% of diabetics. Focal macular edema is caused by foci of vascular abnormalities, primarily micro-aneurysms, which tend to leak fluid whereas diffuse macular edema is caused by dilated retinal capillaries. Non-proliferative diabetic retinopathy and proliferative diabetic retinopathy have incidences of 56% and 29%, respectively. The current standard of care for DR involves photocoagulation and vitrectomy. These are only used in late stage disease and there is a need for earlier pharmacologic intervention. Antiangiogenics, steroids and neuroprotectants may all play a role in the treatment of DR (Hughes et al., 2005).

Vascular endothelial growth Factor (VEGF) seems to be a major contributory factor to vascular leakage and plays an important role in the pathophysiology of neovascular AMD and DR (Kompella et al., 2010). Currently, therapeutic approaches to treat these disorders are limited, although there is a significant interest and research initiative in discovering therapies to combat the progression of these disorders. During the last few years, therapeutic agents have been introduced into the market, to treat DR and AMD i.e. Ozurdex®, an injectable implant containing dexamethasone, an anti-inflammatory corticosteroid (Pacella et al., 2013), Pegatinib (Macugen®), an anti VEGF aptamer, and ranibizumab (Lucentis®) , an anti-VEGF antibody fragment were approved for treating the wet form of AMD in 2004 and 2005, respectively (Ozkiris, 2010). Thus, it is evident that anti-inflammatory agents and VEGF-inhibition are viable therapeutic options for treating DR and AMD (Kompella et al., (2010).

Celecoxib (CCB), a nonsteroidal anti-inflammatory drug (NSAID), is the first selective cyclooxygenase-2 (COX-2) inhibitor used in the treatment of osteoarthritis and rheumatoid arthritis in adult patients. It is also indicated in treatment of acute pain, primary dysmenorrhea, and as an adjuvant in the treatment of familial adenomatous polyposis, a genetic disorder (Davies et al., 2000). In addition, CCB has VEGF inhibitory effects as demonstrated in several anticancer studies using different cell types through the inhibition of COX-2 enzyme and demonstrated to have anti-angiogenic and anti-proliferative effects on several cell types including endothelial cells. Thus, the VEGF inhibitory and anti-proliferative effect activity of CCB could be beneficial in the treatment of the proliferative stages of DR and AMD (Amrite and Kompella, 2008; U. Kompella et al., 2010).

Ayalasomayajula and Kompella (2003) have shown that oral administration of CCB can reduce diabetes-induced retinal VEGF mRNA expression and vascular leakage by inhibiting the activity of COX-2 enzyme in a rat model. Nevertheless, the required dose for these effective levels is very high (50 mg/kg twice a day) resulting in adverse effects and systemic toxicity including cardiovascular problems. Several studies have been performed to overcome the drawbacks of the systemic administration of CCB. The local administration approaches are alternative pathway delivering the drug reach to the posterior segment of the eye. Intravitreal injections can provide high dose of drug concentration to the retina. However, the bolus intravitreal injections can potentially cause retinal toxicity. Moreover, the repeated

intravitreal injections have been associated with complications such as retinal detachment and also endophthalmitis. (Raghava, Hammond, and Kompella, 2004). The other route of administration is subconjunctical injection which has several advantages more than those of the intravitreal route. For instance, the drugs can be administered without directly interfere the vision via subconjunctival routes. In addition, the drug solutions/suspensions volumes can be administered as high as 500–5,000 µl in humans. Ayalasomayajula and Kompella (2004) demonstrated that the 1 mg/ml suspension of CCB injected through subconjunctival administration can be distributed to the retina and other ocular tissues including vitreous, sclera, cornea and lens. Moreover, the retinal availability of CCB following subconjunctival administration in the ipsilateral retina is 54-fold higher compared to intraperitoneal administration. There are several studies regarding to periocular injectable formulation. Amrite *et al*. (2006) demonstrated that CCB-poly(D,L-lactide-co-glycolide) (PLGA) microparticles through subconjunctival administration provided sustained drug level in the retina of rat model for 60 days. Cheruvu et al., (2009) prepared CCB in two injectable forms i.e., CCB suspension in 0.5% carboxymethylcellulose and CCB-loaded poly(L-lactide) (PLA) nanoparticles for trans-scleral drug delivery to the retina which aimed to rapid release and sustained release from both platforms. It can be distributed to vitreous humor and retina. Although these studies showed increased delivery of CCB to the posterior segment of the eye, they all required invasive delivery methods. Both intraocular and periocular routes have inherent risks for ocular infections and tissue damage (Lee et al., 2004). In addition, the invasive methods need ophthalmologist operation leading to high cost of treatment and patient's inconvenient. Currently, there are no safe and patient- friendly drug delivery systems to the posterior segment. Thus, there is a need for development of non-invasive methods as alternative approaches for effective retinal drug delivery (Koevary, 2003; Kurz and Ciulla, 2002).

Conventional ophthalmic dosage forms for non-invasively topical drug delivery to the eye are aqueous solutions, suspensions and ointments. Aqueous solutions are the most common, however; aqueous drug solutions are rapidly removed from the eye surface and drained into the nasolacrimal duct (Urtti and Salminen, 1993). In case of suspensions, the vehicle containing drug particles is an additional factor influencing drug release rate and corneal penetration. However, the development of eye drops may have several limitations that cannot be delivered to the target site. After an eye-

drop instillation, usually less than 5% of an applied dose reaches the intraocular tissues. The small amount of drug can penetrate through the cornea membrane because of drug elimination from the pre-corneal area (pre-cornea clearance) resulting in low ocular drug availability. (Loftssona and Jarvinen, 1999). Current trends in ocular therapeutics and novel drug delivery offer improved biopharmaceutical properties and have the capacity to deliver drugs more precisely to the target sites in the predictable manner.

CCB is a low molecular weight (381.38 Da) hydrophobic molecule with a log P 3.47 at pH 7.4. With a $pK_a$ of 11.1, CCB is neutral at physiologic pH and is practically insoluble. The aqueous solubility of CCB is approximately 2 µg/ml (Ayalasomayajula et al., 2004; Ventura et al., 2005). Due to the poor aqueous solubility of CCB, it hampers the drug permeation resulting in inadequate drug levels to the posterior segment of eye. Thus, the CCB eye drop formulation development should focus on increasing the drug solubility in order to enhance the absorption of the drug into the site of action. There are several techniques used to enhance the drug solubilzation i.e., 1) pH adjustment 2) particle size reduction 3) inclusion complexes/complexation 4) co-solvency 5) miceller solubilization, etc. (Chaudhary, 2012). Using cyclodextrin (CD) as inclusion complex is an alternative approach to enhance the solubility of CCB.

CDs are cyclic oligosaccharides with a hydrophilic outer surface and a lipophilic central cavity comprised ($\alpha$-1,4-)-linked $\alpha$-D-glucopyranose units. The naturally shaped of CD molecules like cones with secondary hydroxy groups protruding from the wider edge and the primary groups from the narrow edge result in this conformation of CD molecules has a hydrophilic outer surface, whereas the lipophilicity of their central cavity. The natural CDs consist of six, seven and eight glucopyranose units are $\alpha$-cyclodextrin ($\alpha$-CD), $\beta$-cyclodextrin ($\beta$-CD) and $\gamma$-cyclodextrin ($\gamma$-CD), respectively. In addition, random substitution of the hydroxy groups such as methoxy functions brings about dramatic improvements in their solubility. CD derivatives of pharmaceutical interest include such as hydroxypropyl-$\beta$-cyclodextrin (HP-$\beta$-CD), hydroxypropyl-$\gamma$-cyclodextrin (HP-$\gamma$-CD), randomly methylated-$\beta$-cyclodextrin (RM-$\beta$-CD), sulfobutylether-$\beta$-cyclodextrin (SBE-$\beta$-CD). The CD has a molecular weight of about 1,000 - 2,000 Da with the negative Log P. Thus, they are poorly absorbed through biological membranes (Loftsson et al., 2005). The various lipophilic drugs can form complex with CD by inserting their moieties or the whole molecules into the cavity

of CD in aqueous solutions resulting in increasing the solubility of lipophilic drugs. Reddy et al., (2004) conducted a study of β-CD complexes of CCB. Regarding to phase- solubility profile, the solubility of CCB can be improved by β-CD. Moreover, the result of this study indicated that the dissolution rate of CCB/β-CD complex in solid-state was significantly increased when compared to free CCB. Additionally, HP-β-CD, the β-CD derivative can enhance the dissolution rate of CCB (Sinha et al., 2011)

Recent studies have shown that the addition of polymer can significantly increase the CD solubilization and complexing abilities by ternary complex formation (Loftsson and Brewster, 1996). In this way, the synergistic effect of water-soluble polymer could decrease in CD amount required to prepare soluble drug/CD complexes (Miranda et al., 2011). Using heating method, autoclaving at 121°C for 20-40 minutes or sonication at a temperature of over 70°C for one hour could accelerate the ternary complexes (Loftsson, Hreinsdottir and Masson, 2005; Maragos et al., 2009). The Influence of hydrophilic polymers on CCB/HP-β-CD complexes as ternary complex was investigated (Chowdary and Srinivas, 2006). It was concluded that polyvinylpyrrolidone (PVP), HPMC and polyethylene glycol (PEG) stabilized the binary complexes consequently to increase the CCB solubility. Besides CDs as solubilizer, they can act as a permeation enhancer by increasing permeability of drug through the lipophilic membrane. Tirucherai and Mitra (2003) studied the effects of HP-β-CD on the solubility and permeability of the acyl ester prodrugs of ganciclovir through the cornea of rabbit eyes. It was found that the permeability of drug increased 2.5 times compared to formulation without HP-β-CD. Since, CDs act as true carriers by keeping and dissolving the hydrophobic drug molecules in solution and delivering them through the mucin layer (aqueous layer) to the surface of the biological membrane similar to increasing drug concentration at the membrane surface lead to increasing permeability. (Loftssona et al., 1999). There was an example of *in-vivo* study using CD as topical eye drop delivering dexamethasone to the anterior and posterior segments of the rabbit eyes. The drug can be absorbed into the eye and distributed to eye tissues such as cornea sclera in large quantities after topical eye drop was applied. It was also found that it could reach to the back of the eye i.e., retina and vitreous, rather than to the intravenous injection (Sigurdsson et al., 2007).

Several researchers have studied the effect of mucoadhesive polymers on lowering drainage rate of instilled eye drop from pre-cornea area. For example,

Saettone et al., (1989) studied mucoadhesive properties of polymer with mucin-coated surfaces model for ophthalmic drug delivery. The good to excellent mucoadhesive properties were found in formulation of tropicamide containing hyaluronic acid which is better than formulation containing polyacrylic acid. Similarly, Felt et al. (1999) have studied regarding precorneal retention in eye rabbits of tobramycin eye drop preparation. At least a 3-fold increasing of the corneal contact time was achieved in the presence of chitosan when compared to a commercially available pharmaceutical product (Tobrex®). It was concluded that the ophthalmic formulation containing mucoadhesive polymer helps to deliver the drugs reach to the site of action by inhibiting drug removal from the pre-corneal area.

Although, these studies showed the increasing retention time of eye drop formulation, the eye drop solution with mucoadhesive polymer are not enough residence time to maintain the drug adhered to the eye surface. Combination of the solid particles and mucoadhesive polymer in eye drop preparations is a promising alternative approach. Jansook et al, (2010) studied the permeation of dorzolamide microsuspension eye drops into the rabbit eyes and intraocular drug levels were determined. After eye drops were instilled, the drug level in the aqueous humor can be maintained more than 24 hours while the formulation of it in solution dosage form (Trusopt®) can be maintained in the aqueous humor for just 8 hours. From this study, the particles in suspension were trapped on the eye surface which increased the contact time leading to prolonged drug absorption. However, the size of the particle in formulation should not exceed 10 microns to prevent irritation of the eyes.

This present study was focused on developing new CCB eye drop formulation containing CD platform. They are expected to provide sustained release and mucoadhesive properties to improve ocular bioavailability which aimed to deliver to the posterior segment of the eye. The effect of CDs and mucoadhesive polymer on CCB solubilization was investigated, CCB eye drop formulations were developed, physicochemical properties were characterized and the mucoadhesive properties and *in-vitro* permeation were evaluated.

## Methods

### 1. Thermal stability of CCB on cycles of autoclaving

The stability of the CCB in the aqueous solution containing 0.5% w/v β-CD was determined by heating method (Loftsson et al., 2005). Excess amount of CCB was dissolved in purified water and equilibrated at 30°C ± 1°C for 24 hrs under constant agitation. The supernatant was filtered through 0.45 µm nylon filter and mixed with 1% w/v β-CD solution at 1:1 ratio. The solution was then divided into three sealed vials that were heated in an autoclave for one, two, and three heating cycles, each cycle consisted of heating to 121◦C for 20 min. The drug concentrations in the vials were then determined by a high-performance liquid chromatographic method (HPLC).

### 2. Quantitative analysis of CCB

***Calibration curve of CCB***

A stock solution was prepared by accurately weighing CCB reference standard (10.0 mg) into 100-ml volumetric flask. Then, it was dissolved and diluted to volume with mixture of acetonitrile:water (55:45 v/v) to a final concentration of 100 µg/ml. This solution was further diluted to give a range of CCB 0.05-16 µg/ml. Each solution was subjected to HPLC analysis in triplicate. Peak area was recorded for all the solutions and the equation was calculated from the relationship between peak area of CCB and their concentrations.

***The sample preparation***

The filtrate of sample was further diluted with mixture of acetonitrile:water (55:45) with appropriate dilution. A portion of the sample was filtered through 0.45 µm nylon filter membrane and subjected to HPLC analysis. The CCB content in the sample was then calculated against the CCB calibration curve.

***HPLC conditions***

The quantitative determination of CCB was performed on a reversed-phase HPLC component system from Shimadzu™ consisting of a LC-20AB binary pump, a

SPD-20A multiple wavelength detectors, a SIL-20ATH auto sampler and LCsolution software. The modified HPLC condition (Ventura et al., 2005) was described as follows:

| | |
|---|---|
| Mobile phase | : Acetonitrile:water (55:45% v/v) |
| Chromatographic column | : Shiseido$^{TM}$ C18 column, 5 µm, 4.6x150 mm ID with C18 guard cartridge column MGII 5µm, 4x10 mm. |
| Flow rate | : 1.0 ml/min |
| Oven temperature | : Ambient temperature |
| UV detector wavelength | : 252 nm |
| Injection volume | : 20 µl |
| Run time | : 15 minutes |

## 3. Validation for the quantitative analysis of CCB

### *Specificity*

The specificity of the analyze method investigated by injecting of CCB and all components i.e., α-CD, β-CD, γ-CD, HP-β-CD, RM-β-CD, chitosan (CS), hyaluronic acid (HA), hydroxypropylmethylcellulose (HPMC), phosphate buffer saline (PBS) pH 7.4, EDTA, Benzalkonium chloride (BAC), and mobile phase to demonstrate the absence of interference with the elution of analyze. The all components were properly diluted before determining by HPLC.

### *Linearity*

Linearity was determined with various amounts of properly diluted stock standard solutions in the range of 0.05-16.0 µg/ml of CCB. For each concentration three measurements were performed and the calibration curves were obtained by plotting the peak area versus nominal concentration expressed in µg/ml of CCB. The slope, intercept and coefficient of determination ($R^2$) of each calibration curve were evaluated.

### *Precision*

*Within run precision*

The within run precision was determined by analyzing five sets of three standard solutions of CCB in the same day. The coefficients of variation of the peak area responses (%CV) for each concentration were determined.

*Between run precision*

The between run precision was determined by comparing each concentration of CCB standard solution which prepared and injected on different days. The percentage coefficient of variation (%CV) CCB of peak area responses from three standard solution on different days was calculated.

***Accuracy***

The recovery of CCB from blank formulation were assessed by spiking blank formulation (all components except the drug) with CCB in triplicate at three level spanning 80-120% of amount of CCB in the formulation. The average recovery and the coefficient of variation (%CV) were calculated**.**

## 4. Phase solubility studies

### *4.1 The effect of CD on CCB solubility*

Excess amount of CCB was added to a solution containing 0 to 15% (w/v) CD (α-CD, β-CD, γ-CD, HP-β-CD and RM-β-CD) in pure water. The drug suspensions were saturated through heating in an autoclave 121°C for 20 min and allowed to cool to room temperature (Loftsson et al., 2005). Then a small amount of solid drug was added to the suspensions to promote drug precipitation. The suspensions were equilibrated at 30°C $\pm$ 1°C for 7 days under constant agitation. After equilibrium was attained, the suspensions were filtered through 0.45 µm syringe filter, the filtrates were diluted with the mobile phase and analyzed by HPLC as described in section 2. Each sample was done in triplicate. The apparent stability constants for CCB/CD complexes ($K_{1:1}$ and/or $K_{1:2}$) and the complexation efficiency (CE) were determined according to the phase-solubility method of Higuchi and Connors (Higuchi & Connors, 1965)

$$\mathrm{CE} = \mathrm{S_o}.\mathrm{K_{1:1}} = \frac{\mathrm{Slope}}{1 - \mathrm{Slope}} \qquad \text{Eq. 1}$$

$$[S_t] - [S_o] = K_{1:1}[S_o][CD] + K_{1:1}K_{1:2}[S_o] \quad \text{Eq. 2}$$

Where $S_0$ is intrinsic solubility of CCB, $S_t$ is the concentration of CCB at CD concentration [CD].

### *4.2 The effect of polymer on solubility of CCB/CD complexes*

The phase solubility of CCB was determined in pure aqueous γ-CD and RM-β-CD solutions containing various water-soluble polymers. HPMC (0.1%), HA (0.01%) or CS (0.1%) (all %w/v) were selected as non-ionic, negatively and positively charged polymer, respectively. The polymer was dissolved in the aqueous solutions containing 0-10% w/v CD. Then the excess of CCB was added to form drug suspensions and then heated in an autoclave 121°C for 20 min. The phase-solubility was determined as described above. $K_{1:1}$, $K_{1:2}$ and CE were calculated.

## 5. Morphology and aggregates particle size analysis

The morphology and the particle size and size distribution of the aggregated in CCB saturated 10%w/v γ-CD and 7.5% RM-β-CD with polymer (0.01 %w/v HA and 0.1%w/v HPMC) mixture thereof were analyzed using transmission electron microscope and dynamic light scattering.

### *5.1 Dynamic light scattering (DLS) measurement*

The particle size of CCB/CD based aggregates in solution was measured by DLS technique (Zetasizer™ Nano-ZS with software version 7.11, Malvern, UK). The samples were properly diluted with purified water. A sample was put in a quartz glass cuvette and placed in the instrument. Measurements were carried out at 25°C, 180 degrees scattering angle. Particle size and size distribution was automatically calculated and analyzed by the curve plotted between size distribution and percentage intensity. Each obtained value was carried out in triplicate.

### *5.2 Transmission electron microscope (TEM) analysis*

The morphology of CCB/CD based aggregates was evaluated by TEM (JEOL, JEM-2100F, USA). Initially, the sample was placed on a formvar-coated grids to permit

the adsorption. After blotting the grid with a filter paper, the grid was transferred onto the drop of negative straining. Aqueous phosphotungstic acid solution (2%) was used as a negative strain. The sample was air dried under room temperature. Finally, the samples were examined with TEM.

## *6.* Preparation and characterization of binary CCB/CD complexes and ternary CCB/CD/polymer complexes

### *6.1 Solid-state characterization*

*6.1.1 Sample preparations*

1:1 molar ratio (m:n; $D_mCD_n$ where m and n represents the total moles of drug and CD, respectively) of CCB and CDs (γ-CD and RM-β-CD) with polymer (0.01%w/v HA and 0.1 %w/v HPMC) mixture thereof were prepared by freeze drying method. The obtained Freeze dried mixture were performed through heating in an autoclave 121°C for 20 min and equilibrated at 30°C ± 1°C for 7 days under constant agitation. The filtrated solutions were frozen and then freeze-dried at -52°C for 48 hrs using a Freeze-dryer (Dura-Dry MP, Canada), yielding a solid complex powder (FD). Identical physical mixtures (PM) were prepared by careful blending CCB and CDs in a mortar with pestle. The samples were characterized in solid state as follows: pure CCB, γ-CD, RM-β-CD, PM and FD of CCB/CD (CCB/γ-CD and CCB/RM-β-CD), and FD ternary complexes i.e., CCB/γ-CD/HA, CCB/γ-CD/HPMC, CCB RM-β-CD/HA, CCB/RM-β-CD/HPMC.

*6.1.2 Differential scanning calorimetry (DSC)*

DSC thermograms were determined in a scanning calorimeter (Mettler Toledo™, DSC822 STAR System, Germany). The samples (3-5 mg) were heated (10°C/min) in sealed aluminium pans under nitrogen. The temperature range was from 30-250°C. An empty aluminium pan was used as reference.

*6.1.3 Powder X-ray diffraction (PXRD) studies*

The PXRD patterns were recorded using Powder X-ray diffractometer (Rigaku™ model MiniFlex II, Japan) and operated at a voltage of 30 kV and a current of 15 mA. The samples were analyzed as the 2θ angle range of 3° to 40° and process parameters were set as follows: step size of 0.020° (2θ), and scan speed of 2° per minute.

*6.1.4 Fourier transform infra-red (FT-IR) spectroscopy*

The FTIR spectra of samples were measured on FT-IR spectrometer (Thermo Scientific$^{TM}$ model Nicolet iS10, USA) using Attenuated Total Reflectance (ATR) technique. The data was obtained in the range of 400-4500 $cm^{-1}$ for each sample. Analyses were performed at room temperature.

### *6.2 Solution state characterization*

*6.2.1 Sample preparations*

The pure compound of CCB, γ-CD, RM-β-CD and their complexes of CCB and CD i.e., CCB/γ-CD and CCB/RM-β-CD, which were prepared by freeze drying method as described in section 6.1.1, were characterized in solution state. The samples were prepared by dissolving in $CD_3OD$:$D_2O$ (50:50 v/v) in a capillary tube and then carried out by proton nuclear resonance spectroscopy measurements.

*6.2.2 Proton nuclear magnetic resonance ($^1$H-NMR) spectroscopy*

$^1$H-NMR spectroscopy measurements was using a 500 MHz $^1$H-NMR spectrometer (BRUKER$^{TM}$ model AVANCE III HD, USA). The spectrum and chemical shift values were recorded. The resonance at 4.6500 ppm due to residual solvent (OD) was used as reference (Ventura et al., 2005).

## 7. Formulation of celecoxib ophthalmic preparation

### *7.1 Celecoxib ophthalmic solution*

Celecoxib ophthalmic solution was prepared by dissolved 0.1% w/v of CCB in aqueous 7.5% w/v RM-β-CD solution. EDTA, BAC and polymer were then added and mixed. The composition of CCB eye drop solutions was given in Table 1. The obtained CCB solution was adjusted pH to 7.4 with sodium hydroxide and adjusted isotonicity with sodium chloride before adjusting to the desired volume with purified water. After that, all formulations were passed through heating process in an autoclave 121°C for 20 min and allowed to cool to room temperature.

*Table 1 Composition of CCB eye drop solutions*

| Ingredient [a] | Formulation (gm) [b] | | | | | |
|---|---|---|---|---|---|---|
| | F1 | F2 | F3 | F4 | F5 | F6 |
| Celecoxib | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| RM-β-CD | 7.5 | 7.5 | 7.5 | 7.5 | 7.5 | 7.5 |
| HPMC | - | - | - | 0.1 | 0.25 | 0.5 |
| HA | 0.1 | 0.25 | 0.5 | - | - | - |
| EDTA | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| BAC | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 |
| Purified water qs. to 100 ml | | | | | | |

[a] RM-β-CD; randomly methylated -β-cyclodextrin, HPMC; Hydroxypropyl methylcellulose, HA; Hyaluronic acid, EDTA; Ethylenediaminetetraacetic acid disodium salt, BAC; Benzalkonium chloride

[b] All formulation was adjusted pH to 7.4 with 1N sodium hydroxide solution and was adjusted with sodium chloride to obtain isotonicity.

### *7.2 Celecoxib ophthalmic suspension (binary complex)*

Celecoxib ophthalmic suspension was prepared by suspending 0.5% w/v of CCB in the aqueous CD solutions. EDTA and BAC were added into the obtained suspension and mixed. The compositions of each formulation are shown in Table 5. The particle size reduction of each sample was performed by mixer mill (RETSCH® MM400, Germany) with 1-mm zirconium bead as media mill, the vibration was set at 25 Hz and was operated for 30 minutes. The mucoadhesive polymers was then added and mixed until the polymer was dissolved. The CCB suspension was adjusted pH to 7.4 with sodium hydroxide and adjusted isotonicity with sodium chloride. Finally, the volume was filled-up with purified water.

*Table 2* Composition of CCB eye drop suspensions2

| Ingredient [a] | Formulation (gm) [b] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | F7 | F8 | F9 | F10 | F11 | F12 | F13 | F14 | F15 | F16 | F17 | F18 |
| Celecoxib | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| RM-β-CD | 7.5 | 7.5 | 7.5 | 7.5 | 7.5 | 7.5 | - | - | - | - | - | - |
| γ-CD | - | - | - | - | - | - | 10 | 10 | 10 | 10 | 10 | 10 |
| HPMC | - | - | - | 0.1 | 0.25 | 0.5 | - | - | - | 0.1 | 0.25 | 0.5 |
| HA | 0.1 | 0.25 | 0.5 | - | - | - | 0.1 | 0.25 | 0.5 | - | - | - |
| EDTA | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| BAC | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 |
| Purified water qs. to 100 ml | | | | | | | | | | | | |

[a] RM-β-CD; randomly methylated-β-cyclodextrin, γ-CD; γ-cyclodextrin, HPMC; Hydroxypropyl methylcellulose HA; Hyaluronic acid, Ethylenediaminetetraacetic acid disodium salt, BAC; Benzalkonium chloride

[b] All formulation was adjusted pH to 7.4 with 1N sodium hydroxide solution and was adjusted with sodium chloride to obtain isotonicity.

### *7.3 Effect of heating method on CCB eye drop suspensions (ternary complex)*

To investigate the effect of heating process to promote the ternary complex of CCB/CD/polymer, CCB eye drop suspensions from the section 7.2 were selected to further study. The CCB ophthalmic suspension was prepared following the procedure described in the section 7.2. Before the adjusting volume, the formulations were sonicated in an ultrasonic bath (GT sonic, China) at 70$^{o}$C for 1 hour.

## 8. Physicochemical and chemical characterizations of CCB eye drop formulations

### *8.1 pH determination*

The pH value of formulations was measured at room temperature with pH meter (METTLER TOLEDO$^{TM}$, SevenCompact, Germany). The equipment was calibrated at pH 4, 7 and 11 using Beckman standard buffer solutions before measurement.

### *8.2 Viscosity determination*

The viscosity measurement of each formulation was determined by using the viscometer (Brookfield LVDV-II+, USA). All measurement samples in triplicate were performed at a 25 $\pm$ 0.1$^{o}$C.

### *8.3 Osmolality measurement*

The osmolality of formulations was measured by Osmometer (Gonotec, OSMOMAT 3000 basic, Germany) at room temperature using freezing point depression principle. The instrument was calibrated with 0.9% sodium chloride solution before the measurement of sample. The clear formulation volume of 50 µl was measured as value for osmolality concentration in mOsmol/kg. Each sample was measured in triplicate.

### *8.4 Sedimentation volume determination*

For CCB (0.5% w/v) eye drop suspensions, the sedimentation volume (F) was measured during storage without agitation in 10-ml cylinder for a period of 10 days

and was recorded at interval time in terms of the ratio of the ultimate settled height (Hu) to the original height (Ho), in the following equation:

$$F = \frac{Hu}{Ho} \qquad \text{Eq.3}$$

***8.5 Re-dispersion time determination***

Celecoxib (0.5% w/v) ophthalmic suspensions were filled in 15-ml colorless glass vials and closured with rubber and aluminium caps. The time required for re-dispersion of the suspensions was measured after standing the vial in an upright position for 5 days. The container was rolled in a horizontal position using a mechanical shaker (Stuart Scientific, Mini Orbital Shaker SO5, UK) at 75 rpm. Re-dispersion time was defined as the required time to become a uniform suspension from the precipitated condition at the bottom of the container. The measurement was done in triplicate and the results are the mean values $\pm$ standard deviation (S.D.).

***8.6 The morphology, particle size and size distribution analysis***

The samples were centrifuged by High speed micro centrifuge (TOMY$^{TM}$, MX-305, Japan) at 3,500 rpm for 40 minutes to separate the supernatant and the solid particles portions. The supernatant of each formulation was performed by DLS as described in the section 5.1 to detect the particle size and size distribution of CCB-CD based aggregates. For solid particles portion, the particle size was investigated by optical microscopy (Nikon$^{TM}$ eclipse E200, Japan). Before the measurement, the ocular micrometer was calibrated with the stage micrometer. The magnification x 400 was used in all experiments. The sample was dropped on the glass slide covered with cover slip and measured the particle size about 300 particles. The mean of particle size values $\pm$ S.D. was recorded. The morphology of particles was observed by scanning electron microscope (SEM, JEOL$^{TM}$ JSM-6610LV, USA). The sample of solid particle portion was layered on the slide and dried overnight in desiccator at room temperature. And then, it was mounted on the stubs. The specimen stubs were coated with a thin layer of gold under argon atmosphere at room temperature. Finally, the samples were observed for their surface morphology by SEM.

### *8.7 Total drug content and dissolved drug content*

Total CCB content was determined by diluting 100-µl of sample with mixture of mobile phase, acetonitrile:water (55:45 v/v), and further diluted to appropriate CCB concentration. For amount of dissolved CCB content in suspension, the sample was centrifuged at 3,500 rpm for 40 minutes. The supernatant was further diluted with mobile phase and analyzed by HPLC which described in section 2. The measurement was done in triplicate. The percentage dissolved content calculated by the following Eq. 4.

$$\%\ \text{dissolved content} = \frac{[\text{Ds}]\text{x}\ 100}{[\text{Dt}]} \qquad \text{Eq.4}$$

Where [Ds] = drug content of supernatant

[Dt] = total drug content

## 9. *In-vitro* mucoadhesive studies

The mucoadhesive properties of the formulations were evaluated using equipment described by Bin Choy et al. (2008). This method was modified as follows. Before the measurement, semipermeable membrane (MWCO 12-14,000 Da) was immersed in an aqueous mucin solution (0.1% w/v mucin from porcine stomach, Type II) for 2 hr. Then, 50-µl of each formulation was dropped in the middle of the membrane. The membrane was rinsed with simulated tear fluid (STF) continuously at the flow rate of 1 ml/min for 1 min. Then the remained drug was washed from the membrane by immersing the membrane in volumetric flask containing the mobile phase. After shaking for 20 min, the solutions were filtered through 0.45 µm nylon filter membrane and the amount of CCB was finally determined by HPLC which described in the section 2. The CCB eye drop formulations without mucoadhesive polymer used as a control were also tested.

## 10. *In-vitro* permeation studies

### *10.1 In-vitro permeation through semipermeable membrane*

The *in-vitro* permeation of CCB formulation through semipermeable membrane (MWCO 12–14,000 Da) was performed by Franz diffusion cell apparatus consisting of a donor and a receptor compartment. The donor and receptor compartments were separated by the semipermeable membrane. The receptor phase consists of the phosphate buffer saline, pH 7.4 and 2.0% (w/v) γ-CD or RM-β-CD. CDs were added in the receptor phase to allow sink condition. Before the experiment, the receptor medium was sonicated to remove dissolved air and the membrane was soaked overnight in the receptor medium. For the experimental condition, the receptor phase was stirred continuously during the experiment at 150 rpm and controlled temperature at 34±0.5°C. The sample (1.5 ml) of each formulation was placed on the donor phase. A 400-µl aliquot of the receptor medium was withdrawn follow suitable time interval for analysis and replaced immediately by an equal volume of fresh receptor medium. The CCB content was determined by HPLC (described in section 2) and the amount of drug permeation was calculated. Each formulation was done in triplicate. The flux *(J)* was calculated from the linear part of each permeation profile. The steady state flux was calculated as the slope of linear plots of the amount of drug in the receptor chamber (*q*) versus time and the apparent permeation coefficient ($P_{app}$) determined from equation Eq.5:

$$J = \frac{dq}{A.dt} = P_{app}.Cd \qquad \text{Eq.5}$$

Where *A* is the surface area of the mounted membrane (2.27 $cm^2$) and *Cd* is the dissolved concentration of the drug in the donor chamber.

### *10.2 In-vitro permeation through simulated artificial vitreous humor*

The study of permeation through artificial vitreous humor model was represented the permeation through vitreous humor which aimed to deliver the drug to the posterior segment of eye i.e., retina and optic nerve. The artificial vitreous humor consisted of 0.25 %w/v HA and 0.2 %w/v agar (Kummer et al., 2007). The donor phase and receptor phase were separated by two-layered of semi-permeable membrane (MWCO 3,500 Da and MWCO 12–14,000 Da) and the artificial vitreous humor was filled inside the compartment. The procedure of this study was done followed by the section 10.1.

## Results and Discussion

### 1. Thermal stability of CCB on cycles of autoclaving

Since the phase solubility studies of CCB determined by heating method (Loftsson et al., 2005), the thermal stability of the CCB/CD complexes in the aqueous solution was determined. Table 3 shows the CCB content in aqueous solution containing 0.5% w/v β-CD after zero to three cycles of autoclaving. Each cycle consisted of heating to 121°C for 20 minutes. The amount of CCB in aqueous β-CD solutions in each cycle of autoclaving was not significantly different ($p<0.05$). This result exhibited CCB was relatively stable which no degradation was observed after three cycles of autoclaving. It was concluded that the CCB/CD complexes could be prepared by heating process. The thermal stability of CCB was confirmed by the study of Srinivasulu et al. (2012). The result was shown that there was no thermal degradation of pure CCB when kept it at the temperature of 105°C for 24 hrs.

*Table 3 CCB content in aqueous solution containing 0.5% w/v β-CD after zero to three cycles of autoclaving*[a]

| No. of autoclaving | Content of CCB (µg/ml) (Mean ± S.D.) |
|---|---|
| 0 cycle | 1.483 ± 0.038 |
| 1 cycle | 1.512 ± 0.068 |
| 2 cycle | 1.525 ± 0.047 |
| 3 cycle | 1.523 ± 0.051 |

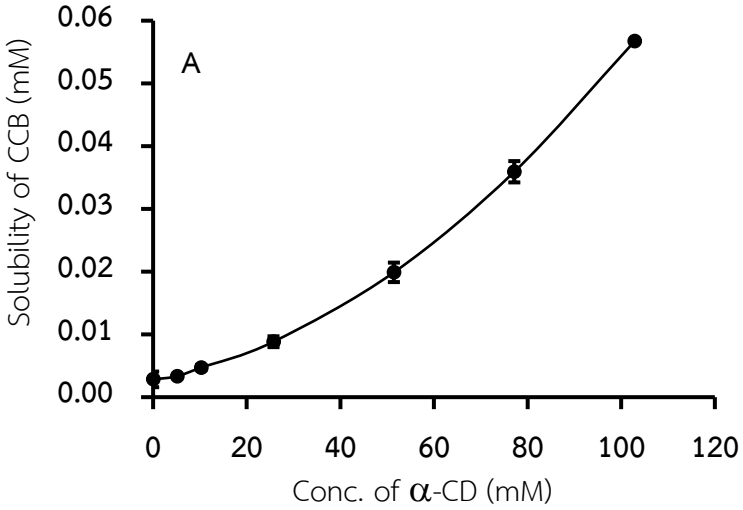
[a]each cycle consisted of 121°C for 20 minutes

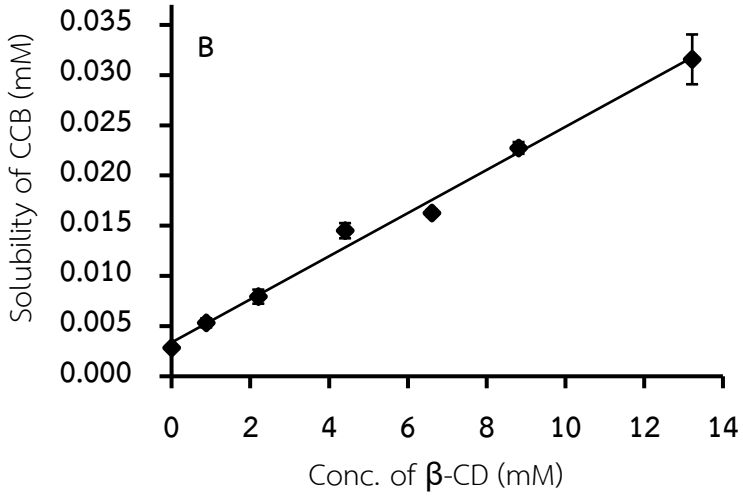
### 2. Phase solubility studies

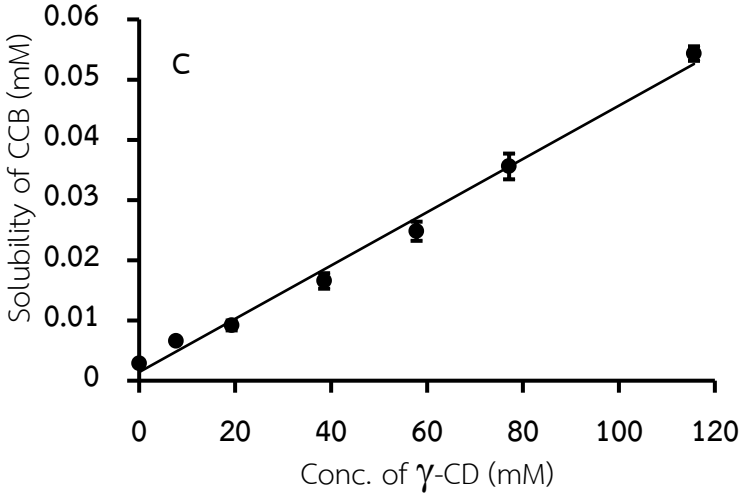
#### *2.1 The effect of CD on CCB solubilization*

Figure 1 displays the phase solubility diagrams of CCB in aqueous CD solutions i.e., α-CD, β-CD, γ-CD, HP-β-CD and RM-β-CD. In case of β-CD, γ-CD and HP-β-CD, they demonstrated that CCB solubility increased linearly with increasing CD concentration and represented an $A_L$-type diagram (Figure 1B, 1C and 1D). Whereas,

α-CD and RM-β-CD showed a positive deviation from linearity represented $A_p$-type profile (Figure 1A and 1E) (Higuchi et al., 1965).



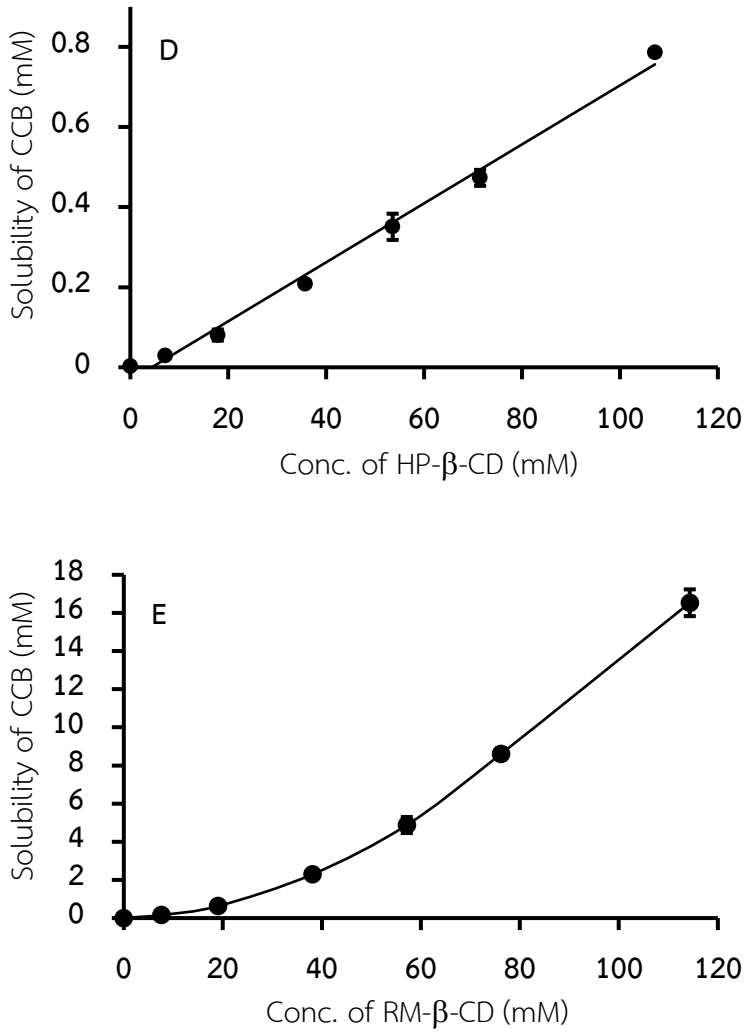



*Figure 1 Phase solubility profiles of celecoxib in aqueous cyclodextrin. $\alpha$-CD solutions (A), $\beta$-CD solutions (B), $\gamma$-CD solutions (C), HP-$\beta$-CD solutions (D) and RM-$\beta$-CD solutions (E).*

According to $A_L$-type phase solubility profile, this linear of drug and CD correlation with slope less than 1 suggested the formation of first-order soluble complex assumed that the increasing solubility of CCB as a result of the formation of a 1:1 molar ratio of CCB/CD inclusion complex (Rawat and Jain, 2004). Table 7 shows apparent stability constant values ($K_{1:1}$ and $K_{1:2}$) and the complexation efficiency (CE) of CCB/CD complexes in pure aqueous CD solutions. For $\alpha$-CD and RM-$\beta$-CD, they indicated that the formation of higher order soluble complexes at high CD concentration. Stability constants (K) for the two complexes i.e., CCB/CD and $CCB/CD_2$ were determined after constructing a plot by using the following Eq.2. They assumed that the 1:1 complex was formed more easily than 1:2 complex.

*Table 4 Apparent stability constant values ($K_{1:1}$ and $K_{1:2}$) and the complexation efficiency (CE) of celecoxib/cyclodextrin complexes in pure aqueous cyclodextrin solutions at 30℃ ± 1℃*

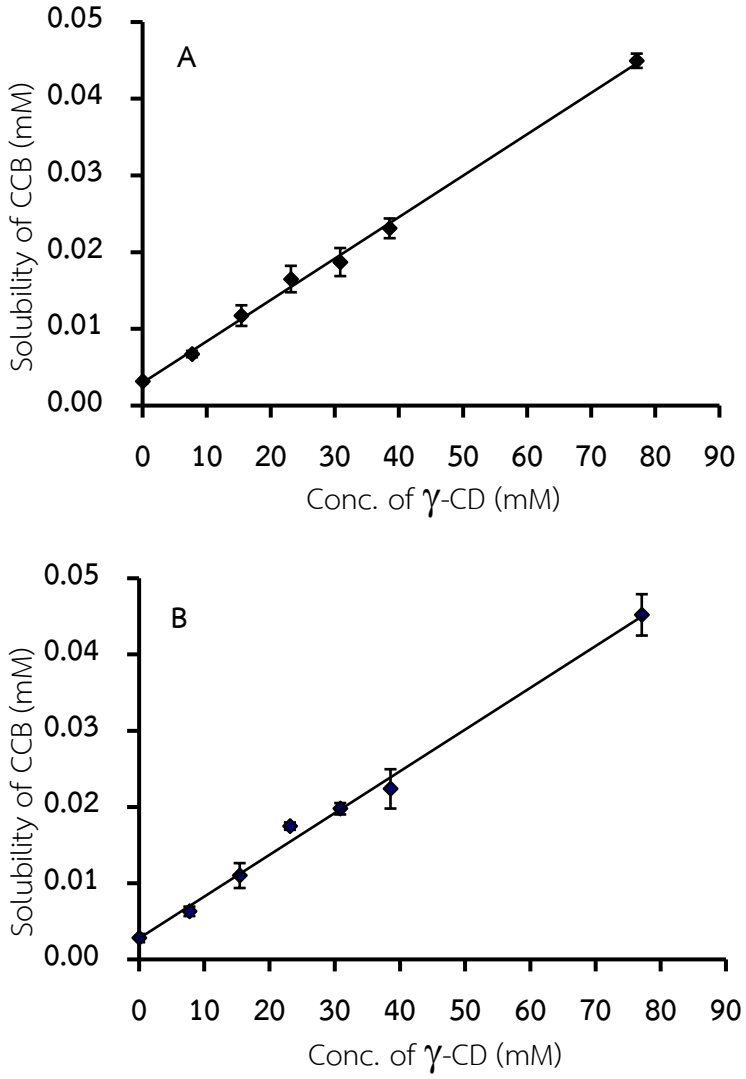
| Cyclodextrin | Type | $K_{1:1}$ ($M^{-1}$) | $K_{1:2}$ ($M^{-1}$) | CE |
|---|---|---|---|---|
| α-CD | $A_p$ | 49.9 | 26.2 | 0.00014 |
| β-CD | $A_L$ | 760.2 | - | 0.00215 |
| γ-CD | $A_L$ | 155.3 | - | 0.00044 |
| HP-β-CD | $A_L$ | 2,630.2 | - | 0.00746 |
| RM-β-CD | $A_p$ | 3,139.9 | 141.0 | 0.00890 |

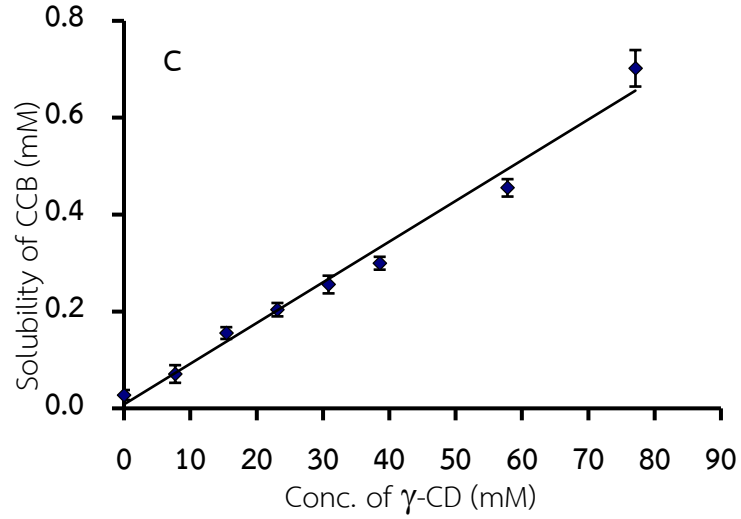
Ventura et al. (2005) investigated that the CCB/dimethyl-β-CD complex was also formed the same stoichiometry complex i.e., 1:1 and 1:2 complex. β-CD displayed the greatest CE among natural CDs. The literature review demonstrated that the cavity diameter of β-CD has been found to be most appropriate size for aromatic and heterocyclic molecules (Del Valle, 2004). However, its aqueous solubility is rather limited due to relatively strong binding of the CD molecules in the crystal state. Random substitution of the hydroxyl groups, even by hydrophobic moieties such as methoxy functions, will result in dramatic improvements in their solubility (Loftsson et al., 2005). This could support that HP-β-CD and RM-β-CD, β-CD derivatives, could enhance the CE when compared with their unmodified ones (Table 4). Among CDs tested, RM-β-CD showed the highest CE value which referred to the great CCB solubilization. However, the safety profiles of CD should be considered. It was reported that the ranking of CD toxicity in human corneal epithelial cell line (HCE) as following: α-CD > DM-β-CD > SBE-β-CD ≈HP-β-CD > γ-CD (Saarinen-Savolainen et al., 1998). γ-CD is well tolerated and possess favorable toxicological profiles but for RM-β-CD could be used at low concentration in topically aqueous eye drop formulation (Loftssona et al., 1999; Saokham and Loftsson, 2017). Therefore, regarding to the data of phase solubility profiles and safety profile aspects, RM-β-CD and γ-CD were selected to further study.

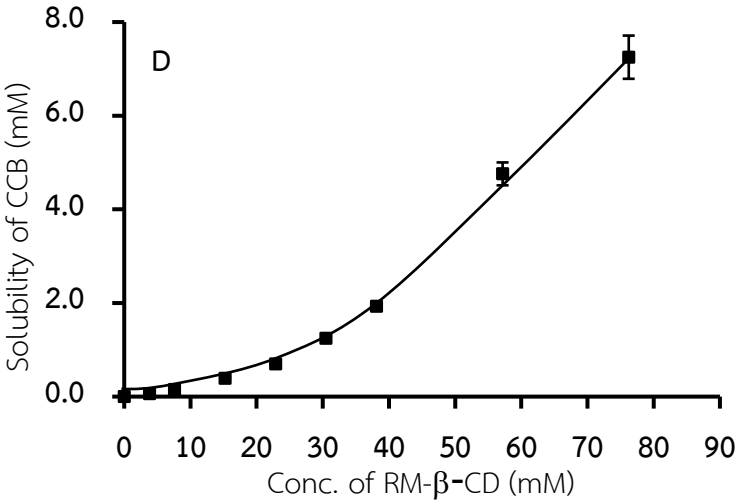
### *2.2 The effect of polymer on CCB/CD complex solubilization*

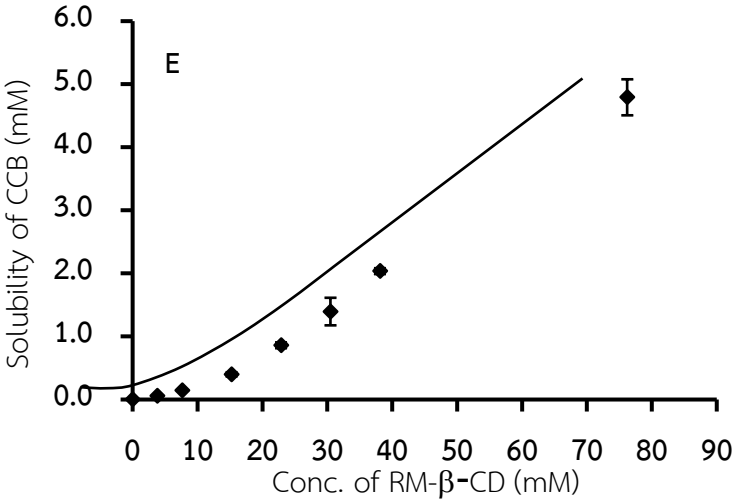
Water-soluble polymers can enhance the CD complexation of drugs and also they can increase the drug permeation through biological membranes (Sigurdardottir

and Loftsson, 1995). This synergistic effect is possible due to the formation of ternary complexes or co-complexes and their mucoadhesive enhancement (Jarho et al., 1998; Loftsson et al., 1999; Jansook et al., 2010). In this study, the effect of polymers i.e., HPMC, hyaluronic acid (HA) and chitosan (CS) (non-ionic, anionic and cationic polymer, respectively) on RM-β-CD and γ-CD solubilization of CCB was investigated. Figure 2 displays the effect of polymers on phase solubility profiles of CCB in aqueous CD solutions and Table 5 shows the apparent stability constant and CE of CCB/CD complexes in the present of polymer. The results exhibited that the addition of polymers in complexing medium did not alter the type of the phase solubility diagram. Thus, the presence of polymers did not change the stoichiometry of the complex formed.




C
Solubility of CCB (mM)
0.0
0.2
0.4
0.6
0.8
0 10 20 30 40 50 60 70 80 90
Conc. of γ-CD (mM)


D
Solubility of CCB (mM)
0.0
2.0
4.0
6.0
8.0
0 10 20 30 40 50 60 70 80 90
Conc. of RM-β-CD (mM)


E
Solubility of CCB (mM)
0.0
1.0
2.0
3.0
4.0
5.0
6.0
0 10 20 30 40 50 60 70 80 90
Conc. of RM-β-CD (mM)

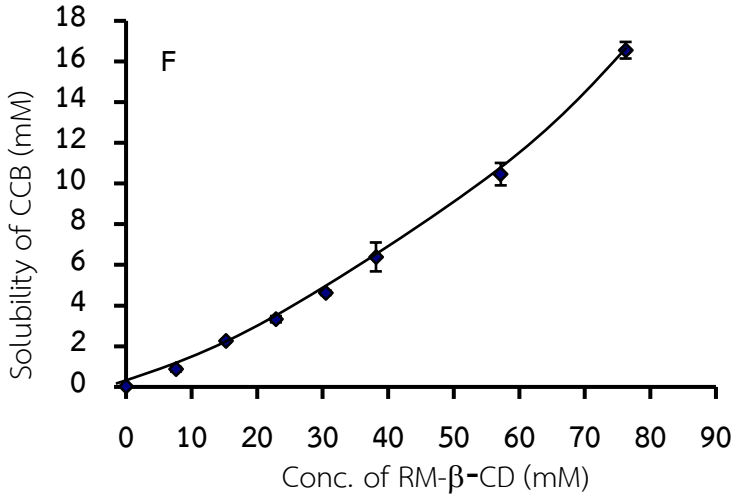



*Figure 2 Phase solubility profile of celecoxib in aqueous cyclodextrin containing polymer. γ-CD solutions containing 0.1% w/v chitosan (A), 0.01% w/v hyaluronic acid (B) and 0.1% w/v HPMC (C), and in aqueous RM-β-CD solutions containing 0.1% w/v chitosan (D), 0.01% w/v hyaluronic acid (E) and 0.1% w/v HPMC (F).*

*Table 5 Apparent stability constant values ($K_{1:1}$ and $K_{1:2}$) and the complexation efficiency (CE) of celecoxib/cyclodextrin complexes in pure aqueous cyclodextrin solutions in the presence of polymer at 30°C ±1°C*

| Cyclodextrin | Polymer | Type | $K_{1:1}$ ($M^{-1}$) | $K_{1:2}$ ($M^{1}$) | CE | CE ratio |
|---|---|---|---|---|---|---|
| **γ-CD** | - | $A_L$ | 155.3 | - | 0.00044 | 1.00 |
| | 0.1 % Chitosan | $A_L$ | 170.9 | - | 0.00048 | 1.10 |
| | 0.01 % HA | $A_L$ | 196.4 | - | 0.00056 | 1.26 |
| | 0.1 % HPMC | $A_L$ | 307.5 | - | 0.00108 | 2.46 |
| **RM-β-CD** | - | $A_p$ | 3,139.9 | 141.0 | 0.00890 | 1.00 |
| | 0.1 % Chitosan | $A_p$ | 2,846.1 | 136.4 | 0.00900 | 1.01 |
| | 0.01 % HA | $A_p$ | 3,532.6 | 119.8 | 0.00990 | 1.11 |
| | 0.1 % HPMC | $A_p$ | 3,564.4 | 18.3 | 0.09820 | 11.03 |

Form this results, the apparent stability constant (K) and CE of both binary CCB/RM-β-CD complex and CCB/γ-CD complex were increased when addition of the small amount of polymer due to the synergistic effect between these components (Loftsson et al., 1994). The effect of polymer on the CE of both CCB/γ-CD complex

and CCB/RM-β-CD complex showed the same trend as follows: HPMC > HA > CS (Table 5). Addition of chitosan to the complexation media has insignificantly effect on the CE of CCB, thus; it was excluded for further study. Interestingly, HPMC showed the highest increments in solubilizing efficiency (CE ratio) of CCB about 11-folds and 2.5-folds increasing in the CE of CCB/RM-β-CD and CCB/γ-CD, respectively. The dramatically increasing CE of CCB/RM-β-CD with HPMC as ternary complex may be possibly caused by the interaction of multi-components. The synergistic effect with polymer might occur of water-soluble polymers interaction with drug molecules through aggregate formation capable of solubilizing drugs. In addition, the polymers plays the important role in stabilizing micelles and other types of aggregates. These reduce the mobility of CD and change the hydration properties of CD molecules, result in enhancing the solubility of drug and their complexes (Loftsson et al., 2005). This observation was similar to the study of Loftsson et al. (2005). They demonstrated that HPMC and PVP can increase the CE as ternary complex of acetazolamide, carbamazepine methazolamide and pregnenolone when the present of HP-β-CD.

### 3. Morphology and aggregates particle size analysis

According to polymer and drug/CD complexes formed aggregates which was able to solubilize the water-insoluble drugs, the aggregated size of ternary complexes i.e., CCB/RM-β-CD/HA, CCB/RM-β-CD/HPMC, CCB/γ-CD/HA, and CCB/γ-CD/HPMC were investigated. Two different techniques were applied to determine aggregation of CCB/CD/polymer complexes, the dynamic light scattering (DLS) technique used to measure the self-assembled aggregates nanoparticles size which was assumed that they have spherical shape, whereas the microscopic methods, transmission electron microscopy (TEM), used to observe the morphology of their aggregates. From the results, the aggregates diameters of the ternary complexes were in the range of 250 - 350 nm (Table 6). As expected, the aggregate diameter of the ternary complexes consisted of CCB/CD containing HPMC were larger than those of CCB/CD with HA. TEM photographs of the ternary complexes are shown in Figure 3. The morphology of all samples was seemed to be spherical in shape and the complex aggregates size was in the same range obtained from the DLS techniques. This was supported that the addition of small amount of water-soluble polymer i.e., HA and HPMC to aqueous

CCB/CD complex solutions can enhance the CE of CD through complex aggregate formation.

*Table 6 The aggregate size and size distribution of CCB/CD/polymer ternary complex*

| Cyclodextrin (%w/v) | Polymer (% w/v) | Mean particle size (nm) |
|---|---|---|
| 10% γ-CD | 0.01% HA | 273 ± 36 |
| | | 1.86 ± 0.02 |
| | 0.1% HPMC | 308 ± 8 |
| | | 2.10 ± 0.14 |
| 7.5% RM-β-CD | 0.01% HA | 283 ± 36 |
| | | 2.05 ± 0.25 |
| | 0.1 %HPMC | 312 ± 29 |
| | | 2.08 ± 0.48 |

In general, in aqueous solutions the relative amount of aggregated CD increases with increasing CD concentration. From the literatures, the diameter of CD aggregates is most frequently between 90 and 300 nm (González-Gaitano et al., 2002). However, CD molecule has its own dynamic behavior resulted from rotational freedom of the glucopyranose unit of CD molecule and the overall molecular mobility. The stabilization of nano-particulate aggregates is regulated by Van der Waals forces and hydrogen bonds interactions, which are at all times in competition resulting in the aggregates are very unstable although the ability of CD to self-assemble to form reversible aggregates is well documented (Ryzhakov et al., 2016).

## 4. Preparation and characterization of binary CCB/CD complexes and ternary CCB/CD/polymer complexes

### *4.1 solid-state characterization*

*4.1.1 Differential scanning calorimetry (DSC)*

DSC thermograms of CCB, CDs, their physical mixture (PM) and their freeze dried (FD) samples are shown in Figure 4 and 5. The thermogram of pure CCB exhibited a sharp endothermic peak at 165.17ºC which represented the melting point of the drug (Figure 4A and 5A). Thermogram of γ-CD showed a very broad endothermic peak in the range of 112ºC (Figure 4B) due to elimination of water of

crystallization (Sinha et al., 2011) while the DSC thermograms of RM-β-CD showed no thermal change (Figure 5B).

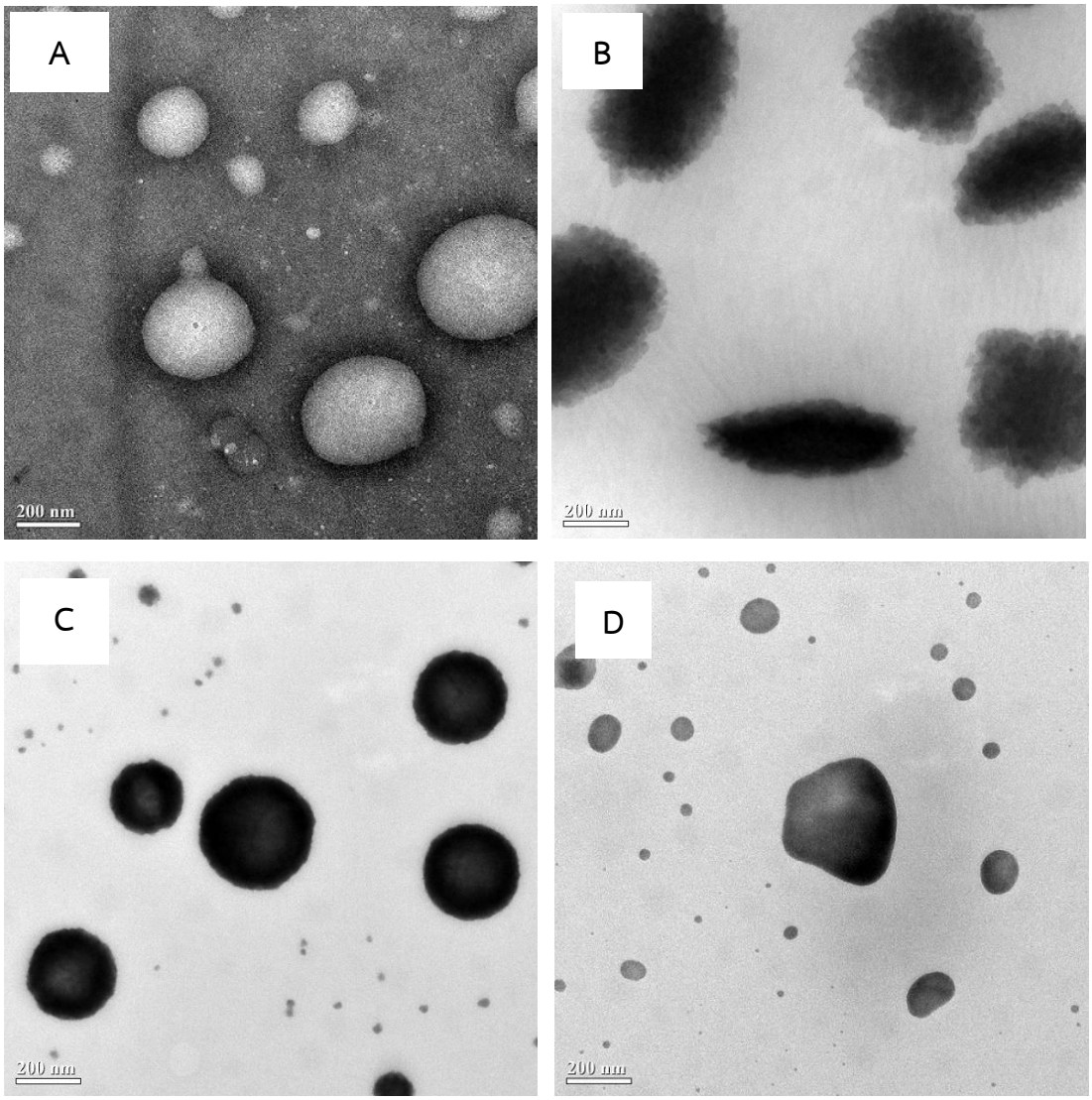



*Figure 3 TEM photographs of saturated solution of CCB in CD solution with polymer. (A) CCB/γ-CD/HA, (B) CCB/γ-CD/HPMC, (C) CCB/RM-β-CD/HA, and (D) CCB/RM-β-CD/HPMC.*

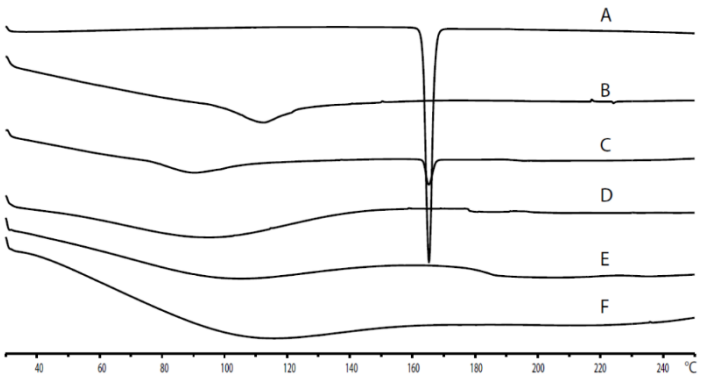



*Figure 1 DSC thermograms. (A) pure CCB, (B) pure γ-CD, (C) CCB/ physical mixture γ-CD, (D) freeze-dried CCB/γ-C, (E) freeze-dried CCB/γ-CD/HA, and (F) freeze-dried CCB/γ-CD/HPMC.*

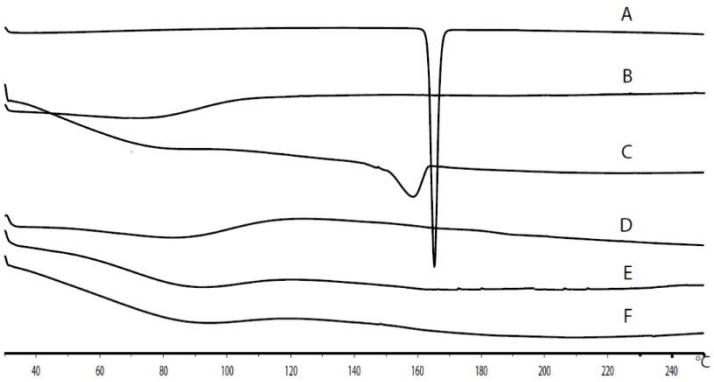



*Figure 5 DSC thermograms. (A) pure CCB, (B) pure RM-β-CD, (C) physical mixture CCB/RM-β-CD, (D) freeze-dried CCB/RM-β-CD, (E) freeze-dried CCB/RM-β-CD/HA, and (F) freeze-dried CCB/RM-β-CD/HPMC.*

In case of PM, the endothermic peak of the CCB in PM CCB/γ-CD was retained at 165.17ºC while the peak of the CCB in PM CCB/RM-β-CD was a broad peak and slightly shifted to lower temperature appeared at 158.67ºC. These observations may be attributed to the presence of weak or no interaction between the pure components in the PM and/or its possibility was an interaction between CCB and RM-β-CD promoted by the heating process in the DSC operation (Nagarsenker et al., 2005). For FD samples, the endothermic peak of binary complex (FD CCB/γ-CD and FD CCB/RM-β-CD) and ternary complex (FD CCB/γ-CD/HA, FD CCB/γ-CD/HPMC, FD

CCB/RM-β-CD/HA and FD CCB/RM-β-CD/HPMC) were absent (Figure 4 and 5). The disappearance of an endothermic peak may be attributed to an amorphous state and/or the inclusion complex between CCB and CD in the solid state (Cappello et al., 2007).

*4.1.2 Powder X-ray diffractometry (PXRD)*

PXRD is used to measure the crystallinity of the formed complexes and the peak position (angle of diffraction) is an indication of a crystal structure. The PXRD spectra of pure CCB, γ-CD, RM-β-CD, their PM, and FD of binary complex and ternary complex are presented in Figure 6 and 7.

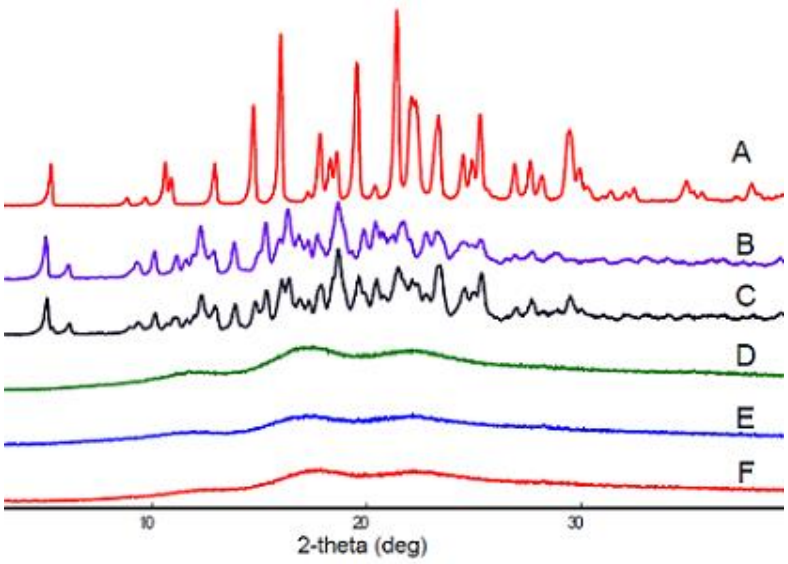



*Figure 6 The PXRD spectra. (A) pure CCB, (B) pure γ-CD, (C) CCB/ physical mixture γ-CD, (D) freeze-dried CCB/γ-C, (E) freeze-dried CCB/γ-CD/HA, and (F) freeze-dried CCB/γ-CD/HPMC.*

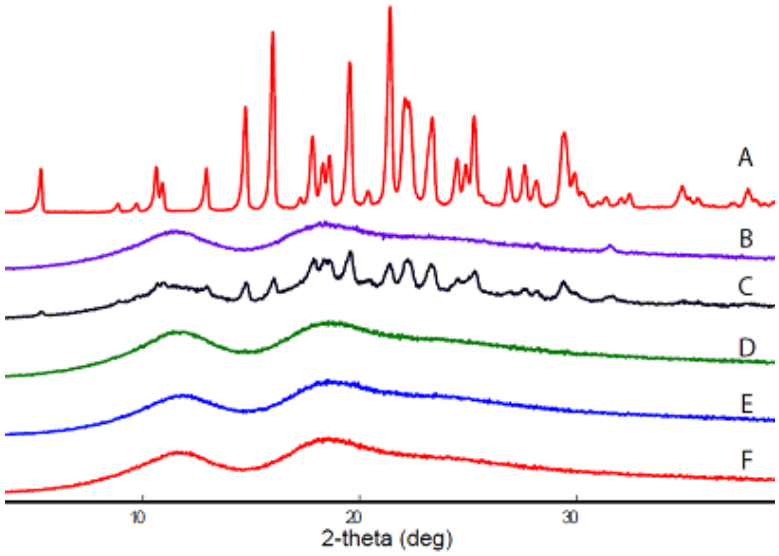



*Figure 7 The PXRD spectra. (A) pure CCB, (B) pure RM-β-CD, (C) physical mixture CCB/RM-β-CD, (D) freeze-dried CCB/RM-β-CD, (E) freeze-dried CCB/RM-β-CD/HA, and (F) freeze-dried CCB/RM-β-CD/HPMC.*

The diffractogram of CCB exhibited a series of intense peaks at 5.3, 10.6, 10.9, 12.9, 14.7, 16.0, 17.8, 18.3, 18.6, 19.5, 20.4, 21.4, 22.1, 23.3, 24.5, 24.9, 25.3, 26.9, 27.7 28.2 and 29.4, which were indicated of their crystallinity (Figure 6A and 7A). The γ-CD exhibited characteristic peaks at 5.0, 10.1, 11.1, 12.2, 13.8, 15.3, 15.9, 16.3, 16.8, 18.7, 20.3, 21.6 22.8 and 23.3 due to its crystalline nature (Figure 6B) while RM-β-CD did not show any peak and exhibited amorphous state (Figure 7B). Most of the principal peaks of CCB were presented in the diffraction patterns of PM of both CCB/γ-CD and CCB/RM-β-CD (Figure 6C and 7C). This indicated that there was no interaction between the pure CCB and respective CDs. In contrast to FD samples of CCB/CD binary complex (FD CCB/RM-β-CD and FD CCB/γ-CD) and ternary complex (CCB/γ-CD/HA, FD CCB/γ-CD/HPMC, FD CCB/RM-β-CD/HA, and FD CCB/RM-β-CD/HPMC), they showed a halo pattern with the disappearance of all the peaks corresponding to CCB. It was demonstrated the transformation of CCB from the crystalline to the amorphous form by possibly formation of inclusion complex with CD (Sinha et al., 2005). However, freeze drying technique preparation may affect to transformation of in solid state when the solvent was completely removed through sublimation which could not be negligible (Einfal, Planinsek, and Hrovat, 2013).

*4.1.3 Fourier transform infra-red (FT-IR) spectroscopy*

Figure 8 and Figure 9 show the FT-IR spectra of pure CCB, γ-CD, RM-β-CD, their PM and FD samples. The FT-IR spectra of CCB are shown in Figure 8A and 9A. Its characteristic peaks at 3,332.4 and 3,229.9 $cm^{-1}$ attributed to N-H stretching vibration of $SO_2NH_2$ group, 1,346.5 and 1,158.2 $cm^{-1}$ for the S=O asymmetric and symmetric stretching and 1,228.7 for C-F stretching. The FT-IR spectrum of γ-CD and RM-β-CD showed a broad absorption band at 3383 $cm^{-1}$ due to -OH stretching and displayed a large band and distinct peak in the region of 1200-900 $cm^{-1}$ (Homayouni et al., 2015). The FT-IR spectrum of CCB in PM sample showed the double peaks of N-H stretching that were slightly shifted to 3330.9 $cm^{-1}$ and 3229.4 $cm^{-1}$ for PM CCB/γ-CD (Figure 8C) and 3336.6 $cm^{-1}$and 3232 $cm^{-1}$ for PM CCB/RM-β-CD (Figure 9C). The S=O stretching vibration was slightly shift to 1347.6 $cm^{-1}$ and 1160.7 $cm^{-1}$ for PM CCB/RM-β-CD while no shift of that in case of CCB/γ-CD. These observations indicated that there was less interaction between CCB and CD in the PM samples.

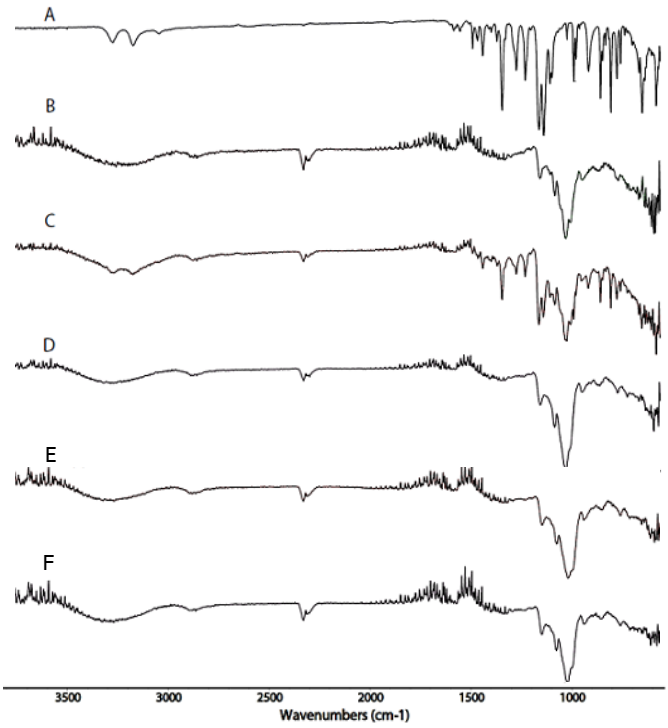



*Figure 2 FT-IR spectra. (A) pure CCB, (B) pure γ-CD, (C) CCB/ physical mixture γ-CD, (D) freeze-dried CCB/γ-C, (E) freeze-dried CCB/γ-CD/HA, and (F) freeze-dried CCB/γ-CD/HPMC.*

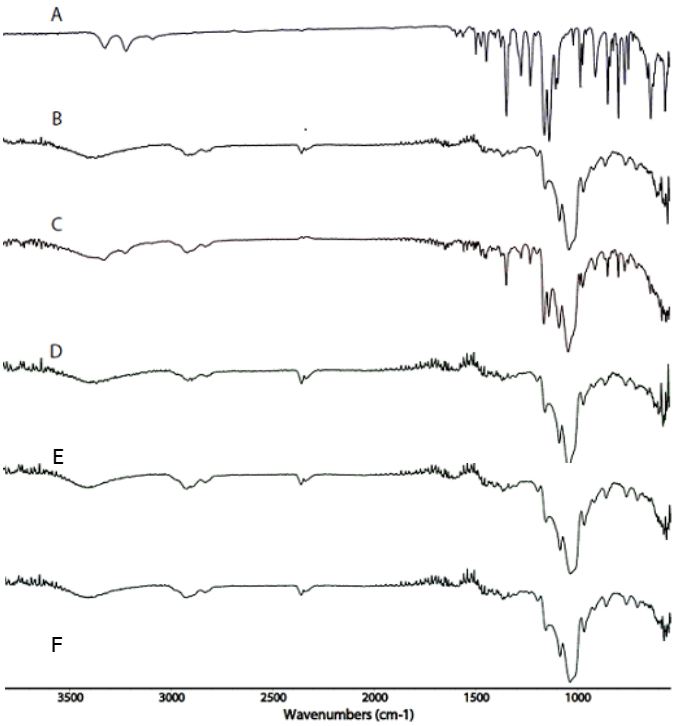



*Figure 9 FT-IR spectra. (A) pure CCB, (B) pure RM-β-CD, (C) physical mixture CCB/RM-β-CD, (D) freeze-dried CCB/RM-β-CD, (E) freeze-dried CCB/RM-β-CD/HA, and (F) freeze-dried CCB/RM-β-CD/HPMC.*

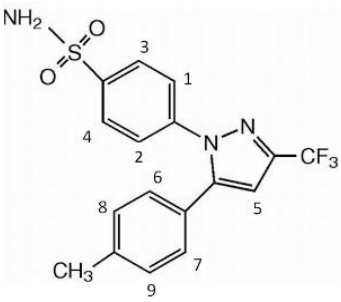
For FD sample of both binary complex and ternary complex, the N-H stretching bands of CCB indicated the masking of characteristic symmetric and asymmetric stretch. Likewise, the C-F stretching band and S=O stretching bands of CCB disappeared in FD samples. These results may be ascribed to the existence of some interaction between functional group (sulfonamide group or -$CF_3$ group) of CCB and functional group in the hydrophobic cavities of CD during inclusion complexes (Sinha et al., 2011).

### *1.2 Solution-state characterization*

Proton nuclear magnetic resonance spectroscopy ($^1$H-NMR) studies provide information of the existence of CCB/CD inclusion complexes and suggest the conformation of guest molecules into the CD cavity. The changes in $^1$H-chemical shifts ($\Delta\delta$) observed for the H5 proton of CCB in the presence of RM-β-CD and γ-CD were 0.045 and 0.043, respectively and displayed significant upfield (Table 7). This behavior

can be associated that the H5 proton of CCB locates close to the oxygen atoms in the CD cavity which is rich in $\pi$ electrons (Ganza-Gonzalez et al., 1994). And also, all aromatic protons of CCB that the presence of γ-CD showed a significant upfield shift. Whereas, all aromatic protons of CCB that solubilized in RM-β-CD displayed downfield shifts demonstrating weaker interactions i.e., Van der Waals forces between CCB and the hydrogen atoms of CD (Ventura et al., 2005).

*Table 7 The $^1$H-chemical shifts of CCB alone and in the presence of individual γ-CD and RM-β-CD.*



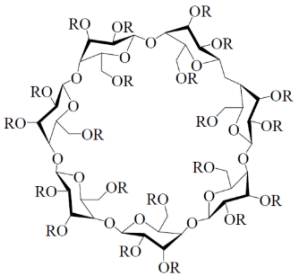

Celecoxib

| Protons | Celecoxib | CCB/γ-CD | Δδ* | CCB/RM-β CD | Δδ* |
|---|---|---|---|---|---|
| -$CH_3$ | 2.206 | 2.183 | -0.023 | 2.217 | 0.011 |
| *H5 | 6.877 | 6.834 | -0.043 | 6.832 | -0.045 |
| H6,H7,H8,H9 | 7.062 | 7.035 | -0.027 | 7.076 | 0.014 |
| H1,H2 | 7.378 | 7.345 | -0.034 | 7.390 | 0.011 |
| H3,H4 | 7.823 | 7.801 | -0.022 | 7.838 | 0.015 |

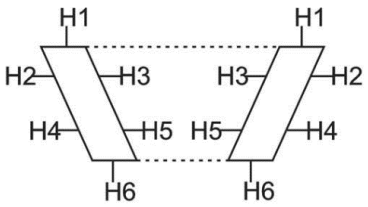
$\Delta\delta^* = \delta_{complex} - \delta_{free}$

$^1$H-NMR chemical shift of RM-β-CD is summarized in Table 8. The H3 and H5 proton of glucose units are facing to the interior of the lipophilic CD cavity. The changes in (Δδ) were generally observed for the upfield of H3 and H5 which is characteristic of the formation of an inclusion complex. As the result, the changes in Δδ of RM-β-CD in the presence of CCB for H3 and H5 proton were 0.092 and 0.042 indicating a significant upfield shifts. In this case, the Δδ* of H3 proton was higher than that of H5 proton. It indicated that RM-β-CD formed partial inclusion of CCB (Greatbanks and Pickford, 1987). In addition, the Δδ* for H1 proton of RM-β-CD was 0.097 displayed significant upfield. This result was agreed with the data obtained by Ventura et al. (2005).

*Table 8 The [1]H-chemical shifts of RM-β-CD alone and in the presence of CCB.*



R= H or $-CH_3$




| Protons | RM-β-CD | CCB/RM-β-CD | Δδ* |
|---|---|---|---|
| H1 | 4.941 | 4.844 | -0.097 |
| H2 | - | - | - |
| *H3 | 3.785 | 3.693 | -0.092 |
| H4 | 3.536 | 3.518 | -0.018 |
| *H5 | 3.577 | 3.536 | -0.042 |
| H6 | - | - | - |
| $CH_3OC2,3$ | 3.438 | 3.451 | 0.013 |
| $CH_3OC6$ | 3.269 | 3.259 | -0.010 |

$\Delta\delta^* = \delta_{complex} - \delta_{free}$

*Table 9 The [1]H-chemical shifts of γ-CD alone and in the presence of CCB.*

| Protons | γ-CD | CCB/γ-CD | Δδ* |
|---|---|---|---|
| **H1** | 4.991 | 4.994 | 0.003 |
| **H2** | 3.523 | 3.499 | -0.024 |
| ***H3** | 3.815 | 3.840 | 0.025 |
| **H4** | 3.469 | 3.480 | 0.011 |
| ***H5** | 3.751 | 3.821 | 0.070 |
| **H6** | 3.732 | 3.715 | -0.017 |

$\Delta\delta^* = \delta_{complex} - \delta_{free}$

The ($\Delta\delta^*$) of γ-CD in the presence of CCB for H3 and H5 proton were 0.025 and 0.070, respectively (Table 9). From the results, the $\Delta\delta^*$ value of H-5 proton > H3 proton

and displayed significant downfield, which indicated that the totally drug molecule inserted inside the hydrophobic cavity of γ-CD.

The proposed conformation structure of CCB/RM-β-CD and CCB/γ-CD inclusion complex are shown in Figure 10. The CCB/RM-β-CD was possibly divided in 2 configuration that were 1:1 inclusion complex and 1:2 inclusion complex. Figure 10A shows the 1:1 inclusion complex; the pyrazole head group of CCB was included through the wide rim of RM-β-CD. Figure 10B shows the 1:2 inclusion complex; the CCB was included into the cavity of the CD dimer in a configuration in which half of CCB molecule was embedded in one monomer and the other half is embedded in the other monomer of CD. It was held in position due to the formation of hydrogen bonds between the hydroxyl groups or methoxy groups of the CD and the fluorine and nitrogen atoms of the CCB. Consequently, significant upfield H1 proton of RM-β-CD may be caused by interaction between hydrogen (H1) at wide rim of CD and CCB molecule during 1:2 inclusion complex formation. It corresponded to the results of phase solubility-profile study that CCB/RM-β-CD displayed $A_P$-type diagram (1:1 and 1:2 complex) (Higuchi et al., 1965). This proposed conformation was supported by the CCB/β-CD complex characterization of Reddy et al. (2004).

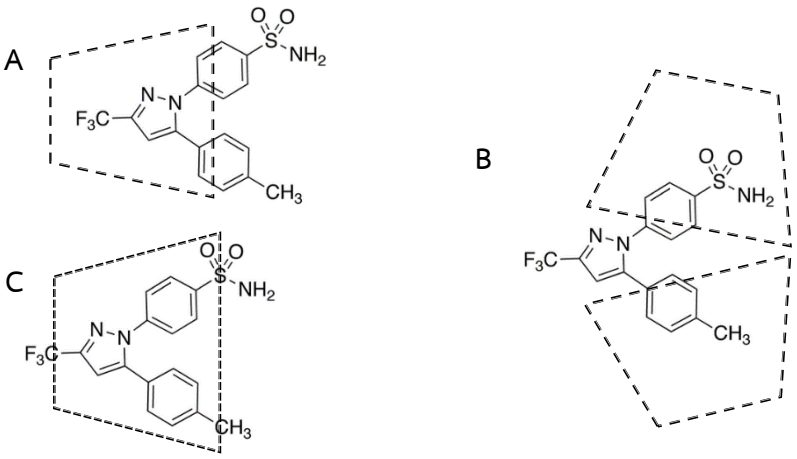



*Figure 10 The proposed conformation of (A) 1:1 CCB/RM-β-CD complex, (B) 1:2 CCB/RM-β-CD complex and (C) 1:1 CCB/γ-CD complex.*

And also, the proposed conformation of CCB/γ-CD is shown in Figure 10C. Due to Δδ* of H5 in both CCB and γ-CD were significantly shift including all aromatic protons were

upfiled shift. It was suggested that CCB was more deeply included into γ-CD cavity than of RM-β-CD cavity. For this reason, it might be due to the inner cavity diameter of γ-CD (7.5-8.3 A$^o$) is bigger than RM-β-CD (6 A$^o$).

## 5. Formulation of celecoxib ophthalmic preparation

### *5.1 Physicochemical and chemical characterizations of formulations*

*Physicochemical characterizations*

The CCB eye drop formulations (F1-F6) showed a clear viscous solution while F7-F18 displayed a milky-white suspension. The pH value, osmolality, viscosity, sedimentation volume and re-dispersion time of each CCB eye drop formulation are given in Table 10 and Table 11.

*Table 10 pH value, osmolality and viscosity of CCB eye drop formulations*

| Formulation | pH | Osmolality (mOsm/Kg) | Viscosity (mPa.s) |
|---|---|---|---|
| F1 | 7.37 ± 0.07 | 291.7 ± 9.3 | 2.43 ± 0.15 |
| F2 | 7.34 ± 0.01 | 305.3 ± 18.8 | 4.90 ± 0.40 |
| F3 | 7.32 ± 0.01 | 300.7 ± 4.7 | 9.03 ± 0.56 |
| F4 | 7.38 ± 0.06 | 294.0 ± 8.2 | 2.66 ± 0.08 |
| F5 | 7.40 ± 0.09 | 297.0 ± 12.5 | 8.43 ± 0.47 |
| F6 | 7.36 ± 0.04 | 308.0 ± 14.1 | 14.67 ± 0.57 |
| F7 | 7.34 ± 0.03 | 296.0 ± 11.5 | 2.72 ± 0.02 |
| F8 | 7.37 ± 0.08 | 285.3 ± 4.9 | 8.83 ± 0.40 |
| F9 | 7.39 ± 0.06 | 292.7 ± 7.8 | 15.67 ± 0.80 |
| F10 | 7.31 ± 0.01 | 297.3 ± 7.1 | 2.47 ± 0.04 |
| F11 | 7.35 ± 0.01 | 291.0 ± 4.4 | 7.57 ± 0.75 |
| F12 | 7.43 ± 0.10 | 287.0 ± 1.0 | 15.13 ± 0.50 |
| F13 | 7.38 ± 0.08 | 290.0 ± 7.5 | 2.36 ± 0.06 |
| F14 | 7.35 ± 0.05 | 292.3 ± 11.2 | 8.20 ± 0.44 |
| F15 | 7.43 ± 0.07 | 306.3 ± 13.9 | 16.87 ± 0.70 |
| F16 | 7.41 ± 0.09 | 284.7 ± 3.1 | 2.58 ± 0.23 |
| F17 | 7.36 ± 0.06 | 295.7 ± 2.5 | 6.90 ± 0.66 |
| F18 | 7.37 ± 0.08 | 295.0 ± 3.6 | 15.13 ± 0.58 |

pH values of all formulations were in the range of 7.3-7.5. The pH value between 7 and 10 are tolerated by the eye without marked discomfort and values of pH less than 6 and above 11 always cause irritations (Kramer, Haber, and Duis, 2002). However, the pH stability of drug in formulation should be considered to avoid precipitation of the drug or its rapid degradation. Srinivasulu et al. (2012) demonstrated that CCB at the pH 7.4 had good stability and no degradation peak was observed by HPLC analysis.

Ideally, an ophthalmic eye drop should have the tonicity value closed to the lacrimal fluid that corresponding to a 0.9% sodium chloride solution (287 mOsm/kg). However, the eye can tolerate a broad range of tonicity values from 0.6 to 2% of sodium chloride solution. Hypertonic eye drop are better tolerated than hypotonic eye drops (Kramer et al., 2002). The osmolalities of all formulations ranged from 280 mOsm/kg to 324 mOsm/kg (Table 10) which were within the acceptable range (260-330 mOsm/kg).

The viscosity of CCB eye drop formulations at 25$^{o}$C are displayed in Table 10. The viscosity inducing agents i.e., HPMC and HA was individual added to the aqueous ophthalmic solution and suspension to increase the viscosity for prolong contact of the drug with the eye tissues. The viscosities of formulations were between 2.36 $\pm$ 0.06 mPa.s and 16.87 $\pm$ 0.70 mPa.s. The viscosity increased with increasing the concentration of polymer. CCB eye drop suspension containing 0.5% w/v HA (F15) had the highest viscosity. However, heating method by autoclaving affected the viscosity of formulation (F1-F3). The viscosity decline from initial might be due to possible degradation of HA after exposure to temperature > 90$^{o}$C for about 1 hour (Lowry and Beavers, 1994). The viscosity for ophthalmic preparation is considered optimal in the range of 15–25 mPa.s (Aldrich et al., 2013). The maximum viscosity to increase contact time should not more than 20 mPa.s. The high viscous formulations usually leave a residue on the eyelid (Kramer et al., 2002).

The good ophthalmic eye drop suspensions are uniform dispersions of solid drug particles in a vehicle. The dispersed particles in suspension should settle slowly and ease to resuspend. The easily re-dispersion of the sediment portion in a suspension is important for the uniformity of dose. A cake formation on setting is not

allowed in appropriate eye drop suspension. In this study, the re-dispersion time and sedimentation volume (F) were used to evaluate the physical characteristics of ophthalmic eye drop suspensions and summarized in Table 11.

*Table 11 The sedimentation volume (F) at 3, 5, 10 days and the re-dispersion time at day 5 measurement of celecoxib eye drop suspensions*

| Formulation | Sedimentation volume (F) | | | Re-dispersion time (s) |
|---|---|---|---|---|
| | Day 3 | Day 5 | Day 10 | |
| F7 | 0.06 ± 0.02 | 0.06 ± 0.02 | 0.06 ± 0.02 | 3.8 ± 0.4 |
| F8 | 0.07 ± 0.01 | 0.07 ± 0.01 | 0.07 ± 0.01 | 11.0 ± 0.7 |
| F9 | 0.09 ± 0.01 | 0.08 ± 0.02 | 0.08 ± 0.02 | 22.8 ± 1.7 |
| F10 | 0.36 ± 0.04 | 0.03 ± 0.01 | 0.03 ± 0.01 | 157.0 ± 11.5 |
| F11 | 0.53 ± 0.03 | 0.05 ± 0.01 | 0.05 ± 0.01 | 406.3 ± 32.7 |
| F12 | 0.90 ± 0.03 | 0.62 ± 0.02 | 0.62 ± 0.02 | 610.7 ± 40.3 |
| F13 | 0.07 ± 0.01 | 0.07 ± 0.01 | 0.07 ± 0.01 | 4.0 ± 1.0 |
| F14 | 0.08 ± 0.02 | 0.08 ± 0.02 | 0.08 ± 0.02 | 7.7 ± 0.6 |
| F15 | 0.08 ± 0.02 | 0.08 ± 0.02 | 0.08 ± 0.02 | 14.3 ± 1.5 |
| F16 | 0.07 ± 0.01 | 0.07 ± 0.01 | 0.07 ± 0.01 | 16.0 ± 1.0 |
| F17 | 0.09 ± 0.01 | 0.09 ± 0.01 | 0.09 ± 0.01 | 14.3 ± 1.5 |
| F18 | 0.10 ± 0.01 | 0.10 ± 0.02 | 0.10 ± 0.02 | 52.3 ± 9.0 |

Form the result, the height of sediment for each suspension was ultimately settled after day 5. F values of F7-F9 and F13-F15 containing HA after storage for a period of 5 days were similar. The sedimentation were rapidly formed, the supernatant were clear and the sediment were easy to re-disperse possibly indicated that they were flocculated suspensions. The flocculated suspensions showed rapid sedimentation creating loose aggregates and formed a network-like structure (Nutan and Reddy, 2010). Whereas, the formulation of F10-F12 and F16-F18 containing HPMC were slowly formed and the supernatant were cloudy. Among the formulations have been performed, F10-F12 formulation spent more time for re-dispersing to become a uniform suspension, especially F12 (Table 11). This phenomenon was called cake formation as shown in Figure 11. Since the sediment is very closely packed

and a hard cake was formed, It indicated that they were deflocculated suspension (Nutan et al., 2010). The re-dispersion time became exponentially longer when % of polymer increased in the suspension. Yasueda et al. (2004) studied the re-dispersability of 0.05% w/v fluorometholone suspensions containing HPMC at the concentration 0.001-0.01 %w/v. It was found that the formulation could re-disperse to uniform suspension. However, the increasing HPMC up to 0.5% w/v, the re-dispersion time became longer and displayed poor re-dispersability. Cake formation is the most serious of all the physical stability problems encountered in suspension. Thus, the formulation of F10, F11 and F12 containing RM-β-CD and HPMC were excluded for further study.

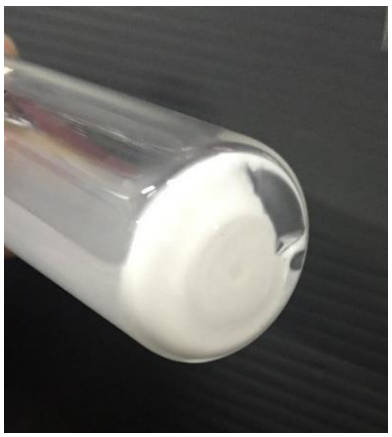


*Figure 11 The appearance of cake formation of F12 after storage at room temperature for 5 days.*

*Chemical characterization*

Table 12 exhibited total CCB content and % dissolved CCB in solution of CCB eye formulation. Regarding to the earlier result, RM-β-CD was more powerful solubilizer than γ-CD. As expected, the dissolved content of the CCB in eye drop formulation containing RM-β-CD (F7-F12) was higher than those of formulations containing γ-CD (F13-F18). On the other hand, the solid drug content (free CCB and solid CCB/CD complex) in formulations containing RM-β-CD obtained about 85-90% while more than 99% were observed in case of formulation containing γ-CD. Again, heating through autoclaving (121$^{o}$C, 20 min) in CCB eye drop solution (F1-F6) lead to accelerate CCB/RM-β-CD complex to completely dissolve. Whereas, unheated

formulations (F7-F12) corresponding to those formulations, 50-70% dissolved drug were existed.

*Table 12 Total drug content and drug dissolved content of celecoxib eye drop formulations*

| Formulation | Total drug content (mg/ml) | Drug dissolved content (µg/ml) | % Total drug content | % dissolved content |
|---|---|---|---|---|
| F1 | 1.052 ± 0.014 | - | 105.3 ± 1.4 | - |
| F2 | 1.056 ± 0.050 | - | 105.7 ± 5.0 | - |
| F3 | 1.059 ± 0.074 | - | 105.9 ± 7.5 | - |
| F4 | 1.061 ± 0.068 | - | 106.2 ± 6.7 | - |
| F5 | 0.993 ± 0.044 | - | 99.4 ± 4.4 | - |
| F6 | 1.052 ± 0.035 | - | 105.3 ± 3.6 | - |
| F7 | 4.687 ± 0.221 | 558.2 ± 18.6 | 93.8 ± 4.4 | 11.16 ± 0.37 |
| F8 | 4.740 ± 0.040 | 497.2 ± 23.3 | 94.8 ± 0.8 | 9.94 ± 0.47 |
| F9 | 4.975 ± 0.113 | 509.0 ± 8.3 | 99.5 ± 2.3 | 10.18 ± 0.17 |
| F10 | 4.856 ± 0.222 | 739.6 ± 45.9 | 97.1 ± 4.5 | 14.8 ± 0.92 |
| F11 | 4.951 ± 0.216 | 707.9 ± 54.4 | 99.0 ± 4.3 | 14.16 ± 1.09 |
| F12 | 4.534 + 0.024 | 632.8 ± 5.8 | 90.7 ± 0.5 | 12.66 ± 0.12 |
| F13 | 4.808 ± 0.139 | 35.3 ± 5.6 | 96.2 ± 2.8 | 0.71 ± 0.12 |
| F14 | 4.677 ± 0.033 | 28.0 ± 6.5 | 93.5 ± 0.7 | 0.56 ± 0.13 |
| F15 | 4.715 ± 0.099 | 33.7 ± 8.0 | 94.3 ± 2.0 | 0.67 ± 0.16 |
| F16 | 4.724 ± 0.189 | 24.7 ± 3.6 | 94.5 ± 3.8 | 0.49 ± 0.07 |
| F17 | 4.848 ± 0.082 | 24.4 ± 2.6 | 97.0 ± 1.7 | 0.49 ± 0.05 |
| F18 | 4.660 ± 0.178 | 23.3 ± 1.8 | 93.2 ± 3.6 | 0.47 ± 0.01 |

### *5.2 In-vitro drug permeation studies*

The effect of CDs and polymer of CCB eye drop formulations on *in-vitro* permeation through a semipermeable membrane (MWCO 12–14,000 Da) were further investigated**.** Since CCB is categorized into BCS Class II which high permeability but poor solubility, the solubility of drug limited ocular bioavailability. The experiment of permeation study would be investigated by the flux pattern that received for membranes with molecular weight cut-off (MWCO 12-14,000 Da) well over the

molecular weight of CDs. Thus, both the drug and drug/CD complexes (excepting their aggregates) were able to permeate the semipermeable membranes into the receptor medium represented to permeate through an aqueous diffusion barrier at the surface of a membranes (Loftsson et al., 2002). Although the complex did not penetrate the biological membrane of eye after diffused across an aqueous diffusion barrier, the drug in the complex was in rapid dynamic equilibrium with the free drug, and increased the drug concentration gradient over the membrane which leaded to increase ocular bioavailability.

Figure 12 and Figure 13 display the permeation flux (J) and apparent permeation coefficient ($P_{app}$) determined from the steady state region of the permeation profiles. The flux values of 0.1% CCB ophthalmic solutions (F1-F6) were superior to those of suspensions (F7-F9) because of their completely dissolved CCB. The flux of CCB containing RM-β-CD formulations (F1-F9) was distinctly greater than γ-CD formulation (F13-F18) due to the great solubilizing efficiency of RM-β-CD. However, the $P_{app}$ of CCB in γ-CD formulations (Figure 13) was slightly higer in all cases which probable due to smaller MW (i.e., smaller hydrodynamic radius) of CCB/γ-CD complex in case of 1:1 complex. In contrast, the inclusion complex fomation of CCB/RM-β-CD were 1:1 complex and 1:2 complex according to $A_p$-type profiles. This behavior presented high MW of CCB/RM-β-CD especially 1:2 complex (MW ≈ 3,005 Da) resulting in slowly premeated through semipermeable membrane. Though the concentration of polymers increases, permeation flux and, $P_{app}$ of the formulation were not decreased. Hence, the viscosity did not influence on the permeation pattern under such circumstances. The definition of viscosity is based on the bulk properties of solutions and does not apply to individual molecules (Loftsson et al., 2002). Although $P_{app}$ of formulations containing RM-β-CD were slighly less than the formulation containing γ-CD (F16-F18), their flux values or the total amount of drug and their complexes were much higher which can increase drug concentration gradient over the absorption surface. Thus, the CCB formulation containing RM-β-CD in both solution and suspension forms were promising to deliver CCB to the posterior segment of the eye.

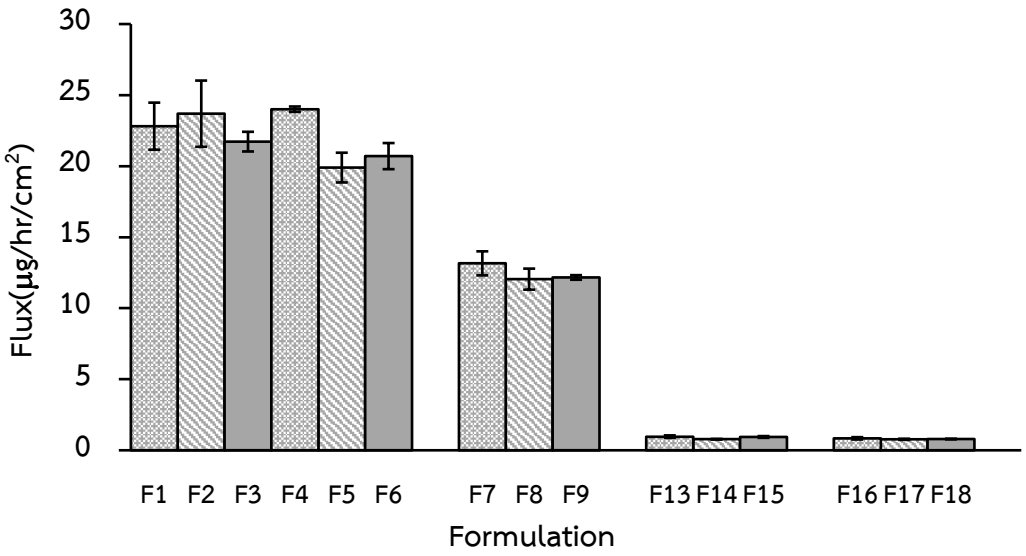



*Figure 12 The permeation flux (J) of celecoxib eye drop formulation through semi-permeable membrane (MWCO 12–14,000 Da)*

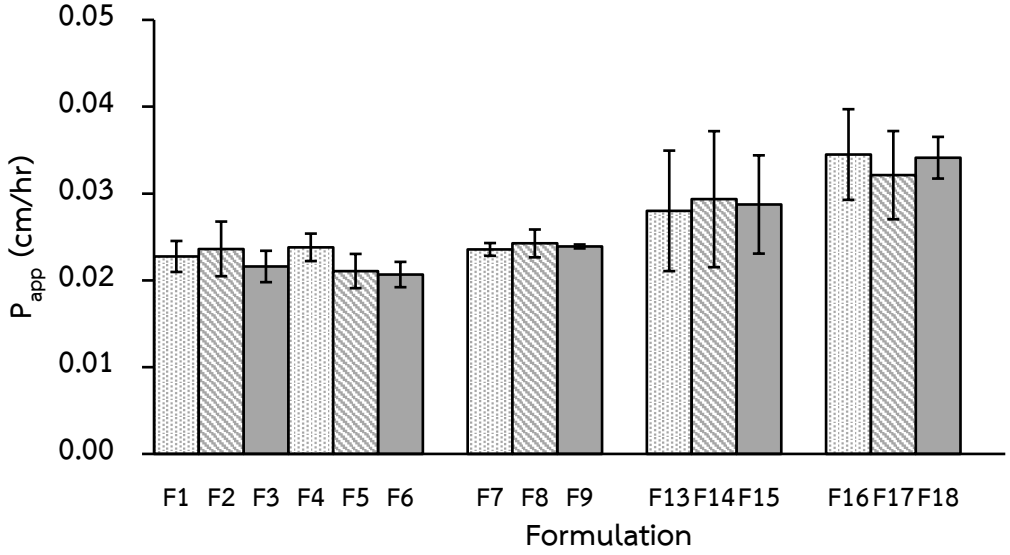



*Figure 13 The apparent permeation coefficient ($P_{app}$) of celecoxib eye drop formulation through semi-permeable membrane (MWCO 12–14,000 Da)*

### *5.3 In-vitro mucoadhesive studies*

Table 13 and 14 demonstrate mucoadhesive properties of CCB-CD eye drop solutions and suspensions containing polymer, respectively. From the result, % drug remained for each formulation of solution (F1-F16) were not significantly different (Table 13). Thus, type of polymer and its concentrations i.e., 0.1-0.5% w/v did not affect mucoadhesive properties of CCB eye drop solution. In contrast, when increasing polymer i.e., 0.25% and 0.5% w/v to the eye drop suspension, they provided the

mucoadhesive characteristics by significantly higher CCB remaining than that of solution (Table 14). This indicated that the eye drop suspensions with polymer ≥ 0.25% w/v that contains solid particles i.e., free CCB and solid CCB/CD complexes would be possibly washed more slowly from the eye surface.

*Table 13 Mucoadhesive properties of celecoxib eye drop solution containing HA and HPMC*

| Formulation | CD | Polymer | % polymer (w/v) | % Drug remained |
|---|---|---|---|---|
| F1 | RM-β-CD | HA | 0.1 | 0.419 ± 0.184 |
| F2 | | | 0.25 | 0.338 ± 0.160 |
| F3 | | | 0.5 | 0.405 ± 0.093 |
| F4 | | HPMC | 0.1 | 0.413 ± 0.183 |
| F5 | | | 0.25 | 0.511 ± 0.108 |
| F6 | | | 0.5 | 0.400 ± 0.135 |

*Table 1 Mucoadhesive properties of celecoxib eye drop suspensions containing HA and HPMC*

| Formulation | CD | Polymer | % polymer (w/v) | % Drug remained | Mucoadhesive Ratio* |
|---|---|---|---|---|---|
| F0R | RM-β-CD | - | - | 0.12 ± 0.009 | 1.0 |
| F7 | | HA | 0.1 | 0.656 ± 0.285 | 5.5 |
| F8 | | | 0.25 | 1.398 ± 0.259 | 11.7 |
| F9 | | | 0.5 | 2.243 ± 0.176 | 18.7 |
| F0G | γ-CD | - | - | 0.115 ± 0.012 | 1.0 |
| F13 | | HA | 0.1 | 0.753 ± 0.291 | 6.5 |
| F14 | | | 0.25 | 1.422 ± 0.153 | 12.4 |
| F15 | | | 0.5 | 3.243 ± 0.320 | 28.2 |
| F16 | | HPMC | 0.1 | 0.476 ± 0.238 | 4.1 |
| F17 | | | 0.25 | 1.317 ± 0.165 | 11.5 |
| F18 | | | 0.5 | 2.335 ± 0.338 | 20.3 |

*Mucoadhesive ratio: Compared with formulation without polymer (F0R and F0G).

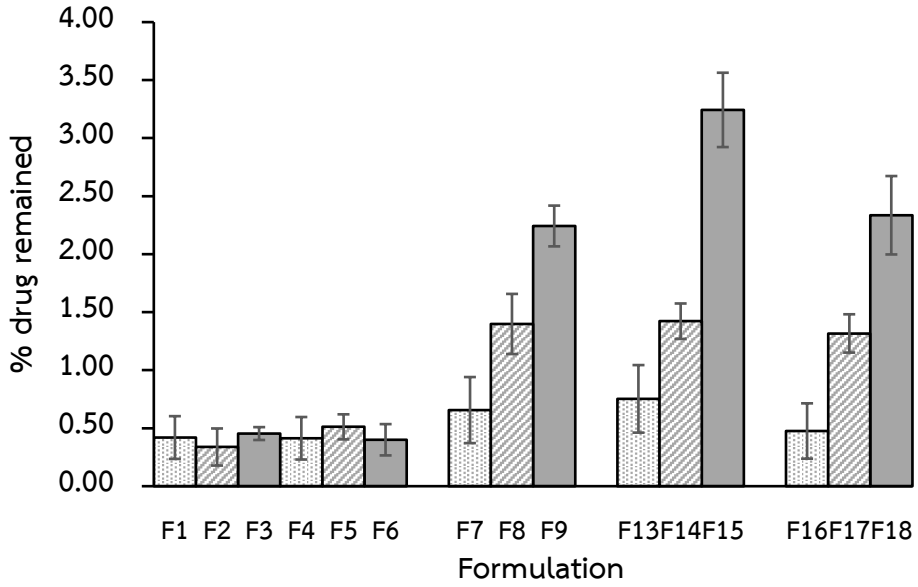



*Figure 14 % drug remained of each celecoxib eye drop formulation on the mucin coated membrane*

In most cases, the CCB remained in eye drop suspensions containing γ-CD was greater than the formulation containing RM-β-CD (Figure 14). This observation was due to that > 99 % CCB solid content in γ-CD formulations were adhered and slowly flushed away from the membrane surface. Regarding to the eye drop suspensions, the role of polymer (HA and HPMC) on mucoadhesive properties is concentration-dependent manner. Especially, eye drop suspension containing γ-CD and 0.5% w/v HA exhibited the greatest mucoadhesion which was 28-folds higher than that of suspension without polymer (F0G) (Table 14). According to the review of Park and Robinson (1987), generally, both anionic and cationic charged polymers showed better mucoadhesive capacity in comparison to nonionic polymer, such as cellulose derivatives or PVA. Moreover, the polyanions polymer are better than polycations polymer in terms of binding efficacy and potential toxicity. Likewise, Saettone et al. (1989) studied mucoadhesive properties of polymer with mucin-coated surfaces model. This study found that the good to excellent mucoadhesive properties were detected in formulation of tropicamide containing HA greater than formulation containing polyacrylic acid. Consequently, HA is the most promising mucoadhesive polymer for ophthalmic drug delivery.

From the above results, it was concluded that CCB-CD suspensions containing 0.5% w/v HA or HPMC (F9, F15 and F18) provided the great mucoadhesive properties

which is possible to increase the residence time, consequently to enhance the ocular bioavailability. Hence, not only permeation flux but also mucoadhesive properties are the main factors to consider to develop the eye drop formulation.

## 6. Effect of heating method on CCB eye drop suspension (ternary complex)

The increasing complexation of CCB-CD with polymer as ternary complex can be accelerated by heating method i.e. using an autoclave (120 to 140ºC) for 20 to 40 minutes, using sonication bath at 70ºC for 1 hour and using microwaves at 40ºC for 5 minutes (Loftsson et al., 2005). Although the heating method by autoclaving is the promising method to enhance CCB-CD complexation discussed earlier in section of phase solubility profiles; in case of eye drop suspension after a cycle of autoclaving, the change in physical appearance of formulation was observed. The solid particle in suspension were agglomerated to the larger size i.e., >2 mm (data not shown). Thus, the heating method by sonication at 70 ºC for 1 hour has been performed. In this study, the formulation of F9, F15 and F18 were selected because they showed excellent mucoadhesion and relatively good permeation parameters. The formulation F9, F15 and F18 passed through heating method were coded as F9so, F15so and F18so, respectively. This study aimed to determine the effect of heating method on physicochemical and chemical properties, particle size and permeation of CCB eye drop formulation compared with the corresponding formulations without heating process.

### *6.1 Physicochemical and chemical properties of heated and unheated CCB/CD suspension*

pH and osmolality and viscosity of F9, F15, F18 with and without heating method are shown in Table 15. The viscosity of formulations containing HA with heating method (F9so and F15so) were slightly decreased when compared with the formulation without heating process. Again, this was due to that HA could be degraded under ultrasonic condition with heating at 70ºC for 1 hour resulting in lowering viscosity value (Drimalova et al., 2005). Table 16 shows the sedimentation volume (F) and re-dispersion time of formulation with and without heating method. There were no changes in both F and re-dispersion time of formulation after heating process.

*Table 2 pH value, osmolality and viscosity of celecoxib eye drop suspension with and without heating method*

| Formulation | pH | Osmolality (mOsm/Kg) | Viscosity (mPa.s) |
|---|---|---|---|
| F9 | 7.39 ± 0.06 | 292.7 ± 7.8 | 15.67 ± 0.80 |
| F15 | 7.43 ± 0.07 | 306.3 ± 13.9 | 16.87 ± 0.07 |
| F18 | 7.37 ± 0.08 | 295.0 ± 3.6 | 15.13 ± 0.58 |
| F9so | 7.36 ± 0.05 | 293.0 ± 7.5 | 13.97 ± 0.57 |
| F15so | 7.45 ± 0.02 | 296.3 ± 7.1 | 13.47 ± 1.06 |
| F18so | 7.38 ± 0.06 | 288.3 ± 3.5 | 16.03 ± 0.57 |

so: heating method by sonication at 70°C for 1 hr.

*Table 16 Sedimentation volume (F) and re-dispersion time of celecoxib eye drop suspension with and without heating method*

| Formulation | Sedimentation volume (F) | | | Re-dispersion time (s) |
|---|---|---|---|---|
| | Day 3 | Day 5 | Day 10 | |
| F9 | 0.09 ± 0.01 | 0.08 ± 0.02 | 0.08 ± 0.02 | 22.8 ± 1.7 |
| F15 | 0.08 ± 0.02 | 0.08 ± 0.02 | 0.08 ± 0.02 | 14.3 ± 1.5 |
| F18 | 0.10 ± 0.0 | 0.10 ± 0.02 | 0.10 ± 0.02 | 52.3 ± 9.0 |
| F9so | 0.09 ± 0.01 | 0.07 ± 0.01 | 0.07 ± 0.01 | 27.3 ± 2.5 |
| F15so | 0.09 ± 0.01 | 0.09 ± 0.01 | 0.09 ± 0.01 | 19.0 ± 3.0 |
| F18so | 0.09 ± 0.01 | 0.09 ± 0.01 | 0.09 ± 0.01 | 35.0 ± 5.0 |

so; heating method by sonication at 70°C for 1 hr.

Table 17 shows total CCB content and drug dissolved CCB content of each formulation with and without heating process. The assay content of all formulations met within the acceptance criteria with 90-110% LA. The percentage dissolved content of F9so, F15so and F18so were 46.9 ± 0.17, 1.33 ± 0.14 and 3.39 ± 0.16 % which greater than 4-folds, 2-fold 7-folds of the corresponding unheated formulations, respectively. This observation was probably due to the interaction between CCB/CD and water-soluble polymer by ion-ion, ion-dipole and dipole-dipole electrostatic bonds, Van der Waals force (Ribeiro et al., 2003). In addition, CD, polymers and their complexes could form aggregates which enhance the solubilization of hydrophobic molecules like CCB. Regarding to accelerate ternary complex formation, it is possible

to activate such interaction bonds between their components during the complexes preparation by heating method (Loftsson et al., 2005). It was shown that the heating method by sonication in water bath at 70°C for 1 hour could improve solubility of CCB probably via through ternary complex formation.

*Table 17 Total drug content and drug dissolved content of celecoxib eye drop suspension with and without heating method*

| Formulation | Drug concentration (mg/ml) | Drug dissolved concentration (µg/ml) | % drug content | % dissolved content |
|---|---|---|---|---|
| F9 | 4.975 ± 0.113 | 509.0 ± 8.3 | 99.5 ± 2.3 | 10.18 ± 0.17 |
| F15 | 4.715 ± 0.099 | 33.7 ± 8.0 | 94.3 ± 2.0 | 0.67 ± 0.16 |
| F18 | 4.660 ± 0.178 | 23.3 ± 1.8 | 93.2 ± 3.6 | 0.47 ± 0.01 |
| F9so | 4.597 ± 0.359 | 2,349.2 ± 113.3 | 91.9 ± 7.2 | 46.98 ± 2.27 |
| F15so | 5.143 ± 0.278 | 66.3 ± 7.2 | 102.9 ± 5.6 | 1.33 ± 0.14 |
| F18so | 5.015 ± 0.156 | 169.5 ± 8.0 | 100.3 ± 3.1 | 3.39 ± 0.16 |

so; heating method by sonication at 70°C for 1 hr.

### *6.2 Particle size analysis*

*The Aggregate size distribution of CCB/CD complex in formulation.*

The particle size and size distribution of the supernatant of CCB eye drop suspensions measured by DLS technique are shown in Figure 15. It demonstrated that all samples have the characteristic of aggregates size. An intensity peak at about 1–2 nm indicated the existing of non-aggregated complex as solubilized component while the second and the third peak referred to the complex aggregates of CCB/CD/polymer. Aggregates sizes of the second and the third intensity peak in formulations ranged from 10 nm to 80 nm and 180 nm to 400 nm, respectively (Table 18). The formulations passed through heating method (F9so, F15so and F18so) displayed the complex aggregates relatively larger than those of unheated formulations. It was suggested that the increasing of aggregate sizes in formulations was influenced by heating method through inclusion and non-inclusion complexes or micelle-like assembles resulting in increasing CCB solubilization. Moreover, the aggregate sizes of formulations containing γ-CD (F15, F15so, F18 and F18so) were

slightly larger than the formulations containing RM-β-CD. It is possible that the larger aggregates were self-aggregates of γ-CD in formulation (Jansook, Moya-Ortega, and Loftsson, 2010). Assuming that the most probable mechanism is the hydrogen bond related self-aggregation of γ-CD monomers (Szente, Szejtli, and Kis, 1998). However, there are various factors such as the formulation components and type of CDs which influence on the aggregate size of complex (Puskás et al., 2013; Ryzhakov et al., 2016). Numerous studies have been investigated the complex aggregates formation. The inclusion complex of CD are able to form larger aggregates in aqueous solution, which solubilized themselves or hydrophobic drugs through non-inclusion complex or micelle-like assembles (Loftsson et al., 2004). Micelle-like structures in aqueous solution consisted of trans-β-carotene β-CD and γ-CD obtained diameter of aggregates more than 200 nm (Mele, Mendichi, and Selva, 1998).

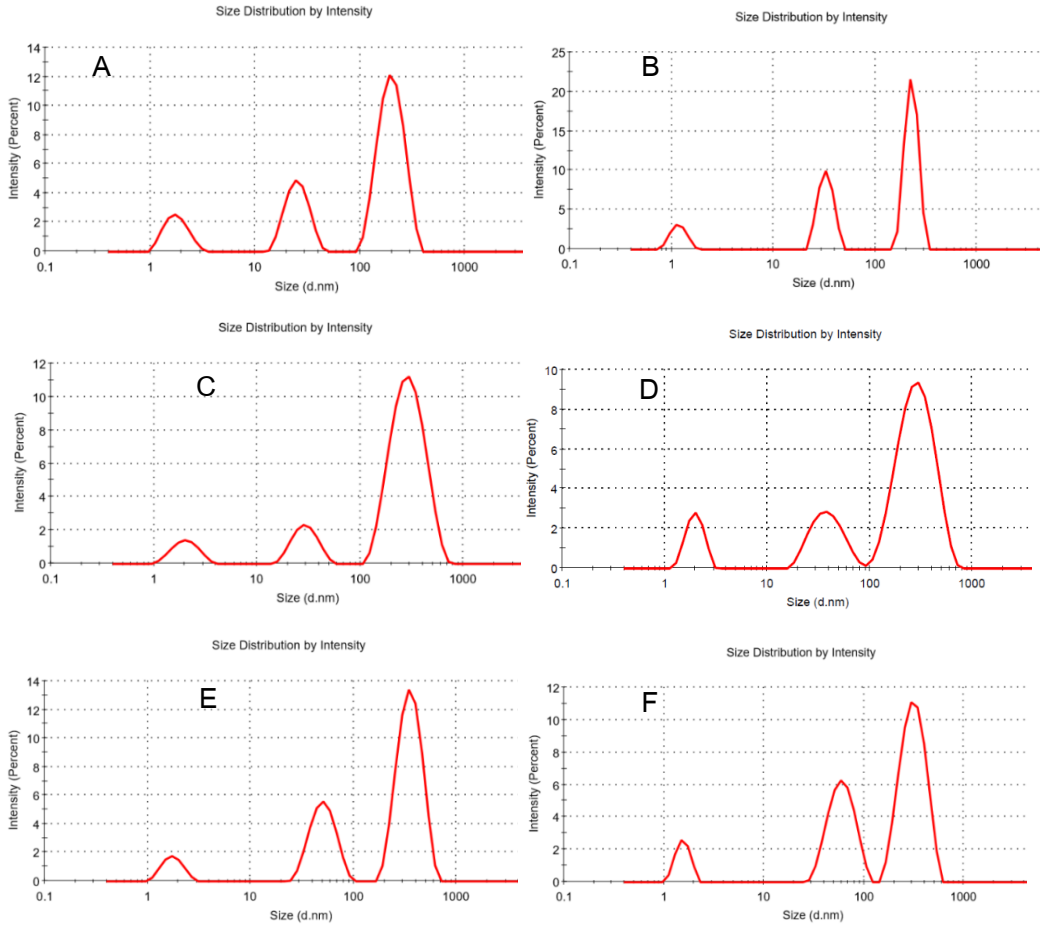



*Figure 15 The aggregate size and size distribution of CCB/CD complex in formulation at 25°C in DI water determined by DLS technique; (A) F9, (B) F9so, (C) F15, (D) F15so, (E) F18, (F) F18so.*

*Table 18 The aggregate size and size distribution of selected CCB-CD eye drop formulations determined by DLS technique.*

| Formulation | Polymer | CD | Particle size (nm) |
|---|---|---|---|
| F9 | 0.5% HA | RM-β-CD | 221.1 ± 18.1<br>29.2 ± 7.2<br>1.92 ± 0.2 |
| F15 | 0.5% HA | γ-CD | 307.2 ± 45.7<br>34.1 ± 5.4<br>2.0 ± 0.2 |
| F18 | 0.5% HPMC | γ-CD | 304.2 ± 64.4<br>45.2 ± 14.9<br>1.7 ± 0.1 |
| F9so | 0.5% HA | RM-β-CD | 230.1 ± 26.7<br>34.0 ± 9.0<br>1.6 ± 0.5 |
| F15so | 0.5% HA | γ-CD | 332.6 ± 32.2<br>48.6 ± 10.7<br>2.0 ± 0.3 |
| F18so | 0.5% HPMC | γ-CD | 350.4 ± 35.3<br>58.1 ± 15.7<br>1.9 ± 0.4 |

so*, Sonication at 70$^{o}$C for 1 hr

γ-CDs are carbohydrates and do self-assemble like other carbohydrates in aqueous solution. It is probable to form larger self-aggregates in aqueous solutions with opalescence and precipitation even at low concentrations. Regarding to RM-β-CD, the phase solubility of triclosan and triclocarban was determined. It displayed $A_p$-type diagram indicating formation of higher-order complexes and their complex aggregates were confirmed by NMR techniques (Duan et al., 2005). In addition, Puskas et al., (2013) studied the effect of glucose, urea, and inorganic salts on the complex formation of RM-β-CD, SBE-β-CD and HP-β-CD containing cholesterol. A

mixture of non-aggregated complexes with a diameter of 1-2 nm and aggregated complexes with a diameter ranging from about 100 to 1000 nm were observed by DLS technique.

*The particle size of suspensions*

Ophthalmic suspensions possibly used to increase the corneal retention time of a drug providing sustained and enhanced ocular bioavailability. The particles in suspensions are desired to be a micronized form (less than 10 µm in diameter) to prevent irritation or scratching of the eye (Kaur and Kanwar, 2002). Suspensions are commonly prepared by dispersing micronized drug powder in a suitable aqueous vehicle. A reduction in particle size usually improves the patient comfort and acceptable suspension (Aldrich et al., 2013). In this study, the particle in suspension was reduced by media milling technique. The particle size of suspensions was measured by optical microscope and their morphology was observed by SEM.

*Table 19 the particle size of celecoxib eye drop suspension with and without heating method determined by optical microscope*

| Formulation | Particle size (µm) (Mean ± S.D.) |
|---|---|
| F9 | 3.90 ± 1.63 |
| F15 | 5.13 ± 2.35 |
| F18 | 4.82 ± 2.18 |
| F9so | 3.77 ± 1.85 |
| F15so | 4.43 ± 2.09 |
| F18so | 4.32 ± 2.10 |

so; Sonication at 70°C for 1 hr.

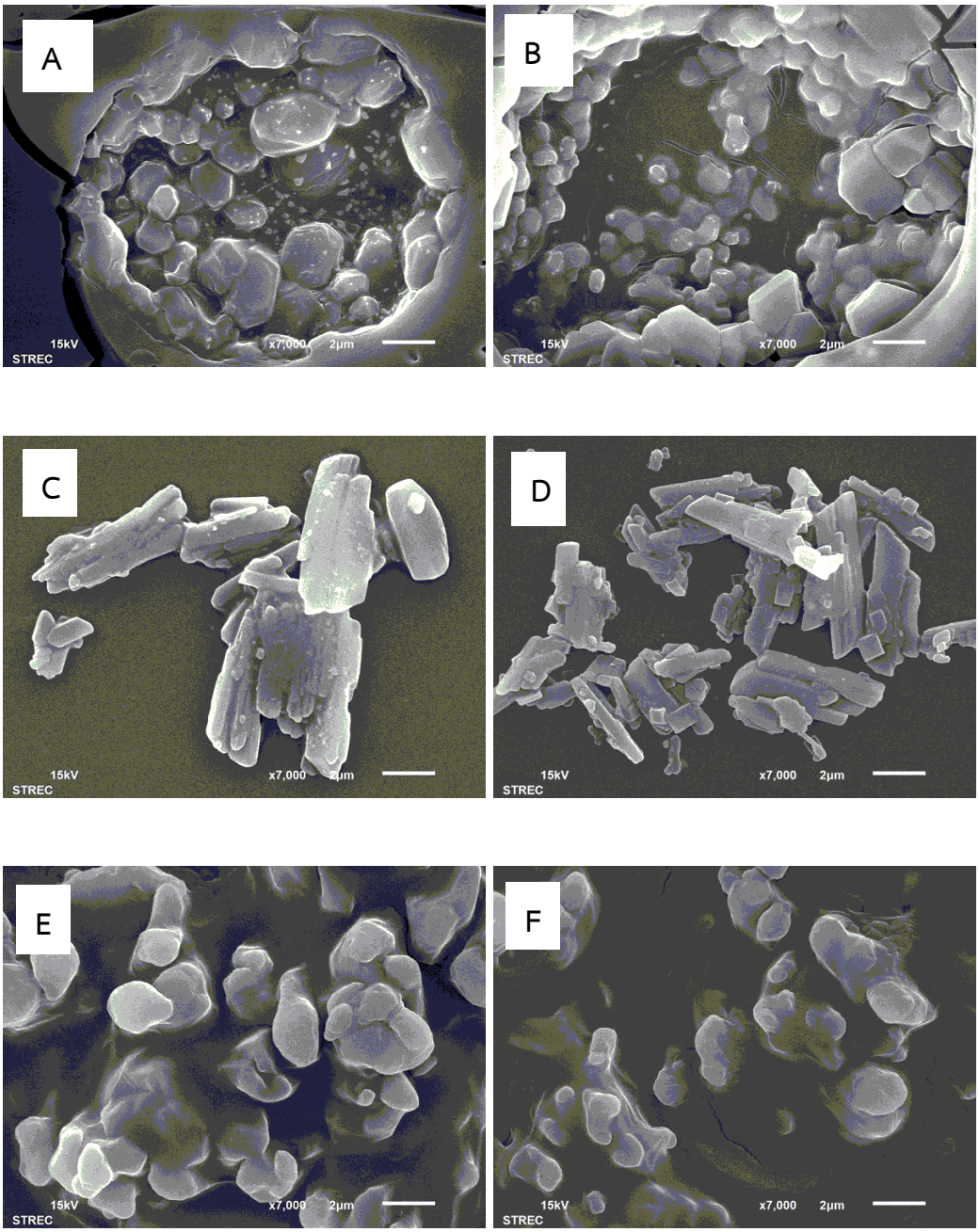



*Figure 3 SEM Photographs of celecoxib eye drop formulation with and without heating method; (A) F9, (B) F9so, (C) F15, (D) F15so, (E) F18, (F) F18so*

Table 19 displays the particle size of CCB eye drop suspensions and Figure 16 shows SEM Photographs of CCB eye drop suspensions. The particles size of F9so, F15so and F18so showed slightly smaller than those of unheated ones. The particle size of all CCB formulations (heated and unheated) not more than 8 μm which was acceptable for eye drop suspension's criteria. Especially, F9so eye drop suspension

(Figure 27B) showed relatively spherical shape with the smallest particle size and narrow size distribution. It ensures that our CCB-CD ophthalmic suspension did not cause to irritate to the eye. Both techniques, media mill and sonication with heating, provide synergistic effect in the particle size reduction.

### *6.3 In-vitro permeation of heated and unheated CCB/CD suspension*

Figure 17 displays the permeation profile of heated and unheated CCB-CD eye drop suspensions through semi-permeable membrane (MWCO 12–14,000 Da). Table 23 shows the flux and $P_{app}$ values of unheated and heated CCB eye drop suspensions (calculated from the slope of permeation profile in Figure 17). It is generalized that the increasing % dissolved CCB content, the higher amount of drug permeation through semi-permeable membrane was observed. And also, when considering to the flux permeation, the flux value of F9so and F18so were 2-folds and 4-folds higher than F9 and F18, respectively. Although % dissolved CCB content of F15so was higher than F15 about 2-folds, the permeation flux of heated formulation was not increased. Among the CCB eye drop suspension tested, CCB eye drop suspension containing RM-β-CD and HA with heating method showed the greatest flux value was observed.

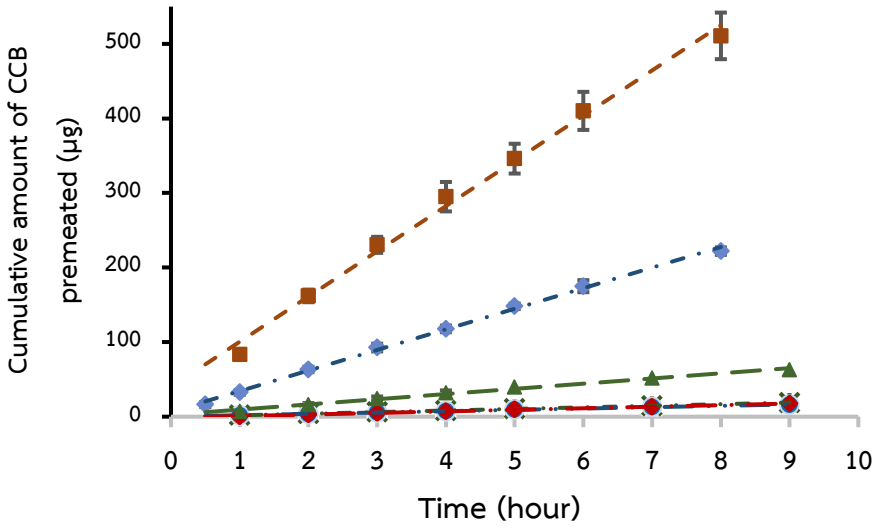



*Figure 17 The permeation profile of CCB-CD eye drop suspension with and without heating method through semi-permeable membrane (MWCO 12–14,000 Da); (◆) F9, (X) F15, (●) F18, (■) F9so, (◆) F15so, (▲) F18so*

*Table 20 Flux (J) and apparent permeation coefficient ($P_{app}$) of celecoxib eye drop suspension with and without heating method through semi-permeable membrane (MWCO 12-14,000 Da)*

| Formulation | Flux (µg/hr/cm$^2$) | $P_{app}$ (cm/hr) |
|---|---|---|
| F9 | 12.17 ± 0.16 | 0.0239 ± 0.0002 |
| F15 | 0.94 ± 0.06 | 0.0287 ± 0.0057 |
| F18 | 0.79 ± 0.02 | 0.0341 ± 0.0024 |
| F9so | 26.72 ± 1.74 | 0.0114 ± 0.0010 |
| F15so | 0.93 ± 0.10 | 0.0141 ± 0.0017 |
| F18so | 3.06 ± 0.14 | 0.0181 ± 0.0008 |

so; heating method by sonication at 70$^o$C for 1 hr.

However, $P_{app}$ of all heated formulations were decreased when compared with the following unheated formulation (Table 20). It is possible that the bonds activation between the components i.e., CCB, CD and water-soluble polymers as ternary complex during the heating method were formed. The increasing interaction between CCB-CD complex and polymer leaded to the increasing the initial dissolved CCB concentration (Cd). However, due to its great interaction and retaining in polymer network resulted in decreasing $P_{app}$ through the membrane.

### *6.4 In-vitro permeation through simulated artificial vitreous humor*

The study of *in-vitro* permeation through artificial vitreous humor model was simulated the permeation of drug through biological membrane such as cornea and conjunctival and through vitreous humor into the posterior segment of eye. In theory, when the drug/CD complex penetrated through aqueous layer barrier, the drug in the complex was rapid dynamic equilibrium release to the “free drug”, and increased the drug concentration gradient over the membrane which was readily absorbed to the site of action. Table 21 displays the permeation flux and apparent permeation coefficient ($P_{app}$) of heated and unheated CCB eye drop suspensions through simulated artificial vitreous humor.

*Table 21* The permeation f*lux (J) and* apparent *permeation coefficient ($P_{app}$) of celecoxib eye drop suspension with and without heating method through simulated artificial vitreous humor*

| Formulation | Flux (µg/hr/cm$^2$) | $P_{app}$ (cm/hr) |
|---|---|---|
| F9 | 1.55 ± 0.20 | 0.0030 ± 0.0004 |
| F15 | 0.11 ± 0.05 | 0.0033 ± 0.0012 |
| F18 | 0.17 ± 0.05 | 0.0075 ± 0.0027 |
| F9so | 2.98 ± 0.16 | 0.0013 ± 0.0001 |
| F15so | 0.14 ± 0.01 | 0.0022 ± 0.0003 |
| F18so | 0.34 ± 0.01 | 0.0020 ± 0.0002 |

so*; heating method by sonication at 70$^o$C for 1 hr.

As expected, because the percentage of dissolved content of suspension containing RM-β-CD were higher, the flux values of the suspension containing RM-β-CD (F9 and F9so) showed greater than suspension containing γ-CD (F15, F15so, F18 and F18so). The flux values of F9so and F18so was 2-folds greater than F9 and F18 while its value of F15 and F15so were slightly different. The polymer enhanced CD complexation of CCB by heating method may lead to higher drug flux through the simulated artificial vitreous humor model. In this study, only free drug and mono- and dimer of CCB/CD complexes were permeated through semipermeable membrane (MWCO 3,500 Da) and artificial vitreous humor to the chamber which almost imitated the delivering of CCB to the posterior segment of eye. On account of limitation of number of CCB molecules to enter and long distance of simulated artificial vitreous humor model resulting in the decreasing of flux permeation and the $P_{app}$ when compared to the earlier *in-vitro* permeation data. It indicated that the MWCO of semi-permeable membrane, the length and its viscosity of vitreous humor model were possibly influenced on the permeation of CCB.

However, Sigurdsson et al. (2007) demonstrated that the topical absorption following of dexamethasone containing CD eye drop application could be reached to the posterior segment of the rabbit eyes. In addition, the concentration of the drug in the retina was higher than the vitreous humor for 3-folds. It possible due to that the non-corneal absorption by diffusion of the drug through the conjunctiva, sclera, choroid as well as systemic absorption from the conjunctival blood vessels and re-entering to the retina were occurred after the topical eye drop application (Boddu et al., 2014).

Therefore, in fact, the concentration in the retina of CCB may be possibly increased via these pathway mechanisms.

From above results, F9so still exhibited the highest flux value of CCB among formulations tested which gave high drug levels in the receptor compartment. Thus, F9so eye drop suspension containing RM-β-CD and HA is the great potential formulation which can deliver CCB into the posterior segment of the eye. Due to the several factors i.e., the eye, barriers, pre-corneal clearances including the absorption pathways for topically eye drop to the posterior segment of the eye, the *in-vivo* study is required to be further study.

## Conclusions

Celecoxib (CCB), a poorly water-soluble drug, its solubility can be enhanced by using cyclodextrin (CD) through inclusion complex formation. The CCB/CD complex in the aqueous solutions has thermal stability and no degradation peak was observed during the heating process.

Among selected CDs tested, RM-β-CD, β-CD derivatives, exhibited the highest complexation efficiency of CCB and presented an $A_P$-type phase solubility diagram. γ-CD was selected due to its safety. The addition of polymer in aqueous CD solutions provided synergistic effect of CD solubilization via ternary complex formation. HPMC showed the highest increments in solubilizing efficiency following HA and chitosan, respectively. The aggregate sizes of CCB/CD/polymer observed by DLS and TEM techniques were in the range of 250-350 nm. It was suggested that these complex aggregates could be capable of solubilizing drugs. The data obtained from the DSC, PXRD, and FT-IR studies demonstrated that CCB formed an inclusion complex with CD. The $^1$H-NMR result indicated that CCB partially included and deeply inserted into the cavity of RM-β-CD and γ-CD, respectively.

The CCB eye drop formulation consisted of RM-β-CD and γ-CD as solubilizers, HPMC and HA as mucoadhesive polymers were developed. The physicochemical and chemical properties of all formulations were determined i.e., pH and osmolality of formulations were closely to lacrimal fluid which possibly not marked discomfort after applied. The solid particle sizes of suspensions shown below 10 μm which indicated that CCB-CD ophthalmic suspension did not irritate the eye. The percentage total drug

content of all formulations met in a limit of 90-110%. In most cases, when applied the heating method, % dissolved drug content was increased. After sedimentation, they were easily re-dispersed to become uniform suspension by gently shaking; except the formulations containing RM-β-CD and HPMC.

No mucoadhesive was observed in the CCB eye drop solution while CCB eye drop suspension containing 0.25-0.5% w/v of polymers i.e., HA and HPMC had mucoadhesive properties. This was due to their solid particles parts which washed more slowly from the mucin-coated membrane. Furthermore, the CCB eye drop formulation containing HA showed the mucoadhesive properties greater than that of HPMC at 0.5% w/v while there were no significantly different in lower concentration of polymer. Thus, these formulations are possible to increase the contact time on eye surface and provide to enhance ocular bioavailability.

The *in-vitro* permeation study was investigated the drug diffuse through a semipermeable membrane. The formulations containing RM-β-CD showed distinguishable difference in greater permeation flux than formulations containing γ-CD due to higher CCB dissolved content. It indicated that the increasing of % drug dissolved content possibly lead to enhance the permeation by increasing the drug concentration gradient over the surface of membrane. Moreover, the permeation of the formulation could be improved by increasing solubility of CCB through heating method. In contrast, in the formulation containing γ-CD, the higher $P_{app}$ was noticed because of their lower CCB/γ-CD complexes molecular weight size. However, the formulation containing RM-β-CD and HA showed the highest flux permeation through semipermeable membrane and also through simulated vitreous humor including relatively good mucoadhesion. Therefore, it can be good candidate for further study to deliver CCB to the posterior segment of the eye.

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**Output ที่ได้จากโครงการ**

1. ได้ผลิตภัณฑ์ยาหยอดตาซิลิคอกซิบ-ไซโคลเดกตรินชนิดติดเยื่อเมือกระดับไมครอน ที่มีแนวโน้มในการนำส่งยังส่วนหลังลูกตาเพื่อรักษาภาวะจอประสาทตาเสื่อมและเบาหวานขึ้นจอตา
2. ได้นำเสนอผลงานวิจัยในรูปแบบโปสเตอร์
   2.1 งานประชุมวิชาการ “นักวิจัยรุ่นใหม่...พบเมธีวิจัยอาวุโส สกว.” ครั้งที่ 17 ประจำปี 2559
   2.2 งานประชุมวิชาการ “นักวิจัยรุ่นใหม่...พบเมธีวิจัยอาวุโส สกว.” ครั้งที่ 18 ประจำปี 2560
   2.3 งานประชุมวิชาการ 6th European Conference on Cyclodextrins – EURO CD 2019 ซึ่งจัดขึ้น ณ เมือง ซานติเอโก ดิ คอมโพสเตลา ประเทศเสปน ระหว่างวันที่ 2-4 ตุลาคม พ.ศ. 2562
3. มีความร่วมมือกับ Professor Dr. Thorsteinn Loftsson คณะเภสัชศาสตร์ มหาวิทยาลัยไอซ์แลนด์ ซึ่งช่วยในการปรับปรุงและแก้ไขต้นฉบับสำหรับตีพิมพ์ในวารสารนานาชาติ
4. ได้ผลงานวิจัยตีพิมพ์ในระดับนานาชาติที่เกี่ยวข้องกับโครงการ จำนวน 2 เรื่องได้แก่
   4.1 Jansook P., Kulsirachote P., Loftsson T. (2018) Cyclodextrin solubilization of celecoxib: solid and solution state characterization. J. Incl. Phenom. Macrocycl. Chem. 90:75–88. (ISI/Scopus Impact factor 1.429/Q2)
   4.2 Jansook P., Kulsirachote P., Asasutjarit R., Loftsson T. Development of celecoxib eye drop solution and microsuspension: A comparative investigation of binary and ternary cyclodextrin complexes. Carbohydr. Polym. (*in press*). (ISI/Scopus Impact factor 5.975/Teir 1)

5. ได้ผลงานวิจัยตีพิมพ์ในระดับนานาชาติที่ไม่ได้เสนอในโครงการวิจัยนี้ แต่ได้สนับสนุนงบวิจัยบางส่วนจากโครงการ จำนวน 1 เรื่อง ได้แก่

   Jansook P., Prajapati M., Pruksakorn P., Loftsson T. Antifungal activity of econazole nitrate/cyclodextrin complex: effect of pH and formation of complex aggregates. Int. J Pharm. (*accepted manuscript*). (ISI/Scopus Impact factor 4.213/Teir 1)

# ภาคผนวก (Appendices)

**เอกสารแนบ**

1. ผลงานวิจัยในรูปแบบโปสเตอร์
   นักวิจัยรุ่นใหม่...พบเมธีวิจัยอาวุโส สกว. ครั้งที่ 17 ประจำปี 2559
   (เอกสารแนบหมายเลข 2.1 ฉบับที่ 1 และ 2)

   นักวิจัยรุ่นใหม่...พบเมธีวิจัยอาวุโส สกว. ครั้งที่ 18 ประจำปี 2560
   (เอกสารแนบหมายเลข 2.2 ฉบับที่ 1 และ 2)

   **6th European Conference on Cyclodextrins – EURO CD 2019**
   (เอกสารแนบหมายเลข 2.3 ฉบับที่ 1 และ 2)

2. ผลงานวิจัยตีพิมพ์ในระดับนานาชาติ
   Jansook P., Kulsirachote P., Loftsson T. (2018) Cyclodextrin solubilization of celecoxib: solid and solution state characterization. J. Incl. Phenom. Macrocycl. Chem. 90:75–88.
   (เอกสารแนบหมายเลข 4.1)

   Jansook P., Kulsirachote P., Asasutjarit R., Loftsson T. Development of celecoxib eye drop solution and microsuspension: A comparative investigation of binary and ternary cyclodextrin complexes. Carbohydr. Polym. (in press).
   (เอกสารแนบหมายเลข 4.2)

   Jansook P., Prajapati M., Pruksakorn P., Loftsson T. Antifungal activity of econazole nitrate/cyclodextrin complex: effect of pH and formation of complex aggregates. Int. J Pharm. (accepted manuscript).
   (เอกสารแนบหมายเลข 5)

# Cyclodextrin Solubilization of Celecoxib: Solid and Solution State Characterization

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## Abstract

Celecoxib (CCB), a nonsteroidal anti-inflammatory drug (NSAID), could be beneficial in the treatment of age-related macular degeneration (AMD) and diabetic retinopathy (DR) through the inhibition of COX-2 enzyme. Currently, the several methods can deliver drugs to the back of the eye. i.e., oral, intravitreal injection, subconjunctival injection and topical. Among routes of administration, topical eye drop is non-invasive method and local effects. However, due to low aqueous solubility of CCB, it hampers ocular bioavailability. Thus, the aim of this study was to enhance the aqueous solubility of CCB by complexation with cyclodextrin (CD) in the presence of water-soluble polymer. CDs, the solubilizer, i.e., αCD, βCD, γCD, HPβCD and RMβCD and mucoadhesive polymers i.e., hydroxypropylmethylcellulose (HPMC) chitosan and hyaluronic acid (HA) were used. The phase solubility profiles and CCB/CD complex characteristics were investigated. RMβCD exhibited the greatest solubilizer among CDs tested. Formation of CCB/RMβCD/HPMC and CCB/γCD/HPMC ternary complexes resulted in 11 and 19-fold enhancement in the apparent complexation efficiency in comparison to their CCB/CD binary complex, respectively. The aggregate size of ternary complexes in solution were found in the range of 250 - 350 nm which could solubilize themselves resulting in increasing CCB solubility. The data obtained from FT-IR, DSC, PXRD and $^{1}$H-NMR indicated that there was interaction of CCB with CD as inclusion complex.

**Keywords:** cyclodextrins, celecoxib, solubility, complexation, aggregates

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# Cyclodextrin Solubilization of Celecoxib: Solid and Solution State Characterization

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## AIM OF STUDY

Due to the very low celecoxib (CCB) aqueous solubility (~2μg/ml) [1], it hampers CCB permeation through biological membranes resulting in low bioavailability and inadequate drug levels at its site of action. Complexation with cyclodextrin (CD) in the presence of water-soluble polymer can enhance the solubility and permeability of drug [2]. Thus, The aim of this study is on evaluation of the ability of different CDs to solubilize CCB and characterization of both the solid state and the dissolved CCB/CD complexes, and their ability to form nano- and microparticle drug delivery systems.

## EXPERIMENTAL METHODS

***Solubility determinations:***

Excess amount of CCB was added to solutions containing from 0 to 15% (w/v) CD in pure water. The drug suspensions were saturated the drug through heating in an autoclave 121°C for 20 min and allowed to cool to room temperature [3]. After equilibrium was attained, the suspensions were filtered through 0.45 μm syringe filter, the filtrates were diluted with the mobile phase and analyzed by HPLC. The apparent stability constants for CCB/CD complexes ($K_{1:1}$ and/or $K_{1:2}$) and the complexation efficiency (CE) were determined according to the phase-solubility method of Higuchi and Connors [4].

$$CE = \frac{Slope}{1-Slope} = \frac{[Drug/CD\ Complex]}{[CD]} = K_{1:1} \cdot S_0 \qquad \text{Eq. 1}$$

$$[S_t] - [S_0] = K_{1:1}[S_0][CD] + K_{1:1}K_{1:2}[S_0][CD]^2 \qquad \text{Eq. 2}$$

where $S_0$ is intrinsic solubility of CCB and $S_t$ is the concentration of CCB at a given CD concentration [CD].

*To determine the effect of polymer on solubility of CCB/CD complexes* the phase solubility of CCB was determined in pure aqueous γCD and RMβCD solutions containing various water-soluble polymers. HPMC (0.1%), HA (0.01%) or CS (0.1%) (all %w/v) were selected as non-ionic, negatively and positively charged polymer, respectively.

***Solid-state characterization:***

Aqueous solutions containing CCB/CD complexes of 1:1 molar ratio with or without polymer (0.01%w/v HA or 0.1 %w/v HPMC) were prepared and then lyophilized (FD). Identical physical mixtures (PM) were prepared by careful blending of CCB and CD in a mortar with pestle. The samples were characterized in solid state as follows: pure CCB, γCD and RMβCD, PM and FD of CCB/CD (CCB/γCD and CCB/RMβCD), and FD ternary complexes CCB/γCD/HA, CCB/γCD/HPMC, CCB/RMβCD/HA and CCB/RMβCD/HPMC. The DSC, PXRD and FT-IR were performed.

***Solution state characterization:***

The pure solid samples of CCB, γCD and RMβCD as well as of the CCB/CD complexes, that is CCB/γCD and CCB/RMβCD, which were prepared by freeze drying as described above, were characterized in a solution. The samples were dissolved in a capillary tube containing $CD_3OD$:$D_2O$ (50:50 v/v) and measured by $^1$H-NMR spectroscopy.

## CONCLUSIONS

- The solubility of celecoxib (CCB), a poorly water-soluble drug, can be enhanced through formation of cyclodextrin (CD) inclusion complexes.
- RMβCD, a relatively lipophilic γCD derivative, exhibited the highest complexation efficiency. HPMC had synergistic effect on the CD solubilization via ternary complex formation and resulted in the highest solubilizing efficiency.
- Formation of CCB/CD and CCB/CD/polymer complexes were verified by solid and solution characterizations especially in NMR studies.

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## RESULTS AND DISCUSSION

### *Solubility determinations*

Apparent stability constant values ($K_{1:1}$ and $K_{1:2}$) and the apparent complexation efficiency (CE) of celecoxib/cyclodextrin complexes in pure aqueous cyclodextrin solutions in the absence or presence of polymer

| Cyclodextrin | Type | $K_{1:1}$ ($M^{-1}$) | $K_{1:2}$ ($M^{-1}$) | CE |
|---|---|---|---|---|
| αCD | $A_p$ | 49.9 | 26.2 | 0.00014[a] |
| βCD | $A_L$ | 760.2 | - | 0.00215 |
| γCD | $A_L$ | 155.3 | - | 0.00044 |
| HPβCD | $A_L$ | 2,630.2 | - | 0.00746 |
| RMβCD | $A_p$ | 3,139.9 | 157.3 | 0.00890[a] |

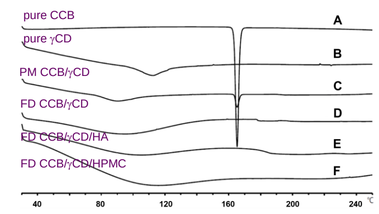
[a]Obtained from the initial linear part of the phase-solubility diagram.

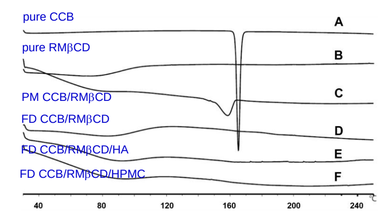
- For γCD and RMβCD, they indicated that the formation of higher order soluble complexes with respect to CD at elevated CD concentrations.
- RMβCD gave the highest CE and γCD is well tolerated and possesses favorable toxicological profile. Thus, they were selected to further studies.

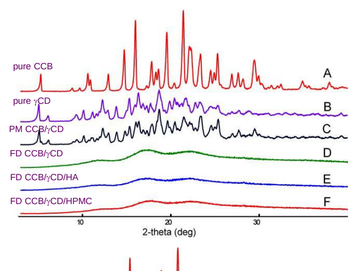
| Cyclodextrin | Polymer (%w/v) | $S_0$ (mM) | Type | $K_{1:1}$ ($M^{-1}$) | $K_{1:2}$ ($M^{-1}$) | CE | CE ratio |
|---|---|---|---|---|---|---|---|
| γCD | - | 0.0028 | $A_L$ | 155.3 | - | 0.00044 | 1.00 |
| | 0.1 % CS | 0.0032 | $A_L$ | 170.9 | - | 0.00054 | 1.23 |
| | 0.01 % HA | 0.0028 | $A_L$ | 196.4 | - | 0.00055 | 1.25 |
| | 0.1 % HPMC | 0.0275 | $A_L$ | 307.5 | - | 0.00847 | 19.24 |
| RMβCD | - | 0.0028 | $A_p$ | 3,140 | 141 | 0.00890 | 1.00 |
| | 0.1 % CS | 0.0032 | $A_p$ | 2,846 | 136 | 0.00900 | 1.01 |
| | 0.01 % HA | 0.0028 | $A_p$ | 3,533 | 120 | 0.00990 | 1.11 |
| | 0.1 % HPMC | 0.0275 | $A_p$ | 3,564 | 18.3 | 0.09820 | 11.03 |

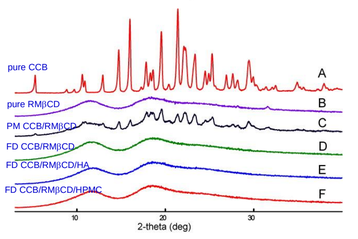
- The addition of polymers to the complexing media did not alter the type of the phase solubility diagram indicating that the presence of polymers did not alter the stoichiometry of the complex formed.
- The effect of polymers on the CE of both complexes showed the same trend: HPMC > HA > CS.
- HPMC showed the highest increments in the apparent solubilizing efficiency (CE ratio) of CCB about 11-folds and 19-folds increasing in the CE of CCB/RMβCD and CCB/γCD, respectively.
- The dramatically increasing CE of CCB/RMβCD with HPMC as ternary complex may be possibly caused by the interaction of multi-components.

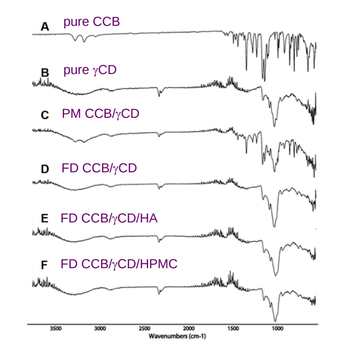
### *Solid-state characterization*

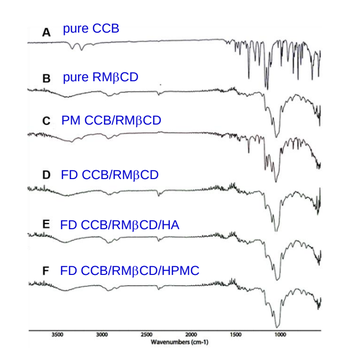



***DSC thermograms:***

- In case of PM, the endothermic peak of the CCB in PM CCB/γCD was retained at 165.17ºC while the peak of the CCB in PM CCB/RMβCD was a broad peak and slightly shifted to lower temperature appeared at 158.67ºC.
- For FD samples, the endothermic peak of binary and ternary complexes were absent. The disappearance of an endothermic peak may be attributed to an amorphous state and/or presence of inclusion CCB/CD complex in the solid state [5].

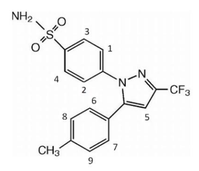
***PXRD:***

- Most of the principal peaks of CCB were presented in the diffraction patterns of PM of both CCB/γCD and CCB/RMβCD. This indicates that there was no interaction between the pure CCB and the respective CDs.
- In contrast FD samples of binary and ternary complexes showed a halo pattern with the disappearance of all the peaks corresponding to CCB. The transformation of CCB from the crystalline to the amorphous form indicates formation of CCB/CD inclusion complexes [6]. However, it should be mentioned that the freeze drying technique applied during sample preparation may affect transformation of the solid state [7].

***FT-IR spectra:***

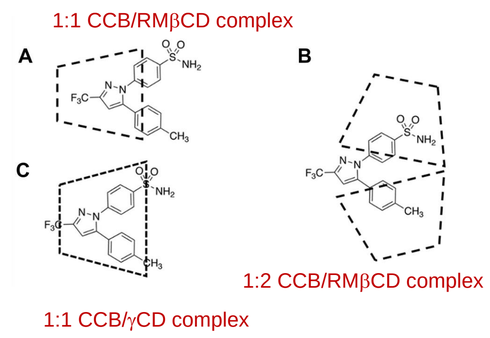
- The FT-IR spectrum of CCB in PM sample showed some peaks were slightly shifted. These observations indicate that there was less interaction between CCB and CD in the PM samples.
- For FD samples of both the binary complexes and the ternary complexes, the N-H stretching bands of CCB indicate masking of characteristic symmetric and asymmetric stretch. Likewise, the C-F stretching band and S=O stretching bands of CCB disappeared in the FD samples.
- These results may be ascribed to the existence of some interaction between functional groups (sulfonamide group or -$CF_3$ group) of CCB and functional groups in the hydrophobic CD cavities in the inclusion complexes [8].

### *Solution-state characterization*










***The proposed conformation structures of CCB/RMβCD and CCB/γCD inclusion complexes:***

**Fig. A.** 1:1 inclusion complex where the pyrazole head group of CCB is included through the wide opening of the RMβCD central cavity.
**Fig. B.** 1:2 inclusion complex where the CCB is included in to the cavity of the CD dimer in a configuration where half of the CCB molecule was embedded in one monomer and another half is embedded in the other RMβCD monomer of CD.
**Fig. C.** The Δδ* of H5 in both CCB and γCD were significantly shifted and all aromatic protons displayed upfield shift. This indicates that CCB is more deeply included into γCD cavity than that of RMβCD, possibly due to the larger inner cavity diameter of γCD (7.5-8.3 Å) in comparison to that of RMβCD (6 Å).

**Acknowledgement:**

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# Development of Celecoxib Eye Drop Solution and Microsuspension for Targeted Delivery to the Posterior Segment of The Eye

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**Abstract**

Celecoxib (CCB), a nonsteroidal anti-inflammatory drug (NSAID), can be beneficial in the treatment of age-related macular degeneration (AMD) and diabetic retinopathy (DR) through the inhibition of COX-2 enzyme. Currently, several methods are used to deliver drugs to the back of the eye, such as oral administration, intravitreal injection, subconjunctival injection and topical application to the eye surface. Of these, the non-invasive topical eye drop administration is preferred. However, the low aqueous solubility of CCB hampers its ocular bioavailability. Thus, the aim of this study was to develop topical eye drop formulations containing cyclodextrin (CD) and polymer. The CCB eye drops formulations were prepared without heating and by a heating method (sonication at the temperature of 70°C for 1 hr). The physicochemical and chemical properties of the aqueous eye drops were determined as well as their mucoadhesive properties and in-vitro drug release. The physicochemical properties, that is their appearance, pH, osmolality and viscosity, were within acceptable range. The mean particle diameters were below 10 μm or well below the particle size irritating threshold for eye drops. The formulations containing hyaluronic acid (HA) displayed excellent mucoadhesive properties. The increasing CCB content of the eye drops, obtained by heat treatment to form ternary CCB/CD/polymer complex, resulted in higher drug permeation through a semipermeable membrane and simulated artificial vitreous humor, especially from the formulation containing randomly methylated βCD and HA (0.5% w/v).

**Keywords:** cyclodextrin, celecoxib, eye drop, microsuspension

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# Development of celecoxib eye drop solution and microsuspension for targeted delivery to the posterior segment of the eye

เอกสารแนบหมายเลข 2.2 ฉบับที่ 2

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## INTRODUCTION

Presently, age-related macular degeneration (AMD) and diabetic retinopathy (DR) are the two predominant causes of vision impairment [1]. Celecoxib (CCB) has VEGF inhibitory and anti-proliferative activity that could be beneficial in the treatment of the proliferative stages of DR and AMD [2,3]. The aim is to provide sustained drug release and mucoadhesive properties to improve ocular drug bioavailability. CCB eye drop formulations (i.e. solutions and suspensions) were developed. The physicochemical properties of the eye drops were characterized as well as their mucoadhesive properties and ability to enhance drug delivery through membrane barriers.

## EXPERIMENTAL METHODS

### *Formulation of CCB ophthalmic preparations:*

**A**:0.1% CCB ophthalmic solutions containing 7.5% RMβCD with polymer (HPMC or HA) were prepared. The formulations went through a heating process in an autoclave (121°C for 20 min).

**B**:0.5% CCB ophthalmic suspensions were prepared by suspending CCB in an aqueous CD solution (7.5% w/v RMβCD or 10% w/v γCD). The particle size reduction by mixer mill.

- **$B_1$**:*Binary CCB/CD complex* - the polymer was then added and mixed until the polymer was dissolved.
- **$B_2$**:*Ternary CCB/CD/polymer complex* - prior to adjust the final volume, the obtained CCB suspensions were sonicated in an ultrasonic bath at 70°C for 1 hour.

### *Physicochemical and chemical characterizations of CCB eye drop formulations:*

pH, viscosity, osmolality, re-dispersion time, total drug content and dissolved drug content were determined.

### *In vitro mucoadhesive studies:*

The mucoadhesive properties of the formulations were evaluated using the equipment described by Bin Choy et. al [4].

### *The morphology, particle size and size distribution analysis:*

The particle size and size distribution was determined by DLS and the morphology was examined by SEM.

### *In vitro permeation studies:*

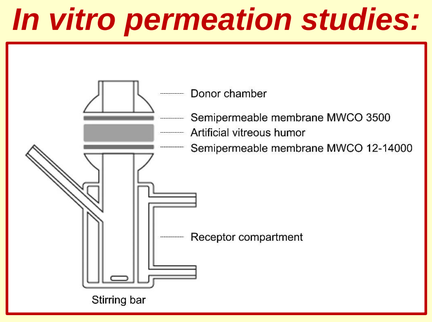


**Fig. 1.** Simulated artificial vitreous humor model.

The *in vitro* permeation of CCB from the test formulations was performed in a Franz diffusion cell. The donor and receptor compartments were separated by the semipermeable membrane MWCO 12–14,000 Da. *In vitro* permeation through simulated artificial vitreous humor was also determined as depicted in Fig. 1.

## CONCLUSIONS

- The solid particle sizes of suspensions were below 10 µm which indicated that CCB/CD ophthalmic microsuspensions will not irritate the eye surface.
- In most cases, when the heating method was applied during preparation of the eye drops, the amount of dissolved drug was increased that lead to enhanced drug permeation through the artificial membranes.
- The microsuspension eye drops containing ternary CCB/RMβCD/HA aggregates showed relatively good mucoadhesion and provided the highest drug flux Therefore, it can be good candidate for further in vivo studies to deliver CCB to the posterior segment of the eye.

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## ACKNOWLEDGEMENT

This work was financially supported by Thailand Research Fund with grant No. RSA5980050 and by the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

## RESULTS AND DISCUSSION

### *Physicochemical and chemical characterizations of CCB eye drop formulations:*

- pH, viscosity, osmolality, re-dispersion time, total drug content of all formulations were within the acceptable range.
- The solid drug content (free CCB and solid CCB/CD complex) in formulations containing RMβCD was determined to be about 85-90% while more than 99% of the drug was in the solid state in formulations containing γCD

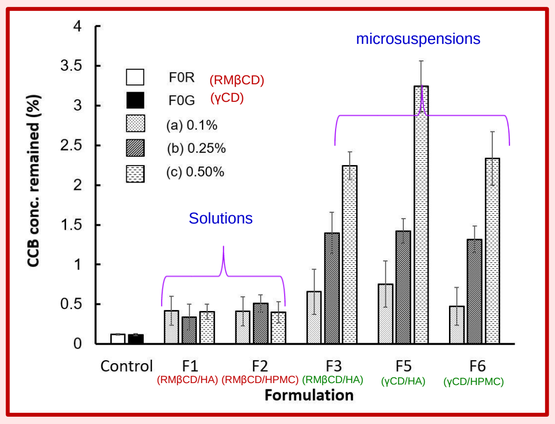



**Fig. 2.** Percent (% w/w) drug remaining in each celecoxib eye drop formulation on the mucin coated membrane; a, b, c: percentage of polymer in formulation.

### *In vitro mucoadhesive studies:*

- The mucoadhesive properties of CCB/CD eye drop solutions (i.e. F1a-F2c) were not statistically different.
- CCB/CD microsuspensions containing 0.5% w/v HA or HPMC (F3c, F5c and F6c) provided good mucoadhesive properties that will increase the drug residence time that can lead to enhance ocular drug bioavailability.

### *The morphology, particle size and size distribution analysis:*

- All eye drop formulations displayed a trimodal particle size distribution. An intensity peak at
  - 1st peak (1–2 nm): dissolved non-aggregated CCB/CD complex (i.e. monomeric complex)
  - 2nd and 3rd peak (10-80 and 180-400 nm, respectively): the complex aggregates of CCB/CD/polymer
- The formulations prepared by the heating method (F3cSO, F5cSO and F6cSO) displayed relatively larger complex aggregates than those of unheated formulations.

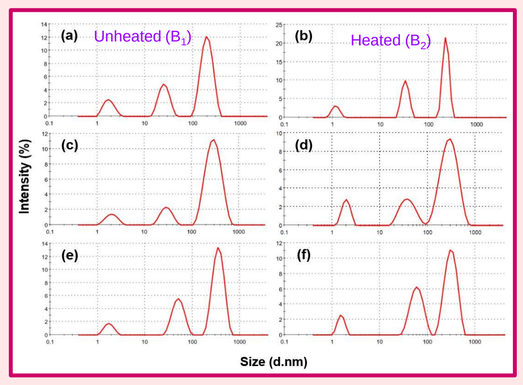



**Fig. 3.** The aggregate size and size distribution of CCB/CD complex particles in the eye drop formulations determined at 25°C by DLS; (a) F3c; (b) F3cSO; (c) F5c; (d) F5cSO, (e) F6c; (f) F6cSO.

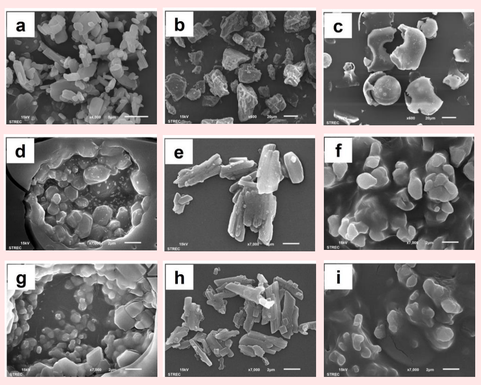



**Fig. 4.** SEM photographs of intact materials and the celecoxib eye drop formulations prepared with and without heating; (a) CCB; (b) γCD; (c) RMβCD; (d) F3c; (e) F5c; (f) F6c; (g) F3cSO, (h) F5cSO; (i) F6cSO.

- Both intact CCB and γCD display crystalline structures while RMβCD is amorphous (Figs 4a-4c).
- The particles size of the heated F3cSO, F5cSO and F6cSO are slightly smaller than those of unheated ones
- The particle size of all CCB formulations tested (heated and unheated) is less than 8 µm, which is acceptable and non-irritating to the eye [5].

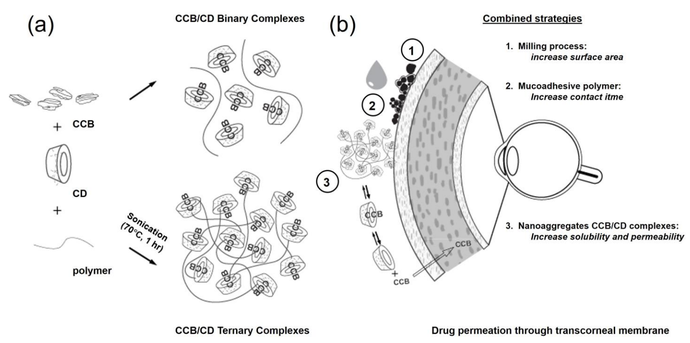
### *In vitro permeation studies:*

**Table 1.** Flux (J) and apparent permeation coefficient ($P_{app}$) of celecoxib from the eye drop suspensions, prepared with and without heating, through semi-permeable membrane (MWCO 12-14,000 Da) and through artificial vitreous humor.

| Formulation | Dissolved CCB content (%) | Through semi-permeable membrane | | Through artificial vitreous humor | |
|---|---|---|---|---|---|
| | | Flux (µg/hr/cm²) | $P_{app}$ (cm/hr) | Flux (µg/hr/cm²) | $P_{app}$ (cm/hr) |
| F3c | 10.18 ± 0.17 | 12.17 ± 0.16 | 0.0239 ± 0.0002 | 1.55 ± 0.20 | 0.0030 ± 0.0004 |
| F5c | 0.67 ± 0.16 | 0.94 ± 0.06 | 0.0287 ± 0.0057 | 0.11 ± 0.05 | 0.0033 ± 0.0012 |
| F6c | 0.47 ± 0.01 | 0.79 ± 0.02 | 0.0341 ± 0.0024 | 0.17 ± 0.05 | 0.0075 ± 0.0027 |
| F3cSo | 46.98 ± 2.27 | 26.72 ± 1.74 | 0.0114 ± 0.0010 | 2.98 ± 0.16 | 0.0013 ± 0.0001 |
| F5cSo | 1.33 ± 0.14 | 0.93 ± 0.10 | 0.0141 ± 0.0017 | 0.14 ± 0.01 | 0.0022 ± 0.0003 |
| F6cSo | 3.39 ± 0.16 | 3.06 ± 0.14 | 0.0181 ± 0.0008 | 0.34 ± 0.01 | 0.0020 ± 0.0002 |

- The flux values from F3cSO and F6cSO were 2-folds and 4-folds larger than from F3c and F6c, respectively.
- $P_{app}$ of CCB from all the heated formulations were lower than from unheated formulation. It is possible that bonds were activated between the different complex components (i.e. CCB, CD and water-soluble polymers) during the heating process.
- Comparison to the semipermeable membrane, about 10-20% of drug passed through artificial vitreous humor. It might be related to CCB formed CD complex aggregates (equal or larger than trimer of CCB/CD complexes) resulting in lower the permeation flux.

Owing to the size reduction provided the large surface area of the particles and the nanoaggregates of the complexes enhanced the CCB solubility, in addition of mucoadhesive polymer could remain adherent to the ocular surface, the CCB bioavailability will be achieved to the targeted tissues.




However, Non-corneal drug absorption by diffusion of drug molecules through the conjunctiva, sclera and choroid, as well as systemic absorption from the conjunctival blood vessels and re-entering to the retina, occur after topical eye drop application [6].

**Fig. 5** Schematic illustration of the drug delivery pathway from ternary CCB/CD/polymer complexes obtained by the combination of milling and heating process to the posterior segment to the eye.

# Development of Celecoxib Eye Drop Solution and Microsuspension for Targeted Delivery to the Posterior Segment of The Eye

Phatsawee Jansook[1,*], Pakin Kulsirachote[1], Thorsteinn Loftsson[2]

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Celecoxib (CCB), a nonsteroidal anti-inflammatory drug (NSAID), can be beneficial in the treatment of age-related macular degeneration (AMD) and diabetic retinopathy (DR) through inhibition of COX-2 enzyme [1,2]. Currently, several methods are used to deliver drugs to the back of the eye, such as oral administration, intravitreal injection, subconjunctival injection and topical application to the eye surface. Of these, the non-invasive topical eye drop administration is preferred. However, the low aqueous solubility of CCB hampers its ocular bioavailability. Thus, the aim of this study was to develop topical eye drop formulations containing cyclodextrin (CD) and polymer. The CCB eye drops formulations were prepared without heating and by a heating method (sonication at the temperature of 70°C for 1 h) [3]. The physicochemical and chemical properties of the aqueous eye drops, their mucoadhesive properties, *in vitro* and *ex-vivo* permeation and the cytotoxicity test were determined. The physicochemical properties, that are their appearance, pH, osmolality and viscosity, were within acceptable range. The mean particle diameters were below 10 µm which well below the particle size irritating threshold for eye drops [4]. The formulations containing hyaluronic acid (HA) displayed excellent mucoadhesive properties. The increasing CCB content of the eye drops, obtained by heat treatment to form ternary CCB/CD/polymer complex, resulted in higher drug permeation through a semipermeable membrane, simulated artificial vitreous humor and rabbit and porcine scleral tissues, especially from the formulation containing randomly methylated βCD and HA (0.5% w/v). The cell viability measured by MTT demonstrated that all selected CCB eye drop formulations were not cytotoxic to human retinal pigment epithelial (RPE) cells.

## Acknowledgments

This work was financially supported by Thailand Research Fund with grant No. RSA5980050 and Faculty of Pharmaceutical Sciences, Chulalongkorn University.

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[4] I. Pal Kaur, M. Kanwar, Drug Dev. Ind. Pharm. 28, 473-493, 2002.

# Development of celecoxib eye drop solution and microsuspension for targeted delivery to the posterior segment of the eye

เอกสารแนบหมายเลข 2.3 ฉบับที่ 2


**Phatsawee Jansook[1*], Pakin Kulsirachote[1], Thorsteinn Loftsson[2]**

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## INTRODUCTION

Presently, age-related macular degeneration (AMD) and diabetic retinopathy (DR) are the two predominant causes of vision impairment [1]. Celecoxib (CCB) has VEGF inhibitory and anti-proliferative activity that could be beneficial in the treatment of the proliferative stages of DR and AMD [2,3]. The aim is to provide sustained drug release and mucoadhesive properties to improve ocular drug bioavailability. CCB eye drop formulations (i.e. solutions and suspensions) were developed. The physicochemical properties of the eye drops were characterized as well as their mucoadhesive properties and ability to enhance drug delivery through membrane barriers.

## EXPERIMENTAL METHODS

### *Formulation of CCB ophthalmic preparations:*

**A**:0.1% CCB ophthalmic solutions containing 7.5% RMβCD with polymer (HPMC or HA) were prepared. The formulations went through a heating process in an autoclave (121°C for 20 min).

**B**:0.5% CCB ophthalmic suspensions were prepared by suspending CCB in an aqueous CD solution (7.5% w/v RMβCD or 10% w/v γCD). The particle size reduction by mixer mill.

- **$B_1$**:*Binary CCB/CD complex* - the polymer was then added and mixed until the polymer was dissolved.
- **$B_2$**:*Ternary CCB/CD/polymer complex* - prior to adjust the final volume, the obtained CCB suspensions were sonicated in an ultrasonic bath at 70°C for 1 hour.

### *Physicochemical and chemical characterizations of CCB eye drop formulations:*

pH, viscosity, osmolality, re-dispersion time, total drug content and dissolved drug content were determined.

### *In vitro mucoadhesive studies:*

The mucoadhesive properties of the formulations were evaluated using the equipment described by Bin Choy et. al [4].

### *The morphology, particle size and size distribution analysis:*

The particle size and size distribution was determined by DLS and the morphology was examined by SEM.

### *Ex-vivo permeation studies:*

The *Ex-vivo* permeation of CCB from the test formulations was performed in a Franz diffusion cell. The donor and receptor compartments were separated by the sclera isolated from rabbit and porcine eyes.

### *Cell viability*

Retina cell line, ARPE-19 was used to study the cell viability by MTT assay. The optical density (OD) of each well was measured at 570 nm and the % cell viability was calculated.

## CONCLUSIONS

- The solid particle sizes of suspensions were below 10 µm which indicated that CCB/CD ophthalmic microsuspensions will not irritate the eye surface.
- In most cases, when the heating method was applied during preparation of the eye drops, the amount of dissolved drug was increased that lead to enhanced drug permeation through the artificial membranes.
- The microsuspension eye drops containing ternary CCB/RMβCD/HA aggregates showed relatively good mucoadhesion and provided the highest drug flux Therefore, it can be good candidate for further *in vivo* studies to deliver CCB to the posterior segment of the eye.

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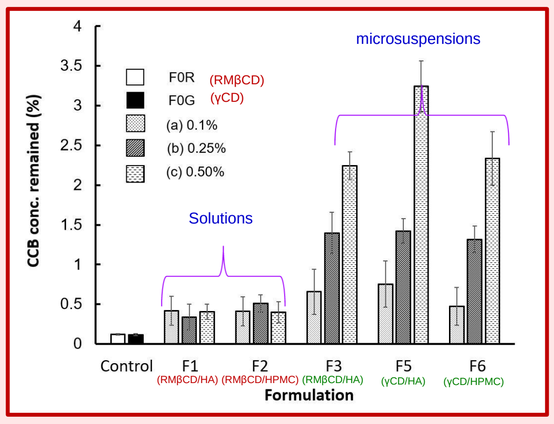


**Fig. 1.** Percent (% w/w) drug remaining in each celecoxib eye drop formulation on the mucin coated membrane; a, b, c: percentage of polymer in formulation.

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- CCB/CD microsuspensions containing 0.5% w/v HA or HPMC (F3c, F5c and F6c) provided good mucoadhesive properties that will increase the drug residence time that can lead to enhance ocular drug bioavailability.

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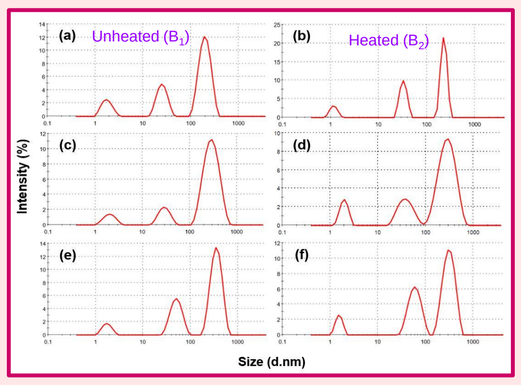


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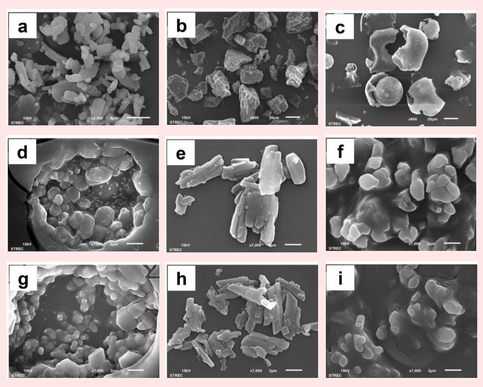


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- Both intact CCB and γCD display crystalline structures while RMβCD is amorphous (Figs 4a-4c).
- The particles size of the heated F3cSO, F5cSO and F6cSO are slightly smaller than those of unheated ones
- The particle size of all CCB formulations tested (heated and unheated) is less than 8 µm, which is acceptable and non-irritating to the eye [5].

### *Ex-vivo permeation studies:*

**Table 1.** Flux (J) and apparent permeation coefficient ($P_{app}$) of celecoxib from the eye drop suspensions, through rabbit and porcine scleral tissues.

| Formulation | Dissolved CCB content (%) | Rabbit | | Porcine | |
|---|---|---|---|---|---|
| | | Flux (µg/hr/cm²) | $P_{app}$ (cm/hr) | Flux (µg/hr/cm²) | $P_{app}$ (cm/hr) |
| F3cSo | 46.98 ± 2.27 | 13.36 ± 2.15 | 0.57 ± 0.01 | 6.08 ± 0.95 | 0.26 ± 0.04 |
| F5cSo | 1.33 ± 0.14 | n.d. [a] | n.d.[a] | 0.12 ± 0.03 | 0.18 ± 0.05 |
| F6cSo | 3.39 ± 0.16 | 1.37 ± 0.27 | 0.80 ± 0.16 | 0.49 ± 0.21 | 0.29 ± 0.12 |

[a] n.d. = not determined

- F3cSo provided the highest flux of CCB followed by F6cSo and F5cSo, respectively.
- These results may represent that the CCB eye drop microsuspensions could have the potential to deliver CCB to the posterior segment of the eye.

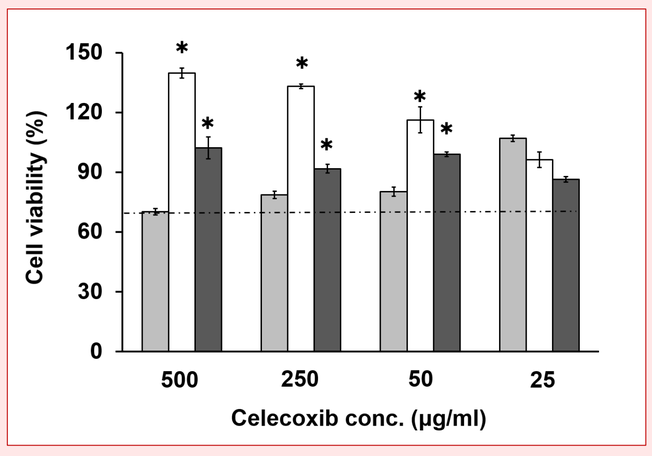


**Fig. 4. Viability of ARPE-19 cells after incubation with celecoxib eye drop micro-suspensions. F3cSO ( ); F5cSO ( ); F6cSO ( )**

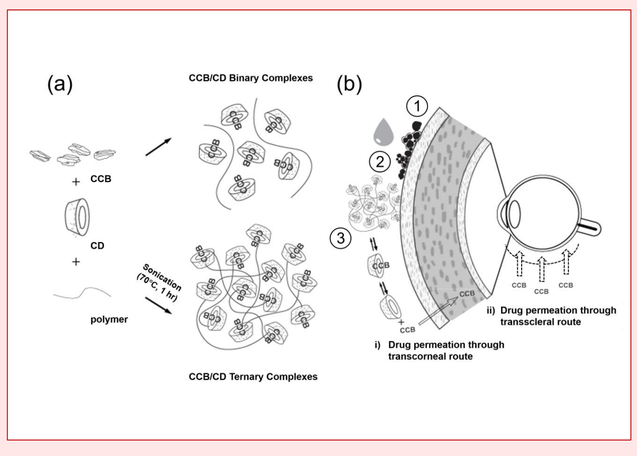


**Fig. 5** Schematic illustration of the drug delivery pathway from ternary CCB/CD/polymer complexes obtained by the combination of milling and heating process to the posterior segment to the eye.

- No cytotoxicity (> 70% cell viability) were noted after 24-hour incubation of CCB eye drop microsuspensions with the cells.
- The CCB eye drop formulations containing γCD (F5cSO and F6cSO) have significantly lower toxicity than the formulation containing RMβCD (F3cSO)

Owing to the size reduction provided the large surface area of the particles and the nanoaggregates of the complexes enhanced the CCB solubility, in addition of mucoadhesive polymer could remain adherent to the ocular surface, the CCB bioavailability will be achieved to the targeted tissues.

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ORIGINAL ARTICLE

# Cyclodextrin solubilization of celecoxib: solid and solution state characterization

**Phatsawee Jansook**[1] · **Pakin Kulsirachote**[1] · **Thorsteinn Loftsson**[2]

Received: 6 June 2017 / Accepted: 21 November 2017 / Published online: 24 November 2017


**Abstract**
The low aqueous solubility of celecoxib (CCB) hampers its oral bioavailability and permeation from aqueous environment through biological membranes. The aim of this study was to enhance the aqueous solubility of CCB by complexation with cyclodextrin (CD) in the presence of water-soluble polymer. The effects of different CDs (αCD, βCD, γCD, 2-hydroxypropyl-β-cyclodextrin and randomly methylated β-cyclodextrin (RMβCD)) and mucoadhesive, water-soluble polymers (hydroxypropyl methylcellulose (HPMC), chitosan and hyaluronic acid) were investigated. The phase solubility profiles and CCB/CD complex characteristics were determined. RMβCD exhibited the greatest solubilizing effect of the two CDs tested. However, γCD was also selected for further investigations due to its safety profile. Addition of polymer to the aqueous CD solutions enhanced the CD solubilization. Formation of CCB/RMβCD/HPMC and CCB/γCD/HPMC ternary complexes resulted in 11 and 19-fold enhancement in the apparent complexation efficiency in comparison to their CCB/CD binary complex, respectively. The size of ternary complex aggregates in solution were determined to be from about 250 to about 350 nm. The data obtained from Fourier transform infra-red, differential scanning calorimetry and powder X-ray diffraction indicated presence of CCB/CD inclusion complexes in the solid state. Proton nuclear magnetic resonance data demonstrated that CCB was partially and totally inserted into the hydrophobic central cavities of RMβCD and γCD.

**Keywords** Cyclodextrins · Celecoxib · Solubility · Complexation · Aggregates

## Introduction

Celecoxib (CCB), a nonsteroidal anti-inflammatory drug (NSAID), is the first selective cyclooxygenase-2 (COX-2) inhibitor used to treat osteoarthritis and rheumatoid arthritis in adult patients. It is also indicated in treatment of acute pain and primary dysmenorrhea as well as an adjuvant in the treatment of familial adenomatous polyposis, a genetic disorder [1]. CCB is a low molecular weight (381.38 Da) hydrophobic molecule with a log P 3.47 at pH 7.4. With a $pK_a$ of 11.1, CCB is neutral at physiologic pH and is practically insoluble. The aqueous solubility of CCB is approximately 2 μg/ml [2, 3]. The very low aqueous solubility hampers CCB permeation through biological membranes resulting in low bioavailability and inadequate drug levels at its site of action. Several techniques are used to enhance aqueous solubility of poorly soluble drugs and their dissolution including (1) pH adjustments, (2) particle size reductions, (3) formation of inclusion complexes, (4) co-solvency and (5) micellar solubilization [4]. Here the usage of cyclodextrins (CDs) as complexing agents to enhance aqueous solubility of CCB was investigated.

CDs are cyclic oligosaccharides consist of (α-1,4-)-linked α-D-glucopyranose units with a hydrophilic outer surface and a lipophilic central cavity. The CD molecules are cone shaped with secondary hydroxy groups protruding from the wider edge and the primary groups from the narrow edge. The three most common natural CDs consist of six, seven and eight glucopyranose units and named α-cyclodextrin (αCD), β-cyclodextrin (βCD) and γ-cyclodextrin (γCD), respectively. In addition, random substitution of the hydroxy groups brings about dramatic improvements in their solubility. CD derivatives of pharmaceutical interest include 2-hydroxypropyl-β-cyclodextrin

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(HPβCD), 2-hydroxypropyl-γ-cyclodextrin (HPγCD), randomly methylated β-cyclodextrin (RMβCD) and sulfobutylether β-cyclodextrin (SBEβCD). The CDs have molecular weights from about 1000 to about 2000 Da and a large negative Log P (i.e. logarithm of the octanol/water partition coefficient). Thus, they are poorly absorbed through biological membranes [5]. Various lipophilic drugs can form inclusion complexes with CDs by inserting some lipophilic moieties of the drug molecules into the lipophilic CD cavity. Formation of such hydrophilic drug/CD inclusion complexes increases the drug solubility in aqueous media. Rawat and Jain [6] conducted a study of CCB/βCD complexes. Based on the reported phase-solubility profile, the solubility of CCB can be improved by βCD. Moreover, this study indicated that the dissolution rate of solid CCB/βCD complexes was significantly increased when compared to pure solid CCB. Furthermore, the water-soluble βCD derivative HPβCD has been shown to enhance the dissolution rate of CCB in aqueous media [7].

Recent studies have shown that the addition of hydrophilic polymers to aqueous complexation media can significantly increase CD solubilization of poorly soluble drugs through ternary complex formation [8]. In a synergistic mode the water-soluble polymers can decrease the amount of CD required to prepare soluble drug/CD complexes and obtain drug solution of a given concentration [9]. Using heating methods, such as autoclaving at 121 °C for 20–40 min or sonication at temperatures above 70 °C for 1 h, can accelerate the ternary complex formation [10, 11]. The influence of hydrophilic polymers on CCB/HPβCD complexes and formation of CCB/HPβCD/polymer ternary complex has been investigated [12]. It was concluded that polyvinylpyrrolidone (PVP), hydroxypropyl methylcellulose (HPMC) and polyethylene glycol (PEG) stabilize the binary CCB/CD complexes with a consequent increase in the CCB solubility. Beside their solubilizing effect, CDs can act as permeation enhancers and increase drug permeation through biological membranes. However, previous studies on CCB/CD complexes have focused on solid dosage forms and the ability of CDs to enhance CCB bioavailability after oral administration. Some drug formulations, such as those for ophthalmic, nasal and parenteral administration, have volume limitations and, thus, frequently require a potent drug solubilizer. The focus of this present study is on evaluation of the ability of different CDs to solubilize CCB and characterization of both the solid state and the dissolved CCB/CD complexes, and their ability to form nano- and microparticle drug delivery systems.

## Materials and methods

### Materials

Celecoxib (CCB) was generously donated by Unison Laboratories Co., Ltd., Thailand. α-Cyclodextrin (αCD), β-cyclodextrin (βCD), γ-cyclodextrin (γCD) and randomly methylated β-cyclodextrin (RMβCD) MS 1.8 (MW 1312 Da) were purchased from Wacker Chemie (Germany), 2-hydroxypropyl-β-cyclodextrin (HPβCD) MS 0.6 (MW 1400 Da) from Roquette (France), chitosan (CS) from Fluka Fine Chemical (Japan), hyaluronic acid (HA) from Soliance (France) and hydroxypropyl methylcellulose 4000 (HPMC) from Sigma (USA). All other chemicals used were of analytical reagent grade purity. Milli-Q (Millipore, USA) water was used for preparation of all solutions.

### Stability evaluation

The stability of CCB in aqueous solution containing 0.5% w/v βCD was determined by heating method [10]. Excess amount of CCB was dissolved in purified water and equilibrated at 30 ± 1 °C for 24 h under constant agitation. The supernatant was filtered through 0.45 μm nylon filter and mixed with 1% w/v βCD solution at 1:1 ratio. The solution was then divided into three sealed vials that were heated in an autoclave for one, two and three heating cycles, each cycle consisted of heating to 121 °C for 20 min. The drug concentrations in the vials were then determined by a high-performance liquid chromatographic method (HPLC).

### Quantitative determinations

Quantitative determinations of CCB were performed on a reversed-phase HPLC component system from Shimadzu consisting of a LC-20AB binary pump, a SPD-20A multiple wavelength detector, a SIL-20ATH auto sampler with LCsolution software, and Shiseido C18 column, 5 μm, 4.6 × 150 mm ID with C18 guard cartridge column MGII 5 μm, 4 × 10 mm. The modified HPLC condition [3] was as follows; mobile phase: acetonitrile:water (55:45); flow rate: 1.0 ml/min; oven temperature: ambient; UV detector wavelength: 252 nm; injection volume: 20 μl; and run time: 15 min. The HPLC method was validated and shown to be consistent, reliable and accurate.

### Solubility determinations

Excess amount of CCB was added to solutions containing from 0 to 15% (w/v) CD (αCD, βCD, γCD, HPβCD or

RMβCD) in pure water. The drug suspensions were saturated the drug through heating in an autoclave 121 °C for 20 min and allowed to cool to room temperature [10]. Then a small amount of solid drug was added to the suspensions to promote drug precipitation. The suspensions were equilibrated at 30 ± 1 °C for 7 days under constant agitation. After equilibrium was attained, the suspensions were filtered through 0.45 µm syringe filter, the filtrates were diluted with the mobile phase and analyzed by HPLC. The determinations were done in triplicate. The apparent stability constants for CCB/CD complexes ($K_{1:1}$ and/or $K_{1:2}$) and the complexation efficiency (CE) were determined according to the phase-solubility method of Higuchi and Connors [13].

$$CE = \frac{Slope}{1 - Slope} = \frac{[Drug/CD\ Complex]}{[CD]} = K_{1:1} \cdot S_0 \quad (1)$$

$$[S_t] - [S_0] = K_{1:1}[S_0][CD] + K_{1:1}K_{1:2}[S_0][CD]^2 \quad (2)$$

where $S_0$ is intrinsic solubility of CCB and $S_t$ is the concentration of CCB at a given CD concentration [CD].

To determine the effect of polymer on solubility of CCB/CD complexes the phase solubility of CCB was determined in pure aqueous γCD and RMβCD solutions containing various water-soluble polymers. HPMC (0.1%), HA (0.01%) or CS (0.1%) (all % w/v) were selected as non-ionic, negatively and positively charged polymer, respectively. The polymer was dissolved in the aqueous solutions containing 0–10% w/v CD. Then the excess of CCB was added to form drug suspensions and the suspensions heated in an autoclave to 121 °C for 20 min. $K_{1:1}$, $K_{1:2}$ and CE were calculated were calculated from the phase-solubility diagrams obtained.

## Morphology, particle size and zeta potential analysis

The morphology, size and size distribution, and zeta potential of the aggregates formed in aqueous complexation media containing 10% (w/v) γCD or 7.5% (w/v) RMβCD, with or without polymer (0.01% w/v HA and 0.1% w/v HPMC), were analyzed using transmission electron microscope and dynamic light scattering.

### Dynamic light scattering (DLS) measurement

The particle size of CCB/CD based aggregates in solution was measured by DLS technique (Zetasizer Nano ZS with software version 7.11, Malvern, UK). The samples were diluted with purified water immediate before analysis. A sample was put in a quartz glass cuvette and placed in the instrument. Measurements were carried out at 25 °C and 180° scattering angle. Particle size and size distribution and zeta potential were automatically calculated and analyzed by the software included within the system. Each measurement was carried out in triplicate.

### Transmission electron microscope (TEM) analysis

The morphology of CCB/CD based aggregates was evaluated by TEM (JEOL, JEM-2100F, USA). Initially, the sample was placed on a formvar-coated grid. After blotting the grid with a filter paper, the grid was transferred onto a drop of negative straining. Aqueous phosphotungstic acid solution (2%) was used as a negative strain. The sample was air dried at room temperature. Finally, the samples were examined with TEM.

## Preparation and characterization of binary CCB/CD complexes and ternary CCB/CD/polymer complexes

### Solid-state characterization

**Sample preparations** Aqueous solutions containing CCB/CD complexes of 1:1 molar ratio (m:n; $D_mCD_n$ where m and n represents the total moles of drug and CD, respectively) with or without polymer (0.01% w/v HA or 0.1% w/v HPMC) were prepared by heating in an autoclave 121 °C for 20 min and equilibrated at 30 ± 1 °C for 7 days under constant agitation. The filtrated solutions were frozen and then lyophilized at −52 °C for 48 h in a freeze-dryer (Dura-Dry MP, Canada), yielding a solid complex powder (FD). Identical physical mixtures (PM) were prepared by careful blending of CCB and CD in a mortar with pestle. The samples were characterized in solid state as follows: pure CCB, γCD and RMβCD, PM and FD of CCB/CD (CCB/γCD and CCB/RMβCD), and FD ternary complexes CCB/γCD/HA, CCB/γCD/HPMC, CCB/RMβCD/HA and CCB/RMβCD/HPMC.

**Differential scanning calorimetry (DSC)** DSC thermograms were determined in a scanning calorimeter (Mettler Toledo, DSC822 STAR System, Germany). The samples (3–5 mg) were heated (10ºC/min) in sealed aluminium pans under nitrogen. The temperature range was from 30 to 250 °C. An empty aluminium pan was used as a reference.

**Powder X-ray diffraction (PXRD) studies** The PXRD patterns were recorded using Powder X-ray diffractometer (Rigaku model MiniFlex II, Japan) and operated at a voltage of 30 kV and a current of 15 mA. The samples were analyzed as the 2θ angle range of 3°–40° and process parameters were set as follows: step size of 0.020° (2θ) and scan speed of 2° per minute.

**Fourier transform infra-red (FT-IR) spectroscopy** The samples were measured in a FT-IR spectrometer (Thermo Scientific model Nicolet iS10, USA) using Attenuated Total

Reflectance (ATR) technique. The data was obtained in the range of 400–4500 $cm^{-1}$. The analyses were performed at room temperature.

### Solution state characterization by proton nuclear magnetic resonance ($^1$H-NMR) spectroscopy

The pure solid samples of CCB, γCD and RMβCD as well as of the CCB/CD complexes, that is CCB/γCD and CCB/RMβCD, which were prepared by freeze drying as described above, were characterized in a solution. The samples were dissolved in a capillary tube containing $CD_3OD$:$D_2O$ (50:50 v/v). $^1$H-NMR spectroscopy measurements were performed using a 500 MHz $^1$H-NMR spectrometer (BRUKER model AVANCE III HD, USA). The spectrum and chemical shift values were recorded. The resonance at 4.6500 ppm due to residual solvent (OD) was used as reference.

### Statistical analysis

The statistical significance of the results was determined by a one-way analysis of variance (ANOVA) with the pairwise multiple comparison procedure (Scheffe) for multiple comparison (SPSS program version, 11.5). A value of $P < 0.05$ was considered statistically significant.

## Results and discussion

### Stability of CCB in aqueous solution

Since the phase-solubility studies of CCB were determined by heating method, the chemical stability of CCB in aqueous solution containing CCB/CD complexes was determined. Table 1 shows the CCB content in aqueous solution containing 0.5% (w/v) βCD after zero to three cycles of autoclaving. Each cycle consisted of heating to 121 °C for 20 min. The amount of CCB in an aqueous βCD solution after each cycle of autoclaving was not significantly different ($P < 0.05$); no degradation was observed after three cycles of autoclaving. The result shows that CCB is relatively stable in aqueous CD solutions. It was concluded that the CCB/CD complexes could be prepared by the heating method. Previously Srinivasulu et al. showed that pure CCB was stable during storage at 105 °C for 24 h [14].

**Table 1** Celecoxib content in aqueous solution containing 0.5% w/v βCD after zero to three cycles of autoclaving

| Autoclaving | CCB content (μg/ml) (Mean ± SD) |
|---|---|
| 0 cycle | 1.483 ± 0.038 |
| 1 cycle | 1.512 ± 0.068 |
| 2 cycles | 1.525 ± 0.047 |
| 3 cycles | 1.523 ± 0.051 |

Each cycle consisted of heating to 121 °C for 20 min

### Solubility determinations

Figure 1 displays phase solubility diagrams of CCB in aqueous CD solutions containing αCD, βCD, γCD, HPβCD or RMβCD. In case of βCD, γCD and HPβCD, the CCB solubility increases linearly with increasing CD concentration to give $A_L$-type diagrams (Fig. 1b–d), whereas αCD and RMβCD showed a positive deviation from linearity represented $A_p$-type diagrams (Fig. 1a, e).

The $A_L$-type phase-solubility diagrams with slope less than 1 suggested the formation of a complex that is first-order with respect to both CCB and CD. In other words, that a CCB/CD 1:1 complex is being formed in the aqueous solution [13]. Table 2 shows the values of the apparent stability constants (i.e. $K_{1:1}$ and $K_{1:2}$) and the complexation efficiency (CE) of CCB/CD complexes in pure aqueous CD solutions. For αCD and RMβCD, they indicated that the formation of higher order soluble complexes with respect to CD at elevated CD concentrations. The stability constants (K) for the two complexes i.e., CCB/CD and CCB/$CD_2$ were determined by fitting the data to Eq. 2. They assumed that the 1:1 complex was formed more easily than the 1:2 complex. Ventura et al. [3] investigated CCB/dimethyl-βCD (DMβCD) complexes and obtained complexes of the same 1:1 and 1:2 stoichiometry.

βCD displayed the greatest CE among natural CDs. Studies have shown that the cavity diameter of βCD is of the most appropriate size for aromatic and heterocyclic molecules [15]. However, its aqueous solubility is rather limited due to relatively strong binding of the βCD molecules in the crystal state. Random substitution of the hydroxyl groups, even by hydrophobic moieties such as methoxy functions, will result in dramatic improvements in their solubility [5]. This indicates that the water-soluble βCD derivatives, HPβCD and RMβCD, could be excellent solubilizers of CCB and indeed they had the highest CE of all CD tested (Table 2). Of those two, RMβCD gave the highest CE. However, in pharmaceutical formulation development the CD safety profiles have to be considered. It was reported that the ranking of CD toxicity in human corneal epithelial cell line (HCE) is as follows: αCD > DMβCD > sulfobutyl ether βCD ≈ HPβCD > γCD [16]. Although RMβCD, the lipophilic methylated CD can act as penetration enhancers by disrupting the lipophilic membranes [17], its toxicological effects are low with no obvious morphological changes of nasal mucosa observed upon administration of 10% (w/v) RMβCD [18, 19]. γCD is well tolerated and possesses favorable toxicological profile but RMβCD can only be used at low concentration in aqueous eye drop formulation

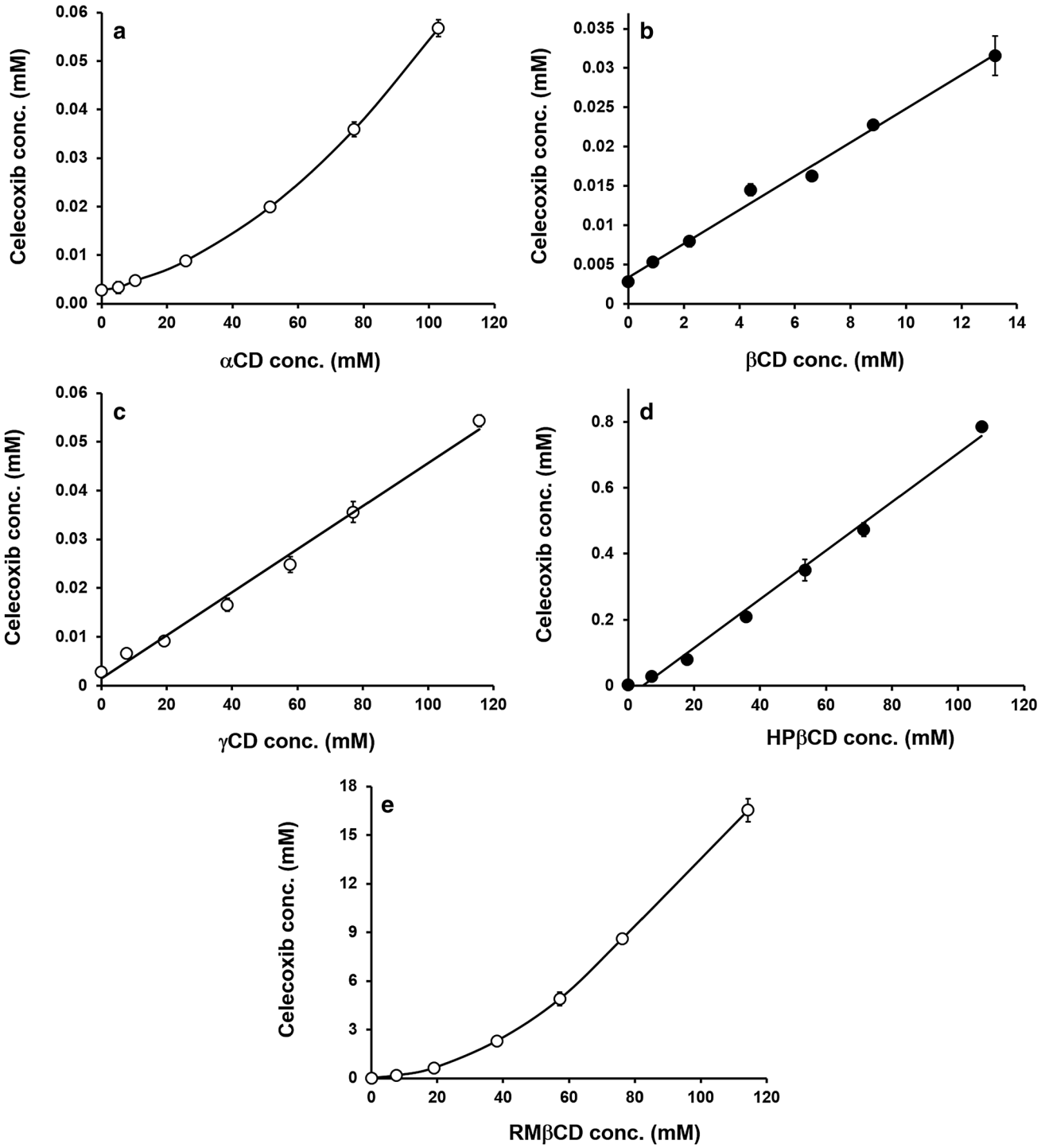



**Fig. 1** Phase solubility profiles of celecoxib in aqueous cyclodextrin. **a** αCD solutions, **b** βCD solutions, **c** γCD solutions, **d** HPβCD solutions and **e** RMβCD solutions

[20, 21]. Based on the phase-solubility profiles and safety, RMβCD and γCD were selected to further studies.

Water-soluble polymers can enhance the CD complexation of drugs and increase the drug permeation from CD containing aqueous vehicles through biological membranes [22]. These effects of polymers on drugs in aqueous CD solutions are possible due to the formation of ternary complexes or co-complexes and their mucoadhesive properties [23–25]. In this study, the effect of polymers, that is HPMC, hyaluronic acid (HA) and chitosan (CS) (non-ionic, anionic

**Table 2** The values of the apparent stability constants (i.e. $K_{1:1}$ and $K_{1:2}$) and the complexation efficiency (CE) of celecoxib/CD complexes in pure aqueous CD solutions at 30 ± 1 °C

| Cyclodextrin | Type | $K_{1:1}$ ($M^{-1}$) | $K_{1:2}$ ($M^{-1}$) | CE |
|---|---|---|---|---|
| αCD | $A_p$ | 49.9 | 26.2 | 0.00014[a] |
| βCD | $A_L$ | 760.2 | – | 0.00215 |
| γCD | $A_L$ | 155.3 | – | 0.00044 |
| HPβCD | $A_L$ | 2630.2 | – | 0.00746 |
| RMβCD | $A_p$ | 3139.9 | 157.3 | 0.00890[a] |

[a]Obtained from the initial linear part of the phase-solubility diagram

and cationic polymer, respectively), on RMβCD and γCD solubilization of CCB was investigated. The amino group in CS has $pK_a$ values from 6.2 to 7.0. With the pH of saturated CCB in aqueous CD solutions containing 0.1% w/v CS was 3.2–4.4. The amine groups are protonated and, thus, CS is a water-soluble cationic polymer in acidic to neutral solutions. Table 3 shows the apparent stability constant and CE of CCB/CD complexes in presence of polymer. The results showed that addition of polymers to the complexing media did not alter the type of the phase solubility diagram indicating that the presence of polymers did not alter the stoichiometry of the complex formed.

Form these results, the apparent stability constant (K) and CE of both binary CCB/RMβCD complex and the binary CCB/γCD complex were increased upon addition of the small amount of polymer. The effect of polymers on the CE of both complexes showed the same trend: HPMC > HA > CS (Table 3). Addition of chitosan to the complexation media has insignificantly effect on the CE of CCB, thus; it was excluded from further studies. Interestingly, HPMC showed the highest increments in the apparent solubilizing efficiency (CE ratio) of CCB about 11-folds and 19-folds increasing in the CE of CCB/RMβCD and CCB/γCD, respectively. The dramatically increasing CE of CCB/RMβCD with HPMC as ternary complex may be possibly caused by the interaction of multi-components. The synergistic effect of the CD-polymer mixture could be due to interaction of the water-soluble polymers with the drug/CD complexes and stabilization of their nano-size aggregates. It is known that polymers stabilize micelles and other types of aggregates [26, 27]. Previous studies have shown that HPMC and PVP can increase the CE as due to formation of ternary HPβCD complexes of acetazolamide, carbamazepine methazolamide and pregnenolone [10].

## Morphology, aggregates particle size and zeta potential analysis

The aggregated size of the ternary CCB/γCD/HA, CCB/γCD/HPMC, CCB/RMβCD/HA and CCB/RMβCD/HPMC complexes were investigated. Two different techniques were applied, DLS and TEM. The DLS technique determines the hydrodynamic diameter of the CCB/CD/polymer aggregates that assuming they have spherical shape. The instrument reports size distribution of particles in an aqueous complexation medium. With the TEM technique the shape of the nanoparticles (i.e. the complex aggregates) become visible and their morphology can be observed. The samples displayed bimodal size distribution that existed the unaggregated CCB/CD complexes (diameter approximately 2 nm) and ternary complex aggregates with diameter between about 250 and 350 nm (Table 4). As expected, the aggregate diameter of the ternary complexes consisted of CCB/CD containing HPMC were larger than those of CCB/CD with HA. It was supported by the zeta potential data that the former ternary complexes had lower zeta potential than those of the latter ones (Table 4). This confirmed that HPMC, non-ionic polymer enhanced CCB solubility by forming large CCB/CD complex aggregates. TEM photographs of the ternary complexes are shown in Fig. 2. The HA containing nanoparticles were spherical but the larger HPMC containing nanoparticles had somewhat irregular shape. The diameter of the ternary complex aggregates observed by TEM was within the same range as those observed by the DLS. This supported the notion that addition of small amount of water-soluble polymer to aqueous CCB/CD complex solutions can enhance the CE of CD through promotion of complex aggregate formation.

**Table 3** Apparent stability constant values ($K_{1:1}$ and $K_{1:2}$) and the apparent complexation efficiency (CE) of celecoxib/cyclodextrin complexes in pure aqueous cyclodextrin solutions in the presence of polymer at 30 ± 1 °C

| Cyclodextrin | Polymer (% w/v) | $S_0$ (mM) | Type | $K_{1:1}$ ($M^{-1}$) | $K_{1:2}$ ($M^{-1}$) | CE | CE ratio |
|---|---|---|---|---|---|---|---|
| γCD | – | 0.0028 | $A_L$ | 155.3 | – | 0.00044 | 1.00 |
| | 0.1% CS | 0.0032 | $A_L$ | 170.9 | – | 0.00054 | 1.23 |
| | 0.01% HA | 0.0028 | $A_L$ | 196.4 | – | 0.00055 | 1.25 |
| | 0.1% HPMC | 0.0275 | $A_L$ | 307.5 | – | 0.00847 | 19.24 |
| RMβCD | – | 0.0028 | $A_p$ | 3140 | 157 | 0.00890 | 1.00 |
| | 0.1% CS | 0.0032 | $A_p$ | 2846 | 136 | 0.00900 | 1.01 |
| | 0.01% HA | 0.0028 | $A_p$ | 3533 | 120 | 0.00990 | 1.11 |
| | 0.1% HPMC | 0.0275 | $A_p$ | 3564 | 18.3 | 0.09820 | 11.03 |

**Table 4** The size distribution and zeta potential of celecoxib/cyclodextrin/ polymer ternary complex aggregates

| Cyclodextrin (% w/v) | Polymer (% w/v) | Mean particle size (nm) | Zeta potential (mV) |
|---|---|---|---|
| 10% γCD | 0.01% HA | 273 ± 36<br>1.86 ± 0.02 | −16.40 ± 2.10 |
| | 0.1% HPMC | 308 ± 8<br>2.10 ± 0.14 | −9.90 ± 2.33 |
| 7.5% RMβCD | 0.01% HA | 283 ± 36<br>2.05 ± 0.25 | −9.27 ± 1.35 |
| | 0.1%HPMC | 312 ± 29<br>2.08 ± 0.48 | −3.46 ± 0.06 |

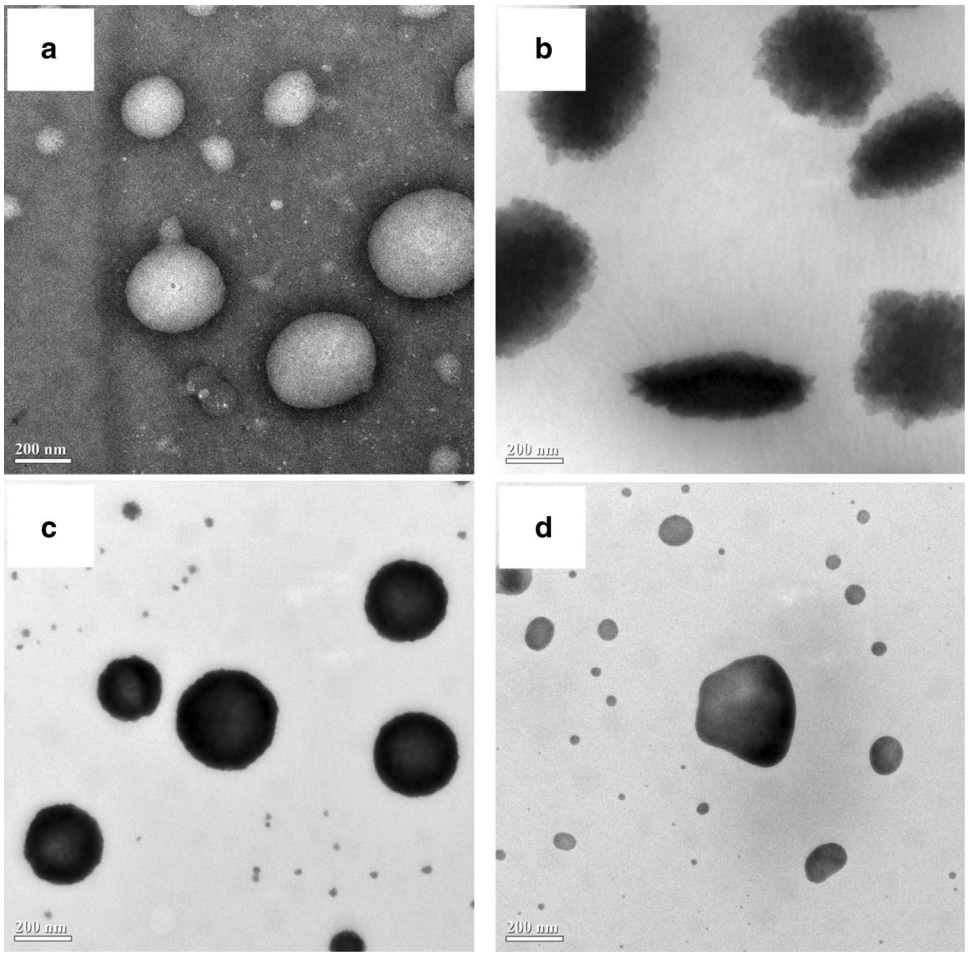



**Fig. 2** TEM photographs of an aqueous drug/cyclodextrin/polymer complex solution saturated with celecoxib. **a** CCB/γCD/HA, **b** CCB/γCD/HPMC, **c** CCB/RMβCD/HA, and **d** CCB/RMβCD/HPMC

In general, in aqueous solutions the relative amount of aggregated CD increases with increasing CD concentration. From the literature, the diameter of CD aggregates is most frequently between 90 and 300 nm [28]. However, CD molecules have their own dynamic behavior resulted from rotational freedom of the glucopyranose unit of CD molecule and the overall molecular mobility. The stabilization of nano-particulate aggregates is regulated by van der Waals forces and hydrogen bonds interactions, which are at all time in competition resulting in very unstable aggregates, although the ability of CD to self-assemble to form reversible aggregates is well documented [29].

## Solid-state characterization

DSC thermograms of CCB, CDs, their PM and FD samples are shown in Figs. 3 and 4. The thermogram of pure CCB exhibited a sharp endothermic peak at 165.17 °C which represented the melting point of the drug (Figs. 3a, 4a). Thermogram of γCD showed a very broad endothermic

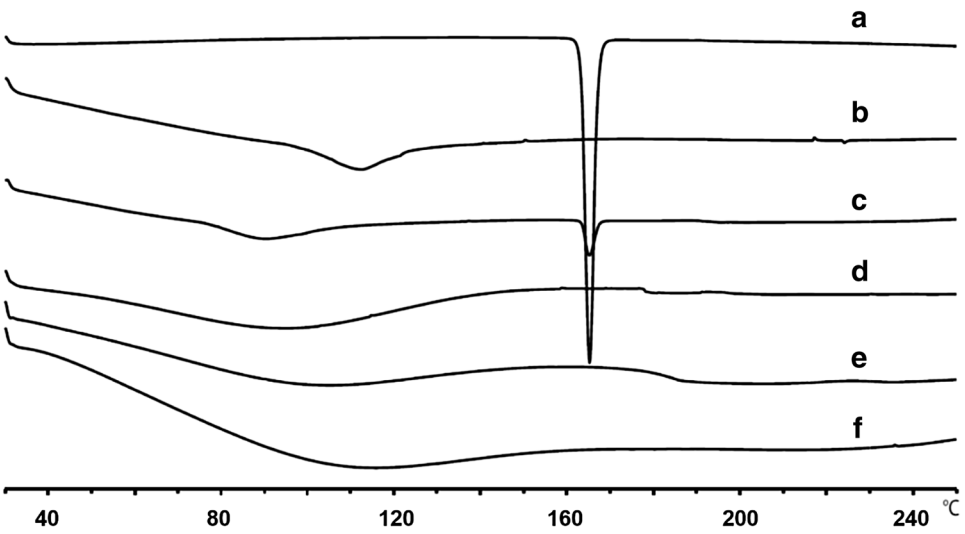



**Fig. 3** DSC thermograms of (*a*) pure CCB, (*b*) pure γCD, (*c*) PM CCB/γCD, (*d*) FD CCB/γCD, (*e*) FD CCB/γCD/HA, and (*f*) FD CCB/γCD/HPMC

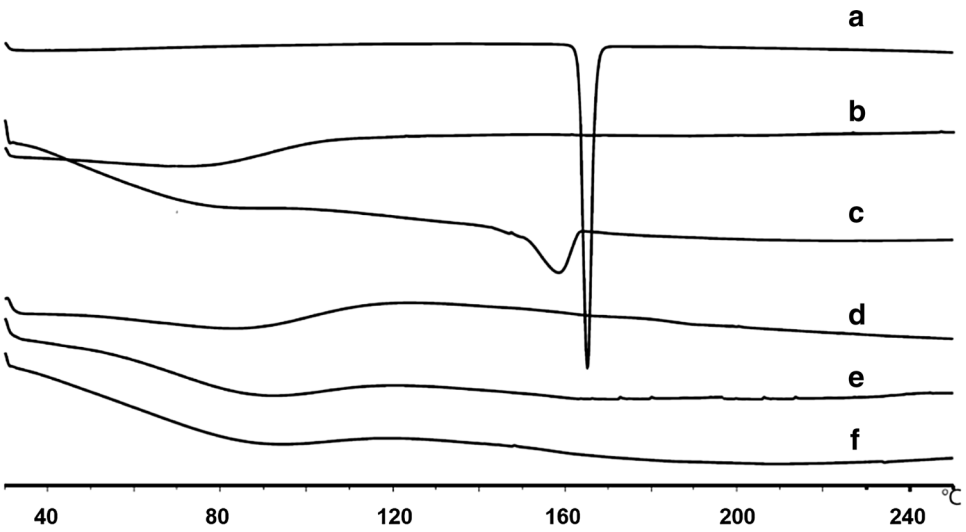



**Fig. 4** DSC thermograms of (*a*) pure CCB, (*b*) pure RMβCD, (*c*) PM CCB/RMβCD, (*d*) FD CCB/RMβCD, (*e*) FD CCB/RMβCD/HA, and (*f*) FD CCB/RMβCD/HPMC

peak in the range of 112 °C (Fig. 3b) due to elimination of water of crystallization [7] while the DSC thermograms of RMβCD showed no thermal change (Fig. 4b). In case of PM, the endothermic peak of the CCB in PM CCB/γCD was retained at 165.17 °C while the peak of the CCB in PM CCB/RMβCD was a broad peak and slightly shifted to lower temperature appeared at 158.67 °C. These observations may be attributed to the presence of weak or no interaction between the pure components in the PM and/or possible interaction between CCB and RMβCD that was promoted by the heating process in the DSC operation [30]. For FD samples, the endothermic peak of binary complex (i.e. FD CCB/γCD and FD CCB/RMβCD) and ternary complex (i.e. FD CCB/γCD/HA, FD CCB/γCD/HPMC, FD CCB/RMβCD/HA and FD CCB/RMβCD/HPMC) were absent (Figs. 3, 4). The disappearance of an endothermic peak may be attributed to an amorphous state and/or presence of inclusion CCB/CD complex in the solid state [31].

PXRD is used to measure the crystallinity of the formed complexes and the peak position (angle of diffraction) is an indication of a crystal structure. The PXRD spectra of pure CCB, γCD, RMβCD, their PM and FD binary and ternary complex are presented in Figs. 5 and 6. The diffractogram of CCB exhibited a series of intense peaks at 5.3, 10.6, 10.9, 12.9, 14.7, 16.0, 17.8, 18.3, 18.6, 19.5, 20.4, 21.4, 22.1, 23.3, 24.5, 24.9, 25.3, 26.9, 27.7 28.2 and 29.4, which were indicated of their crystallinity (Figs. 5a, 6a). The γCD exhibited characteristic peaks at 5.0, 10.1, 11.1, 12.2, 13.8, 15.3, 15.9, 16.3, 16.8, 18.7, 20.3, 21.6 22.8 and 23.3 due to its crystalline nature (Fig. 5b), while RMβCD did not show any peak and exhibited amorphous state (Fig. 6b). RMβCD is obtained by random substitution of the hydroxy moieties of the βCD molecule and, thus, is an amorphous mixture

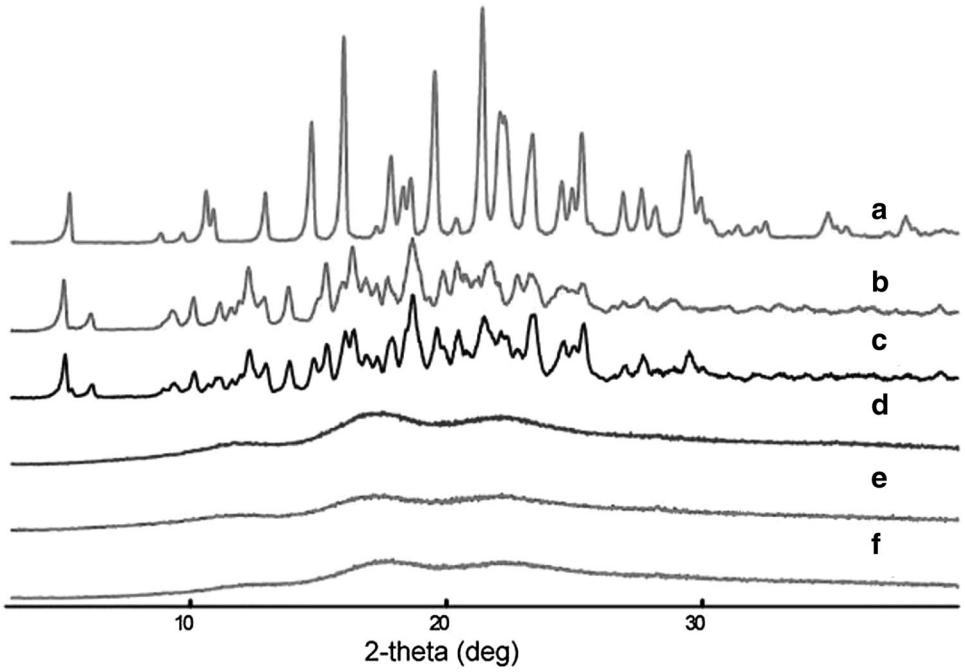



**Fig. 5** The PXRD spectra of (*a*) pure CCB, (*b*) pure γCD, (*c*) PM CCB/γCD, (*d*) FD CCB/γCD, (*e*) FD CCB/γCD/HA, and (*f*) FD CCB/γCD/HPMC

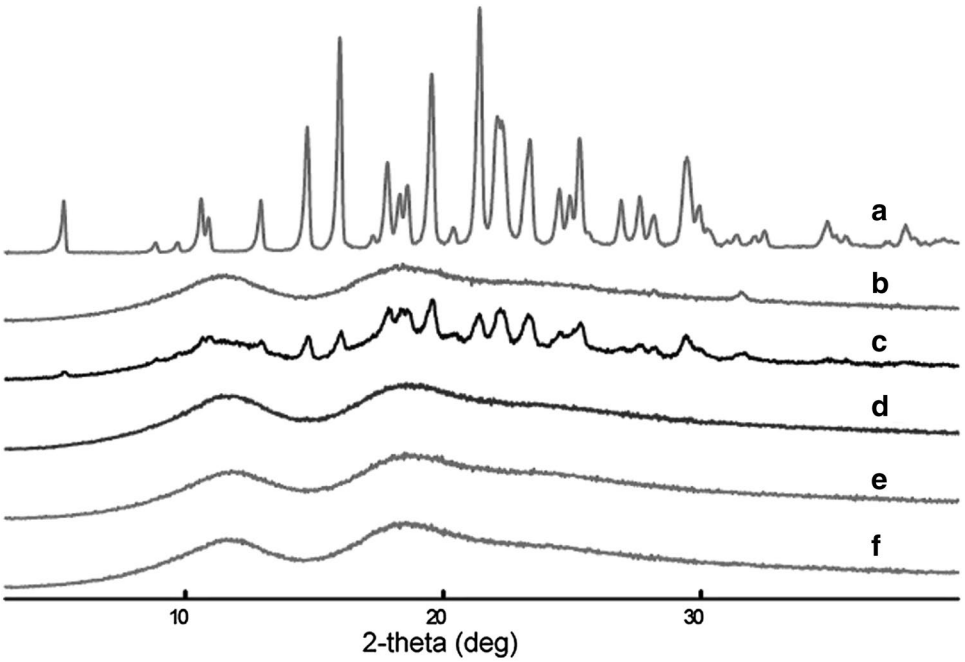



**Fig. 6** The PXRD spectra of (*a*) pure CCB, (*b*) pure RMβCD, (*c*) PM CCB/RMβCD, (*d*) FD CCB/RMβCD, (*e*) FD CCB/RMβCD/HA, and (*f*) FD CCB/RMβCD/HPMC

of isomers. Most of the principal peaks of CCB were presented in the diffraction patterns of PM of both CCB/γCD and CCB/RMβCD (Figs. 5c, 6c). This indicates that there was no interaction between the pure CCB and the respective CDs. In contrast FD samples of CCB/CD binary complexes (i.e. FD CCB/RMβCD and FD CCB/γCD) and ternary complexes (i.e. FD CCB/γCD/HA, FD CCB/γCD/HPMC, FD CCB/RMβCD/HA and FD CCB/RMβCD/HPMC) showed a halo pattern with the disappearance of all the peaks corresponding to CCB. The transformation of CCB from the crystalline to the amorphous form indicates formation of CCB/CD inclusion complexes [32]. However, it should be mentioned that the freeze drying technique applied during sample preparation may affect transformation of the solid state [33].

Figures 7 and 8 show the FT-IR spectra of pure CCB, γCD, RMβCD, their PM and FD samples. The FT-IR spectra of CCB are shown in Figs. 7a and 8a. Its characteristic peaks

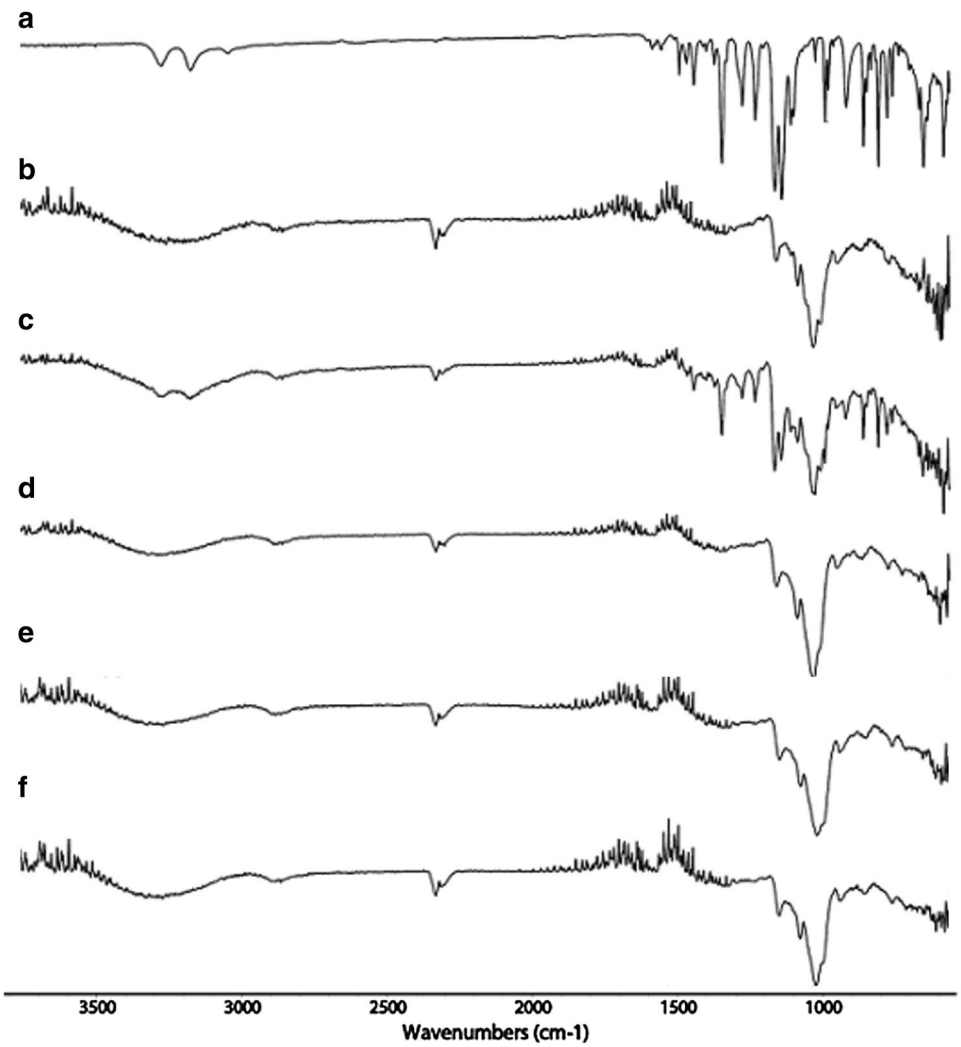



**Fig. 7** FT-IR spectra of (*a*) pure CCB, (*b*) pure γCD, (*c*) PM CCB/γCD, (*d*) FD CCB/γCD, (*e*) FDCCB/γCD/HA, and (*f*) FD CCB/γCD/HPMC

at 3332.4 and 3229.9 $cm^{-1}$ attributed to N–H stretching vibration of $SO_2NH_2$ group, 1346.5 and 1158.2 $cm^{-1}$ for the S=O asymmetric and symmetric stretching and 1228.7 for C–F stretching. The FT-IR spectrum of γCD and RMβCD showed a broad absorption band at 3383 $cm^{-1}$ due to –OH stretching and displayed a large band and distinct peaks in the region of 1200–900 $cm^{-1}$ [34]. The FT-IR spectrum of CCB in PM sample showed the double peaks of N–H stretching that were slightly shifted to 3330.9 and 3229.4 $cm^{-1}$ for PM CCB/γCD (Fig. 7c) and 3336.6 and 3232 $cm^{-1}$ for PM CCB/RMβCD (Fig. 8c). The S=O stretching vibration was slightly shifted to 1347.6 and 1160.7 $cm^{-1}$ for PM CCB/RMβCD while no shift was observed in case of the CCB/γCD complex. These observations indicate that there was less interaction between CCB and CD in the PM samples. For FD samples of both the binary complexes and the ternary complexes, the N–H stretching bands of CCB indicate masking of characteristic symmetric and asymmetric stretch. Likewise, the C–F stretching band and S=O stretching bands of CCB disappeared in the FD samples. These results may be ascribed to the existence of some interaction between functional groups (sulfonamide group or $–CF_3$ group) of CCB and functional groups in the hydrophobic CD cavities in the inclusion complexes [7].

## Solution-state characterization

$^1$H-NMR studies provide information on the existence of CCB/CD inclusion complexes and suggest the conformation of guest molecules in the CD cavity. The changes in $^1$H-chemical shifts (Δδ) observed for the H5 proton of CCB in the presence of RMβCD and γCD were 0.045 and 0.043, respectively and displayed significant upfield shift (Table 5). This behavior can indicate that the H5 proton of CCB is located close to the oxygen atoms in the CD cavity which is rich in $\pi$ electrons [35]. Also, all aromatic protons of CCB showed a significant upfield shift in the presence of γCD. Whereas all aromatic protons of CCB dissolved in aqueous

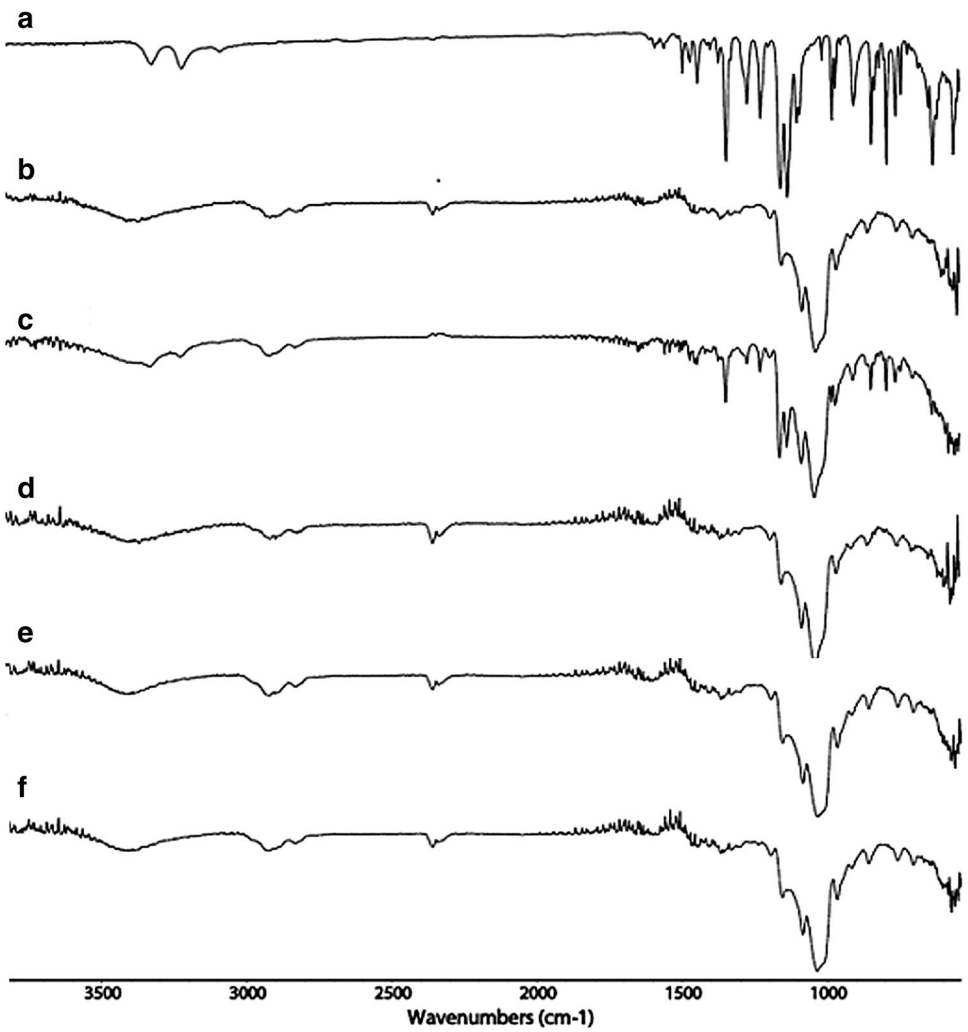
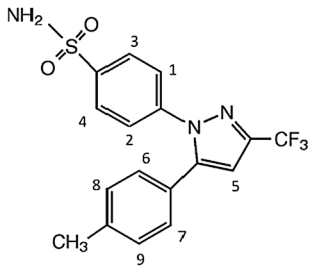



**Fig. 8** FT-IR spectra of (*a*) pure CCB, (*b*) pure RMβCD, (*c*) PM CCB/RMβCD, (*d*) FD CCB/RMβCD, (*e*) FD CCB/RMβCD/HA, and (*f*) FD CCB/RMβCD/HPMC

RMβCD solution displayed downfield shifts demonstrating weaker interactions or van der Waals forces between CCB and the hydrogen atoms of CD [3]. $^{1}$H-NMR chemical shift of RMβCD is summarized in Table 6. The H3 and H5 protons of the glucose units are facing to the interior of the lipophilic CD cavity. The observed changes in (Δδ) for H3 (−0.092) and H5 (−0.042) were upfield which is characteristic for formation of an inclusion complex. The Δδ* of the H3 proton was higher than that of the H5 proton indicating partial inclusion of CCB into the RMβCD cavity [36]. The results are consistent with the data obtained by Ventura et al. [3]. The γCD Δδ* in presence of CCB for the H3 and H5 protons were 0.025 and 0.068, respectively (Table 7). The fact that the Δδ* value of H5 proton is greater than that of the H3 proton, and that they displayed significant downfield shift, indicates the drug molecule occupies the total volume of the hydrophobic γCD cavity.

The proposed conformation structures of CCB/RMβCD and CCB/γCD inclusion complexes are shown in Fig. 9. The CCB/RMβCD complex can form both 1:1 and 1:2 CCB/RMβCD inclusion complexes. Figure 9a shows the 1:1 inclusion complex where the pyrazole head group of CCB is included through the wide opening of the RMβCD central cavity. Figure 9b shows the 1:2 inclusion complex where the CCB is included in to the cavity of the CD dimer in a configuration where half of the CCB molecule was embedded in one monomer and another half is embedded in the other RMβCD monomer of CD. The complex was kept together by formation of hydrogen bonds between the hydroxyl groups or methoxy groups of RMβCD and the fluorine and nitrogen atoms of CCB. Consequently, significant upfield H1 proton of RMβCD may be caused by interaction between hydrogen (H1) at wide rim of the RMβCD molecule and CCB molecule during 1:2 inclusion complex formation. This corresponds to the phase solubility-profile study of the CCB/RMβCD $A_P$-type diagram (1:1 and 1:2 complex). The proposed complex structure is supported by the CCB/βCD complex characterization of Reddy et al. [37]. The proposed conformation of the CCB/γCD complex is shown in Fig. 9c. The Δδ* of H5 in both CCB and

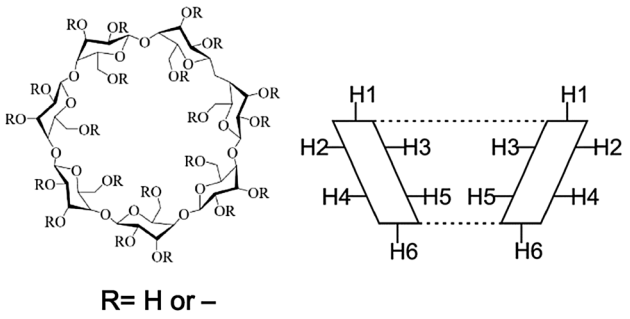
**Table 5** The $^1$H-chemical shifts of celecoxib alone and in the presence of γCD or RMβCD




| Protons | CCB | CCB/RMβCD | Δδ* | CCB/γCD | Δδ* |
|---|---|---|---|---|---|
| $-CH_3$ | 2.206 | 2.217 | +0.011 | 2.183 | − 0.023 |
| H5 | 6.877 | 6.832 | − 0.045 | 6.834 | − 0.043 |
| H6,H7,H8,H9 | 7.062 | 7.076 | +0.014 | 7.035 | − 0.027 |
| H1,H2 | 7.378 | 7.390 | +0.011 | 7.345 | − 0.034 |
| H3,H4 | 7.823 | 7.838 | +0.015 | 7.801 | − 0.022 |

$\Delta\delta^* = \delta_{complex} - \delta_{free}$

**Table 6** The $^1$H-chemical shifts of RMβCD alone and in the presence of celecoxib




| Protons | RMβCD | CCB/RMβCD | Δδ* |
|---|---|---|---|
| H1 | 4.941 | 4.844 | −0.097 |
| H2 | – | – | – |
| H3 | 3.785 | 3.693 | −0.092 |
| H4 | 3.536 | 3.518 | −0.018 |
| H5 | 3.577 | 3.536 | −0.042 |
| H6 | – | – | – |
| $CH_3OC2,3$ | 3.438 | 3.451 | +0.013 |
| $CH_3OC6$ | 3.269 | 3.259 | −0.010 |

$\Delta\delta^* = \delta_{complex} - \delta_{free}$

γCD were significantly shifted and all aromatic protons displayed upfield shift. This indicates that CCB is more deeply included into γCD cavity than that of RMβCD, possibly due to the larger inner cavity diameter of γCD (7.5–8.3 Å) in comparison to that of RMβCD (6 Å).

**Table 7** The $^1$H-chemical shifts of γCD alone and in the presence of celecoxib

| Protons | γCD | CCB/γCD | Δδ* |
|---|---|---|---|
| H1 | 4.991 | 4.994 | +0.003 |
| H2 | 3.523 | 3.499 | −0.024 |
| H3 | 3.815 | 3.840 | +0.025 |
| H4 | 3.469 | 3.480 | +0.011 |
| H5 | 3.732 | 3.800 | +0.068 |
| H6 | 3.751 | 3.821 | +0.070 |

$\Delta\delta^* = \delta_{complex} - \delta_{free}$

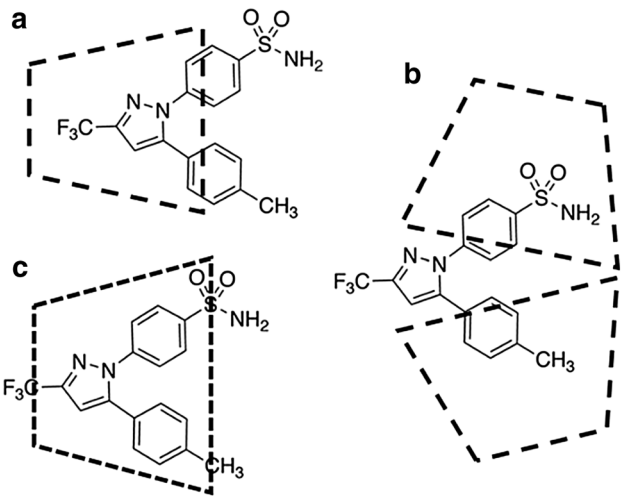



**Fig. 9** The proposed conformation of **a** 1:1 CCB/RMβCD complex, **b** 1:2 CCB/RMβCD complex and **c** 1:1 CCB/γCD complex

## Conclusions

The solubility of celecoxib (CCB), a poorly water-soluble drug, can be enhanced through formation of cyclodextrin (CD) inclusion complexes. CCB is stable in the aqueous CD solutions and no degradation peaks were observed during autoclaving. RMβCD, a relatively lipophilic βCD derivative, exhibited the highest complexation efficiency. HPMC had synergistic effect on the CD solubilization via ternary complex formation and resulted in the highest solubilizing efficiency followed hyaluronic acid and chitosan. The CCB/CD/polymer complexes formed aggregates and participation of the polymers in the aggregate formation is thought to explain the enhanced solubilization. Formation

of CCB/CD and CCB/CD/polymer complexes were verified my NMR studies. The obtained results suggested that CD can enhance solubility of CCB and that such solutions can be sterilized by autoclaving.

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ORIGINAL ARTICLE

# Cyclodextrin solubilization of celecoxib: solid and solution state characterization

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**Abstract**
The low aqueous solubility of celecoxib (CCB) hampers its oral bioavailability and permeation from aqueous environment through biological membranes. The aim of this study was to enhance the aqueous solubility of CCB by complexation with cyclodextrin (CD) in the presence of water-soluble polymer. The effects of different CDs (αCD, βCD, γCD, 2-hydroxypropyl-β-cyclodextrin and randomly methylated β-cyclodextrin (RMβCD)) and mucoadhesive, water-soluble polymers (hydroxypropyl methylcellulose (HPMC), chitosan and hyaluronic acid) were investigated. The phase solubility profiles and CCB/CD complex characteristics were determined. RMβCD exhibited the greatest solubilizing effect of the two CDs tested. However, γCD was also selected for further investigations due to its safety profile. Addition of polymer to the aqueous CD solutions enhanced the CD solubilization. Formation of CCB/RMβCD/HPMC and CCB/γCD/HPMC ternary complexes resulted in 11 and 19-fold enhancement in the apparent complexation efficiency in comparison to their CCB/CD binary complex, respectively. The size of ternary complex aggregates in solution were determined to be from about 250 to about 350 nm. The data obtained from Fourier transform infra-red, differential scanning calorimetry and powder X-ray diffraction indicated presence of CCB/CD inclusion complexes in the solid state. Proton nuclear magnetic resonance data demonstrated that CCB was partially and totally inserted into the hydrophobic central cavities of RMβCD and γCD.

**Keywords** Cyclodextrins · Celecoxib · Solubility · Complexation · Aggregates

## Introduction

Celecoxib (CCB), a nonsteroidal anti-inflammatory drug (NSAID), is the first selective cyclooxygenase-2 (COX-2) inhibitor used to treat osteoarthritis and rheumatoid arthritis in adult patients. It is also indicated in treatment of acute pain and primary dysmenorrhea as well as an adjuvant in the treatment of familial adenomatous polyposis, a genetic disorder [1]. CCB is a low molecular weight (381.38 Da) hydrophobic molecule with a log P 3.47 at pH 7.4. With a $pK_a$ of 11.1, CCB is neutral at physiologic pH and is practically insoluble. The aqueous solubility of CCB is approximately 2 μg/ml [2, 3]. The very low aqueous solubility hampers CCB permeation through biological membranes resulting in low bioavailability and inadequate drug levels at its site of action. Several techniques are used to enhance aqueous solubility of poorly soluble drugs and their dissolution including (1) pH adjustments, (2) particle size reductions, (3) formation of inclusion complexes, (4) co-solvency and (5) micellar solubilization [4]. Here the usage of cyclodextrins (CDs) as complexing agents to enhance aqueous solubility of CCB was investigated.

CDs are cyclic oligosaccharides consist of (α-1,4-)-linked α-D-glucopyranose units with a hydrophilic outer surface and a lipophilic central cavity. The CD molecules are cone shaped with secondary hydroxy groups protruding from the wider edge and the primary groups from the narrow edge. The three most common natural CDs consist of six, seven and eight glucopyranose units and named α-cyclodextrin (αCD), β-cyclodextrin (βCD) and γ-cyclodextrin (γCD), respectively. In addition, random substitution of the hydroxy groups brings about dramatic improvements in their solubility. CD derivatives of pharmaceutical interest include 2-hydroxypropyl-β-cyclodextrin

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(HPβCD), 2-hydroxypropyl-γ-cyclodextrin (HPγCD), randomly methylated β-cyclodextrin (RMβCD) and sulfobutylether β-cyclodextrin (SBEβCD). The CDs have molecular weights from about 1000 to about 2000 Da and a large negative Log P (i.e. logarithm of the octanol/water partition coefficient). Thus, they are poorly absorbed through biological membranes [5]. Various lipophilic drugs can form inclusion complexes with CDs by inserting some lipophilic moieties of the drug molecules into the lipophilic CD cavity. Formation of such hydrophilic drug/CD inclusion complexes increases the drug solubility in aqueous media. Rawat and Jain [6] conducted a study of CCB/βCD complexes. Based on the reported phase-solubility profile, the solubility of CCB can be improved by βCD. Moreover, this study indicated that the dissolution rate of solid CCB/βCD complexes was significantly increased when compared to pure solid CCB. Furthermore, the water-soluble βCD derivative HPβCD has been shown to enhance the dissolution rate of CCB in aqueous media [7].

Recent studies have shown that the addition of hydrophilic polymers to aqueous complexation media can significantly increase CD solubilization of poorly soluble drugs through ternary complex formation [8]. In a synergistic mode the water-soluble polymers can decrease the amount of CD required to prepare soluble drug/CD complexes and obtain drug solution of a given concentration [9]. Using heating methods, such as autoclaving at 121 °C for 20–40 min or sonication at temperatures above 70 °C for 1 h, can accelerate the ternary complex formation [10, 11]. The influence of hydrophilic polymers on CCB/HPβCD complexes and formation of CCB/HPβCD/polymer ternary complex has been investigated [12]. It was concluded that polyvinylpyrrolidone (PVP), hydroxypropyl methylcellulose (HPMC) and polyethylene glycol (PEG) stabilize the binary CCB/CD complexes with a consequent increase in the CCB solubility. Beside their solubilizing effect, CDs can act as permeation enhancers and increase drug permeation through biological membranes. However, previous studies on CCB/CD complexes have focused on solid dosage forms and the ability of CDs to enhance CCB bioavailability after oral administration. Some drug formulations, such as those for ophthalmic, nasal and parenteral administration, have volume limitations and, thus, frequently require a potent drug solubilizer. The focus of this present study is on evaluation of the ability of different CDs to solubilize CCB and characterization of both the solid state and the dissolved CCB/CD complexes, and their ability to form nano- and microparticle drug delivery systems.

## Materials and methods

### Materials

Celecoxib (CCB) was generously donated by Unison Laboratories Co., Ltd., Thailand. α-Cyclodextrin (αCD), β-cyclodextrin (βCD), γ-cyclodextrin (γCD) and randomly methylated β-cyclodextrin (RMβCD) MS 1.8 (MW 1312 Da) were purchased from Wacker Chemie (Germany), 2-hydroxypropyl-β-cyclodextrin (HPβCD) MS 0.6 (MW 1400 Da) from Roquette (France), chitosan (CS) from Fluka Fine Chemical (Japan), hyaluronic acid (HA) from Soliance (France) and hydroxypropyl methylcellulose 4000 (HPMC) from Sigma (USA). All other chemicals used were of analytical reagent grade purity. Milli-Q (Millipore, USA) water was used for preparation of all solutions.

### Stability evaluation

The stability of CCB in aqueous solution containing 0.5% w/v βCD was determined by heating method [10]. Excess amount of CCB was dissolved in purified water and equilibrated at 30 ± 1 °C for 24 h under constant agitation. The supernatant was filtered through 0.45 μm nylon filter and mixed with 1% w/v βCD solution at 1:1 ratio. The solution was then divided into three sealed vials that were heated in an autoclave for one, two and three heating cycles, each cycle consisted of heating to 121 °C for 20 min. The drug concentrations in the vials were then determined by a high-performance liquid chromatographic method (HPLC).

### Quantitative determinations

Quantitative determinations of CCB were performed on a reversed-phase HPLC component system from Shimadzu consisting of a LC-20AB binary pump, a SPD-20A multiple wavelength detector, a SIL-20ATH auto sampler with LCsolution software, and Shiseido C18 column, 5 μm, 4.6 × 150 mm ID with C18 guard cartridge column MGII 5 μm, 4 × 10 mm. The modified HPLC condition [3] was as follows; mobile phase: acetonitrile:water (55:45); flow rate: 1.0 ml/min; oven temperature: ambient; UV detector wavelength: 252 nm; injection volume: 20 μl; and run time: 15 min. The HPLC method was validated and shown to be consistent, reliable and accurate.

### Solubility determinations

Excess amount of CCB was added to solutions containing from 0 to 15% (w/v) CD (αCD, βCD, γCD, HPβCD or

RMβCD) in pure water. The drug suspensions were saturated the drug through heating in an autoclave 121 °C for 20 min and allowed to cool to room temperature [10]. Then a small amount of solid drug was added to the suspensions to promote drug precipitation. The suspensions were equilibrated at 30 ± 1 °C for 7 days under constant agitation. After equilibrium was attained, the suspensions were filtered through 0.45 µm syringe filter, the filtrates were diluted with the mobile phase and analyzed by HPLC. The determinations were done in triplicate. The apparent stability constants for CCB/CD complexes ($K_{1:1}$ and/or $K_{1:2}$) and the complexation efficiency (CE) were determined according to the phase-solubility method of Higuchi and Connors [13].

$$CE = \frac{Slope}{1 - Slope} = \frac{[Drug/CD\ Complex]}{[CD]} = K_{1:1} \cdot S_0 \quad (1)$$

$$[S_t] - [S_0] = K_{1:1}[S_0][CD] + K_{1:1}K_{1:2}[S_0][CD]^2 \quad (2)$$

where $S_0$ is intrinsic solubility of CCB and $S_t$ is the concentration of CCB at a given CD concentration [CD].

To determine the effect of polymer on solubility of CCB/CD complexes the phase solubility of CCB was determined in pure aqueous γCD and RMβCD solutions containing various water-soluble polymers. HPMC (0.1%), HA (0.01%) or CS (0.1%) (all % w/v) were selected as non-ionic, negatively and positively charged polymer, respectively. The polymer was dissolved in the aqueous solutions containing 0–10% w/v CD. Then the excess of CCB was added to form drug suspensions and the suspensions heated in an autoclave to 121 °C for 20 min. $K_{1:1}$, $K_{1:2}$ and CE were calculated were calculated from the phase-solubility diagrams obtained.

## Morphology, particle size and zeta potential analysis

The morphology, size and size distribution, and zeta potential of the aggregates formed in aqueous complexation media containing 10% (w/v) γCD or 7.5% (w/v) RMβCD, with or without polymer (0.01% w/v HA and 0.1% w/v HPMC), were analyzed using transmission electron microscope and dynamic light scattering.

### Dynamic light scattering (DLS) measurement

The particle size of CCB/CD based aggregates in solution was measured by DLS technique (Zetasizer Nano ZS with software version 7.11, Malvern, UK). The samples were diluted with purified water immediate before analysis. A sample was put in a quartz glass cuvette and placed in the instrument. Measurements were carried out at 25 °C and 180° scattering angle. Particle size and size distribution and zeta potential were automatically calculated and analyzed by the software included within the system. Each measurement was carried out in triplicate.

### Transmission electron microscope (TEM) analysis

The morphology of CCB/CD based aggregates was evaluated by TEM (JEOL, JEM-2100F, USA). Initially, the sample was placed on a formvar-coated grid. After blotting the grid with a filter paper, the grid was transferred onto a drop of negative straining. Aqueous phosphotungstic acid solution (2%) was used as a negative strain. The sample was air dried at room temperature. Finally, the samples were examined with TEM.

## Preparation and characterization of binary CCB/CD complexes and ternary CCB/CD/polymer complexes

### Solid-state characterization

**Sample preparations** Aqueous solutions containing CCB/CD complexes of 1:1 molar ratio (m:n; $D_mCD_n$ where m and n represents the total moles of drug and CD, respectively) with or without polymer (0.01% w/v HA or 0.1% w/v HPMC) were prepared by heating in an autoclave 121 °C for 20 min and equilibrated at 30 ± 1 °C for 7 days under constant agitation. The filtrated solutions were frozen and then lyophilized at −52 °C for 48 h in a freeze-dryer (Dura-Dry MP, Canada), yielding a solid complex powder (FD). Identical physical mixtures (PM) were prepared by careful blending of CCB and CD in a mortar with pestle. The samples were characterized in solid state as follows: pure CCB, γCD and RMβCD, PM and FD of CCB/CD (CCB/γCD and CCB/RMβCD), and FD ternary complexes CCB/γCD/HA, CCB/γCD/HPMC, CCB/RMβCD/HA and CCB/RMβCD/HPMC.

**Differential scanning calorimetry (DSC)** DSC thermograms were determined in a scanning calorimeter (Mettler Toledo, DSC822 STAR System, Germany). The samples (3–5 mg) were heated (10ºC/min) in sealed aluminium pans under nitrogen. The temperature range was from 30 to 250 °C. An empty aluminium pan was used as a reference.

**Powder X-ray diffraction (PXRD) studies** The PXRD patterns were recorded using Powder X-ray diffractometer (Rigaku model MiniFlex II, Japan) and operated at a voltage of 30 kV and a current of 15 mA. The samples were analyzed as the 2θ angle range of 3°–40° and process parameters were set as follows: step size of 0.020° (2θ) and scan speed of 2° per minute.

**Fourier transform infra-red (FT-IR) spectroscopy** The samples were measured in a FT-IR spectrometer (Thermo Scientific model Nicolet iS10, USA) using Attenuated Total

Reflectance (ATR) technique. The data was obtained in the range of 400–4500 $cm^{-1}$. The analyses were performed at room temperature.

### Solution state characterization by proton nuclear magnetic resonance ($^1$H-NMR) spectroscopy

The pure solid samples of CCB, γCD and RMβCD as well as of the CCB/CD complexes, that is CCB/γCD and CCB/RMβCD, which were prepared by freeze drying as described above, were characterized in a solution. The samples were dissolved in a capillary tube containing $CD_3OD$:$D_2O$ (50:50 v/v). $^1$H-NMR spectroscopy measurements were performed using a 500 MHz $^1$H-NMR spectrometer (BRUKER model AVANCE III HD, USA). The spectrum and chemical shift values were recorded. The resonance at 4.6500 ppm due to residual solvent (OD) was used as reference.

### Statistical analysis

The statistical significance of the results was determined by a one-way analysis of variance (ANOVA) with the pairwise multiple comparison procedure (Scheffe) for multiple comparison (SPSS program version, 11.5). A value of $P < 0.05$ was considered statistically significant.

## Results and discussion

### Stability of CCB in aqueous solution

Since the phase-solubility studies of CCB were determined by heating method, the chemical stability of CCB in aqueous solution containing CCB/CD complexes was determined. Table 1 shows the CCB content in aqueous solution containing 0.5% (w/v) βCD after zero to three cycles of autoclaving. Each cycle consisted of heating to 121 °C for 20 min. The amount of CCB in an aqueous βCD solution after each cycle of autoclaving was not significantly different ($P < 0.05$); no degradation was observed after three cycles of autoclaving. The result shows that CCB is relatively stable in aqueous CD solutions. It was concluded that the CCB/CD complexes could be prepared by the heating method. Previously Srinivasulu et al. showed that pure CCB was stable during storage at 105 °C for 24 h [14].

**Table 1** Celecoxib content in aqueous solution containing 0.5% w/v βCD after zero to three cycles of autoclaving

| Autoclaving | CCB content (μg/ml) (Mean ± SD) |
|---|---|
| 0 cycle | 1.483 ± 0.038 |
| 1 cycle | 1.512 ± 0.068 |
| 2 cycles | 1.525 ± 0.047 |
| 3 cycles | 1.523 ± 0.051 |

Each cycle consisted of heating to 121 °C for 20 min

### Solubility determinations

Figure 1 displays phase solubility diagrams of CCB in aqueous CD solutions containing αCD, βCD, γCD, HPβCD or RMβCD. In case of βCD, γCD and HPβCD, the CCB solubility increases linearly with increasing CD concentration to give $A_L$-type diagrams (Fig. 1b–d), whereas αCD and RMβCD showed a positive deviation from linearity represented $A_p$-type diagrams (Fig. 1a, e).

The $A_L$-type phase-solubility diagrams with slope less than 1 suggested the formation of a complex that is first-order with respect to both CCB and CD. In other words, that a CCB/CD 1:1 complex is being formed in the aqueous solution [13]. Table 2 shows the values of the apparent stability constants (i.e. $K_{1:1}$ and $K_{1:2}$) and the complexation efficiency (CE) of CCB/CD complexes in pure aqueous CD solutions. For αCD and RMβCD, they indicated that the formation of higher order soluble complexes with respect to CD at elevated CD concentrations. The stability constants (K) for the two complexes i.e., CCB/CD and CCB/$CD_2$ were determined by fitting the data to Eq. 2. They assumed that the 1:1 complex was formed more easily than the 1:2 complex. Ventura et al. [3] investigated CCB/dimethyl-βCD (DMβCD) complexes and obtained complexes of the same 1:1 and 1:2 stoichiometry.

βCD displayed the greatest CE among natural CDs. Studies have shown that the cavity diameter of βCD is of the most appropriate size for aromatic and heterocyclic molecules [15]. However, its aqueous solubility is rather limited due to relatively strong binding of the βCD molecules in the crystal state. Random substitution of the hydroxyl groups, even by hydrophobic moieties such as methoxy functions, will result in dramatic improvements in their solubility [5]. This indicates that the water-soluble βCD derivatives, HPβCD and RMβCD, could be excellent solubilizers of CCB and indeed they had the highest CE of all CD tested (Table 2). Of those two, RMβCD gave the highest CE. However, in pharmaceutical formulation development the CD safety profiles have to be considered. It was reported that the ranking of CD toxicity in human corneal epithelial cell line (HCE) is as follows: αCD > DMβCD > sulfobutyl ether βCD ≈ HPβCD > γCD [16]. Although RMβCD, the lipophilic methylated CD can act as penetration enhancers by disrupting the lipophilic membranes [17], its toxicological effects are low with no obvious morphological changes of nasal mucosa observed upon administration of 10% (w/v) RMβCD [18, 19]. γCD is well tolerated and possesses favorable toxicological profile but RMβCD can only be used at low concentration in aqueous eye drop formulation

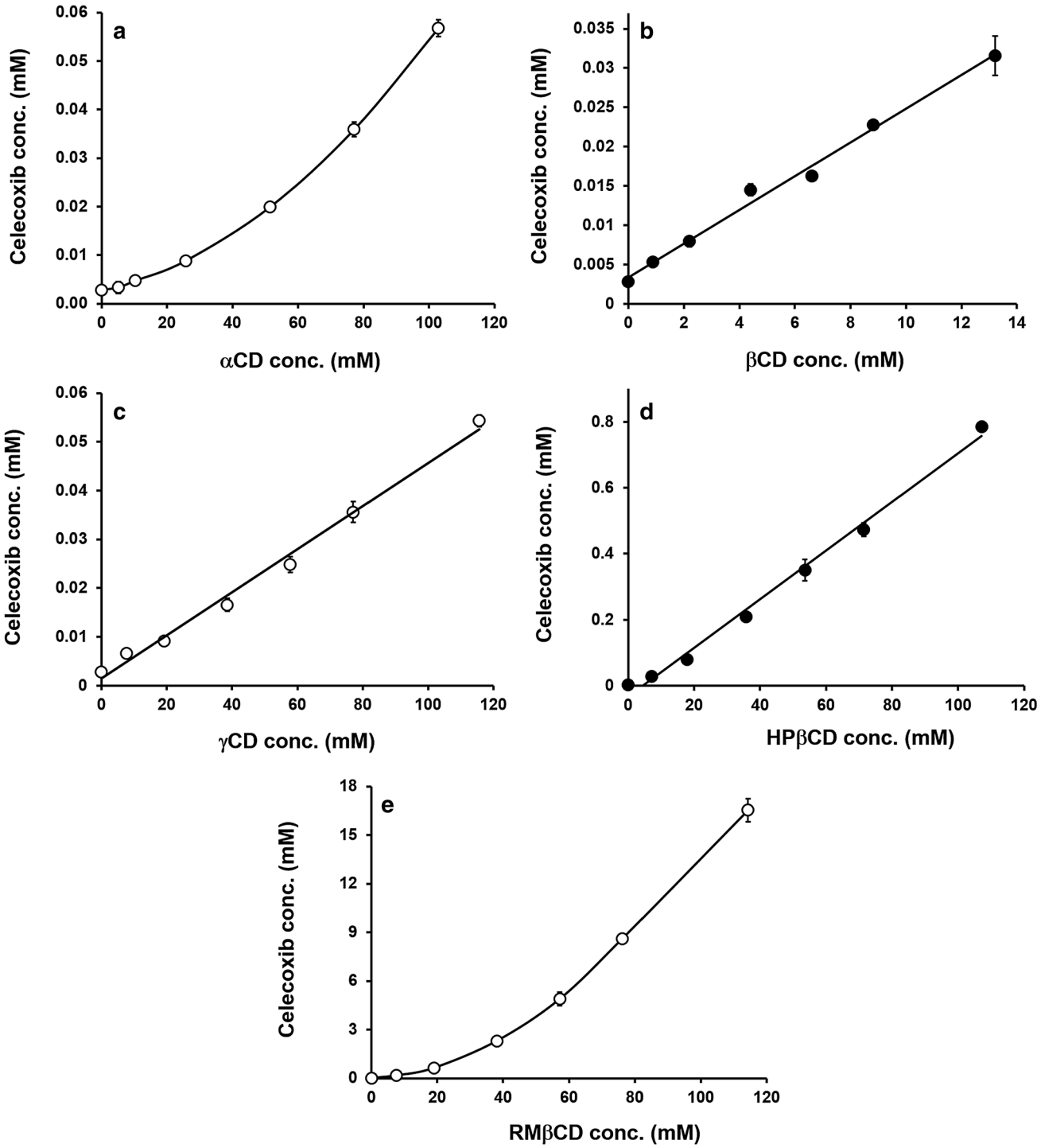



**Fig. 1** Phase solubility profiles of celecoxib in aqueous cyclodextrin. **a** αCD solutions, **b** βCD solutions, **c** γCD solutions, **d** HPβCD solutions and **e** RMβCD solutions

[20, 21]. Based on the phase-solubility profiles and safety, RMβCD and γCD were selected to further studies.

Water-soluble polymers can enhance the CD complexation of drugs and increase the drug permeation from CD containing aqueous vehicles through biological membranes [22]. These effects of polymers on drugs in aqueous CD solutions are possible due to the formation of ternary complexes or co-complexes and their mucoadhesive properties [23–25]. In this study, the effect of polymers, that is HPMC, hyaluronic acid (HA) and chitosan (CS) (non-ionic, anionic

**Table 2** The values of the apparent stability constants (i.e. $K_{1:1}$ and $K_{1:2}$) and the complexation efficiency (CE) of celecoxib/CD complexes in pure aqueous CD solutions at 30 ± 1 °C

| Cyclodextrin | Type | $K_{1:1}$ ($M^{-1}$) | $K_{1:2}$ ($M^{-1}$) | CE |
|---|---|---|---|---|
| αCD | $A_p$ | 49.9 | 26.2 | 0.00014[a] |
| βCD | $A_L$ | 760.2 | – | 0.00215 |
| γCD | $A_L$ | 155.3 | – | 0.00044 |
| HPβCD | $A_L$ | 2630.2 | – | 0.00746 |
| RMβCD | $A_p$ | 3139.9 | 157.3 | 0.00890[a] |

[a]Obtained from the initial linear part of the phase-solubility diagram

and cationic polymer, respectively), on RMβCD and γCD solubilization of CCB was investigated. The amino group in CS has $pK_a$ values from 6.2 to 7.0. With the pH of saturated CCB in aqueous CD solutions containing 0.1% w/v CS was 3.2–4.4. The amine groups are protonated and, thus, CS is a water-soluble cationic polymer in acidic to neutral solutions. Table 3 shows the apparent stability constant and CE of CCB/CD complexes in presence of polymer. The results showed that addition of polymers to the complexing media did not alter the type of the phase solubility diagram indicating that the presence of polymers did not alter the stoichiometry of the complex formed.

Form these results, the apparent stability constant (K) and CE of both binary CCB/RMβCD complex and the binary CCB/γCD complex were increased upon addition of the small amount of polymer. The effect of polymers on the CE of both complexes showed the same trend: HPMC > HA > CS (Table 3). Addition of chitosan to the complexation media has insignificantly effect on the CE of CCB, thus; it was excluded from further studies. Interestingly, HPMC showed the highest increments in the apparent solubilizing efficiency (CE ratio) of CCB about 11-folds and 19-folds increasing in the CE of CCB/RMβCD and CCB/γCD, respectively. The dramatically increasing CE of CCB/RMβCD with HPMC as ternary complex may be possibly caused by the interaction of multi-components. The synergistic effect of the CD-polymer mixture could be due to interaction of the water-soluble polymers with the drug/CD complexes and stabilization of their nano-size aggregates. It is known that polymers stabilize micelles and other types of aggregates [26, 27]. Previous studies have shown that HPMC and PVP can increase the CE as due to formation of ternary HPβCD complexes of acetazolamide, carbamazepine methazolamide and pregnenolone [10].

## Morphology, aggregates particle size and zeta potential analysis

The aggregated size of the ternary CCB/γCD/HA, CCB/γCD/HPMC, CCB/RMβCD/HA and CCB/RMβCD/HPMC complexes were investigated. Two different techniques were applied, DLS and TEM. The DLS technique determines the hydrodynamic diameter of the CCB/CD/polymer aggregates that assuming they have spherical shape. The instrument reports size distribution of particles in an aqueous complexation medium. With the TEM technique the shape of the nanoparticles (i.e. the complex aggregates) become visible and their morphology can be observed. The samples displayed bimodal size distribution that existed the unaggregated CCB/CD complexes (diameter approximately 2 nm) and ternary complex aggregates with diameter between about 250 and 350 nm (Table 4). As expected, the aggregate diameter of the ternary complexes consisted of CCB/CD containing HPMC were larger than those of CCB/CD with HA. It was supported by the zeta potential data that the former ternary complexes had lower zeta potential than those of the latter ones (Table 4). This confirmed that HPMC, non-ionic polymer enhanced CCB solubility by forming large CCB/CD complex aggregates. TEM photographs of the ternary complexes are shown in Fig. 2. The HA containing nanoparticles were spherical but the larger HPMC containing nanoparticles had somewhat irregular shape. The diameter of the ternary complex aggregates observed by TEM was within the same range as those observed by the DLS. This supported the notion that addition of small amount of water-soluble polymer to aqueous CCB/CD complex solutions can enhance the CE of CD through promotion of complex aggregate formation.

**Table 3** Apparent stability constant values ($K_{1:1}$ and $K_{1:2}$) and the apparent complexation efficiency (CE) of celecoxib/cyclodextrin complexes in pure aqueous cyclodextrin solutions in the presence of polymer at 30 ± 1 °C

| Cyclodextrin | Polymer (% w/v) | $S_0$ (mM) | Type | $K_{1:1}$ ($M^{-1}$) | $K_{1:2}$ ($M^{-1}$) | CE | CE ratio |
|---|---|---|---|---|---|---|---|
| γCD | – | 0.0028 | $A_L$ | 155.3 | – | 0.00044 | 1.00 |
| | 0.1% CS | 0.0032 | $A_L$ | 170.9 | – | 0.00054 | 1.23 |
| | 0.01% HA | 0.0028 | $A_L$ | 196.4 | – | 0.00055 | 1.25 |
| | 0.1% HPMC | 0.0275 | $A_L$ | 307.5 | – | 0.00847 | 19.24 |
| RMβCD | – | 0.0028 | $A_p$ | 3140 | 157 | 0.00890 | 1.00 |
| | 0.1% CS | 0.0032 | $A_p$ | 2846 | 136 | 0.00900 | 1.01 |
| | 0.01% HA | 0.0028 | $A_p$ | 3533 | 120 | 0.00990 | 1.11 |
| | 0.1% HPMC | 0.0275 | $A_p$ | 3564 | 18.3 | 0.09820 | 11.03 |

**Table 4** The size distribution and zeta potential of celecoxib/cyclodextrin/ polymer ternary complex aggregates

| Cyclodextrin (% w/v) | Polymer (% w/v) | Mean particle size (nm) | Zeta potential (mV) |
|---|---|---|---|
| 10% γCD | 0.01% HA | 273 ± 36<br>1.86 ± 0.02 | −16.40 ± 2.10 |
| | 0.1% HPMC | 308 ± 8<br>2.10 ± 0.14 | −9.90 ± 2.33 |
| 7.5% RMβCD | 0.01% HA | 283 ± 36<br>2.05 ± 0.25 | −9.27 ± 1.35 |
| | 0.1%HPMC | 312 ± 29<br>2.08 ± 0.48 | −3.46 ± 0.06 |

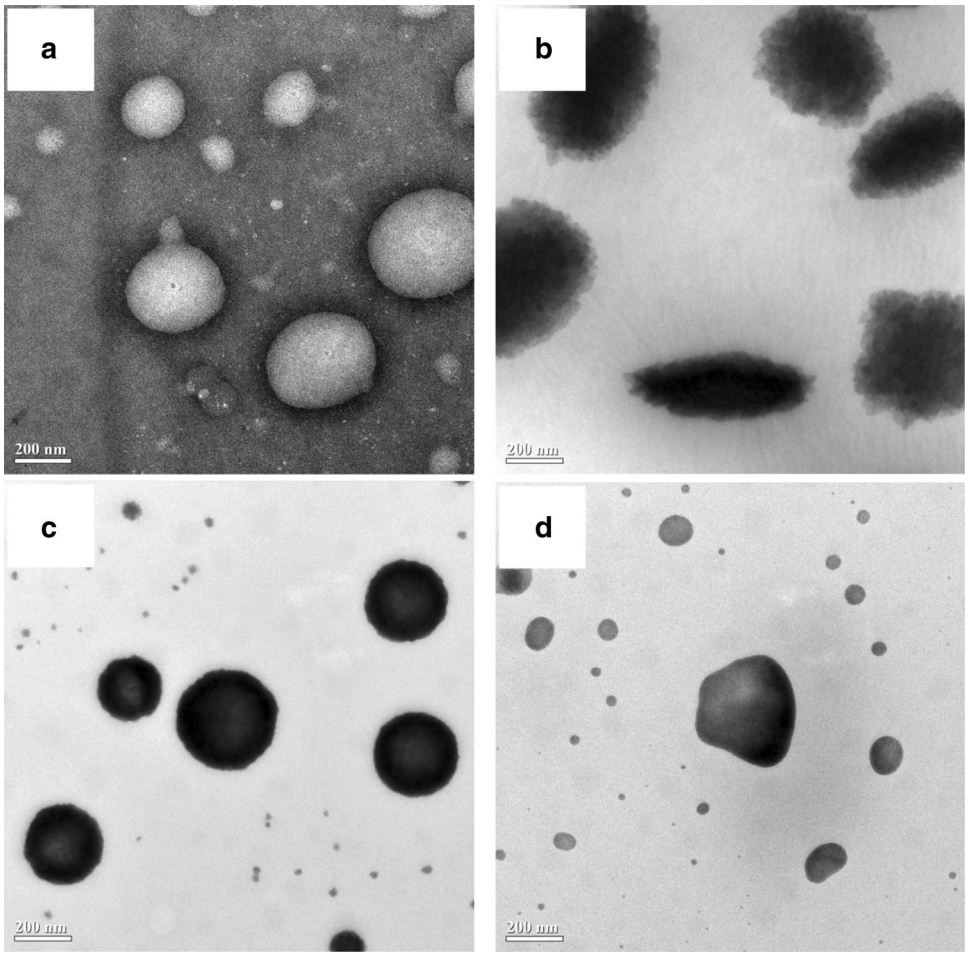



**Fig. 2** TEM photographs of an aqueous drug/cyclodextrin/polymer complex solution saturated with celecoxib. **a** CCB/γCD/HA, **b** CCB/γCD/HPMC, **c** CCB/RMβCD/HA, and **d** CCB/RMβCD/HPMC

In general, in aqueous solutions the relative amount of aggregated CD increases with increasing CD concentration. From the literature, the diameter of CD aggregates is most frequently between 90 and 300 nm [28]. However, CD molecules have their own dynamic behavior resulted from rotational freedom of the glucopyranose unit of CD molecule and the overall molecular mobility. The stabilization of nano-particulate aggregates is regulated by van der Waals forces and hydrogen bonds interactions, which are at all time in competition resulting in very unstable aggregates, although the ability of CD to self-assemble to form reversible aggregates is well documented [29].

## Solid-state characterization

DSC thermograms of CCB, CDs, their PM and FD samples are shown in Figs. 3 and 4. The thermogram of pure CCB exhibited a sharp endothermic peak at 165.17 °C which represented the melting point of the drug (Figs. 3a, 4a). Thermogram of γCD showed a very broad endothermic

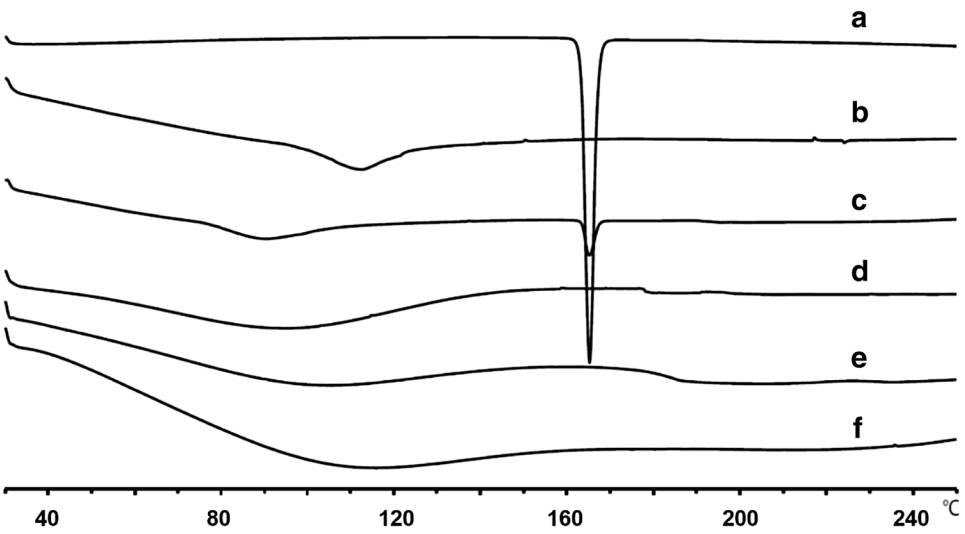



**Fig. 3** DSC thermograms of (*a*) pure CCB, (*b*) pure γCD, (*c*) PM CCB/γCD, (*d*) FD CCB/γCD, (*e*) FD CCB/γCD/HA, and (*f*) FD CCB/γCD/HPMC

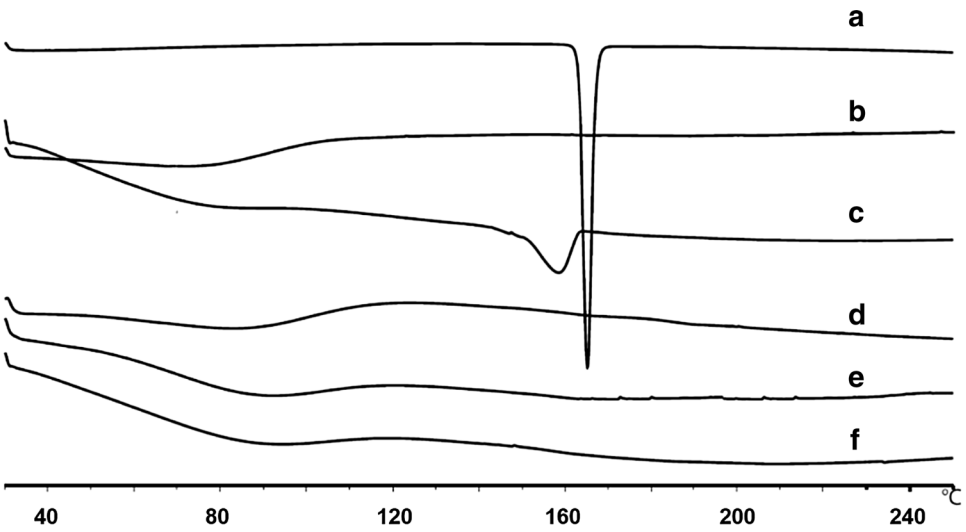



**Fig. 4** DSC thermograms of (*a*) pure CCB, (*b*) pure RMβCD, (*c*) PM CCB/RMβCD, (*d*) FD CCB/RMβCD, (*e*) FD CCB/RMβCD/HA, and (*f*) FD CCB/RMβCD/HPMC

peak in the range of 112 °C (Fig. 3b) due to elimination of water of crystallization [7] while the DSC thermograms of RMβCD showed no thermal change (Fig. 4b). In case of PM, the endothermic peak of the CCB in PM CCB/γCD was retained at 165.17 °C while the peak of the CCB in PM CCB/RMβCD was a broad peak and slightly shifted to lower temperature appeared at 158.67 °C. These observations may be attributed to the presence of weak or no interaction between the pure components in the PM and/or possible interaction between CCB and RMβCD that was promoted by the heating process in the DSC operation [30]. For FD samples, the endothermic peak of binary complex (i.e. FD CCB/γCD and FD CCB/RMβCD) and ternary complex (i.e. FD CCB/γCD/HA, FD CCB/γCD/HPMC, FD CCB/RMβCD/HA and FD CCB/RMβCD/HPMC) were absent (Figs. 3, 4). The disappearance of an endothermic peak may be attributed to an amorphous state and/or presence of inclusion CCB/CD complex in the solid state [31].

PXRD is used to measure the crystallinity of the formed complexes and the peak position (angle of diffraction) is an indication of a crystal structure. The PXRD spectra of pure CCB, γCD, RMβCD, their PM and FD binary and ternary complex are presented in Figs. 5 and 6. The diffractogram of CCB exhibited a series of intense peaks at 5.3, 10.6, 10.9, 12.9, 14.7, 16.0, 17.8, 18.3, 18.6, 19.5, 20.4, 21.4, 22.1, 23.3, 24.5, 24.9, 25.3, 26.9, 27.7 28.2 and 29.4, which were indicated of their crystallinity (Figs. 5a, 6a). The γCD exhibited characteristic peaks at 5.0, 10.1, 11.1, 12.2, 13.8, 15.3, 15.9, 16.3, 16.8, 18.7, 20.3, 21.6 22.8 and 23.3 due to its crystalline nature (Fig. 5b), while RMβCD did not show any peak and exhibited amorphous state (Fig. 6b). RMβCD is obtained by random substitution of the hydroxy moieties of the βCD molecule and, thus, is an amorphous mixture

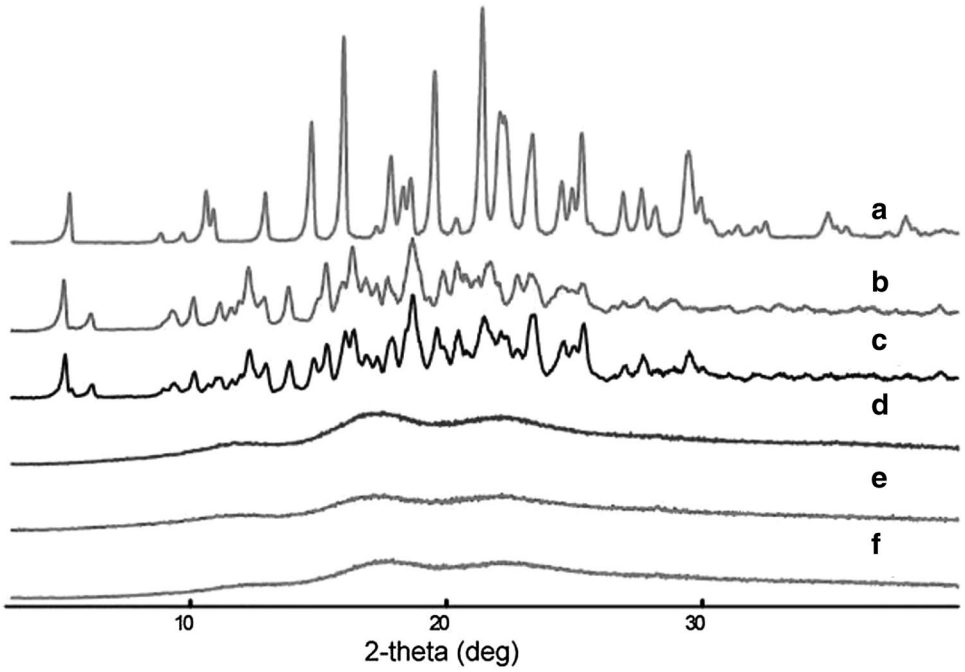



**Fig. 5** The PXRD spectra of (*a*) pure CCB, (*b*) pure γCD, (*c*) PM CCB/γCD, (*d*) FD CCB/γCD, (*e*) FD CCB/γCD/HA, and (*f*) FD CCB/γCD/HPMC

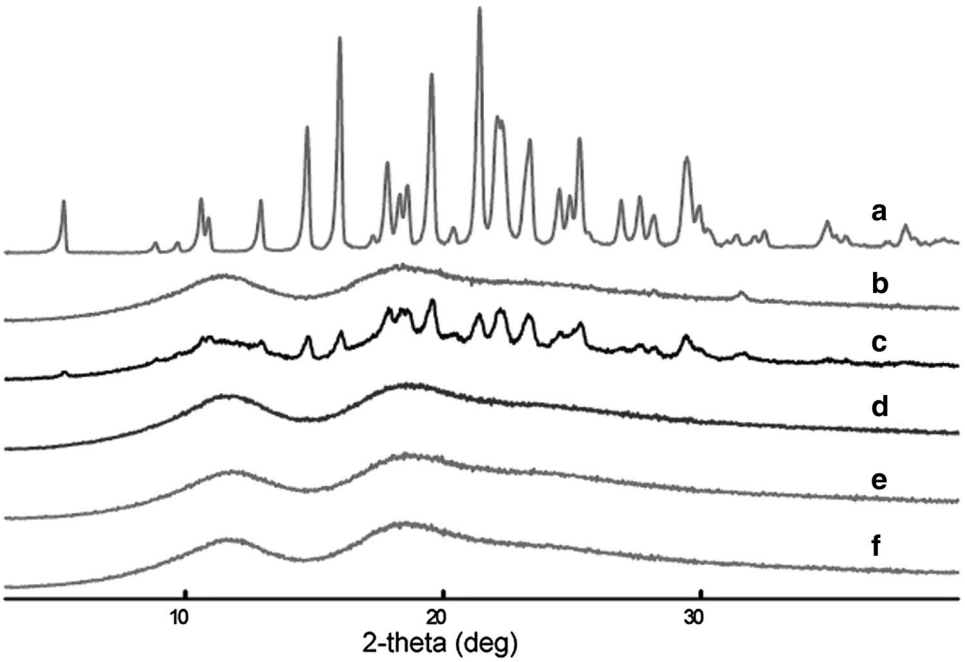



**Fig. 6** The PXRD spectra of (*a*) pure CCB, (*b*) pure RMβCD, (*c*) PM CCB/RMβCD, (*d*) FD CCB/RMβCD, (*e*) FD CCB/RMβCD/HA, and (*f*) FD CCB/RMβCD/HPMC

of isomers. Most of the principal peaks of CCB were presented in the diffraction patterns of PM of both CCB/γCD and CCB/RMβCD (Figs. 5c, 6c). This indicates that there was no interaction between the pure CCB and the respective CDs. In contrast FD samples of CCB/CD binary complexes (i.e. FD CCB/RMβCD and FD CCB/γCD) and ternary complexes (i.e. FD CCB/γCD/HA, FD CCB/γCD/HPMC, FD CCB/RMβCD/HA and FD CCB/RMβCD/HPMC) showed a halo pattern with the disappearance of all the peaks corresponding to CCB. The transformation of CCB from the crystalline to the amorphous form indicates formation of CCB/CD inclusion complexes [32]. However, it should be mentioned that the freeze drying technique applied during sample preparation may affect transformation of the solid state [33].

Figures 7 and 8 show the FT-IR spectra of pure CCB, γCD, RMβCD, their PM and FD samples. The FT-IR spectra of CCB are shown in Figs. 7a and 8a. Its characteristic peaks

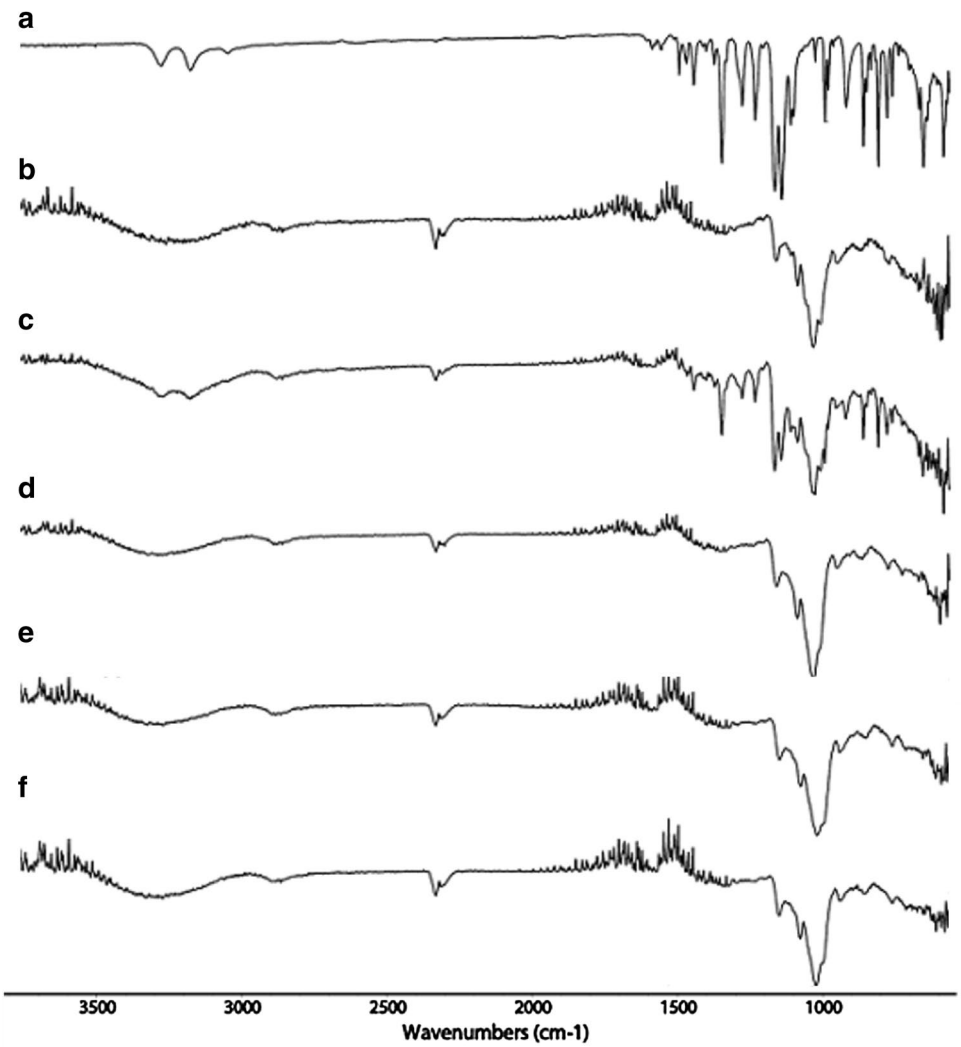



**Fig. 7** FT-IR spectra of (*a*) pure CCB, (*b*) pure γCD, (*c*) PM CCB/γCD, (*d*) FD CCB/γCD, (*e*) FDCCB/γCD/HA, and (*f*) FD CCB/γCD/HPMC

at 3332.4 and 3229.9 $cm^{-1}$ attributed to N–H stretching vibration of $SO_2NH_2$ group, 1346.5 and 1158.2 $cm^{-1}$ for the S=O asymmetric and symmetric stretching and 1228.7 for C–F stretching. The FT-IR spectrum of γCD and RMβCD showed a broad absorption band at 3383 $cm^{-1}$ due to –OH stretching and displayed a large band and distinct peaks in the region of 1200–900 $cm^{-1}$ [34]. The FT-IR spectrum of CCB in PM sample showed the double peaks of N–H stretching that were slightly shifted to 3330.9 and 3229.4 $cm^{-1}$ for PM CCB/γCD (Fig. 7c) and 3336.6 and 3232 $cm^{-1}$ for PM CCB/RMβCD (Fig. 8c). The S=O stretching vibration was slightly shifted to 1347.6 and 1160.7 $cm^{-1}$ for PM CCB/RMβCD while no shift was observed in case of the CCB/γCD complex. These observations indicate that there was less interaction between CCB and CD in the PM samples. For FD samples of both the binary complexes and the ternary complexes, the N–H stretching bands of CCB indicate masking of characteristic symmetric and asymmetric stretch. Likewise, the C–F stretching band and S=O stretching bands of CCB disappeared in the FD samples. These results may be ascribed to the existence of some interaction between functional groups (sulfonamide group or $–CF_3$ group) of CCB and functional groups in the hydrophobic CD cavities in the inclusion complexes [7].

## Solution-state characterization

$^1$H-NMR studies provide information on the existence of CCB/CD inclusion complexes and suggest the conformation of guest molecules in the CD cavity. The changes in $^1$H-chemical shifts (Δδ) observed for the H5 proton of CCB in the presence of RMβCD and γCD were 0.045 and 0.043, respectively and displayed significant upfield shift (Table 5). This behavior can indicate that the H5 proton of CCB is located close to the oxygen atoms in the CD cavity which is rich in $\pi$ electrons [35]. Also, all aromatic protons of CCB showed a significant upfield shift in the presence of γCD. Whereas all aromatic protons of CCB dissolved in aqueous

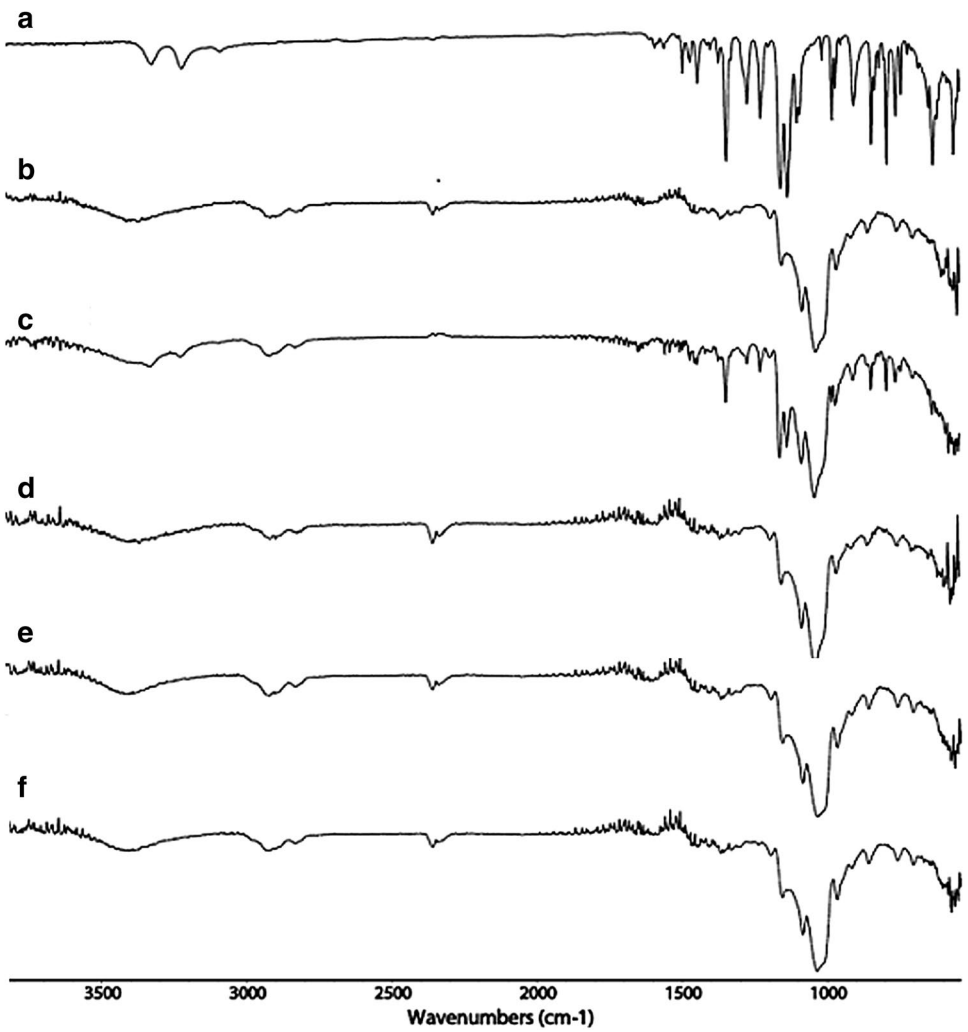
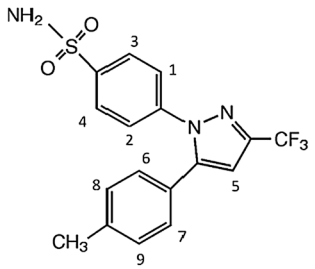



**Fig. 8** FT-IR spectra of (*a*) pure CCB, (*b*) pure RMβCD, (*c*) PM CCB/RMβCD, (*d*) FD CCB/RMβCD, (*e*) FD CCB/RMβCD/HA, and (*f*) FD CCB/RMβCD/HPMC

RMβCD solution displayed downfield shifts demonstrating weaker interactions or van der Waals forces between CCB and the hydrogen atoms of CD [3]. $^{1}$H-NMR chemical shift of RMβCD is summarized in Table 6. The H3 and H5 protons of the glucose units are facing to the interior of the lipophilic CD cavity. The observed changes in (Δδ) for H3 (−0.092) and H5 (−0.042) were upfield which is characteristic for formation of an inclusion complex. The Δδ* of the H3 proton was higher than that of the H5 proton indicating partial inclusion of CCB into the RMβCD cavity [36]. The results are consistent with the data obtained by Ventura et al. [3]. The γCD Δδ* in presence of CCB for the H3 and H5 protons were 0.025 and 0.068, respectively (Table 7). The fact that the Δδ* value of H5 proton is greater than that of the H3 proton, and that they displayed significant downfield shift, indicates the drug molecule occupies the total volume of the hydrophobic γCD cavity.

The proposed conformation structures of CCB/RMβCD and CCB/γCD inclusion complexes are shown in Fig. 9. The CCB/RMβCD complex can form both 1:1 and 1:2 CCB/RMβCD inclusion complexes. Figure 9a shows the 1:1 inclusion complex where the pyrazole head group of CCB is included through the wide opening of the RMβCD central cavity. Figure 9b shows the 1:2 inclusion complex where the CCB is included in to the cavity of the CD dimer in a configuration where half of the CCB molecule was embedded in one monomer and another half is embedded in the other RMβCD monomer of CD. The complex was kept together by formation of hydrogen bonds between the hydroxyl groups or methoxy groups of RMβCD and the fluorine and nitrogen atoms of CCB. Consequently, significant upfield H1 proton of RMβCD may be caused by interaction between hydrogen (H1) at wide rim of the RMβCD molecule and CCB molecule during 1:2 inclusion complex formation. This corresponds to the phase solubility-profile study of the CCB/RMβCD $A_P$-type diagram (1:1 and 1:2 complex). The proposed complex structure is supported by the CCB/βCD complex characterization of Reddy et al. [37]. The proposed conformation of the CCB/γCD complex is shown in Fig. 9c. The Δδ* of H5 in both CCB and

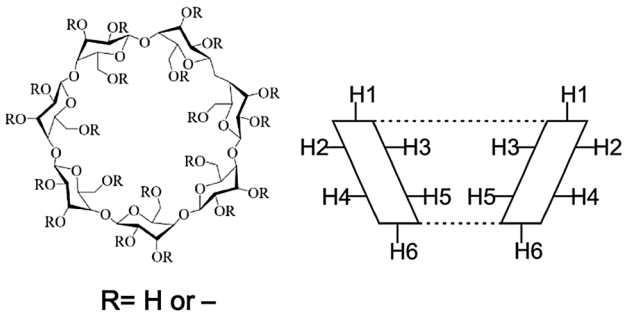
**Table 5** The $^1$H-chemical shifts of celecoxib alone and in the presence of γCD or RMβCD




| Protons | CCB | CCB/RMβCD | Δδ* | CCB/γCD | Δδ* |
|---|---|---|---|---|---|
| $-CH_3$ | 2.206 | 2.217 | +0.011 | 2.183 | − 0.023 |
| H5 | 6.877 | 6.832 | − 0.045 | 6.834 | − 0.043 |
| H6,H7,H8,H9 | 7.062 | 7.076 | +0.014 | 7.035 | − 0.027 |
| H1,H2 | 7.378 | 7.390 | +0.011 | 7.345 | − 0.034 |
| H3,H4 | 7.823 | 7.838 | +0.015 | 7.801 | − 0.022 |

$\Delta\delta^* = \delta_{complex} - \delta_{free}$

**Table 6** The $^1$H-chemical shifts of RMβCD alone and in the presence of celecoxib




| Protons | RMβCD | CCB/RMβCD | Δδ* |
|---|---|---|---|
| H1 | 4.941 | 4.844 | −0.097 |
| H2 | – | – | – |
| H3 | 3.785 | 3.693 | −0.092 |
| H4 | 3.536 | 3.518 | −0.018 |
| H5 | 3.577 | 3.536 | −0.042 |
| H6 | – | – | – |
| $CH_3OC2,3$ | 3.438 | 3.451 | +0.013 |
| $CH_3OC6$ | 3.269 | 3.259 | −0.010 |

$\Delta\delta^* = \delta_{complex} - \delta_{free}$

γCD were significantly shifted and all aromatic protons displayed upfield shift. This indicates that CCB is more deeply included into γCD cavity than that of RMβCD, possibly due to the larger inner cavity diameter of γCD (7.5–8.3 Å) in comparison to that of RMβCD (6 Å).

**Table 7** The $^1$H-chemical shifts of γCD alone and in the presence of celecoxib

| Protons | γCD | CCB/γCD | Δδ* |
|---|---|---|---|
| H1 | 4.991 | 4.994 | +0.003 |
| H2 | 3.523 | 3.499 | −0.024 |
| H3 | 3.815 | 3.840 | +0.025 |
| H4 | 3.469 | 3.480 | +0.011 |
| H5 | 3.732 | 3.800 | +0.068 |
| H6 | 3.751 | 3.821 | +0.070 |

$\Delta\delta^* = \delta_{complex} - \delta_{free}$

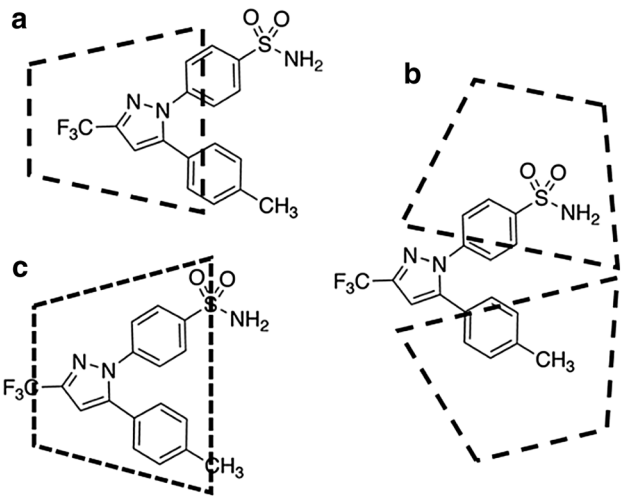



**Fig. 9** The proposed conformation of **a** 1:1 CCB/RMβCD complex, **b** 1:2 CCB/RMβCD complex and **c** 1:1 CCB/γCD complex

## Conclusions

The solubility of celecoxib (CCB), a poorly water-soluble drug, can be enhanced through formation of cyclodextrin (CD) inclusion complexes. CCB is stable in the aqueous CD solutions and no degradation peaks were observed during autoclaving. RMβCD, a relatively lipophilic βCD derivative, exhibited the highest complexation efficiency. HPMC had synergistic effect on the CD solubilization via ternary complex formation and resulted in the highest solubilizing efficiency followed hyaluronic acid and chitosan. The CCB/CD/polymer complexes formed aggregates and participation of the polymers in the aggregate formation is thought to explain the enhanced solubilization. Formation

of CCB/CD and CCB/CD/polymer complexes were verified my NMR studies. The obtained results suggested that CD can enhance solubility of CCB and that such solutions can be sterilized by autoclaving.

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Journal Pre-proofs


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# Antifungal Activity of Econazole Nitrate/Cyclodextrin Complex: Effect of pH and Formation of Complex Aggregates

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## Abstract

Econazole nitrate (ECN) is a weakly basic drug with very low aqueous solubility that hampers its permeation through biological membranes and results in low ECN bioavailability. Formation of drug/cyclodextrin (drug/CD) inclusion complexes is a formulation technology that can be applied to enhance drug solubility in aqueous media. The aim of this study was to determine the effect of CD complexation and pH adjustments on the ECN solubility. The ECN pH-solubility and ECN/CD phase-solubility profiles were determined. The solubility of ECN in aqueous acidic solutions containing α-cyclodextrin (αCD) was relatively high and much higher than in aqueous

γ-cyclodextrin (γCD) solutions under same conditions. The complexation efficiency of the ECN/CD complex was relatively low for the unionized drug. Formation of ECN/CD inclusion complex was verified by proton nuclear magnetic resonance spectroscopy. Formation of ECN/CD complexes enhanced the drug stability during autoclaving. γCD complexes self-assembled to form nano- and microparticles whereas αCD complexes had negligible tendency to self-assemble. Formation of CD complex nano- and microparticles was investigated by dynamic light scattering and by drug permeation through semipermeable membranes of different molecular weight cut-off. The largest aggregate fraction was observed for the unionized ECN in aqueous pH 7.5 solution containing high CD concentration, that is 10% (w/v) CD. It was shown that in acidic solutions ECN/αCD can enhance the antifungal activity to filamentous fungi. This was associated with the increased ECN solubility and increase of readily available ECN molecules in aqueous αCD solutions.

*Keywords:*

Cyclodextrins

Econazole

Solubility

Complexation

Antifungal

## 1. Introduction

Various pharmaceutical techniques can be applied to enhance solubility and permeability of poorly water-soluble drugs, such as pH adjustments, use of cosolvents, size reduction (e.g. micronization), complexation and formation of solid dispersions (Kawabata et al., 2011; Khadka et al., 2014). One of the more promising technologies is drug - cyclodextrin (CD) complexation. CDs are cyclic oligosaccharides consisting of $\alpha$-D-glucopyranose units with hydrophilic outer surface and somewhat lipophilic central cavity. They increase the aqueous solubility of hydrophobic drugs through formation of water-soluble inclusion complexes. In general, uncharged hydrophobic molecules or their moieties, that is molecules that generally have poor aqueous solubility, have affinity for the CD cavity (Brewster and Loftsson, 2007; Jansook et al., 2018).

Saturated aqueous drug/CD complex solutions frequently contain mixtures of inclusion and non-inclusion complexes (Loftsson et al., 2004). Both the natural CDs and their derivatives form CD aggregates. The diameter of the parent CD aggregates (i.e. of αCD, βCD and γCD) have been reported to be between about 200 and 300 nm by dynamic light scattering (DLS) (González-Gaitano et al., 2002). Various analytical techniques, such as DLS, nuclear magnetic resonance (NMR) spectroscopy, transmission electron microscopy (TEM) and permeation through different molecular weight cut-off (MWCO) semipermeable membranes, are commonly used to detect and characterize the aggregates (Duan et al., 2005; Jansook et al., 2010a; Messner et al., 2010; Witte and Hoffmann, 1996).

Econazole nitrate (ECN) is an imidazole antifungal drug. Its antifungal activity appears to be associated with disruption of cell membranes (Dyas and Delargy, 1994).

Less than 1% of applied dose is absorbed into the general blood circulation after topical administration and, thus, currently ECN is mainly used to treat skin infections (Heel et al., 1978; Kalus, 2017). The low aqueous solubility of ECN (<1 mg/ml at 20ºC) limits ECN permeation through biological membranes and results in, for example, low oral bioavailability (Al-Marzouqi et al., 2009; Dyas and Delargy, 1994). Among natural CDs, αCD has the highest solubilizing effect on ECN whereas γCD has almost negligible effect (Díaz-Tomé et al., 2018; Mura et al., 1999). Various methods to enhance the complexing efficacy (CE) have been reviewed (Loftsson and Brewster, 2012; Loftsson and Duchêne, 2007). For example, ternary drug/CD complexes with hydroxyl-acids as third component enhanced ECN solubility with consequent improved drug dissolution (Jug et al., 2014; Mura et al., 2001; Mura et al., 1999). The entrapment of ECN in chitosan/SBEβCD nanoparticles provided mucoadhesive effect and sustained antifungal activity (Mahmoud et al., 2011). However, pH adjustments or drug ionization that increases the drug solubility can result in enhanced CE of ECN/CD complex. The aim of this study was to determine the effect of pH on the CD solubilization of ECN and thermal stability of ECN, and to investigate the influence of pH on formation of ECN/CD complex aggregates. The antifungal activity of the ECN/CD complexes was also evaluated.

## 2. Materials and methods

### *2.1 Materials*

Econazole nitrate (ECN) was purchased from Fagron Group (Amsterdam, Netherlands). α-Cyclodextrin (αCD) and γ-cyclodextrin (γCD) were purchased from Wacker Chemie (Burghausen, Germany), and semi-permeable cellophane membranes (SpectaPor®, molecular weight cut-off (MWCO) 3500, 6-8000 and 12-

14000 Da) from Spectrum Europe (Breda, Netherlands). All other chemicals used were of analytical reagent grade purity. Milli-Q (Millipore, USA) water was used for preparation of all solutions.

*2.2 pH solubility profiles*

Excess amount of ECN was added to the unbuffered aqueous pH medium (pH from 2 to 9) without and with CD (5% w/v αCD or γCD). This pH range covered the ionized and unionized forms of ECN molecules. The desired pH was obtained by dropwise titration of the medium with concentrated aqueous sodium hydroxide or hydrochloric acid solutions. The suspension formed was agitated at room temperature (22-23°C) for 7 days and the pH was readjusted, if necessary. After equilibration the samples were removed and filtered through 0.45 μm nylon membrane filter. Finally, the filtrate was diluted with a mixture of acetonitrile and water (30:70 v/v) and the amount of dissolved drug determined by a high-performance liquid chromatographic (HPLC) method.

*2.3 Thermal stability of econazole*

The stability of ECN in aqueous solutions containing 5% (w/v) αCD or γCD, as well as in purified water, was determined by heating in an autoclave (Loftsson et al., 2005). Heating of an aqueous drug suspension can promote complex formation and enhance the complexation efficiency (CE) (Loftsson and Brewster, 2012). Excess amount of ECN was dissolved in the aqueous complexation media and the pH adjusted with concentrated hydrochloric acid or sodium hydroxide to 3.0, 5.0 or 7.5. The samples were equilibrated at 22-23°C for 24 h under constant agitation. The supernatant was filtered through 0.45 μm nylon membrane filter. The clear filtrate

was then divided into three sealed vials that were heated in an autoclave for one, two and three heating cycles, each cycle consisted of heating to 121°C for 20 min. Then the ECN concentration in the vials was determined by HPLC.

*2.4 Solubility determinations*

Excess amount of ECN was added to aqueous solutions containing from 0 to 12% (w/v) αCD or 0 to 15% (w/v) γCD. The pH of the saturated drug suspensions formed was adjusted with concentrated hydrochloric acid and sodium hydroxide to 3.0±0.2, 5.0±0.2 or 7.5±0.2 and then heated in an autoclave (121°C for 20 min) followed by cooling to room temperature (Loftsson et al., 2005). Then a small amount of solid drug was added to the suspensions to promote drug precipitation. The suspensions were equilibrated at 22-23°C for 7 days under constant agitation. The pH was monitored and readjusted if necessary. After equilibrium was attained, the suspensions were filtered through 0.45 μm syringe filter, the filtrates were diluted with mixture of acetonitrile and water (70:30 v/v) and analyzed by HPLC. The determinations were done in triplicate. The apparent stability constants for the ECN/CD complexes ($K_{1:1}$ and/or $K_{1:2}$), the CE and the ECN:CD molar ratio were determined by Eqs.1-3 (Loftsson et al., 2007).

$$\mathrm{CE} = S_0.K_{1:1} = \frac{\mathrm{Slope\_}}{1 - \mathrm{Slope}} \qquad \text{Eq. 1}$$

$$[S_t] - [S_o] = K_{1:1}[S_o][CD] + K_{1:1}K_{1:2}[S_o] \qquad \text{Eq. 2}$$

$$\text{ECN:CD molar ratio} = \frac{1 + CE}{CE} \qquad \text{Eq. 3}$$

where $S_0$ is the ECN solubility in the aqueous complexation media when no CD is present, $S_t$ is the total amount of dissolved ECN, slope means the corresponding slope of the phase-solubility diagrams, $K_{1:1}$ and $K_{1:2}$ are the stability constants of the 1:1 and 1:2 inclusion complexes.

*2.5 Quantitative determinations*

*2.5.1 Econazole analysis*

ECN content analysis was performed on an ultra HPLC (UHPLC) component system Ultimate 3000 Series from Dionex Softron GmbH (Germering, Germany) consisting of a DGP-3600A pump with a degasser, WPS-3000TLS well plate sampler, TCC-3100 column compartment, photodiode array detector with Chromeloen software version 7.2.8 and Phenomenex Luna C18(2) 5 µm column (4.6x150 mm) with C18 security guard cartridge. The HPLC condition was as follows:

| | |
|---|---|
| Mobile phase: | 50 mM aqueous ammonium acetate:acetronitrile (35:65% v/v) |
| Flow rate: | 1.0 ml/min |
| Oven temperature: | ambient |
| UV detector wavelength: | 220 nm |
| Injection volume: | 20 µl |
| ECN retention: | 6 min |

*2.5.2 αCD and γCD analysis*

Quantitative determinations of αCD and γCD in the samples obtained from phase solubility studies were performed on a reverse-phase UHPLC. Ultimate 3000 series system from Dionex Softron GmbH (Germering, Germany) consisting of LPG-3400SD pump with a degasser, a WPS-3000 autosampler, a TCC-3100 column

compartment operated at 30°C, and a Corona™ ultraRS™ CAD. Phenomenex Luna C18 (150x4.60 mm) 5 μm column with Security Guard (Phenomenex, Cheshire, UK) were used. Chromeleon™, version 7.2 SR4 chromatography data system (CDS) software (ThermoScientific) was used to analysis. The mobile phase consisted of acetonitrile and water (25:75 %v/v), the flow rate was 1.0 ml/min, and the injection volume was 10 μl.

*2.6 $^{1}$H-NMR spectroscopy*

Solutions of the pure compounds (i.e. ECN, αCD, γCD) and the CD complexes of 1:1 stoichiometry ( i.e., ECN/αCD and ECN/γCD) were prepared by dissolving in DMSO-$d_6$:$D_2O$ (90:10 v/v) and equilibrated at 22-23°C under constant agitation for 24 h. Their spectrum and chemical shift values were recorded by using a 400 MHz $^{1}$H-NMR spectrometer (BRUKER™ model AVANCE III HD, Brucker Biospin GmbH, Karlsruhe, Germany). The resonance at 2.5000 ppm, due to residual solvent (DMSO-$d_6$), was used as internal reference. $^{1}$H-NMR chemical shift change (Δδ) was calculated as $\Delta\delta = \delta_{complex} - \delta_{free}$.

*2.7 Determinations of ECN/CD complexes aggregates*

*2.7.1 Dynamic light scattering (DLS) measurement*

The particle size of ECN/CD based aggregates in solution was measured by DLS technique using Nanotrac Wave particle size analyzer (Microtrac Inc., Philadelphia, PA). The samples, that is ECN saturated aqueous αCD or γCD solutions (CD conc. 1, 5 and 10% w/v) of various pH, were placed in the cell holder. The wavelength of the laser beam was set at 780 nm with the scattering angle of 180°. The particle size and percentage of volume were recorded. Each measurement was

conducted at 25 ± 0.5°C and carried out in triplicate. The mass distribution was calculated, assuming that the particle of the complexes and complex aggregates are spherical, according to Eq. 4:

$$M_i = \frac{A_i / R_i^a}{\sum A_i / R_i^a} x100 \quad \text{Eq. 4}$$

where $M_i$ is the mass distribution percentage, $A_i$ is the intensity area, $R_i$ is the hydrodynamic radius of the size population *i* and a is the shape parameter which equals to 3, assuming the spherical particles (Bonini et al., 2006; González-Gaitano et al., 2002).

*2.7.2 Permeation studies*

To investigate the effect of pH and CD concentration on the size of ECN/CD complex aggregates, the *in vitro* ECN permeation from ECN saturated 1, 5 and 10% (w/v) αCD and γCD solutions at pH of 3, 5 and 7.5 through semi-permeable membranes was studied in a Franz diffusion cell apparatus consisting of a donor compartment and a receptor compartment (12 ml). The donor and receptor compartments were separated by the semi-permeable membrane with MWCO 3500, 6-8000 or 12–14,000 Da. The receptor phase consists of 2.5% (w/v) αCD or γCD in pure water. CD was added in the receptor phase to allow for sink condition. Two milliliters of each sample were placed in the donor compartment. The receptor phase was kept at ambient temperature (22-23°C) and stirred continuously (300 rpm) during the experiment. A 150-µl aliquot of the receptor medium was withdrawn at 1, 2, 3, 4 and 6 h for analysis and replaced immediately by an equal volume of fresh receptor medium. The ECN content was determined by HPLC and the amount of

drug permeation was calculated. Each sample was done in triplicate. The steady state flux (*J*) was calculated as the slope of linear plots of the amount of drug in the receptor chamber (*q*) versus time and the apparent permeation coefficient ($P_{app}$) determined from Eq. 5:

$$J = \frac{dq}{A.dt} = P_{app}.Cd \qquad \text{Eq. 5}$$

where *A* is the surface area of the mounted membrane (1.77 $cm^2$) and *Cd* is the concentration of dissolved drug in the donor chamber.

*2.8 Antifungal susceptibility test*

The methods for antifungal susceptibility testing were modified according to Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards (NCCLS) document M27-A3 and M38-A2 of yeasts and filamentous fungi, respectively (CLSI, 2008a; 2008b). Three isolates fungal organisms i.e., *Candida albicans* (DMST 15317), *Aspergillus flavus* (13-56-29897) *and Fusarium solani* (13-61-03470) were tested by broth microdilution assay. Briefly, the isolated organisms were subcultured onto sabouraud dextrose agar (SDA) slants and stored as suspensions at 2-8 °C. The suspensions of tested organisms were diluted with 0.85% saline to the density of $1x10^6$ - $5x10^6$ cells/ml. The turbidity of the supernatant was determined by UV-VIS spectroscopy (Model UV-1601, Shimadzu, Japan) at a wavelength of 530 nm. The tests were conducted in 96-well culture plates. Each well was inoculated with 50 µl of two-fold dilution inoculum suspension. An aliquot of 50 µl of test samples was placed in separate wells in triplicate after appropriate dilution with the tested media. DMSO and the ECN-free medium were

included as growth control. The plates were incubated at 35 °C for 24 h (*C. albicans*) and for 96-120 h (*A. flavus* and *F. solani*).

Minimum inhibitory concentrations (MICs) were read and defined as the lowest ECN concentration at which no growth could be observed. After MIC readings, 10 μl aliquots were removed from each growth-negative well and were spread on SDA petri dishes. The plates were incubated in 35°C, and the fungal colonies grown were counted after 2 days and approximately 4-7 days of incubation for yeast and filamentous fungi, respectively. The minimum fungicidal concentrations (MFCs) were defined as the lowest drug concentration from which no colonies were visible on the agar plate.

## 3. Results and discussion

### *3.1 pH solubility profiles*

The pH solubility profiles of ECN in aqueous solutions without and with CD (5% w/v αCD or γCD) are shown in Fig. 1. The desired pH of the aqueous solutions was adjusted with diluted hydrochloric acid or sodium hydroxide. The aqueous solubility of ECN is less than 1 mg/ml (Abd El-Gawad et al., 2017; Pedersen et al., 1993). ECN has $pK_a$ of 6.6. At pH ranging from 2 to 5, ECN molecules are protonated and somewhat soluble in water. In contrast, at pH above 7.5 the ECN molecules are mainly in their unionized form and very poorly soluble in water (Fig. 2). Addition of CD increases the ECN solubility. αCD is a better solubilizer of ECN than γCD. ECN had affinity to αCD resulting in significantly enhanced ECN solubility through formation of water-soluble ECN/αCD complex. It has been reported that αCD is the best CD solubilizer for ECN of all CDs tested (Díaz-Tomé et al., 2018; Mura et al., 2001). To

evaluate the effect of pH on CD complexation with ECN, the pH of solutions i.e., 3.0, 5.0 and 7.5 were selected for further studies. Of these the pH, ECN molecules exhibit fully ionized, partially ionized and unionized forms, respectively.

*3.2 Thermal stability of econazole on cycles of autoclaving*

The thermal stability of ECN in aqueous solutions without and with CD (αCD or γCD, 5% w/v) at the pH 3, 5 and 7.5 was determined by heating in an autoclave (each heating cycle: 121$^{o}$C for 20 min). Table 1 shows the drug remaining in the complexation media after zero to three cycles of autoclaving. The drug content at pH 3 with and without CD did not decrease upon heating for up to 3 cycles. At pH 5, the drug concentration in pure water or aqueous solution containing 5% w/v γCD decreased after heating for 2 cycles while it was stable in aqueous solutions containing αCD. However, upon increasing the pH to 7.5 the drug degraded rapidly in the αCD solution or from 5% to 18% after 1 to 3 cycles of autoclaving. The ECN content after heating for 3 cycles was significantly decreased when compared to that of no autoclaving ($P$<0.05). The protonated drug is thermally stable while the unionized drug is somewhat unstable. The degradation products of ECN would be produced by hydrolysis of the ether linkage in parent ECN molecule (Dyas and Delargy, 1994). Baker et al. (2016) have found the degradation product of ECN after heating with 5% hydrogen peroxide at 90 °C for 2 h. CD can insulate labile compounds from the environment to prevent drug hydrolysis and oxidation; for examples, doxorubicin, aspirin and β-lactam antibiotics (Loftsson and Brewster, 1996; Popielec et al., 2016). The stabilizing effect of CD depends on the type and concentration of CD, the degree of the complex formation and the rate of

degradation within the complex (Loftsson et al., 2005). In this case, addition of αCD may shield the ECN molecules by encapsulating them against the hydrolysis degradation process.

*3.3 Solubility determinations*

The phase solubility diagrams of ECN in αCD solutions were of type A (Fig. 3a) while their diagrams in γCD solutions were of $B_s$-type (Fig. 3b) according to Higuchi-Connor classification system (Higuchi and Connors, 1965). At the pH lower than the $pK_a$ value (i.e., pH 3.0 and 5.0), the ECN/αCD phase solubility diagram is of $A_N$-type. However, the stoichiometry of the ECN/αCD complex formed is 1:1. In other words, one ECN molecule forms a complex with one αCD molecule. At pH higher than the $pK_a$, the unionized form of ECN forms second or higher order complexes with αCD and $A_P$-type phase solubility diagram is observed. In case of γCD, the drug solubility was increased with increasing γCD concentration with a maximum solubility at 3% (w/v) γCD at pH 3 and 1% w/v γCD at pH 5, with a following decreased solubility at higher γCD concentrations. It indicates that the ECN forms complexes and complex aggregates with γCD which have limited solubility in aqueous solutions. At pH 7.5 the solubility of the ECN/γCD complex was below the limit of quantitation (LOQ). Table 2 shows the apparent stability constants ($K_{1:1}$ and $K_{1:2}$), the complexation efficiency (CE) which was calculated from the slopes of the initial linear sections of the diagrams, and the molar ratio (ECN:CD). The determined intrinsic solubilities of ECN at the pH of 3 were 10 and 1000 times higher than at pH of 5 and 7.5, respectively. ECN is a weak base and, thus the protonized form is somewhat more soluble in water than the unionized form. Regarding to apparent stability constant (K), ECN had stronger binding affinity to αCD. The strong association is probably due to fit of ECN molecule into CD

cavity. The number of αCD and γCD molecules needed to solubilize one ECN molecule (i.e. the molar ratio) was calculated from the CE values. The commercial 1% (w/w) econazole cream contains 10 mg of econazole nitrate per one gram. The results show that it is possible to develop aqueous econazole hydrogel containing CD at low pH (pH 3 or 5) by including 80-100 mg of αCD or 140-280 mg of γCD per one gram hydrogel but at pH 7.5 about 550 mg αCD/gram will be needed to solubilize ECN which is not practical.

*3.4 Quantitative analysis of αCD and γCD*

The presence of ECN decreased the aqueous solubility of γCD while ECN has negligible effect on the αCD solubility (Fig. 4). According to the phase-solubility profiles, precipitation of the ECN/γCD complexes resulted in limited ECN solubility at elevated γCD concentrations. If formation of 1:1 ECN/γCD complex is assumed, the number of γCD molecules in the saturated aqueous ECN solutions should be proportional to the ECN solubility profiles. However, it was found that the observed γCD solubility did slightly deviated from the theoretical values (Fig. 4b). This might indicate that γCD self-aggregates at elevated γCD concentrations. This self-aggregation strongly affected the ECN solubility. The observations were in accordance with previous reports (Jansook et al., 2010b; Messner et al., 2011). The media pH did not have any significant effect on the CD solubility in saturated ECN solutions.

*3.5 $^{1}$H-NMR analysis*

$^{1}$H-NMR chemical shifts of αCD and γCD are summarized in Table 3. The H3 and H5 protons of the glucose units are facing the interior of the lipophilic CD cavity. The changes in $^{1}$H-chemical shifts (Δδ) of the H3 proton of αCD and γCD in the

presence of ECN were -0.176 and -0.064, respectively, displaying significant upfield shift, while the H5 proton of both CDs exhibited insignificant chemical shift (i.e. +0.028 and -0.027 for αCD and γCD, respectively). The Δδ* of the H3 proton was higher than that of the H5 proton indicating the partial inclusion of ECN into the CD cavity (Greatbanks and Pickford, 1987). In comparison, the lipophilic moiety of ECN was more efficiently inserted into αCD cavity than into that of γCD. This observation supported the obtained phase solubility data and the fact that αCD is better solubilizer of ECN than γCD. The results are consistent with the 1D and 2D NMR data obtained by Díaz-Tomé et al. (Díaz-Tomé et al., 2018). They suggested that the imidazole ring of ECN was included into the CD cavity.

*3.6 Determinations of ECN/CD complexes aggregates*

Table 4 shows the size and size distribution data of ECN/CD complexes in aqueous solutions at different pH. The particle size distributions varied, ranging from mono-, bi- to trimodal distribution. In most cases, no complex aggregates were seen in the saturated ECN aqueous solutions containing 1% w/v CD. In most cases, small and large aggregates were observed when the CD concentration was increased to 5% and 10% w/v. The influence of pH on the ECN/CD complex aggregation was also examined. It was noticed that the aggregate size and the size distribution increased with increasing pH. This was probably due to ECN protonation at low pH and the consequent charge-charge repulsion. Especially, at the pH 3 there was insignificant difference among CD concentrations in the tendency to form aggregates. In other words, the unionized ECN/CD complex present at high pH has greater tendency to self-association to form complex aggregates than complexes of the ionized drug.

Permeation of drug through semi-permeable membranes of different MWCO can be used to observe aggregation of drug/CD complexes (Messner et al., 2010). According to the phase solubility profiles, the stoichiometry of ECN/CD complex can be assumed to be 1:1. Thus, the ECN/αCD 1:1 complex dimers, tetramers and octamers can pass through semipermeable membranes of MWCO 3500, 6-8000 and 12-14000 Da, respectively. γCD has higher MW and hence, the 1:1 ECN/γCD complex monomers, trimers and hexamers able to permeate these membranes, respectively. Figure 5 displays the flux and the $P_{app}$ of ECN from ECN saturated aqueous CD solutions at various pH. As expected, both ECN permeation flux and $P_{app}$ increased with increasing membrane MWCO. Also, the aggregate formation and their size increase with increasing CD concentration. The ECN flux from aqueous ECN saturated αCD solutions was higher in all cases than from comparable γCD solutions due to the higher ECN $C_d$ in the αCD complexing medium. In case of ECN in saturated aqueous αCD solutions, the flux of ECN from ECN saturated αCD solutions did not increase proportionally with increasing αCD concentrations as would be expected from the observed A-type phase solubility profile. This shows that at higher αCD concentrations the solubilized ECN was partly present in soluble aggregates that could not permeate the membrane. The ratio of the flux of ECN permeated through the incremental MWCO values (i.e., 6-8 kDa/3.5 kDa and 12-14 kDa/3.5 kDa) are shown in Table 5. The fraction of small ECN/CD complex aggregates (i.e., tetramers to dimers and trimers to monomers in case of αCD and γCD, respectively) increased with increasing CD concentrations. At pH 7.5 the small aggregates were predominant. Especially, the highest fraction of small aggregates (3-times higher) was observed in 10% αCD solution saturated with ECN (Table 5). In other words, the decreased ability to form ECN/CD complex aggregates at low pH (i.e. pH 3 and 5) was most probably

due to the repulsion force between positively charged protonated ECN. This result was confirmed by the DLS data. In all cases, formation of ECN/γCD complex aggregates was less than in the case of αCD. Normally, in aqueous solutions γCD tends to self-aggregate at high γCD concentrations (Jansook et al., 2010b; Saokham and Loftsson, 2017). This phenomenon may hamper formation of ECN/γCD complexes and solubilization of ECN through formation of water-soluble complex aggregates. Thus, the saturated aqueous solutions containing complexes aggregates of ECN/αCD were selected to evaluate the antifungal activity.

*3.7 Antifungal susceptibility test*

The MICs and MFCs of ECN in saturated aqueous αCD solutions at pH 3 and 5 against *C. albicans*, *A. flavus* and *F. solani* were obtained by broth microdilution technique (Table 6). ECN solubilized in aqueous αCD solutions had MIC and MFC ranging between 5-45 µg/ml and 0.7-11 µg/ml against *C. albicans* and filamentous fungi i.e., *A. flavus* and *F. solani*, respectively. Aqueous 10% w/v αCD solution saturated with ECN inhibited the growth of tested pathogenic fungi at the same ECN concentration or less than solutions without αCD. ECN/αCD solutions at pH 3 and 5 did not display significantly different antimycolytic activity. In comparison to the uncomplexed ECN, the ECN/αCD inclusion complex showed higher fungicidal activity especially against *A. flavus* (MFC 0.7 µg/ml) followed by *F. solani* (MFC 11.0 µg/ml) while the CD complex did not enhance the antifungal activity of ECN against *C. albicans* (MFC ~44 µg/ml). The effect of pH on *in vitro* susceptibility of *C. glabrata* and *C. albicans* was investigated by Danby et al. (2012). It was found that *C. albicans* strains had reduced susceptibility to azole antifungal agents at pH 4. However, the

exact mechanism of the pH induced reduction of the antifungal activity has not been established.

Schär et al. (1976) reported that ECN was more active against filamentous fungi such as *Aspergillus* and *Rhizopus spp*. The results indicated that the CD complexation could improve antifungal activity in terms of both MIC and MFC to filamentous fungi. Gao et al. (2019) summarized that the inclusion complex of CD and chlorothalonil had better fungicidal activity than original chlorothalonil. Thus, ECN/αCD complex is a potential therapeutic alternative that can be further developed to pharmaceutical preparation for the treatment of infectious diseases (Díaz-Tomé et al., 2018; Jacobsen et al., 1999).

In general, the ECN concentration must exceed the MIC in the epidermis and as deep as the middle region of the dermis for dermatophytes. Due to their size and hydrophilicity, CDs are not able to penetrate biological membranes. CDs enhance topical drug delivery by increasing the drug availability at the barrier surface and can only enhance topical drug delivery in the presence of water (Loftsson and Masson, 2001). In addition, the CD complex aggregates may hamper the drug permeability through the lipophilic membrane. To overcome the difficulties, ECN/CD can be incorporated into hydrogel contained water-rich structure or lipid nanoparticles; for example, liposome is possible to enhance the topical drug permeation (Chen et al., 2014).

## 4. Conclusions

The very low aqueous solubility of ECN is the main obstacle in ECN product development. CD complexation with pH adjustments can improve the ECN solubility. ECN has relatively high affinity to the αCD cavity, especially in acidic solutions. αCD

can enhance the ECN solubility in aqueous solutions and increase the thermal stability of ECN. γCD cavity is too large to give a good fit with ENC molecule and γCD and its complexes have higher tendency to self-associate to form aggregates that limits the ECN solubility. The enhanced ECN solubility might be due to CD complex aggregates formation which was observed by DLS and permeation studies. At pH 3 and 5 the ECN saturated aqueous αCD solutions had antifungal activity against the tested filamentous fungi. The results show that αCD complexation can improve antifungal activity of ECN.

## Acknowledgements

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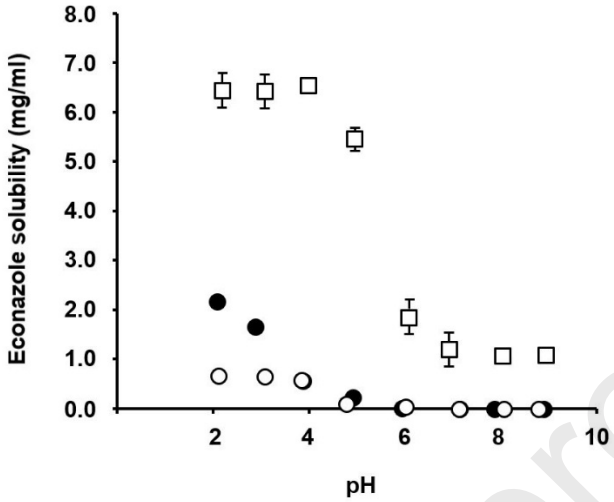



**Fig. 1.** The pH-solubility profiles of econazole in pure water and in aqueous solutions containing 5% w/v αCD or γCD at 22-23°C; pure water (○), αCD (□), γCD (●).

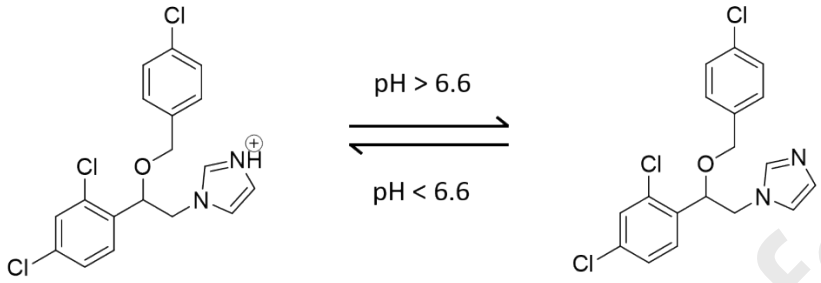



**Fig. 2.** The unionized and ionized form of econazole, pKa = 6.6.

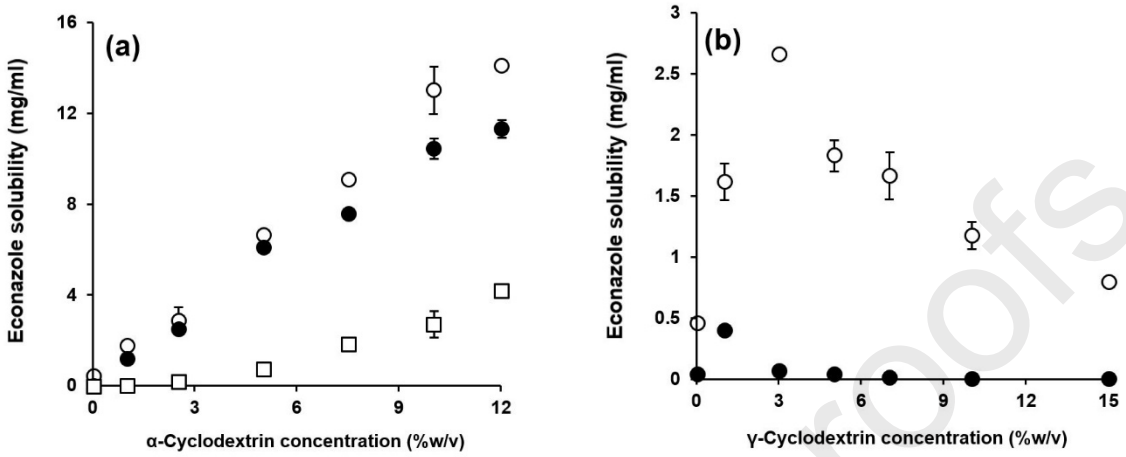



**Fig. 3.** Phase-solubility profiles of econazole in aqueous solutions containing αCD (a) or γCD (b) at different pH (22-23°C); pH 3 (○), pH 5 (●), pH 7.5 (□).

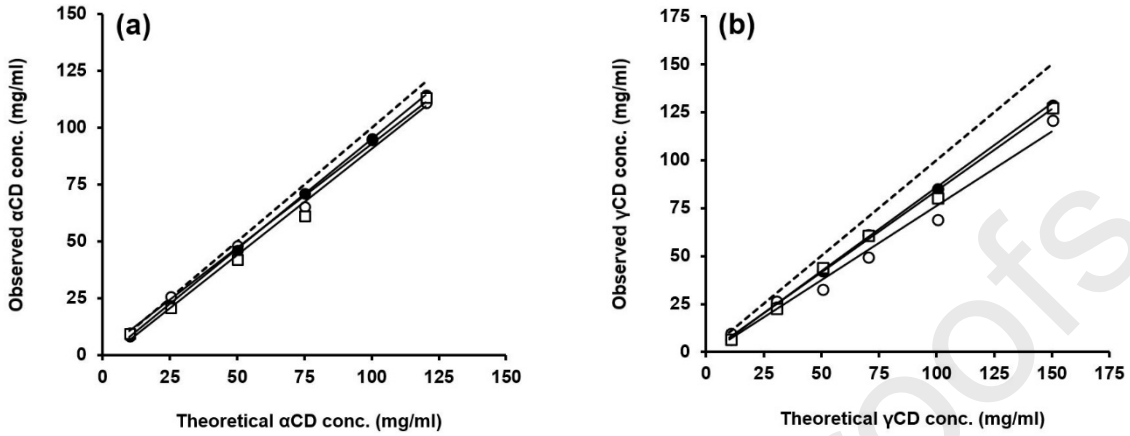



**Fig. 4.** The theoretical and observed CD solubility in aqueous solution saturated with ECN at different pH. The theoretical CD concentrations means the amount of CD added to the aqueous media while the observed CD concentrations were derived from HPLC determination. Theoretical (----); observed (—); pH 3 (○); pH 5 (●); pH 7.5 (□).

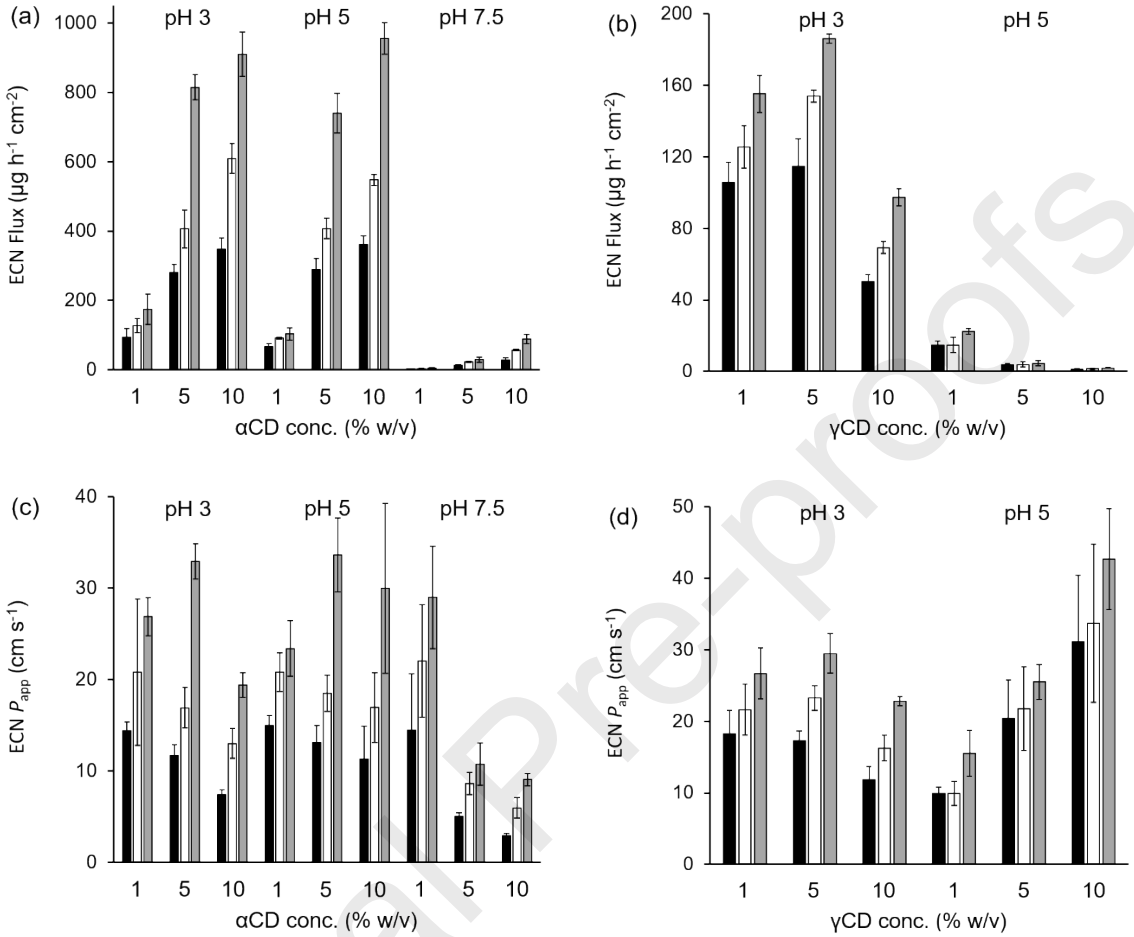



**Fig. 5.** Permeation flux and apparent permeability coefficient ($P_{app}$) of econazole saturated 1, 5 and 10% w/v CD solutions at different pH through semipermeable membranes of various molecular weight cut-off (MWCO)**.** ECN flux against CD concentrations; αCD (a); γCD (b), ECN $P_{app}$ against CD concentrations; αCD (c); γCD (d); MWCO 3500 Da (■); MWCO 6-8,000 Da (□) MWCO 12-14,000 Da (■)**.**

TABLE LEGENDS

**Table 1.** Econazole content in aqueous solution with and without 5% w/v CD at different pH after zero to three cycles of autoclaving.

**Table 2**. The values of the apparent stability constants (i.e. $K_{1:1}$ and $K_{1:2}$) and the complexation efficiency (CE) of econazole/CD complexes in aqueous CD solutions with different pH at 22-23°C.

**Table 3.** The $^{1}$H-chemical shifts of αCD or γCD alone and in the presence of econazole.

**Table 4.** The DLS results of aqueous solution of ECN saturated in aqueous CD solutions at 25°C±0.5°C. Data reported as the means of three determinations, the hydrodynamic diameter (*d*), intensity area (*%A*) and, mass distribution (*%M*). The samples were filtered through 0.45 μm membrane filter prior to analysis.

**Table 5.** The fraction of size population at different cyclodextrin solutions containing saturated econazole which pass through the various MWCO of semipermeable membranes.

**Table 6.** *In vitro* activities of saturated aqueous ECN/αCD solutions in different pH against important medical fungi (Mean, n=3).

**Table 1** Econazole content in aqueous solution with and without 5% w/v CD at different pH after zero to three cycles of autoclaving.[a]

| Sample | ECN content (mg/ml) (Mean ± S.D.) | | | |
|---|---|---|---|---|
| | 0 cycle | 1 cycle | 2 cycles | 3 cycles |
| *No CD* | | | | |
| pH 3 | 0.402 ± 0.039 | 0.395 ± 0.039 | 0.396 ± 0.039 | 0.402 ± 0.042 |
| pH 5 | 0.160 ± 0.034 | 0.159 ± 0.046 | 0.136 ± 0.041 | 0.127 ± 0.042 |
| pH 7.5 | -[b] | -[b] | -[b] | -[b] |
| *5% w/v αCD* | | | | |
| pH 3 | 2.824 ± 0.120 | 2.933 ± 0.108 | 3.019 ± 0.064 | 3.000 ± 0.057 |
| pH 5 | 2.072 ± 0.080 | 2.036 ± 0.099 | 2.101 ± 0.056 | 2.099 ± 0.063 |
| pH 7.5 | 0.342 ± 0.009 [c] | 0.325 ± 0.016 | 0.285 ± 0.035 | 0.279 ± 0.024 [c] |
| *5% w/v γCD* | | | | |
| pH 3 | 0.759 ± 0.021 | 0.761 ± 0.015 | 0.761 ± 0.033 | 0.772 ± 0.022 |
| pH 5 | 0.108 ± 0.008 | 0.106 ± 0.011 | 0.094 ± 0.064 | 0.089 ± 0.018 |
| pH 7.5 | -[b] | -[b] | -[b] | -[b] |

[a]Each cycle consisted of heating to 121°C for 20 minutes.
[b]Could not determined (ECN conc. below LOQ).
[c]$P < 0.05$. $P < 0.05$ was considered statistically significant between 3 cycles of autoclaving and no cycle of autoclaving.

**Table 2** The values of the apparent stability constants (i.e. $K_{1:1}$ and $K_{1:2}$) and the complexation efficiency (CE) of econazole/CD complexes in aqueous CD solutions with different pH at 22-23°C.

| Cyclodextrin | pH | $S_0$ (mM) | Type | $K_{1:1}$ ($M^{-1}$) | $K_{1:2}$ ($M^{-1}$) | CE | Molar ratio |
|---|---|---|---|---|---|---|---|
| αCD | 3 | 1.05 | $A_L$ | 354.5 | - | 0.371 | 1:4 |
| | 5 | $1.13x10^{-1}$ | $A_L$ | 2597.5 | - | 0.293 | 1:5 |
| | 7.5 | $1.11x10^{-3}$ | $A_p$ | 870.2 | 15.0 | 0.041 [a] | 1:25 |
| γCD | 3 | 1.05 | $B_s$ | 246.7 | - | 0.258 [a] | 1:5 |
| | 5 | $1.13x10^{-1}$ | $B_s$ | 1032.8 | - | 0.117 [a] | 1:10 |
| | 7.5 | $1.11x10^{-3}$ | - [b] | - [b] | - [b] | - [b] | - [b] |

[a]Obtained from the initial linear part of the phase-solubility diagram.
[b]Could not determined (ECN conc. below LOQ).

**Table 3.** The $^{1}$H-chemical shifts of αCD or γCD alone and in the presence of econazole

| Protons | CD | ECN/CD | Δδ* |
|---|---|---|---|
| *αCD* | | | |
| H1 | 4.984 | 4.955 | - 0.029 |
| H2 | 3.563 | 3.546 | - 0.017 |
| H3 | 3.895 | 3.719 | - 0.176 |
| H4 | 3.515 | 3.496 | - 0.019 |
| H5 | 3.794 | 3.822 | +0.028 |
| γ*CD* | | | |
| H1 | 5.043 | 5.016 | - 0.027 |
| H2 | 3.572 | 3.581 | +0.009 |
| H3 | 3.835 | 3.771 | - 0.064 |
| H4 | 3.491 | 3.504 | +0.013 |
| H5 | 3.781 | 3.745 | - 0.027 |

$\Delta\delta^* = \delta_{complex} - \delta_{free}$

**Table 4.** The DLS results of aqueous solution of ECN saturated in aqueous CD solutions at 25°C±0.5°C. Data reported as the means of three determinations, the hydrodynamic diameter (*d*), intensity area (*%A*) and, mass distribution (*%M*). The samples were filtered through 0.45 μm membrane filter prior to analysis.

| Sample | pH | Peak summary | | |
|---|---|---|---|---|
| | | *d* (nm) | *%A* | *%M* |
| *αCD(%w/v)* | | | | |
| 1 | 3 | 1.07±0.04 | 100 | 100 |
| 5 | | 1.07±0.08 | 100 | 100 |
| 10 | | 1.05±0.12 | 79.93 | 97.78 |
| | | 2.35±0.97 | 20.07 | 2.22 |
| 1 | 5 | 1.00±0.01 | 100 | 100 |
| 5 | | 0.97±0.01 | 100 | 95.99 |
| | | 1.64±1.43 | 78.47 | 4.01 |
| | | 334.03±6.13 | 15.77 | - |
| 10 | | 0.97±0.02 | 81.00 | 98.37 |
| | | 2.04±0.41 | 12.40 | 1.63 |
| | | 464.37±157.83 | 6.60 | - |
| 1 | 7.5 | 1.05±0.01 | 100 | 100 |
| 5 | | 0.96±0.02 | 67.37 | 100 |
| | | 239.70±47.23 | 3.20 | - |
| | | 518.33±87.65 | 29.43 | - |
| 10 | | 0.96±0.01 | 51.20 | 96.14 |
| | | 1.83±0.79 | 13.57 | 3.66 |
| | | 4.83±1.49 | 13.67 | 0.20 |
| | | 303.90±55.18 | 21.57 | - |
| *γCD (%w/v)* | | | | |
| 1 | 3 | 1.28±0.07 | 100 | 100 |
| 5 | | 1.15±0.11 | 100 | 100 |
| 10 | | 1.00±0.03 | 97.03 | 100 |
| | | 390.93±111.97 | 2.97 | - |
| 1 | 5 | 1.01±0.02 | 98.53 | 99.66 |
| | | 1.66±0.63 | 1.48 | 0.34 |
| 5 | | 1.15±0.06 | 91.97 | 98.64 |
| | | 2.13±0.38 | 8.03 | 1.36 |
| 10 | | 1.20±0.02 | 47.71 | 95.38 |
| | | 2.24±0.67 | 14.97 | 4.62 |
| | | 335.63±42.25 | 37.32 | - |

**Table 5.** The fraction of size population at different cyclodextrin solutions containing saturated econazole which pass through the various MWCO of semipermeable membranes.

| MWCO (kDa) | αCD conc. (%w/v) | | | γCD conc. (%w/v) | | |
|---|---|---|---|---|---|---|
| | 1 | 5 | 10 | 1 | 5 | 10 |
| *pH 3* | | | | | | |
| (6-8)/3.5 | 1.37 | 1.45 | 1.75 | 1.19 | 1.34 | 1.38 |
| (12-14)/3.5 | 1.86 | 2.90 | 2.61 | 1.47 | 1.62 | 1.93 |
| *pH 5* | | | | | | |
| (6-8)/3.5 | 1.37 | 1.41 | 1.51 | 1.01 | 1.06 | 1.10 |
| (12-14)/3.5 | 1.55 | 2.56 | 2.64 | 0.53 | 0.16 | 0.28 |
| *pH 7.5* | | | | | | |
| (6-8)/3.5 | 1.54 | 1.70 | 1.98 | - | - | - |
| (12-14)/3.5 | 2.03 | 2.14 | 3.05 | - | - | - |

**Table 6.** *In vitro* activities of saturated aqueous ECN/αCD solutions in different pH against important medical fungi (Mean, n=3).

| Samples | *C. albicans* | | *A. flavus* | | *F. solani* | |
|---|---|---|---|---|---|---|
| | MIC | MFC | MIC | MFC | MIC | MFC |
| ECN | 5.0 | 20.0 | 1.25 | 1.25 | 5.0 | 20.0 |
| ECN/αCD, pH 3 | 5.5 | 43.8 | 0.7* | 0.7* | 5.5 | 11.0* |
| ECN/αCD, pH 5 | 5.7 | 45.5 | 0.7* | 0.7* | 5.7 | 11.0* |

[a] ECN: econazole nitrate; ECN/αCD: saturated ECN in aqueous solution containing 10% w/v αCD

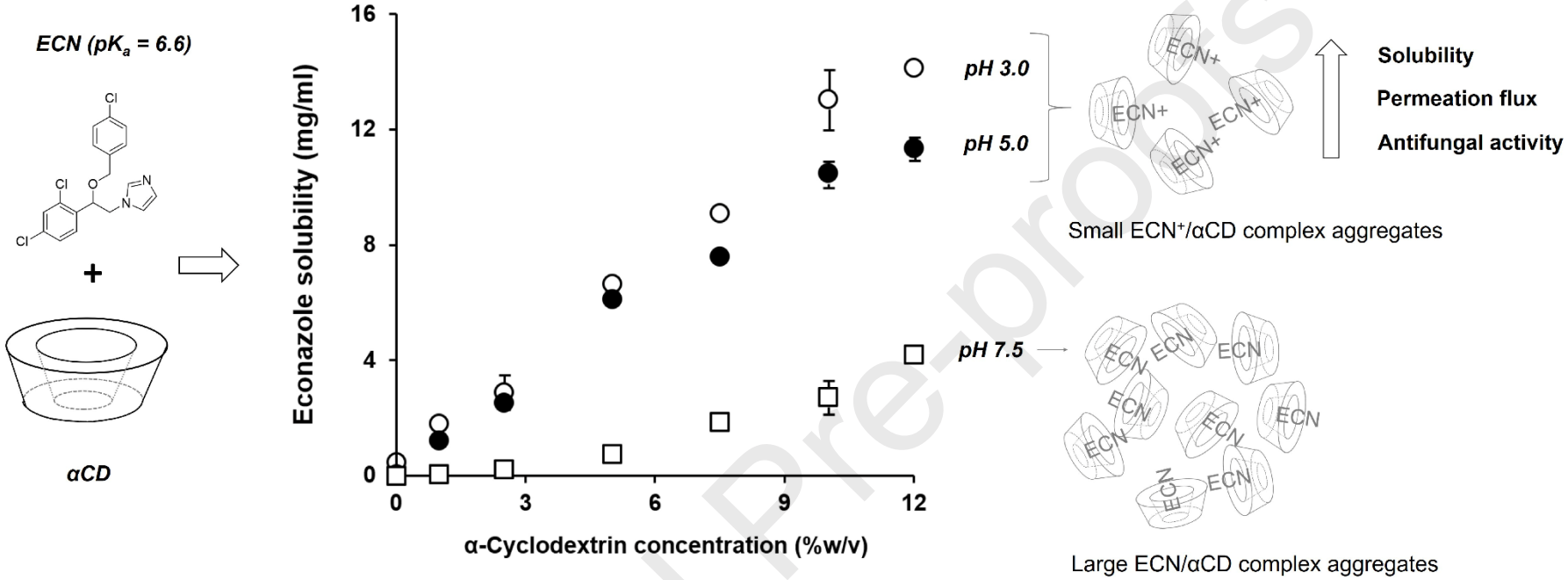
[b] MIC, MFC (μg/ml); * more effective than ECN itself when half strength was given.

**Authors' contributions**

| Name | Location | Role | Contribution |
|---|---|---|---|
| Phatsawee Jansook | Faculty of Pharmaceutical Sciences, Chulalongkorn University, 254 Phyathai rd., Bangkok, 10330 Thailand | Author | Design and conceptualized study; carried out the experiments; interpret and analysis data; drafted manuscript and revision of manuscript |
| Manisha Prajapati | Faculty of Pharmaceutical Sciences, University of Iceland, Hofsvallagata 53, IS-107 Reykjavik, Iceland | Author | Conducted the experiment; interpret and analysis data |
| Patamaporn Pruksakorn | Medical Life Sciences Institute, Department of Medical Sciences, Ministry of Public Health, 88/7 Tiwanon rd., Nonthaburi,11000 Thailand | Author | Carried out the antifungal activity study |
| Thorsteinn Loftsson | Faculty of Pharmaceutical Sciences, University of Iceland, Hofsvallagata 53, IS-107 Reykjavik, Iceland | Author | Supervision and correction of final manuscript; acquisition of the financial support |

**CRediT author statement**

**Phatsawee Jansook**: Conceptualization, Methodology, Investigation, Writing-Original draft preparation.: **Manisha Prajapati**: Resources; Investigation.: **Patamaporn Pruksakorn**: Resources; Investigation.: **Thorsteinn Loftsson**: Supervision; Writing - Review & Editing; Funding acquisition.




**Phase-solubility profiles of ECN in aqueous solutions containing αCD**

**Declaration of interests**

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: