INCREASING LEVODOPA AND CARBIDOPA LOADING QUANTITY IN ORAL PULLULAN THIN FILM

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เนื่องจากปัญหาการใช้ยาของผู้ป่วยพาร์กินสันทั้งในเด็กและผู้สูงอายุซึ่งเป็นผลที่ต่อเนื่องมาจากการบริหารยารูปแบบยาเม็ด ดังนั้นแนวทางเพื่อแก้ไขปัญหาการใช้ยานี้โดยการพัฒนายาลีโวโดปาและคาบิโดปาในรูปแบบแผ่นฟิล์มชนิดบางแบบรับประทานจึงเกิดขึ้น การศึกษานี้มุ่งเน้นการเพิ่มการละลายของลีโวโดปาและคาบิโดปาเพื่อการปรับเพิ่มปริมาณบรรจุยาสำหรับแผ่นฟิล์มชนิดบางรวมถึงการพัฒนาผลิตภัณฑ์และการศึกษาความคงตัวระยะสั้น เทคนิคการเพิ่มการละลายด้วยวิธีไอออไนเซชันจึงถูกเลือกนำมาใช้ ปริมาณลีโวโดปาและคาบิโดปาที่ละลายในสารละลายกรดชนิดต่าง ๆ แสดงให้เห็นว่าค่าการละลายสูงสุดของยาทั้งสองชนิดพบในสารละลายผสมระหว่างกรดไฮโดรคลอริกความเข้มข้น 0.1 โมลาร์ ร่วมกับกรดซิตริก 0.1 โมลาร์ พีเอช 1.5 การใช้ตัวทำละลายร่วมและสารลดแรงตึงผิวและการเพิ่มความเข้มข้นของโพลิเมอร์ได้ถูกนำมาศึกษาด้วยเช่นกัน ผลการศึกษาพบว่าวิธีทั้งหมดข้างต้นไม่สามารถส่งเสริมการเพิ่มละลายของลีโวโดปาและคาบิโดปาในสารละลายกรดได้ แผ่นฟิล์มชนิดบางแบบรับประทานซึ่งบรรจุลีโวโดปาและคาบิโดปาถูกเตรียมขึ้นด้วยวิธีการหล่อจากสารละลายโดยใช้พูลลูแลนเป็นพอลิเมอร์ กลีเซอรีนและกรดแอสคอร์บิกถูกใช้เป็นพลาสติไซเซอร์และสารต้านอนุมูลอิสระในผลิตภัณฑ์ตามลำดับ สูตรตำรับแผ่นฟิล์มชนิดบางแบบรับประทานถูกประเมินคุณสมบัติทางกายภาพเชิงกลด้านต่างๆ แผ่นฟิล์มชนิดบางแบบรับประทานที่ไม่มีกลีเซอรีนจะแสดงลักษณะที่แตกหักง่ายในขณะที่ปริมาณกลีเซอรีนที่ความเข้มข้นร้อยละ 5 และ 10 โดยมวลให้แผ่นฟิล์มที่เหนียวเกินไป แต่อย่างไรก็ตามปริมาณความเข้มข้นของกลีเซอรีนที่ปริมาณร้อยละ 1 หรือ 2 โดยมวลให้แผ่นฟิล์มชนิดบางแบบรับประทานที่เหมาะสม ถึงแม้ว่าปริมาณพูลลูแลนความเข้มข้นสูงร้อยละ 8 โดยมวลจะให้แผ่นฟิล์มชนิดบางแบบรับประทานที่มีคุณสมบัติที่ยอมรับได้แต่สามารถเพิ่มการบรรจุยาได้เพียงเล็กน้อยเท่านั้น ดังนั้นผลิตภัณฑ์แผ่นฟิล์มชนิดบางแบบรับประทานที่พัฒนาผ่านตามข้อกำหนดประกอบด้วยพูลลูแลนความเข้มข้นร้อยละ 8 โดยมวลผลิตภัณฑ์ร่วมกับกลีเซอรีนปริมาณร้อยละ 1 หรือ 2 โดยมวลของพูลลูแลน หากพิจารณาความคงตัวของผลิตภัณฑ์ที่พัฒนาที่กล่าวข้างต้น ไม่พบสูตรตำรับใดเลยที่แสดงความน่าพึงพอใจในด้านความคงตัวซึ่งแสดงให้เห็นจากการลดลงอย่างมีนัยสำคัญของปริมาณลีโวโดปาและคาบิโดปาในระหว่างเวลาการจัดเก็บ ดังนั้นจึงควรมีการพัฒนาสูตรตำรับเพิ่มขึ้นโดยมุ่งเน้นที่สารเติมแต่งเฉพาะและกระบวนการเตรียมผลิตภัณฑ์

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Kitisak Krisai : INCREASING LEVODOPA AND CARBIDOPA LOADING QUANTITY IN ORAL PULLULAN THIN FILM. Advisor: WANCHAI CHONGCHAROEN, Ph.D. Co-advisor: Asst. Prof. Dusadee Charnvanich, Ph.D.

Due to the non-compliance of Parkinson's patient, both youth and elderly patients commonly suffer from the consequent of tablet administration. Therefore, the way to solve this patient's non-compliance is the development of levodopa (LD) and carbidopa (CD) as an oral thin film (OTF) formulation. The current study focuses on the increasing solubility of LD and CD in order to improve the loading quantity for OTF preparation including formulation development and short-term stability investigation. The technique of solubilization by ionization approach was selected. Quantitative determination of solubilized LD and CD in various acid solvents showed the highest solubility of both drugs in 0.1 M HCl acid/0.1 M citric acid pH 1.5. The utilization of cosolvency, surfactant and high polymer concentration were also applied. The result revealed that they were not provide synergistic effect on the improving of LD and CD solubility in acid solvent. OTFs containing LD and CD were prepared by solvent casting method using pullulan as polymeric material. Glycerin and ascorbic acid were used as a plasticizer and antioxidant, respectively. The OTF formulation was assessed according to various physico-mechanical properties. The OTF produced without glycerin was breakable whereas 5% and 10%w/w addition provided tackier film. However, small level of glycerin at 1% and 2%w/w were successfully applied to gain appropriate OTF. Although high pullulan content of 8 %w/w was remarkably showed acceptable film characteristic, loading content of both drugs was negligibly improved. Therefore, the developed OTFs that met the requirements was found to be composed of 8 %w/w of pullulan with either glycerin added of 1% or 2 %w/w of dry pullulan. When considering the stability of mentioned products, they were not exhibited appreciable stability as shown with the significant disappearing of LD and CD content during storage. Further formulation development should be conducted by pointing out with specific additives and method of preparation.

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LIST OF ABBREVIATIONS

AADC	aromatic amino acid decarboxylase
API	active pharmaceutical ingredient
BBB	blood-brain barrier
°C	degree celsius (centigrade)
CD	carbidopa
Co., Ltd.	company limited
COMT	catechol-omethyltransferase
cm	centimeter (s)
cm^2	square centimeter (s)
CMC	critical micelle concentration
CNS	central nervous system
conc	concentration
DI water	deionize water
E	Young's modulus
e.g.	exampli gratia, for example
et.al	et alii, and others
etc.	et cetera
eq	equation
g	gram (s)
g/L	gram per liter
hr	hour
HLB	hydrophilic-lipophilic balance
HPLC	high performance liquid chromatography
l	liter (s)
%LA	percentage label amount
LD	levodopa
L-NAA	large neutral amino acid transporter
M	molarity
mg	milligram (s)
mg/ cm^2	milligram per square centimeter

mg/ml	milligram per milliliter
ml	milliliter (s)
ml/min	milliliter per minute (s)
mm	millimeter (s)
mol	mole
mPa	millipascal
Mw	molecular weight
ND	not detected
No.	numero, numero sign
nm	nanometer (s)
N/m^2	newton per square meter
OTF	oral thin film
PEG400	polyethylene glycol 400
pH	potential of hydrogen ion
Phe	phenylalanine
pKa	dissociation constant
q.s. to	add until
R^2	coefficient of variation
%RH	percentage of relative humidity
rpm	revolutions per minute
%RSD	percentage of relative standard deviation
s	second (s)
SEM	scanning electron microscopy
SD	standard deviation
T_g	glass transition temperature
U.S.P.	United States Pharmacopeia
UV	ultraviolet
UW	ultrapure water
v/v	volume by volume
w/v	weight by volume
w/w	weight by weight
%	percentage

µl	microliter (s)
µm	micrometer (s), micron (s)
σ	tensile stress
ε	strain
γ	activity coefficient
3-O-MD	3-O-methyldopa

CHAPTER I

INTRODUCTION

Nowadays, neurological disorder is one of the most important cause of disability globally. More than 5 million people across the world have suffered from Parkinson's disease. They have been estimated as 0.3 percent of the world's population. People aged 60 or over are diagnosed with Parkinson disease at around 1 to 2 percent and will rapidly increases to 5 percent in the aged over 85. As mentioned earlier, valid information indicated that aging is a pivotal risk factor of such disease. The prevalence of Parkinson's disease in Thailand is approximately 242.57 per 100,000 people or around 0.24% (1). Therefore, Parkinson seems to be continuously arising until it may become the significant issue on quality of life of next era.

Parkinson's disease is a disease that caused by the degeneration of central nervous system (CNS). The pathogenesis is remaining unknown but it is suspected that neurons do not properly function and eventually resulting in less dopamine neurotransmitters in ganglion region. This key neurotransmitter signifies the connection between thalamus and cortex which directly handles the motor function or symptoms. Hence, these symptoms include tremor, rigidity, and loss of spontaneous movement and lack of balance will be occurred.

The main purpose of Parkinson's treatment is governing with the delay of disease progression. Furthermore, it should be brought the patient back to normal condition and improve quality of life. This disease cannot properly treat with only medicine. Thus, three main methods of treatment consist of physical therapy, deep implantation surgery in the brain and drug treatment are combined and employed as standard therapy (2, 3). Even though drug treatment does not allow the recovering of damaging neurons, it can directly increase the dopamine level in the CNS to be more sufficient for the progression of signals transmission. In general, dopamine is a chemical messenger that relates to the conduct of signal for the body movement. If dopamine is administered directly into the body, it does not provide the desired outcome because it does not come across blood-brain barrier (BBB) into the CNS. However, the molecule that is able to pass the BBB should be more favorable to the precursor of

dopamine. Theoretically, the precursor of dopamine goes to L-dihydroxyphenylalanine (levodopa, LD). It is transported by facilitated diffusion mechanism. It has been used as the drug of first choice or as a part of the gold standard treatment for Parkinson's disease. LD is well absorbed via small intestine and transported into CNS by active transport through a large neutral amino acid transporter (L-NAA), where it is converted to dopamine. However, if patient receives only LD, mostly of LD would be altered outside the CNS by either aromatic amino acid decarboxylase (AADC) or catechol-omethyltransferase (COMT) to 3-O-methyldopa (3-O-MD) that eventually inactive form. There is only 1% of LD able to pass through the CNS or BBB. Therefore, administration of parent compound of LD is not effective provided the effective LD level in CNS. The combination of LD with another inhibitor can reduce the breakdown of LD outside the CNS and yield the higher amount of LD before passing through the CNS (4). It is well known and accepted that the combination of LD and carbidopa (CD) is a medicine for the standard treatment of Parkinson's disease.

The ratio between LD and another inhibitor are designed as proportional dosage unit. The available drugs in the market are existed in the form of fixed dose combination such as Sinemet® 100/25, Sinemet® 250/25 etc. However, the dosage that the patients usually administered at the beginning of treatment is only ¼ or ½ of fixed dose tablet which sufficient to control their symptoms. If the standard dose of treatment is not able to control the symptoms, dose titration should be applied in order to optimized desired outcome with less side effect. In case of patients who may be received larger dose of LD than required, a group of such symptoms e.g. nausea, vomit, dizziness and low blood pressure will be expressed. In serious rare cases, even at high dose of LD may causes the confusion, hallucination and sleep problem (1). Therefore, if the drug tablet is divided inaccurately, the patient might be gained an overdosing with more serious side effect (5, 6).

Generally, youth and elderly patients always suffer from the problem relating to the holding of tablet, tablet dividing and tablet administration. The possible way to solve such problems is the using of alternative dosage forms (such as standardized unit micro tablets or solution) that able to improve patient compliance. The standardized unit micro tablets mean the small size tablet containing micro dose of active

pharmaceutical ingredient (API). It will provide an advantage particularly with the prescription of very low dose of drugs. Low dose administration according to the requirement of medical practitioner would be carried out by either dividing the regular tablet or using a several number of micro tablets. (7) Cutting or dividing the regular tablet is commonly performed in daily life but several problems have been occurred as mention earlier. Another way of the utilization of standardized unit micro tablets might be the best practice mode. Nevertheless, many of applied micro tablet was able to generated more specific issue of Parkinson's patient who suffer from hand tremor and chocking. Thus, LD and CD products in the solution form provide more benefit than those of other dosage forms. Anyway, the stability and patient adherence of solution should be reconsidered. Instability of LD and CD solution had been investigated (8, 9) including the difficulty of product handling is also found. An efficient way to overcome many mentioned problems will be the development of oral thin film (OTF) containing LD and CD. OTF can be handled or carried more convenient than tablets. Furthermore, patients with trembling or who have difficulty in holding tablets or chock should more appreciated with them. OTF can disintegrate rapidly in the oral cavity with the aid of small amount of saliva. By the way, the dose titration of OTF that equivalent to ¼ or ½ of tablet strength could be done easily. It could be prepared by calculating the specified area of OTF that contain required dose of API. Theoretically, clear OTFs preparation consisted of molecularly distributed of drugs throughout of thin film of OTF. That is an important the reason why, no matter what area of OTF that has been taken out the drug will be equal. Consequently, the accuracy of dose administration will be achieved and eventually reduces the side effects synchronously. Moreover, an OTF dosage form does not perturb the absorption pathway compared with tablet dosage form. This is because an OTF can dissolve rapidly in the oral cavity and automatically swallow. The absorption via oral cavity is not possibly happened due to the less of retaining drugs in oral cavity. In addition, LD and CD are absorbed mainly through the small intestine using large neutral amino acid transporter. Therefore, absolutely unabsorbed LD and CD from OTFs was proposed. OTF contain LD and CD should provide the nearly identical absorption pattern compared to immediate release tablet formulation.

An OTF can be described as the thin sheet of polymeric material that can easily disintegrate in oral cavity with the aid of small amount of saliva. Generally, the size of OTF is properly defined at around 5 to 20 square centimeters (cm^2) (10). It should contain drugs together with other essential additives. OTF is commonly able to contain API in the range of 1-25% of its weight depending on the value of drug-polymer solubility. Sujaritnarakorn (2016) reported that pullulan OTF containing LD and CD could be fabricated with the loading quantity of 40 mg and 4 mg of LD and CD in 90 cm^2 (11) . Such loading quantity was quite low due to the lower drug solubility. Then, the developed product was found to be larger in term of the dimension which caused the difficulty of patient administration consequently. Therefore, an increasing of higher loading quantity of both LD and CD in OTF that closes to tablet formulation should be achieved. Subsequently, the size of OTF should eventually be reduced according to higher drug loading.

Since LD and CD have low intrinsic water solubility. Their loading quantity in hydrophilic polymer (like pullulan) was commonly less than the target level of drug loading. However, these two drugs were more favorable dissolving in an acidic condition such as hydrochloric acid, acetic acid or formic acid solution including certain buffer solution of citric acid (12, 13). Therefore, both drugs provide more solubility in low pH conditions. In addition, the stability of both LD and CD in acid environment was well accepted. Adjusting of pH of solution should be employed for improving the solubility of both drugs. There are other techniques involving the increasing of drug loading in polymeric thin film such as the adding of surfactant and/or cosolvent. They will include in this study in order to elevate the drug loading capacity.

Therefore, the objective of this study is to increase the solubility of LD and CD in order to improve the loading quantity of both drugs for OTF preparation by using the low pH condition, adding of surfactant and/or cosolvent, increasing the quantity of polymer. Later on, the stable formulation of pullulan OTF containing LD and CD by with appropriated additives will be developed.

CHAPTER II

LITERATURE REVIEWS

1. OTF composition

According to the US FDA guideline, film is defined as a thin layer or coating in which has been categorized along with the its release characteristic. Pallavi (2014) also classify the film product into 3 types based on the release pattern as shown in Table 1 (14).

Table 1 The classification of thin film product using release pattern as a platform (Pallavi, 2014)

Property	flash release	mucoadhesive melt release	mucoadhesive sustain release
Area (cm^2)	2-8	2-7	2-4
Thickness (μm)	20-70	50-500	50-250
Structure	Film single layer	Single or multilayer	multilayer system
Excipient	Soluble, highly hydrophilic polymer	Soluble, hydrophilic polymer	Low/non-soluble polymer
Drug phase	Solid solution	Solid solution/suspended drug particle	Suspension or solid solution
Application	Tongue (upper plate)	Gingival or buccal region	Gingival (or another region of oral cavity)
Dissolution	Maximum 60 seconds	Disintegration in few minutes, forming gel	Maximum 8-10 hours
Site of action	Systemic or local	Systemic or local	Systemic or local

An OTF was first introduced in 1970s. It has been used to overcome the problem related to tablets and capsules swallowing. The development of OTF was based on transdermal patch technology. Another name of OTF appeared as oro-flash release film, oral soluble film, wafer, oral strip, orodispersible film, buccal film and mucoadhesive film. It was definitely described as the "thin sheet of polymeric material" that can easily disintegrate in oral cavity with the aid of small amount of saliva. It is able to be dissolved within 30 seconds (or maximum 60 seconds) in the oral cavity when placed on tongue. Generally, the proper size of OTF used should be in the range of 5 to 20 cm^2 **(**10**)**.

Composition of OTF formulation

The API is incorporated with hydrophilic polymeric material and other excipient such as plasticizer, coloring agents, flavoring agents, sweetener and masking agent. Commonly, the three major components of OTF formulation were API, hydrophilic polymeric material and plasticizer (Table 2). Typical concentration used were also suggested as a range and shown in Table 2.

Table 2 General composition and concentration used of the major ingredients in OTF formulation

Composition	Concentration (%w/w)
API	1 -25
Polymer	40-65
Plasticizer	0-20
Additive	0-40

1.1 Active Pharmaceutical Ingredient

In general, the API should be a low dose drug because the size and thickness of OTF always thin and small that not able to hold up more drug content as appeared in high dose formulation. Unacceptable bitter taste of certain drug substances should be a huge problem in OTF preparation. It was due to the fact that the drug will completely dissolved and molecularly impregnated into polymeric network. When it has been administered, free drug will directly contact with the taste bud in buccal cavity.

Normally, the group of drugs that have been formulated in OTF form is anti-histamine, anti-diarrheal, anti-depressants, vasodilators, anti-asthmatic and anti-emetic etc. (15). For example, salbutamol sulfate, rizatriptan benzoate, verapamil, ondansetron, dexamethasone, rofecoxib, cetirizine, pilocarpine, tianeptine sodium and indomethacin etc. has been formulated in OTF form. (16).

The couple of the drugs between LD and CD (Figure 1, Figure 3) in the therapeutic regimen of Parkinson were a great of interest. Parkinson's patient who may suffered from tablet holding, crushing or the consequence of tablet administration e.g. trembling, choking including the need of water for intake commonly received fixed dose combination in solid dosage form. It was found that several problems from dose titration per individual patient of fixed dose combination tablet had been reported. Thus, tablet of LD and CD should not be appropriately prescribed if another dosage form with more convenient has been generated such as OTF.

The physical characteristics of LD are white crystalline powder, odorless and tasteless. Its chemical property contained the melting point in the range of 284 to 286 °C. It contained low intrinsic water solubility of 5000 mg/L at 20 °C. However, it dissolves well in acids such as hydrochloric acid, acetic acid solution, formic acid solution and buffer solution of citric acid due to the dissociation constant (pKa1) at 2.32 (Figure 2) (9). Nevertheless, LD poorly dissolves in ethanol, benzene, chloroform and ethyl acetate. In term of its stability, oxidative reaction is able to occur immediately under severe moisture and temperature exposure. It will also decomposes to nitrogen oxide when heated (12).

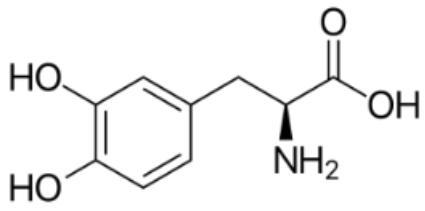


Figure 1 Chemical structure of LD

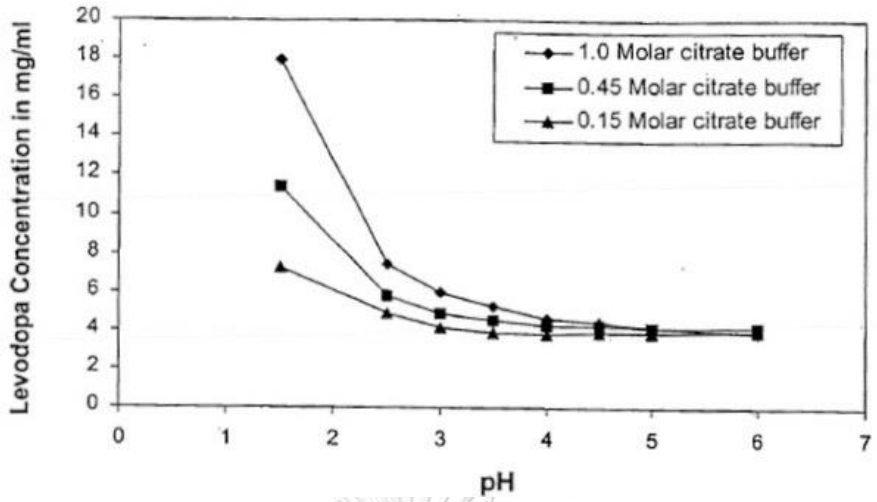


Figure 2 pH solubility profile of LD in citrate buffer solution

For CD, the physical characteristic is a crystalline solid. Its chemical properties showed the melting point in the range of 206-208 °C and dissolved well in acids such as hydrochloric acid. However, it is poorly dissolved in both water and methanol but not in alcohol, acetone, chloroform and ether. In addition, CD contained low intrinsic water solubility at 3.8 mg/mL and the pKa1 of CD is 2.35 at 25 °C (13). Therefore, it will gain the higher solubility at low pH conditions as same as LD.

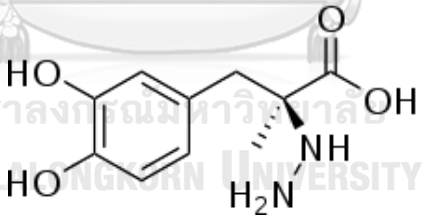


Figure 3 Chemical structure of CD

Due to the physical characteristics and chemical properties of both drugs, the most suitable technique for increasing the solubility of both drugs in OTF is the adjusting of low pH condition. In addition, not only the increasing of drugs solubility but the stability of both drugs also be the important factor. It is due to the fact that LD and CD are unstable when being exposes to air, light, and high temperatures including alkaline pH. An oxidation reaction of LD can cause dopaquinone, leukodopachrome

and red dopachrome, respectively. It is eventually turned to melanin which is the dark color of product (Figure 4) (17).

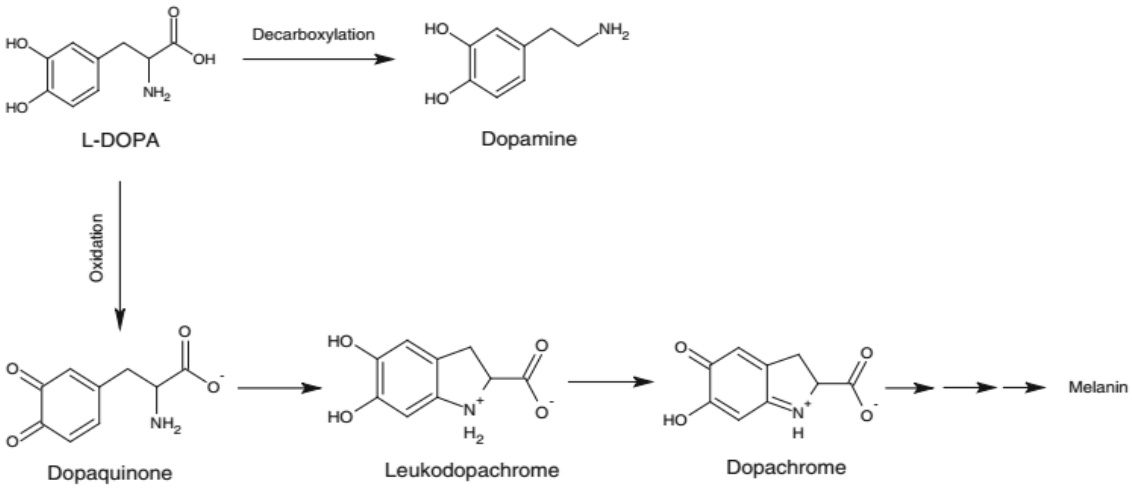


Figure 4 The pathway of LD degradation by oxidative reaction.

Consequently, it was essential for the OTF containing LD and CD to be prevented from light, control the temperature and prepared under acidic condition (at low pH). The initial degradation of LD when exposed to inappropriate condition can be observed by the product discoloration which eventually becomes reddish-brown and black from melanin.

1.2 Polymer or film forming agent

The polymer used mostly be hydrophilic polymer in OTF preparation. It acts as good film formers for OTF since it provides the rapid disintegration and good mechanical properties. Irfan (2016) stated that the productive development of OTF depended on the appropriate selection of the type and concentration of hydrophilic polymers starting material. Furthermore, the OTF's mechanical properties are intensely associated with above mentioned factors (10). They can be utilized either alone or mixing with another one to provide the suitable film properties. Polymer concentration used in OTF formulation is typically around 45% w/w of its total weight of OTF and it can be up to 65% w/w to obtain the desired attributes and characteristics of OTF. The hydrophilic polymer commonly used in film preparation can be divided into 2 major types as shown in Table 3 (18).

Table 3 The type of common hydrophilic polymers in OTF

polymer type	example
natural	starch, pullulan, sodium alginate, pectin, gelatin, and maltodextrins
synthetic	polyvinyl alcohol, polyvinyl pyrrolidone, hydroxy propyl methyl cellulose and hydroxy propyl cellulose

One of the natural polymers which has the appropriate properties for OTF is pullulan. Pullulan powder is white to off white, odorless and tasteless. It is highly soluble in water, dilute alkali but insoluble in alcohol and other organic solvents expect dimethylsulphoxide and formamide. The viscous non-hygroscopic solution is able to be formed by 5-10% pullulan concentration in water (19).

Pullulan is a non-ionic polysaccharide of glucose which produced from the fermentation of black yeast-like *Aureobasidium pullulans* fungus. Its molecular weight is around 1,000 to 2,000,000 daltons depending on the growth conditions of the organism *Aureobasidium pullulans.* It is well known from its biodegradable, biocompatibility, non-toxicity, odorless, tasteless, non-hygroscopic, impermeable to oxygen and highly water-soluble polymer. Pullulan is easily soluble in hot and cold water making clear and viscous solution. It also contained high adhesion and film forming abilities but low viscosity. The viscosity of pullulan solution does not change with heat but rather from pH, and metal ions including sodium chloride (20).

The historical background of pullulan, Cooke (1959) revealed that pullulan was discovered in 1866 by De Bary. Firstly, he described the species of *Dermatium pullulans*. After that in 1891, the *Aureobasidium pullulans* genus was discovered by Viala and Bowyer (21). Leathers (2003) suggested that the biological production and application of pullulan was started in 1938 by Bauer. In 1958, the exopolysaccharide of pullulan was isolated to monopolysaccharide (D-glucose) via acid hydrolysis process. In 1959, α-D-glucan with α-(1-4) linkages and chemical formula $(C_6H_{10}O_5)_n$ were discovered in pullulan structure. After that, its basic structure was determined (1960). Bender (1961) discovered the enzyme pullulanase which specifically hydrolyzed the α-(1-6) linkages in pullulan and converted the polysaccharide almost quantitatively to maltotriose. Accordingly, pullulan is described as α-(1-6) linkages

linked polymer of maltotriose. Up to date, the commercial production of pullulan began by the Hayashibara Company, Ltd in 1976 (22).

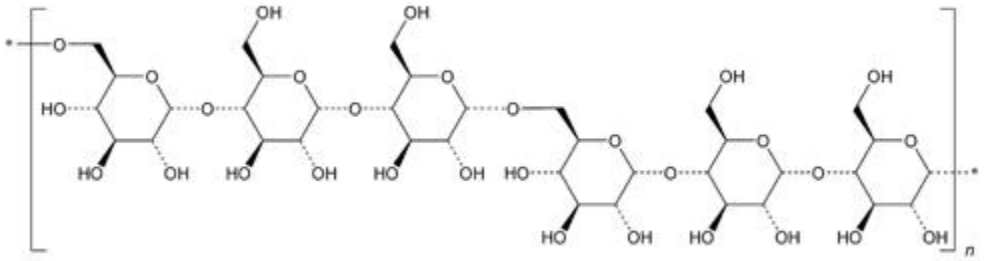


Figure 5 The basic structure of pullulan

Pullulan is a linear homopolysaccharide of glucose. The polymer is α-D-glucan in which α-(1-4) linkages predominate (Figure 5). Pullulan is essentially a linear glucan containing α-(1-4) and α-(1-6) linkages in a ratio of 2:1. The α bond (1-4) linkages connect the glucose to formation the maltose sugar and the α-(1-6) linkages between the maltotriose unit to pullulan formation (Figure 6) (23).

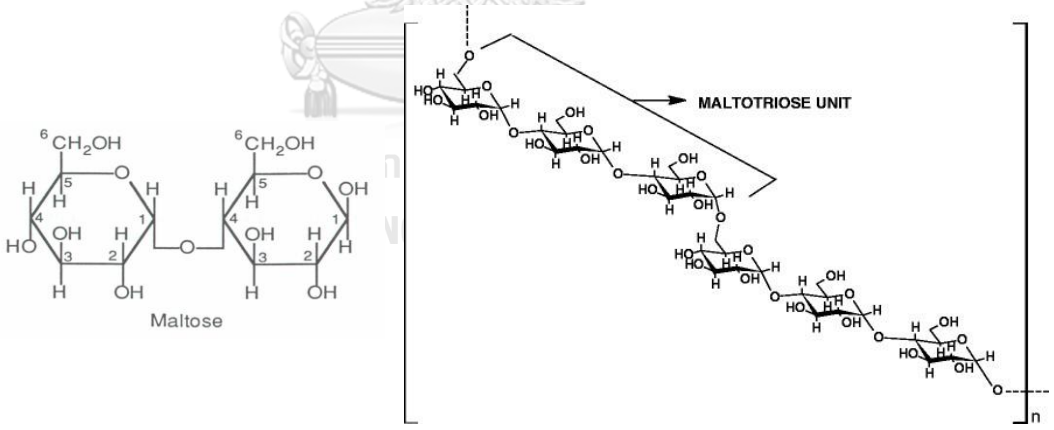


Figure 6 The basic structure of maltose and maltotriose unit.

1.3 Plasticizer

The mechanical properties of OTF such as tensile strength and strain are improved by adding plasticizer. The plasticizer's concentration generally ranges from 0% to 20% w/w of polymer dry weight (24). The common plasticizers are found to be

PEG, glycerol, diethyl phthalate, triethyl citrate and tributyl citrate (25). Pallavi (2014) suggested that plasticizer improves the mechanical properties of OTF such as tensile strength and strain by reducing the glass transition temperature of the polymer. It reduces the OTF's fragility and eventually improves its flexibility (14). However, inappropriate utilization of plasticizer may lead to film breaking or tacky of film.

1.4 Additive

1.4.1 Solubilizing and wetting agents

The solubilizing and wetting agent is used to produce the OTF rapid disintegration. Poloxamer 407 has been used with the widely used surfactants of Tween 80, sodium lauryl sulphate and benzalkonium chloride for the purpose of drug loaded solubilization.

1.4.2 Saliva stimulating agents

The saliva stimulating agent is frequently utilized in OTFs because the increasing the rate of saliva creation will be acquired. Higher rate of saliva secretion lead to higher amount of disintegration medium in oral cavity and will make OTFs rapidly disintegrate after administration. Joshua (2016) suggested the saliva stimulating agent can be used alone or in combination within the range of 2-6%. Typical saliva stimulating agents are indicated as citric acids, malic acid, lactic acid, ascorbic acid and tartaric acid (26).

2 Conventional approaches for manufacturing of OTF

To manufacture the OTF, following methods are generally used:

2.1 Solvent casting method

The most popular utilized technique for OTF preparation is solvent casting method. It is normally used in case of the water-soluble excipients such as hydrophilic polymer, plasticizer and drugs that dissolve in water. Pallavi (2014) demonstrated that the solvent casting method is the process that stirred up the hydrophilic polymer and plasticizer in solution for 2 hours and kept away for taking out the air bubbles. At the same time, other excipients and API were dissolved and stirred for 30 minutes. Both solutions were then combined together. The homogenous mixture was achieved by

applying high shear forces of magnetic stirrer and poured into petri plate or a suitable plastic mold to form an OTF (14).

2.2 Hot melt extrusion

The hot melt extrusion method implicates shaping a mixture of drug, polymer and excipients into a film through the heating process rather than the conventional solvent casting methods. Panda (2012) suggested that the hot melt extrusion was the method that blended the API with other excipients in a dry state, then exposed to the heating process and extruded out in melted state by extruder. The melt of mixture was then transformed into films by the dies (27).

2.3 Semisolid casting

The semisolid casting method was the method that firstly prepared the solution of polymer. The acid insoluble polymer (e.g. cellulose acetate phthalate) which was prepared in ammonium or sodium hydroxide in the solution was carried out. The ratio of the acid insoluble polymer to polymer should be 1:4. They were blended together. After that, the appropriate amount of plasticizer was incorporated to reach the demanded gel mass. Then, the gel mass was rolled or casted in the films or ribbon by heat-controlled drum (28).

2.4 Rolling method

The manufacturing of OTF rolling method firstly prepared the solution or suspension of drug by using the aqueous solvent or hydroalcoholic solvent. The solution or suspension of drug was combined with polymer and then taken into the roller. Irfan (2016) suggested that it was necessary for the solution or suspension to contain specific rheological consideration. The solvent in the mixture of drug solution or suspension with polymer was evaporated and finally dried. The film was formed and cut in to desired sizes (10).

2.5 Solid dispersion extrusion

The solid dispersion method was provided by mixing the API with suitable solvent and incorporated into PEG. The mixture was added to the immiscible solid amorphous hydrophilic polymer. The solid dispersions were later formed into films by dies (10).

2.6 Spray technique

The API, polymer, plasticizer and excipients are dissolved in a solvent until the clear solution is gained. After that, the solution is sprayed over the suitable carrier. Panda (2012) suggested that the suitable material was glass, polyethylene film of non-siliconized Kraft paper and Teflon sheet (27).

3 Characterization and evaluation of OTF

The OTF sample is generally characterized according to the physical, mechanical, quantity and stability aspects in various related guideline.

3.1 Physical properties

3.1.1 Morphology

The organoleptic evaluation or sensory evaluation of OTF is performed by human feeling for screening in transparency, clarity, smoothness, fragility and further investigated are made by the investigation of the presence of insoluble precipitated solid particle.

3.1.2 Disintegration time

OTF's disintegration time is the time that an OTF starts to break or disintegrate from the patch structure when it contacts with water or saliva. There are no official guideline on the procedure or the range of disintegration time for OTF. Nevertheless, Bala (2013) suggested that the disintegration period should be in range of 5-30 s for the fast dissolving film (25).

3.1.3 Moisture content

The hygroscopicity of OTF is determined by the percentage of moisture content. Karki (2016) points out the quantity of moisture in the OTF was able to be the influencer of the mechanical strength, adhesive properties, and friability of OTF (29). The OTF's moisture content is came from several factors such as hygroscopic properties of polymer, API, the solvent of the formulation and the manufacturing method of OTF. Generally, the moisture content is calculated by weighting the differential of OTF at initial weight and final dried weight.

3.1.4 Thickness

The OTF's thickness is usually measured by vernier caliper, micrometer screw gauge, electronic digital micrometer and scanning electron microscopy (SEM) images. It related to the amount of excipients such as polymer, API or plasticizer and the uniformity of film product as dose accuracy of the film. Pallavi (2014) recommended that the thickness of OTF should be in the range of 20 to 250 μm (14). Nair (2013) argued that the thickness of OTF could be up to 1000 μm (30). Therefore, the thickness of OTFs should depend on the purpose and type of film.

3.2 Mechanical properties

3.2.1 Tensile stress

Tensile stress of OTF is a measurement of the force that required to pull the sample to the point where it breaks. In another word, it refers to the maximum quantity of tensile stress before the film sample is raptures. The measurement of tensile stress can be calculated by dividing force at breaking point or sample raptures point with cross-sectional area of film.

3.2.2 Strain

OTF's strain refers to the deviation of the length of OTF from its initial length. The measurement of strain is expressed as the ratio of OTF's length changing over the initial length. Strain is significantly related to the amount of plasticizer in sample**.** Normally**,** the increasing of sample length is the result of increasing plasticizer concentration in formulation.

3.2.3 Young's modulus

Young's modulus or elastic modulus is a mechanical property that signified the measurement of the stiffness or the resistance to elastic deformation of OTF under force applied. Young's modulus is the rate of change in stress to strain in the elastic deformation area. The higher the Young's modulus the more the film strength.

3.3 Quantitative determination

The content of API of OTF or %label amount is determined by appropriate assay method that specifics and validates the method for individual drug substance.

3.4 Stability study

Joshua (2016) stated that stability study is principally done to assess whether the drug substance in prepared sample that formulated in OTFs platform is stable or not. It is also used for the determination the effect of temperature and humidity on the stability of the drug in OTFs for the proper storage (26). Bala (2013) and Joshua (2016) established that the storage conditions that the OTFs samples are kept should be stored at 30ºC / 60 % RH and 45ºC / 75 % RH for 3 months. During the study period, the prepared samples are taken out at three sampling intervals i.e. 0, 1 and 3 month and the sample should be evaluated for physical changes and drug content synchronously (25, 26).

4 Factor affecting OTFs

Glass transition temperature (T_g) of polymeric material base component is one of the important properties on the quality attribute of OTF produced. It is the temperature region where the polymer transitions from hard characteristic (or glassy material or crystalline state) to soft characteristic (or rubbery material or amorphous state). The modification of the T_g of system may be use as an effective approach for improving or gaining the desired characteristic OTF. Plasticizer plays a key role on T_g modification. However, Jadhav (2009) pointed out that not only the incorporation of plasticizer but other additives can alter the T_g. The occurance of new T_g should improve the dissolution, bioavailability, processing, handling qualities of material and physical stability (31).

4.1 Plasticizer

In case of the incorporation of plasticizer, it will greatly get into the void space between the polymeric chains and eventually obstruct the interaction among polymeric chain. Consequently, the polymer is so softened by lowering its T_g after kept at the same storage temperature. It is due to the fact that less polymeric chain interaction of lower T_g pretend to the easily motion of polymer chain. Vuddanda (2017) investigated the effect of plasticizers on the physico-mechanical properties of pullulan based pharmaceutical OTF. The studies showed that the plasticizer could change the mechanical property of pullulan based OTF as tensile strength, elastic modulus and elongation at break implying the structure property, processing and quality of polymeric

films. The tensile strength exhibited the decreasing tendency as the plasticizer concentration increased. The increasing glycerol concentration up to 30% w/w based on weight of pullulan showed negative effects on the mechanical properties of pullulan OTFs. Even though, higher plasticizer of glycerin directly impacted on the mechanical properties of OTF, the disintegrations were not obviously significant difference and not depended upon the content of plasticizer. Conclusively, the hydrophilic nature of pullulan appeared to be a dominate factor over the incorporation of plasticizer (32).

4.2 Water or moisture content

The increasing moisture content of polymer leads to the increasing frees volume and distance between polymer chains. It is due to the polymer chain's ability of hydrogen bond formation with water. To summarize, the increasing frees volume and distance between polymer chains results in decreasing of T_g (31).

4.3 Polymer film thickness

The increasing of film thickness decreases the polymer's molecular mobility and increases the compaction. Therefore, the increasing of film thickness results in an increasing of T_g. On the other hand, T_g is decreased according to the decreasing of film thickness (31).

4.4 Polymer solution and solvent used

The changing of T_g depends on the polymer solution formulation and solvent used in formulation. The solvent additive and the increasing of solvent concentration are able to cause the decreasing of T_g because of the plasticization effect.

5 Techniques for increasing the loading quantity of drug in OTF

The commonly used techniques for increasing the loading quantity of drug in OTF are described herein as follows:

5.1 Increase surfactant or cosolvent in the formulation

The poorly water-soluble drugs are increased the solubility by adding the water miscible solvent as surfactant and/or cosolvent. The adding surfactant and/or cosolvent is wildly used for increasing the solubility of drug because of the simply

approach. The example of typical cosolvents which are frequently used for improving the solubility are PEG 400, propylene glycol, Tween80, glycerin or ethanol (33).

Adding of surfactant and/or cosolvent are carried out in order to increase the solubility of API with the expectation of higher drug loading in particular with OTF. Either cosolvency or the addition of surfactant solubilization techniques would be suggested**.** It can also be used in conjunction with other solubilization techniques to maximize the enhancing solubility.

5.2 Changing the acidity of the formulation

The pH-adjustment method is frequently considered as effective approach for increasing the solubility of an ionizable compound. Ionized drug's solubility is found to be depends on pH of the formulation. Normally, the higher ionizable species of weak acid drug will be occurred under the pH of formulation becomes more basic and vice versa.

Amino acid is an ionizable compound in aqueous solution (Figure 7). It appears in neutral, zwitterion, cationic and anionic, respectively. It's aqueous solubility favorable depended on the pH of solution. The higher proton concentration or lower pH of the acid incorporation is able to generate a cation from the ionization process of its carboxyl group. In the other word, the zwitterion species of amino acid can donate the proton to form anion via its amino group after basic compound was added. Therefore, the solubility of amino acid molecule commonly increases when the significant deviation of proton concentration from its isoelectric point occurred in both situation of higher and lower its pKa (34).

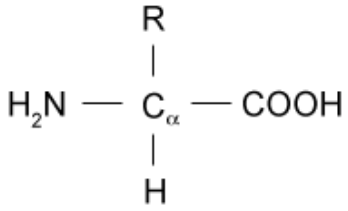


Figure 7 General amino acid chemical structures

5.3 Increase concentration of polymer in a thin film

Marsac (2006) and Huang (2014) said that drugs may be molecularly dissolved or dispersed in homogeneous manner in polymer network. In the case of high drug loading or non-compatible drug polymer system, the drug might be presented in an amorphous or crystalline state (35, 36). Therefore, the higher amount of polymer could hold the higher drug content since the larger interactive space among polymeric chains from higher the cross link of the polymer. It wound be then allows more API to be distributed thoroughly.

In addition, most of polymers have an amphiphilic in nature. They have contained both of hydrophobic and hydrophilic sites that allow them interact with low solubility drug compound. Their interaction will be dominate happened through the hydrophobic molecular region. Their solubilizing structures like micelles, colloids and ionic complexes will be formed to enhance drug solubility. Loftsson (1995) suggested that the polymers can interact with drug by electrostatic bonds or other types of forces, such as van der Waals' forces and hydrogen bridges (37). Thus, the higher the polymer chain the higher the drug loading.

6 Advantage of Pullulan as the OTF polymeric material

From the proper properties of pullulan, Singh (2012) pointed out that pullulan and its derivative is introduced as a food additive for a certain period time. It had been further found with several applications in the specific fields of pharmaceutical and medical device (38). They were chosen as film forming agent, drug delivery system and medical devise because of its remarkable properties such as edible, biodegradable, biocompatibility, non-toxicity, odorless, tasteless, non-hygroscopic, impermeable to oxygen and high water soluble (39-41). There are a lot of researches (42, 43) regarding the application of Pullulan but only film forming properties of pullulan in pharmaceutical area is the main aim of this chapter.

Pullulan has a physical characteristic that suitable for OTF such as transparency, clarity, smoothness, toughness and easy to handle. Galgatte (2013) investigated the various types of polymers, plasticizers and super-disintegrating agents in alone and combination for using in OTF. It was found that the films using pullulan show good

film-forming capacity, transparent and smooth including rapid disintegrate (less than 45 seconds) (44).

Many previous investigations indicated that pullulan was able to contain several types of drugs such as anti-Parkinson, antifungal, antihypertensive and anti-migraine drug. Panchal (2012) prepared the mouth dissolving film formulation of ropinirole hydrochloride comprised of pullulan with PEG 400. It was not any physicochemical interaction amidst ropinirole and its additives. The formulation was stable and illustrated the quick onset of action (45). Moreover, Krull (2016) also studied the preparation and characterization of fast dissolving pullulan film containing griseofulvin. It shown that all pullulan-based film had the excellent content uniformity, fast immediate drug release and further defined pullulan as an acceptable stabilizer (46). Gherman (2016) recommended that pullulan itself was able to use as an effective polymer for enalapril maleate film preparation. The film preparations exhibited a good physicochemical properties as well as a high dissolution rate which make these formulations usable as the effective mucoadhesive buccal film (47). Prajapati (2018) suggested that the pullulan based OTF formulation of zolmitriptan by solvent casting method used PEG 400 and sucralose as a plasticizer and sweetener, respectively. It presented excellent mechanical properties, easy to handle, smooth mouth feels and excellent stability in closed aluminum sachet at 40 ± 2 ◦C and 75 ± 5% RH (6). In addition, Wadetwar (2019) revealed the formulation and evaluation of fast dissolving film of paroxetine prepared with pullulan. It was found that pullulan film had a good physical property, rapid disintegration, good mouth feels and mechanical properties (48). Pullulan polymer was not only used alone but it also in combination with another polymer. Ezim, (2019) studied the dihydroergotamine mesylate sublingual film that was prepared using pullulan and maltodextrin as a film forming polymers and propylene glycol as plasticizer. The suitable formulation using pullulan and maltodextrin was free from air bubbles, cuttings or cracks. It was fast onset of action and high bioavailability that offered for clinical use of emerging migraine(49).

CHAPTER III

MATERIALS AND METHOD

MATERIALS

- Levodopa (Batch No. 20151205), Tunshun pharm and chem Co., Ltd., China
- Carbidopa (Batch No. CAA-20150904), Tunshun pharm and chem Co., Ltd., China
- pullulan (Cosmetic grade, Batch No. 4I01), Hayashibara Co., Ltd., Okayama, Japan
- ascorbic acid (Batch No. DY0261621427) obtained from S. Tong chemical Co., Ltd., Nonthaburi, Thailand
- glycerin U.S.P. (Batch No. 52020) obtained from Srichand united dispensary Co., Ltd., Bangkok, Thailand
- ethanol (Batch No. D2C170207), Apex alco Co., Ltd., Bangkok, Thailand
- PEG400 (Batch No. X15228) obtained from S. Tong chemical Co., Ltd., Nonthaburi, Thailand
- Tween80 (Batch No. 1/2167550) obtained from S. Tong chemical Co., Ltd., Nonthaburi, Thailand
- citric acid monohydrate (Batch No. J008G09), RFCL Limited, New Delhi, India
- tri-sodium citrate (Batch No. AJA467-500G), Ajax finechem, Auxland, New Zealand
- hydrochloric acid (Batch No. A137-2.5L GL), Ajax finechem, Auxland, New Zealand
- potassium chloride (Batch No. A383-1KG), Ajax finechem, Auxland, New Zealand
- sodium chloride (Batch No. 2785521215), Fisher chemical, India
- potassium dihydrogen orthophosphate (Batch No. P167L08), RFCL Limited, New Delhi, India
- sodium phosphate dibasic (Batch No. AJA621-500G), Ajax finechem, Auxland, New Zealand

- acetonitrile, HPLC Grade (Batch No. 1705301801), Avantor performance materials, Poland
- orthophosphoric acid 85%, HPLC Grade (Batch No. V6M675216M), Carlo erba reagent S.A.S, France
- ultrapure water (UW)
- deionize (DI) water

Equipment

- volumetric flask 10, 25, 50, 100, 150, 250, 1000, 2000 ml
- petri dish dimension 9 cm
- magnetic stirrer and bar
- micropipette and tip
- cellulose acetate membrane 0.45 µm
- rectangular plastic mold (6x5x1.5 cm)
- aluminum bag

Instrument

- balance (Model ME403, Mettler Toledo, USA)
- pH meter (Model Sevencompact, Mettler Toledo, USA)
- magnetic stirrer (Model Big squid, IKA, Germany)
- hot air oven (Model Beschickung-Loading Modell 100-800, Memmert, Germany)
- UV-Vis spectrophotometer (Model UV-1800 distributed by Bara scientific Co., Ltd., Thailand)
- vernier caliper (Model Digital caliper within 300mm)
- optical microscope (Nikon eclipse E200) equipped with polarizer, Hollywood international Nikon, Thailand
- moisture analyzer (Model HR83, Mettler Toledo, USA)
- dissolution tester (Model VanKel VK 7000 distributed by Meditop Co., Ltd., Thailand)
- universal testing machine (Model LR10K, LLOTD instruments, England)
- sonicator bath (Model GT SONIC-D13, GT Sonic, China)
- high performance liquid chromatography (Model Agilent HPLC 1260 Infinity II, Agilent, USA)

METHOD

The study was composed of 2 experimental parts.

- Part 1 was performed in order to investigate the solubility of LD and CD in various solvent systems.

- Part 2 was designed for preparing the pullulan OTF containing LD and CD using the appropriated solvent system from the conclusion of Part 1.

Part 1 Solubility determination of LD and CD in various acid solvents

An excess amount of either LD or CD powder were separately suspended in solvents of interested using magnetic stirring at specified conditions. They were agitated at 300 rpm with the controlled temperature of 30±2 °C for 48 hours (50). They should also be protected from light and excessive heat due to the instability of LD and CD substances.

Clear supernatant of suspended sample was collected at 1, 2, 4, 8, 16, 32 and 48 hours, respectively. It was further filtered through cellulose acetate membrane 0.45 µm. The obtained solution was then transferred to 10 ml volumetric flask and diluted with DI water reached at appropriate concentration. Dissolved LD or CD was then quantitated using validated UV spectrophotometry. The investigation of UV absorption was determined at the maximum wavelength of 280 and 279 nm represented the maximum absorptivity of LD and CD, respectively (Appendix A, Figure 27 and 28). DI water was used as a blank. Assay of samples were done in triplicate. The relationship between dissolved drug concentrations against time was constructed. Linear relationship should be obtained within suitable concentration range. The saturated solubility of both drugs was therefore determined as of the concentration at plateau.

The acid solvents of interest for LD and CD were described herein below. They were chosen based on the type and molar concentration including the acidity (pH) related to both drugs physicochemical properties.

- 0.2 M hydrochloric acid/ 0.1 M citric acid pH 1.5
- 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5
- 0.1 M hydrochloric acid/ 0.1 M potassium chloride pH 1.5
- 0.1 M citric acid pH 2

- 0.1 M citric/citrate buffer pH 2.5
- 0.1 M citric/citrate buffer pH 3.0
- 0.1 M citric/citrate buffer pH 3.5

The acid solvent system that provided the highest solubility of LD and CD was brought to the next experiment for employing as a part of OTF formulation.

1.1 Effect of cosolvent on the solubility of LD and CD in acid solvent system

Cosolvency is another great of concern for improving LD and CD aqueous solubility. The selected cosolvents in this study were ethanol, PEG400, and glycerin. However, Tween 80 which was frequently used for solubility improvement of several drugs is also an interesting material (51). Thus, the potential effect of cosolvent and surfactant on the increment of solubilized LD and CD would be investigated. They were incorporated together with the appropriated acid solvent of 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5. Quantitative determination of solubilized either LD or CD was carried out with the same validated UV spectrophotometry as mention earlier (Part 1). In addition, the effect of various concentrations of such cosolvents and surfactant were also observed. The specified range concentrations of solvent used were set up at 5, 10 and 20 %v/v, respectively.

1.2 Effect of pullulan content on the solubility of LD and CD

Normally, the higher polymer chain or cross-linking network able to hold on or entrapped more drug molecule. Hence, the effect of amount of pullulan should be investigated on the ability to increase the solubility of LD and CD in the appropriated acid solvent of 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5. The range of pullulan concentrations used were 6, 8 and 10 %w/v, respectively.

Pullulan was directly dispersed in 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5 solution at the desired concentration. Clear solution was later obtained after mixing for a period of 2 hours. Excess amount of either LD or CD powder was later introduced to above solution with gently mixing by magnetic stirrer for specified time. At each time point of collection (1, 2, 4, 8, 16, 32 and 48 hours), solubilized LD or CD was then separately quantified using validated UV spectrophotometry as mention

earlier. Saturated solubility was thus indicated using the concentration at steady state of solubilization.

Part 2 Formulation development of pullulan OTF containing LD and CD

2.1 Preparation of pullulan OTF containing LD and CD

Preliminary prototype formulation of OTF was modified from the conclusion of Sujaritnarakorn (2016). It was composed of pullulan as a film former, glycerin as a plasticizer, ascorbic acid as antioxidant and suitable acid solvent obtained from Part 1 of this studies. It was shown herein after. (Table 4)

Table 4 Pullulan OTF prototype formulation containing LD and CD

Ingredients	Concentration (%w/w)
pullulan	6.0000
glycerin	0.6000
LD	0.6250
CD	0.1563
ascorbic acid	0.7980
selected solvent q.s. to	100.0000

Solvent casting method was used as the method of OTF preparation. Briefly, LD 150 mg and CD 37.5 mg were separately dissolved in 12.5 and 6.25 ml of selected acid solvents, respectively. The clear solution of CD was directly poured into a clear LD solution with continuous stirring until clear mixture was obtained. Later on, glycerin was added with continuous blending. Ascorbic acid powder was then incorporated and vigorously agitated at 500 rpm for 30 minutes. Pullulan was later dispersed with continuous stirring until clear and homogeneous solution without any bubbles was obtained. The solution should be avoided from excessive heat and protected from light. Approximate six grams of sample solution was gently introduced into 30 cm^2 plastic mold (6x5x1.5 cm) and evenly spread over. It was dried by subjecting into hot air oven, at the controlled temperature of 40±2 °C with continuous air flow. Dry film is carefully taken out from plastic mold. It was overwrapped with aluminum foil sheet before kept in tight and light resistance aluminum pouch.

2.2 Evaluation of pullulan OTF containing LD and CD

The dry film sample was characterized according to the physical and mechanical aspects as followed. The characterizations were done in triplicate, average with % relative standard deviation of measuring value was reported. Statistical data analysis was employed using t-test for comparative between groups of treatment whereas multiple groups' comparison used ANOVA at α-level 0.05.

2.2.1 Physical properties

A piece of sample film was prepared in the form of rectangular shape with the dimension of 4x5 cm (20 cm^2). It was then overwrapped with aluminum foil sheet and enclosed in aluminum foil pouch. It would be further kept in air tight and light resistant container unless otherwise specify before testing.

2.2.1.1 Physical appearance

Film sample was inspected by sensory evaluation (organoleptic test) for smoothness, fragility, tackiness. In term of the transparency, further investigation of the presence of insoluble precipitated solid particle was taken into consideration. Preliminary observation with naked eye was carried out. In depth observation was later done by polarized light microscope.

The smaller piece of rectangular film sample (1x1 cm) was cut and placed over glass slide. It was then subjected into a set of polarizer and analyzer in conjunction with optical microscope. Photomicrograph was then taken and recorded. Birefringence of any appeared particles should be an additional investigation.

2.2.1.2 Thickness

The rectangular shape of film sample was measured in term of the thickness by using vernier caliper. The sample was measured in triplicate at five different positions: four corners and a center of film sheet as shown in Figure 8 (8). The consistency of whole sample film thickness was consequently determined. % relative standard deviation (%RSD) of not more than 5% was acceptable.

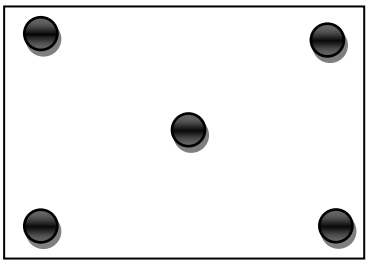


Figure 8 The thickness measuring point of OTF sample film sheet

2.1.1.1 Weight variation

Weight variation was studied by individually weigh the rectangular OTF sample in triplicate and calculating the average and %RSD. It was then determined by using analytical balance. The 20 cm^2 film sample weight should not remarkable deviate from its average weight. %RSD of not more than 5% of its average weight conformed to the requirement.

2.1.1.2 Moisture content

The moisture content of rectangular shape with the dimension of 4x5 cm film sample was tested by using moisture analyzer at 105ºC. The film sample was placed on the sample holder. Heating from room temperature reached 105 ºC was performed in order to impede the retained moisture of film sample. Constant weight loss of film was recorded and further calculated based on initial weight. It was tested in triplicate. The moisture of sample should be consistent with each other. %RSD of sample test was not more than 5%.

2.1.1.3 In vitro disintegration time

Disintegration time of dry film sample was determined by modified method (52). Mounting the dry film sample to plastic frames of 4x5 cm was carried out (Figure 9). It was attached to the paddle of dissolution testing machine. The paddle was then immersed into disintegrating medium and promptly spun at the speed of 10 rpm and 37±0.5 ºC. Disintegration medium was simulated saliva pH 6.8 that comprised of the ingredients as follow (53): sodium chloride 8 g/L, potassium phosphate monobasic 0.19 g/L and sodium phosphate dibasic 2.38 g/L. In order to prepare simulated saliva, all materials were dissolved in DI water. The pH of solution

was monitored and controlled at 6.8 with phosphoric acid. Three hundred mL of simulated saliva was employed for this study. The disintegration time that was not more than 30 seconds (s) was acceptable for OTF.

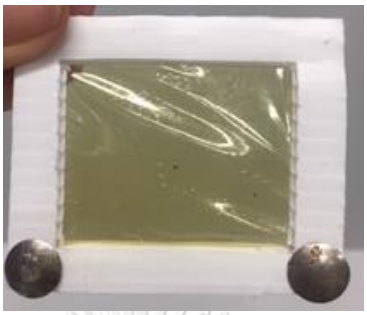


Figure 9 Modified plastic frame attached with the paddle of dissolution apparatus for measuring the disintegration time of film sample.

2.1.1 Mechanical properties

Mechanical properties of OTF were measured by universal testing machine for tensile stress, strain and Young's modulus, respectively. Methods of testing were modified from ASTM D882-Standard test method for tensile properties of thin film sheet and ASTM D638, Standard Test Method for Tensile Properties of Plastics. The tensile stress, strain and Young's modulus were calculated from equation (1), (2) and (3), respectively. The maximum force to break the OTF sample means the tensile stress. It was calculated by dividing the force at breaking point with total area of film. Strain is expressed as the ratio of change between the length of the film sample after pull force applied and the initial length of film. Meanwhile, Young's modulus is a measure of film strength, which is the rate of change in stress to strain and calculated follow equation 3.

The OTF sample was cut into the dumbbell shape (gauge length 20 mm, width 7 mm, length overall 60 mm and width overall 15 mm) as shown in Figure 10. The dimension of the dumbbell shape sample was modified from ASTM D638 specimen dimension type 4 and 5. When the measuring would be start, it was clamped by the lower and upper grips and the experimental force was then applied. The operating conditions of the machine was equipped with 10 kN load cell. The cross-head speed was controlled at 2 mm/min. The sample was measured in triplicate.

$$\text{Tensile stress } (\sigma) = \frac{\text{Force}}{\text{Area}} = \frac{F}{A} \ N/m^2 \quad (1)$$

$$\text{Strain } (\varepsilon) = \frac{\text{Length of the film changed}}{\text{Initial length of the film}} = \frac{\Delta L}{L} . \quad (2)$$

$$\text{Young's modulus (E)} = \frac{\text{Tensile stress } (\sigma)}{\text{Strain } (\varepsilon)} \ N/m^2 \quad (3)$$

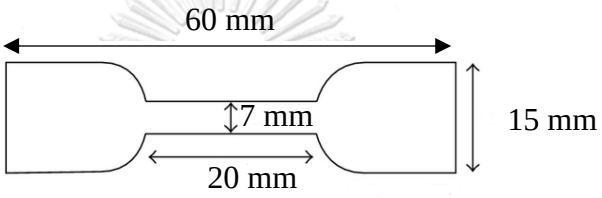


Figure 10 The dimension of the dumbbell shape of OTF sample

2.3 Effect of additives on OTF containing LD and CD formulation

The effect of certain additives, plasticizer and polymer, on the formulation of OTF containing LD and CD was proposed. In general, plasticizer should be used for OTF in order to improve the mechanical properties of film produced. Moreover, the amount of polymer also played a key role on film product characteristic. Thus, both factors were included in this study.

2.3.1 Effect of glycerin content on OTF containing LD and CD

Glycerin was chosen as plasticizer of OTF containing LD and CD due to a preliminary study and the literature review (11). The concentration of glycerin over the range of 0, 1, 2, 5 and 10 %w/w of polymer dry weight was selected. They were equivalent to 0, 0.06, 0.12, 0.30, 0.60 %w/w of the pullulan OTF containing LD and CD formulation, respectively. All formulations containing a glycerin were presented in Table 5.

Table 5 Pullulan OTF formulation containing LD and CD at different concentration of glycerin

Ingredients	concentration (%w/w in acid solution)				
	1	2	3	4	5
pullulan	6.0000	6.0000	6.0000	6.0000	6.0000
glycerin	0.0000	0.0600	0.1200	0.3000	0.6000
LD	0.6250	0.6250	0.6250	0.6250	0.6250
CD	0.1563	0.1563	0.1563	0.1563	0.1563
ascorbic acid	0.7980	0.7980	0.7980	0.7980	0.7980
selected solvent q.s. to	100.0000	100.0000	100.0000	100.0000	100.0000

2.3.2 Effect of pullulan content on OTF containing LD and CD

Various concentration of pullulan in the range of 6, 8, 10, 12 %w/w of the OTF containing LD and CD formulation with constant concentration of glycerin (data from 2.3.1) are formulated and displayed as follow (Table 6).

Table 6 Pullulan OTF containing LD and CD formulation at different concentration of pullulan

Ingredients	concentration (%w/w in acid solution)			
	1	2	3	4
pullulan	6.0000	8.0000	10.0000	12.0000
glycerin	suitable content from previous study 2.3.1			
LD	0.6250	0.6250	0.6250	0.6250
CD	0.1563	0.1563	0.1563	0.1563
ascorbic acid	0.7980	0.7980	0.7980	0.7980
selected solvent q.s. to	100.0000	100.0000	100.0000	100.0000

2.4 Short term stability of the OTF containing LD and CD

Short term stability of the OTF containing LD and CD formulation was determined in triplicated. The stability study condition was over 3 months under ambient condition (30±2 °C, 75±5 %RH) and accelerated condition (40±2 °C, 75±5 %RH) with controlled moisture of environment at 75±5 %RH (54). Testing parameters were described and shown in Table 7.

Table 7 Testing parameter and requirement for stability investigation of pullulan OTF containing LD and CD at both ambient and accelerated conditions

Testing parameter	Requirement
Physical appearance	
- Product color	Light yellow
- Transparency	Clear
- Smoothness	Smooth
- Fragility	Unbreakable
- Precipitated solid particle	Not presence
- Tackiness of film	Non-tacky
Assay content of	
- LD	90-110 %LA
- CD	90-110 %LA

The dry film sample was characterized according to the physical appearance and the quantitative determination of LD and CD content in OTF as followed below:

2.4.1 Physical appearance

The physical appearance of pullulan OTF containing LD and CD was examined by product color, transparency, smoothness, fragility, and tackiness. The transparency testing was done by polarized light microscope for investing the appeared of insoluble precipitated solid particle after exposing to controlled environment at specified time of storage. The acceptance criteria of OTF product should be clear, smooth and easy to handle. In addition, none of any precipitated solid particle should be found.

2.4.2 Quantitative determination of LD and CD content in OTF

Assay of drug content of LD and CD in OTF preparations were determined at initial and 1 month storage under ambient and accelerated condition. Sample preparation was carried out by cutting the 2x5 cm (10 cm^2) film sample as rectangular shape. It was transferred in to a 100 milliliters volumetric flask and the solvent mixture of 0.1 M orthophosphoric acid and acetonitrile at 92.5:7.5 volume ratio was added to volume. It was then agitated and clear solution was acquired. It was further filtered through 0.45 µm cellulose acetate membrane filter. The content of both drugs was measured by high performance liquid chromatography (HPLC). The flow rate was indicated at 0.8 ml/min with the injection volume of 20 µl. The analytical method validation of HPLC were shown in Appendix C. The standard curve between drug concentrations and absorbance was constructed for each drug. The content of LD and CD was therefore calculated. The labeled claim of LD and CD in OTF preparation were based on theoretical loading of 1.25 and 0.31 mg/cm^2, respectively. The acceptance limit of LD and CD assay were found to be “not less than 90.00 and not more than 100.00 %LA”. Statistical data analysis was employed using t-test for comparative between %LA at initial time and 1 month storage.

CHAPTER IV

Result and Discussion

The result was composed of 2 consecutive parts. Part 1 was directly related to the solubility of LD and CD in various acid solvent systems whereas Part 2 mainly focused on the development of the pullulan OTF containing LD and CD using appropriated selected solvents concluded from Part 1.

Part 1 Solubility determination of LD and CD in various acid solvent

The quantitative determinations of LD or CD were investigated in different acidity (pH) and molar concentration of either acid or buffers at controlled temperature of 30±2 °C (Figure 11). As the line graph suggests the relationship between the concentration of LD or CD dissolved in acid and buffer solutions over the pH range of 1.5 to 3.5. They showed the same solubility profile's pattern. The concentration of LD and CD increased sharply within the first hour. After that, they remained constant at the steady state over 48 hours. The top three suitable solvents which provided the maximum solubility of both LD and CD were found to be 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5, 0.1 M citric acid pH 2 and 0.1 M citric/citrate buffer pH 2.5, respectively. The highest solubility of LD and CD were found in 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5 with the value around 12 and 6 mg/ml, respectively. On the other hand, the lowest solubility of LD and CD were around 5 mg/ml and 1.7 mg/ml in the 0.1 M citric/citrate buffer solvent (pH 3 and 3.5), respectively.

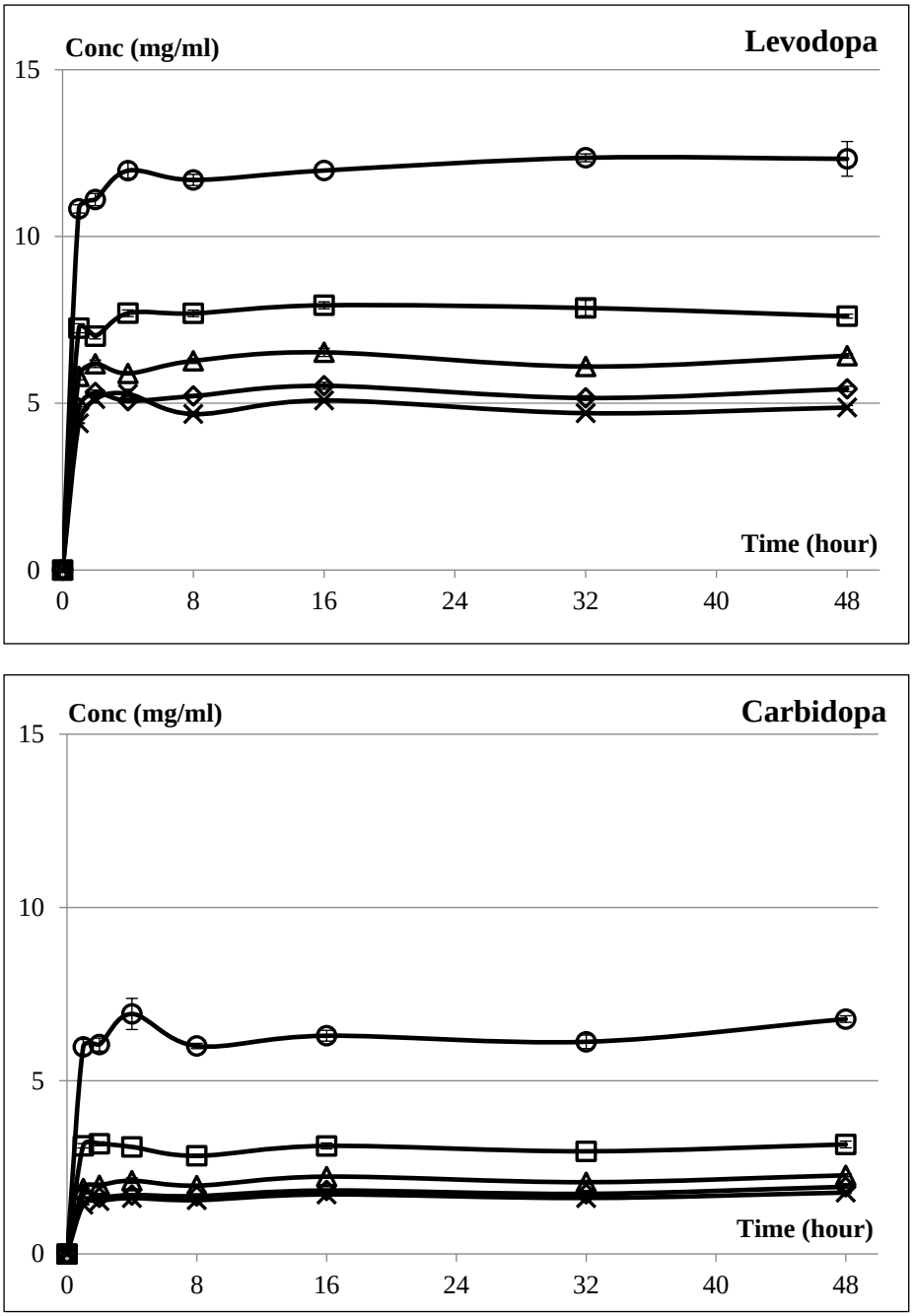


Figure 11 The solubility profile of LD and CD in acid and buffer solutions at the pH range of 1.5 to 3.5; (O) 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5; (□) 0.1 M citric acid pH 2; (Δ) 0.1 M citric/citrate buffer pH 2.5; (◊) 0.1 M citric/citrate buffer pH 3.0; (×) 0.1 M citric/citrate buffer pH 3.5

In term of the acidity of solvent systems, the equilibrium solubility of both drugs was statistically significant increased at the pH of lower than 3 ($p<0.05$). Meanwhile, the solvent at pH of 3.5 revealed the nearly identical solubility of both drugs compared to that of pH 3. It was due to the molecular structure of LD and CD that contain primary amine and carboxyl group as could be seen in Figure 12. According to the pKa1 of LD and CD that nearly identical around 2.3 (12, 13) the amino and carboxyl structure are able to ionize in the solution state at higher proton concentration (particularly at the state of lower pH than their pKa1) as could be seen in Figure 12 (17). Their zwitterion will be converted to positive charge by the protonation at carboxylic group and eventually presenting in form of cation of the overall structure. Positive charged structure of both drugs was then formed and was later induced to dissolve in aqueous system according to "like dissolves like" (55). In addition, Remenar (2005) also observed the solubility of LD in citrate buffer at varying pH and buffer strength system. The results demonstrated that concentration of LD at lower pH was found to be higher than that of the higher pH at the same buffer molar concentration (9). It would be a well corresponded evidence to support our finding. However, the protonation should not be occurred at the pH higher than their pKa1. As a result of above phenomena, zwitterion ion structure would be existed (34) and revealed lower solubility compared to those of the lower pH systems. In conclusion, the lower the pH (lower than pKa1 of 2.3), the higher aqueous solubility was observed.

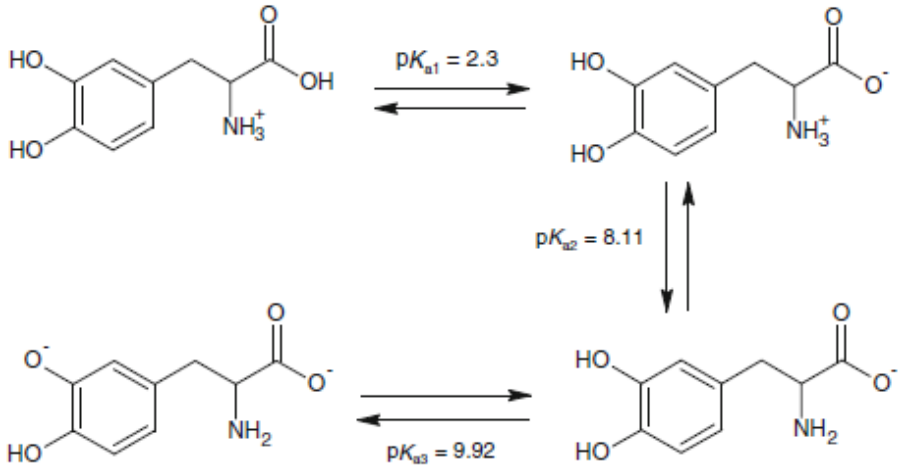


Figure 12 Zwitterion conversion of LD under the solution state at different pH

In order to clearly understand the higher solubility of LD and CD as a function of more acidity environment, Tseng (2009) explained that amino acid like structure molecule (Figure 13) in aqueous solution appears in several forms such as neutral, zwitterion, cationic and anionic. Their aqueous solubility directly governed with proton concentration. When the acid is added to aqueous solution; the higher concentration of proton or lower pH is able to form a cation via the expression of ionization of its carboxyl group. The solubility of amino acid liked molecules commonly increases as the changing of proton concentration or pH that deviates from its isoelectric point (34).

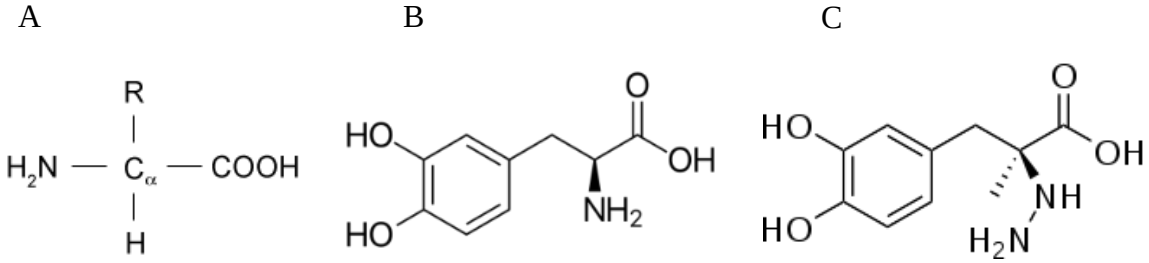


Figure 13 Molecular structure of (A) Amino acid structures; (B) LD; (C) CD

Furthermore, the solubility profile of LD and CD in acid solutions with respect to the different common ion were carried out. The solubility profiles of LD and CD in 0.1M hydrochloric acid/0.1M citric acid and 0.1M hydrochloric acid/0.1M potassium chloride at the same pH condition (pH 1.5) were shown in Figure 14. As can be seen, the solubility of both LD and CD in 0.1M hydrochloric acid/0.1M citric acid was significantly higher than that of 0.1M hydrochloric acid/0.1M potassium chloride ($p<0.05$). However, the pattern of solubility profile was also the same. It was due to the effect of common ion effect of chloride ion in the acid solvent. The acid solvent system consisted of potassium chloride shared common chloride ion with hydrochloric acid that resulted in the lower solubility of LD and CD (56).

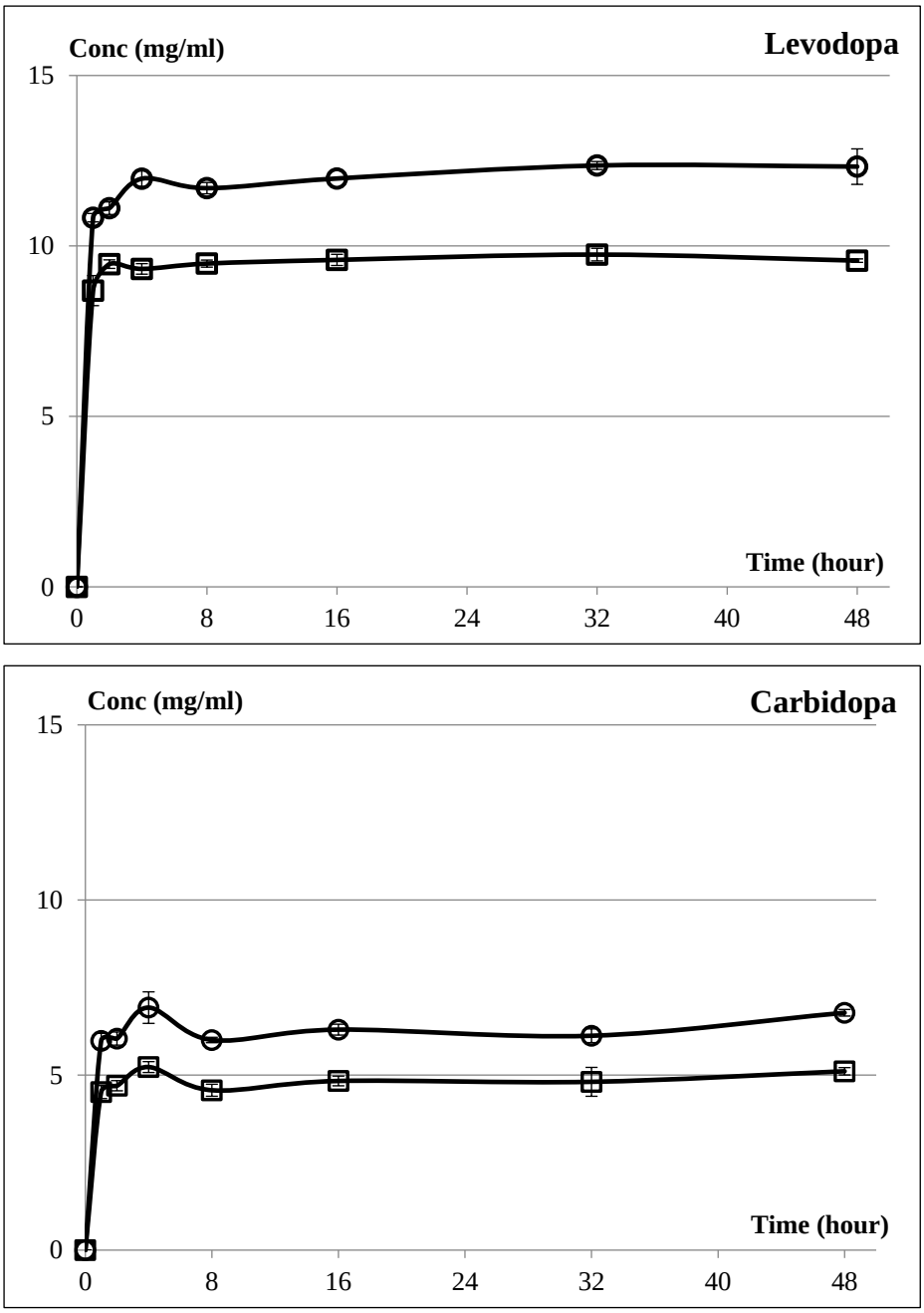


Figure 14 The solubility of LD and CD in acid solutions containing different ions at the controlled pH of 1.5; (O) 0.1 M hydrochloric acid/ 0.1 M citric acid; (□) 0.1 M hydrochloric acid/ 0.1 M potassium chloride

Not only the pH of solution and ion type of solvent used, the molar concentration also was an important part on the solubility determination of LD and CD. It can be seen from the Figure 15 that the solubility of LD and CD in 0.2M hydrochloric acid/0.1M citric acid was significantly higher than 0.1M hydrochloric acid/0.1M citric acid at the

same pH ($p<0.05$). The solubility of LD and CD from 0.2 M hydrochloric acid/0.1 M citric acid was around 12.6 and 6.3 mg/ml at pH 1.5, respectively whereas the solubility in 0.1 M hydrochloric acid/0.1 M citric acid was around 11.8 and 5.8 mg/ml at pH 1.5, respectively.

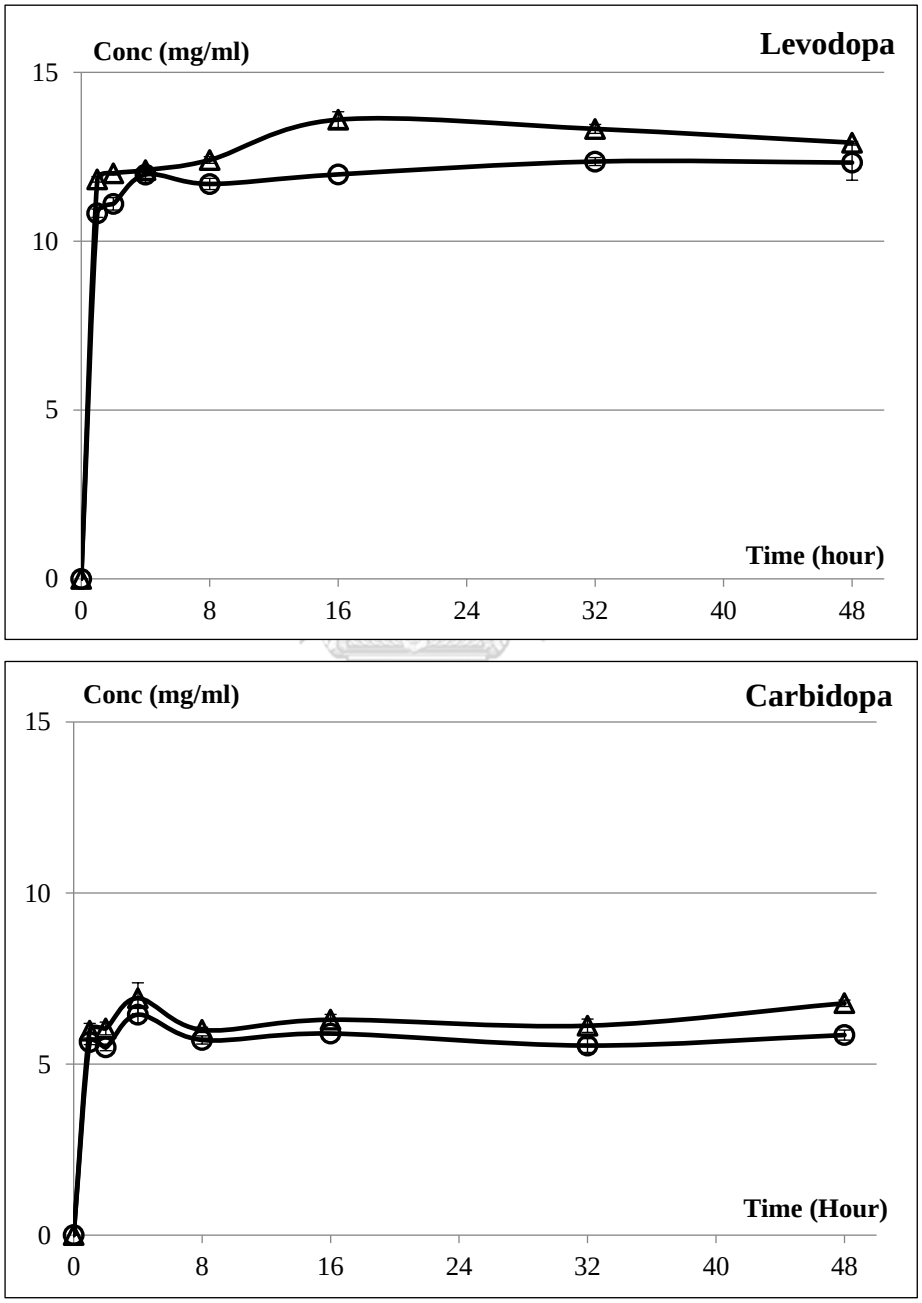
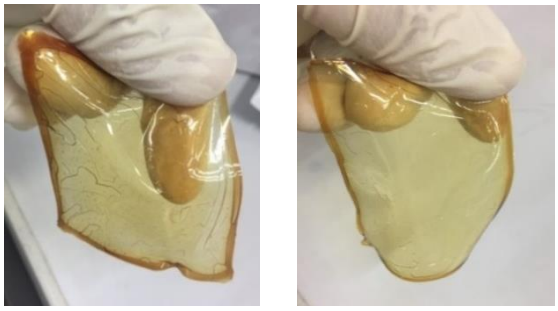


Figure 15 The solubility profile of LD and CD in hydrochloric acid and citric acid; (O) 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5; (Δ) 0.2 M hydrochloric acid/ 0.1 M citric acid pH 1.5

Thus, the higher molar concentration of 0.2M hydrochloric acid in acid solvent system provided the higher solubility of LD and CD when compared to 0.1 M hydrochloric acid because of the increasing of ionic strength. The ionic strength of a solution is a measure of the concentration of ions in that solution (57). Due to the relationship of activity coefficient (γ) with respect to the solubility from Tseng (2009), γ is inversely related to the solubility. Tseng (2009) and Hitchcock (1924) pointed out that the solubility of amino acid like structure molecule increased while the activity coefficient decreased (34, 58). In addition, Remenar (2005) revealed that the solubility of LD in citrate buffer at higher molar concentration was greater than lower molar concentration (9). Further supporting explanation would be gained from the Debye-Huckel relationship that focusing on ionic aspect. When considering with our study, solvent comprised of higher molar concentration yielded higher ionic strength with lower γ. Therefore, 0.2 M hydrochloric acid/ 0.1 M citric acid pH 1.5 should provide the higher solubility of LD and CD.

Two appropriated acid solvents that was able to maximize the solubility of LD and CD from this study was found to be 0.2 M hydrochloric acid/0.1 M citric acid and 0.1 M Hydrochloric acid/0.1 M citric acid at pH 1.5. They were then employed as the acid solvent of preliminary study of OTF containing LD and CD preparation. The prototype OTF containing LD and CD using above different acid solvents were formulated and characterized (Figure 16). OTF evaluation parameters and results were tabulated and shown in Table 8.



A B

Figure 16 Physical appearance of the prototype OTFs containing LD and CD using 0.2 M hydrochloric acid/0.1 M citric acid pH 1.5 (A) and 0.1 M Hydrochloric acid/0.1 M citric acid pH 1.5 (B)

Table 8 Evaluation of the prototype OTF containing LD and CD formulation using 0.2 M hydrochloric acid/0.1 M citric acid pH 1.5 or 0.1 M Hydrochloric acid/0.1 M citric acid pH 1.5

Characterization	0.2M hydrochloric acid/ 0.1M citric acid pH 1.5	0.1M hydrochloric acid/ 0.1M citric acid pH 1.5
Physical properties		
- Transparency	Clear	Clear
- Smoothness	Smooth	Smooth
- Fragility	Unbreakable	Unbreakable
- Precipitated solid particle	Not presence	Not presence
- Tackiness of film	Tacky	Slightly-tacky
- Thickness [millimeter]*	0.115±0.009*(7.842)	0.098±0.004*(4.081)
- Weight [gram]*	0.336±0.004*(1.239)	0.316±0.003* (0.837)
- Moisture content [%]	5.433±1.540 (28.353)	4.127±0.102 (2.475)
- Disintegration time [second]	25.967±5.648	23.467±3.495
Mechanical properties		
- Tensile stress [mPa]	0.675±0.260	0.989±0.313
- Strain	2.992±0.934	2.130±0.660
- Young's modulus [mPa]*	0.232±0.075*	0.480±0.126*

Mean ± SD, %RSD is shown in parentheses

$p<0.05$

Both OTF samples were found to be clear and transparent without any precipitated solid crystal under polarized light microscope (Appendix B, Figure 31 and Figure 16). It should be concluded that all components in the film formulation were molecularly miscible and resulting in homogeneous polymeric thin film. However, they exhibited some different properties such as thickness, weight per unit, tackiness of film and mechanical properties. Result of statistical analysis between two samples using t-test are displayed in Table 8. It indicated that the utilization of 0.1 M hydrochloric acid/0.1 M citric acid pH 1.5 solvent systems provided the film product with less tackiness, thinner film sheet, less weight per unit and higher Young's modulus than that

of 0.2 M hydrochloric acid/0.1 M citric acid pH 1.5 product ($p<0.05$). As can be realized from mentioned results, the tensile stress and Young's modulus increased when molar concentration of hydrochloric acid decreased. It was due to the higher solid content of hydrochloric acid in 0.2 M hydrochloric acid/0.1 M citric acid pH 1.5. It affected on polymer network formation. Higher solid fraction prone to effect on the completeness of polymer network formation after drying. That is because the higher level of certain additives able to act as the plasticizer (31) and resulted in less structural integrity of polymer network in OTF formulation. Moreover, the value of strain deviated directly as a function of solid content. It would agree well with the incorporation of more additives that able to entangle within the polymer chain and obtained the less integrity of polymer network.

Further comparison regarding with the drug loading ability between our current prototype OTF formulation and the OTF formulation developed by Sujaritnarakorn (2016) was made and evaluation (11). The result showed that three times higher loading of LD and eight times of CD loading in 0.1 M hydrochloric acid/0.1 M citric acid pH 1.5 was determined. The result is summarized and tabulated in Table 9. Thus, it should be clearly showed that our developed prototype formulation was an efficient platform for OTF formulation development.

Table 9 Comparative determination of the loading quantity of LD and CD per area of OTF using different solvent system

solvent	loading quantity (mg/cm^2)	
	LD	CD
0.0205 M citric / 0.0045 M citrate buffer pH 3.0*	0.444	0.044
0.1 M hydrochloric acid/0.1 M citric acid pH 1.5	1.250	0.313

* Sujaritnarakorn (2016)

In summary, OTF produced with 0.1 M hydrochloric acid/0.1 M citric acid pH 1.5 provided more stiffness or more resistance to elastic deformation under force applied comparing with 0.2 M hydrochloric acid/0.1 M citric acid pH 1.5. However, its physical appearance and mechanical properties were not acceptable for the larger scale of manufacture. Consequently, the current OTF formulation that consisted of pullulan

6 %w/w in 0.1 M hydrochloric acid/0.1 M citric acid pH 1.5 with glycerin of 10 %w/w had been further developed in order to acquire more drug loading by employing cosolvents and higher polymer content, respectively.

1.1 Effect of cosolvents on the solubility of LD and CD in acid system solvent

LD and CD commonly show two major moieties in their molecular structure. The primary important moiety is going to zwitterionic of carboxyl and amino function group. On the other hand, the bulky group of benzene ring attached with hydrocarbon backbone that represents the hydrophobic characteristic of non-polar moiety. It may also play a pivotal role on their intrinsic solubilities since it does not able to form hydrogen bond with water. Theoretically, three different solubilization techniques of pH adjustment, cosolvency and surfactant are able to apply for improving the solubility of both drugs. The pH adjustment had already been conducted in the previous experimental section and found to be the most empowering method for increasing the solubility. Therefore, the increasing solubility by aiming to the bulky group was then additional objectives.

According to the principle of the usage of cosolvency, cosolvent can reduce the interfacial tension between solvent and solute of system. Firstly, cosolvent will be working by forming the hydrophilic hydrogen bonds with water to ensure the water miscibility. Then, the hydrophobic hydrocarbon area of cosolvent will interfere the hydrogen bonding network of water. It will weaken the intermolecular hydrogen bonding network of water. As a result of two above mechanisms, cosolvent is able to increase the miscibility between low water solubility drug with water (59). The criteria of cosolvent selection was based on the dielectric constant of drugs. Since there were no clear evidence data of the dielectric constant value of our drugs, predominantly non-polar part of LD and CD (benzene ring attached with hydrocarbon backbone) should primarily be considered for estimating their dielectric constant. They were classified as the moderate dielectric constant value. The dielectric constant of LD was estimated around 20 at room temperature (60). Hence, cosolvents with moderate level of dielectric constant was appropriated and chosen. They were PEG400, ethanol and glycerin that would have the dielectric constants of 12.4, 25 and 42.5, respectively (59). PEG400

tends to be non-polar material while ethanol and glycerin tend to be semi-polar materials.

Tween 80 was also included in this case because it is widely used as surfactant. We will point out at the bulky non-polar of benzene ring and hydrocarbon backbone in the LD and CD. Micellar formation through such non-polar part was proposed. Tween 80 is a nonionic surfactant and emulsifier from polyethoxylated sorbitan and oleic acid. The hydrophilic-lipophilic balance (HLB) value of Tween 80 is 15.0 ± 1.0 which means that it is a hydrophilic or water miscible substance (61, 62). It is more often used as emulsifier, solubilizer and wetting agent in pharmaceutical formulation. Tween 80 can form the micelle in water to hold non-polar molecules inside (63) and eventually provides the more effective solubilization of several drugs.

1.1.1 Effect of PEG400 on the solubility of LD and CD

Figure 17 shows dissolved LD and CD in acid solvents with and without PEG400 of 5, 10 and 20%v/v, respectively. All solubility profiles provided the nearly identical pattern. The solubility profile increased rapidly at initial period of solubilization and remain unchanged at steady state through 48 hours. It can be seen that all solubility profiles were not statistically significant ($p<0.05$). Therefore, the addition of PEG400 at three different concentrations of 5, 10 and 20%v/v were not able to increase the solubility of LD and CD. Generally, the hydrophobic interaction between the methylene groups of PEG400 and the bulky group of non-polar part of both drugs should play an important role on the increasing of the solubility. Sasahara (1993) revealed that the hydrophobic interaction between PEG and drug was increased with the increasing of the size of drug's hydrophobic group. Phenylalanine (Phe) is an amino acid containing the comparable size of non-polar bulky group to LD and CD, its hydrophobic interaction with PEG 400 was determined that could be observed from slightly higher solubility in aqueous-PEG system. Nevertheless, Phe was less soluble. So, the solubility of both drugs in PEG solution at all concentration might be similar to its solubility in aqueous solution (64). It was due to the fact that the size of non-polar group was not huge enough to significantly increase the solubility.

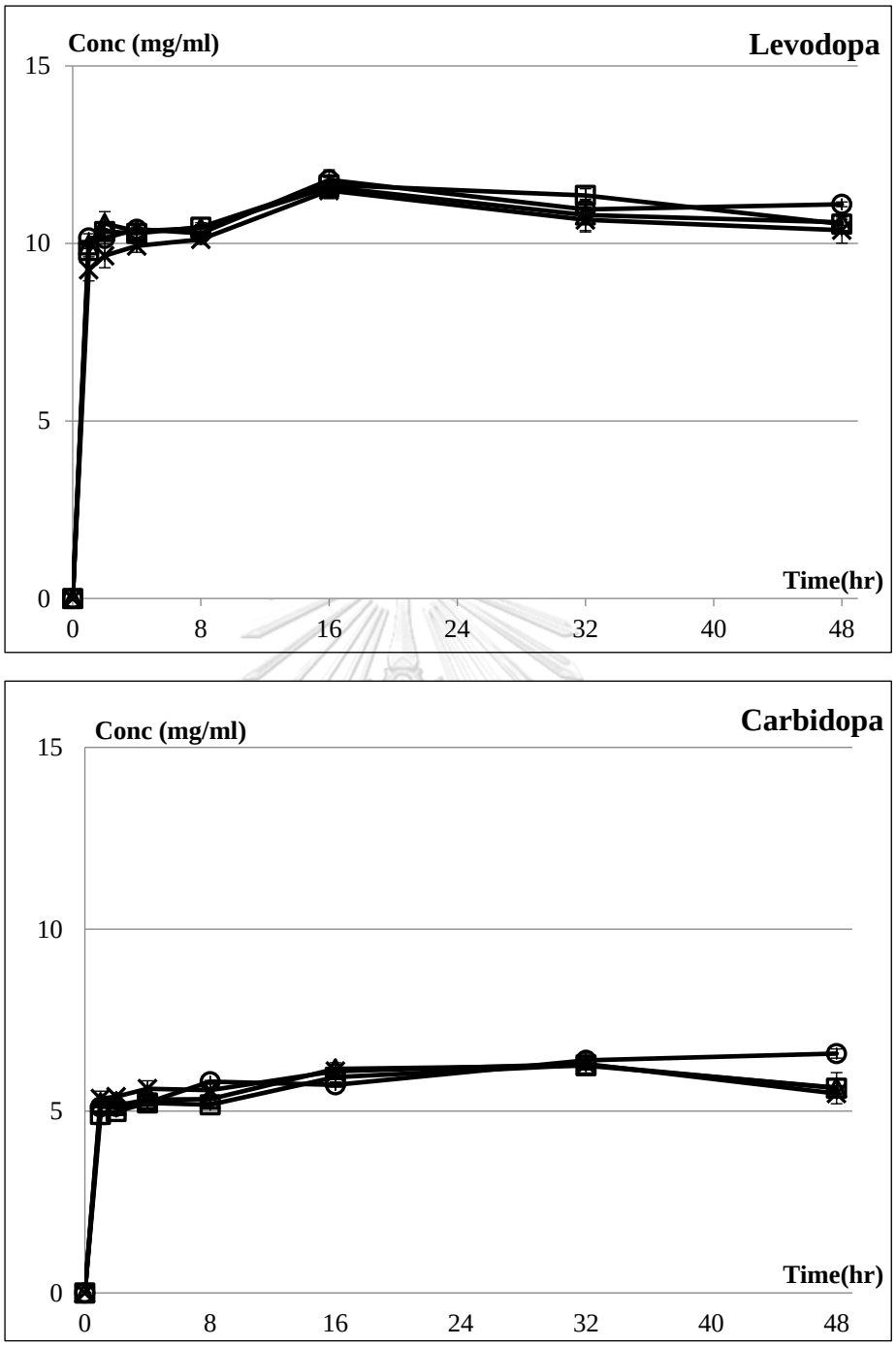


Figure 17 The solubility of LD and CD in acid solvent with and without PEG400; (O) 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5; (Δ) 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5 with PEG400 5% v/v; (×) 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5 with PEG400 10% v/v; (□) 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5 with PEG400 20% v/v

1.1.2 Effect of ethanol content on the solubility of LD and CD

All solubility profiles of LD in acid solvent with and without ethanol were significantly different. However, the solubility profile of CD in acid solution without ethanol significantly higher than those of others contained ethanol ($p<0.05$). The concentration of LD tended to decrease with the increase of ethanol concentration from 5 to 20 %v/v (Figure 18). Positive charged and zwitterion structure of LD and CD in acid solution was theoretically formed due to the protonation of enriched proton of acid solvent. They should be dissolved in polar solvent according to "like dissolved like" (55). Needham (1970) suggested that the addition of semi-polar liquid (such as ethanol and glycerin) into polar aqueous solution would be expected as a cause of solubility deviation by providing the downward shift of polarity (65). In addition, Dey (1985) investigated the solubility of Phe in ethanol and water mixture at various volume ratio. The result revealed that the solubility of Phe was reduced by the increasing of alcohol content (66). It was the clearly supporting evidences regarding the lower solubility of LD in aqueous ethanol system. Furthermore, the solubility profile of LD and CD tended to be gradually decreased as function of time. It was due to the salting out effect related to ethanol incorporation. Miscibility between a part of water with ethanol was presumably occurred that eventually decreased the number of available interactive water molecule to interact with polar part of LD and CD.

However, the tendency of the solubility of CD was not obey along with LD case. In general, ethanol able to change the solubility of CD as same as LD. Nevertheless, CD itself had low intrinsic solubility. Therefore, the changing of the magnitude of solubility was not remarkably observed. Conclusively, the adding of ethanol at 5, 10 and 20 %v/v as a cosolvent were not appropriate for increasing the solubility of LD and CD.

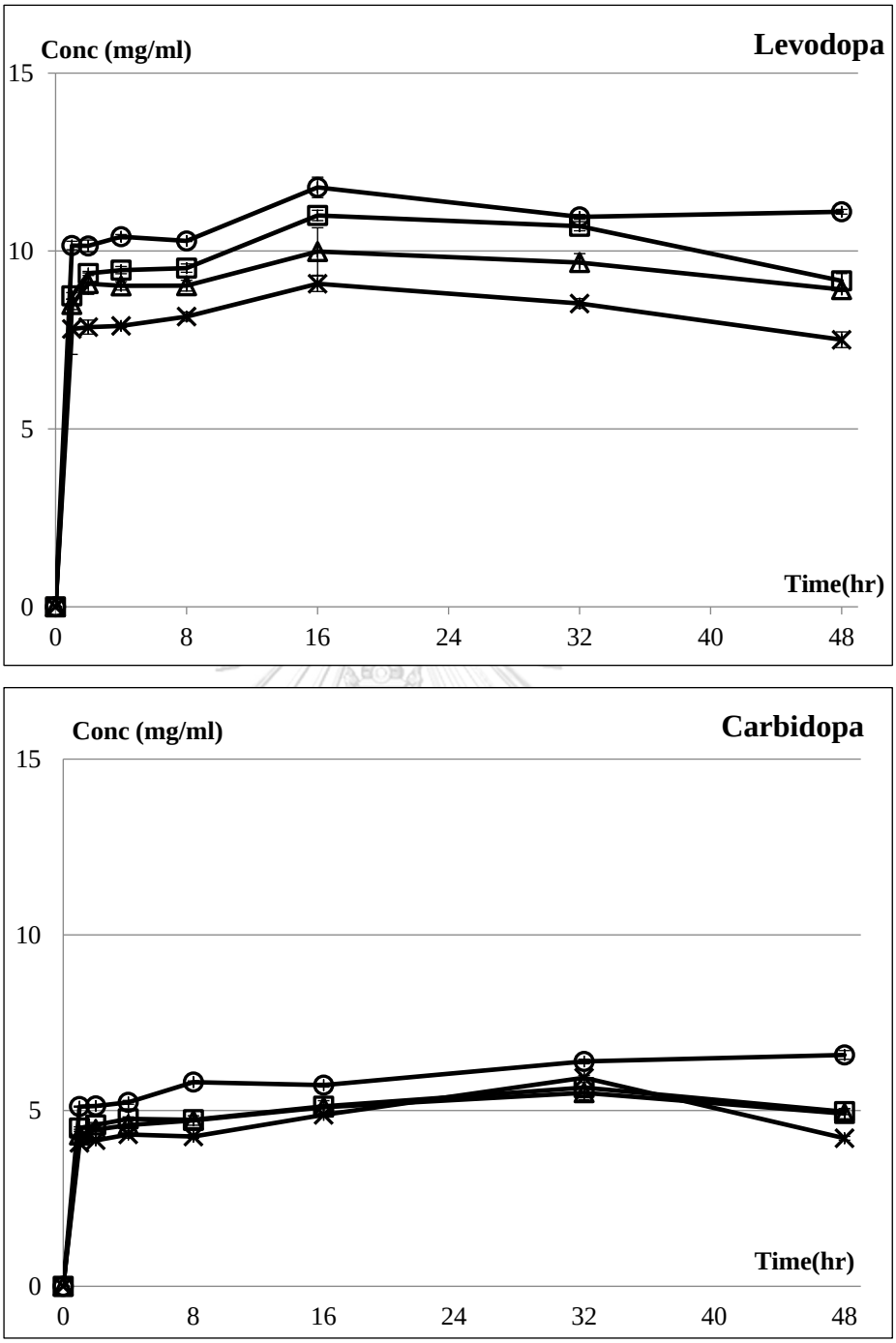


Figure 18 The solubility of LD and CD in acid solvent with and without ethanol; (O) 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5; (□) 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5 with ethanol 5% v/v; (Δ) 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5 with ethanol 10% v/v; (×) 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5 with ethanol 20% v/v

1.1.3 Effect of glycerin content on the solubility of LD and CD

The addition of various concentration of glycerin showed an interesting result. Addition of 5, 10 and 20%v/v obviously suppressed the solubility of LD (Figure 19). However, it was not concentration dependence for the lowering of dissolved LD. Therefore, the added glycerin at any concentration is not appropriate for increasing the solubility of LD in solution. It was due to the fact that glycerin, a semi-polar liquid solvent, showed the same behavior as ethanol on the decreasing of the polarity of solution. Gekko (1981) showed that Phe solubility in water was higher than those of aqueous glycerin solvent over the range of 10 to 40 %w/v. Hence, the lower LD dissolved should be found with higher concentration of glycerin. At the lower concentration used in this experiment (5 %v/v of glycerin), it should be expected to gain very low dissolved LD. Gekko revealed that the addition of glycerin did not greatly reduce the polarity of solution because of the higher dielectric constant of glycerin (67). Therefore, it could be seen that glycerin was not effectively enough to enhance the solubility of LD. In term of the solubility of CD with and without glycerin, they were negligibly different because of the slightly changes of dielectric constant of the system. Therefore, it was not obviously seen the difference of solubility profile of CD.

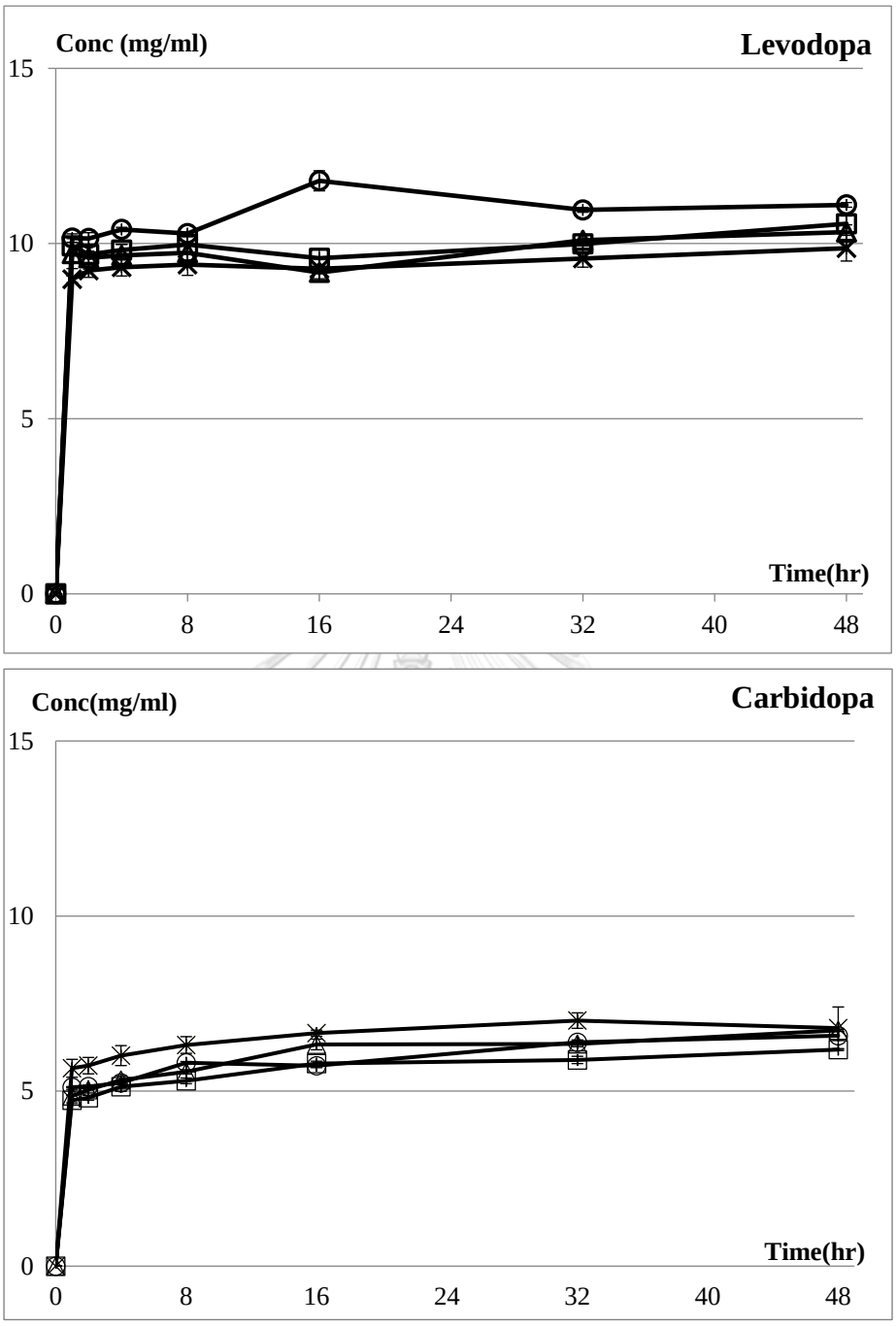


Figure 19 The solubility of LD and CD in acid solvent with and without glycerin; (O) 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5; (□) 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5 with glycerin 5% v/v; (Δ) 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5 with glycerin 10% v/v; (×) 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5 with glycerin 20% v/v

1.1.4 Effect of Tween80 content on the solubility of LD and CD

As could be seen from the solubility profile incorporated with Tween 80, the addition of Tween 80 over the range of 5 to 20 %V/V did not give any positive result. The dissolved LD in acid solvent containing Tween 80 was lower compared to pure acid solvent (Figure 20). Tween 80 typically increases the solubility of drug substance via micelle formation. Minimum concentration of surfactant that provide micelle structure should be indicated as critical micelle concentration (CMC). If the higher amount of surfactant is introduced to system, micelle and interfacial barrier will be organized. Mingzhong (2013) additionally explained that Tween80 could increase the solubility of drug in solution but its solubilization capacity was limited. The large molecule sizes of Tween 80 and its aggregates in solution could be formed as the interfacial barrier to prevent the moving of drug molecules into bulk solution (68). It would hence act as the suppressor of solubilization. In this case, the experimental concentration of Tween80 was pretty much higher than its CMC that would be around 0.016 %v/v in water at room temperature (69). Thus, not only micelle but interfacial barrier for LD dissolved would also occurred. It should be end up with the lower dissolvable of LD in aqueous Tween 80 mixture at concentration of over 5%v/v.

On the other hand, the concentration of CD dissolved did not differ significantly among various concentration of Tween 80 ($p<0.05$). It was due to the low solubility of CD. The amount of dissolving molecule of CD is thus less and had been negligibly impacted from the interfacial barrier. Therefore, the added of Tween80 was not suitable for increasing the solubility of LD and CD in 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5.

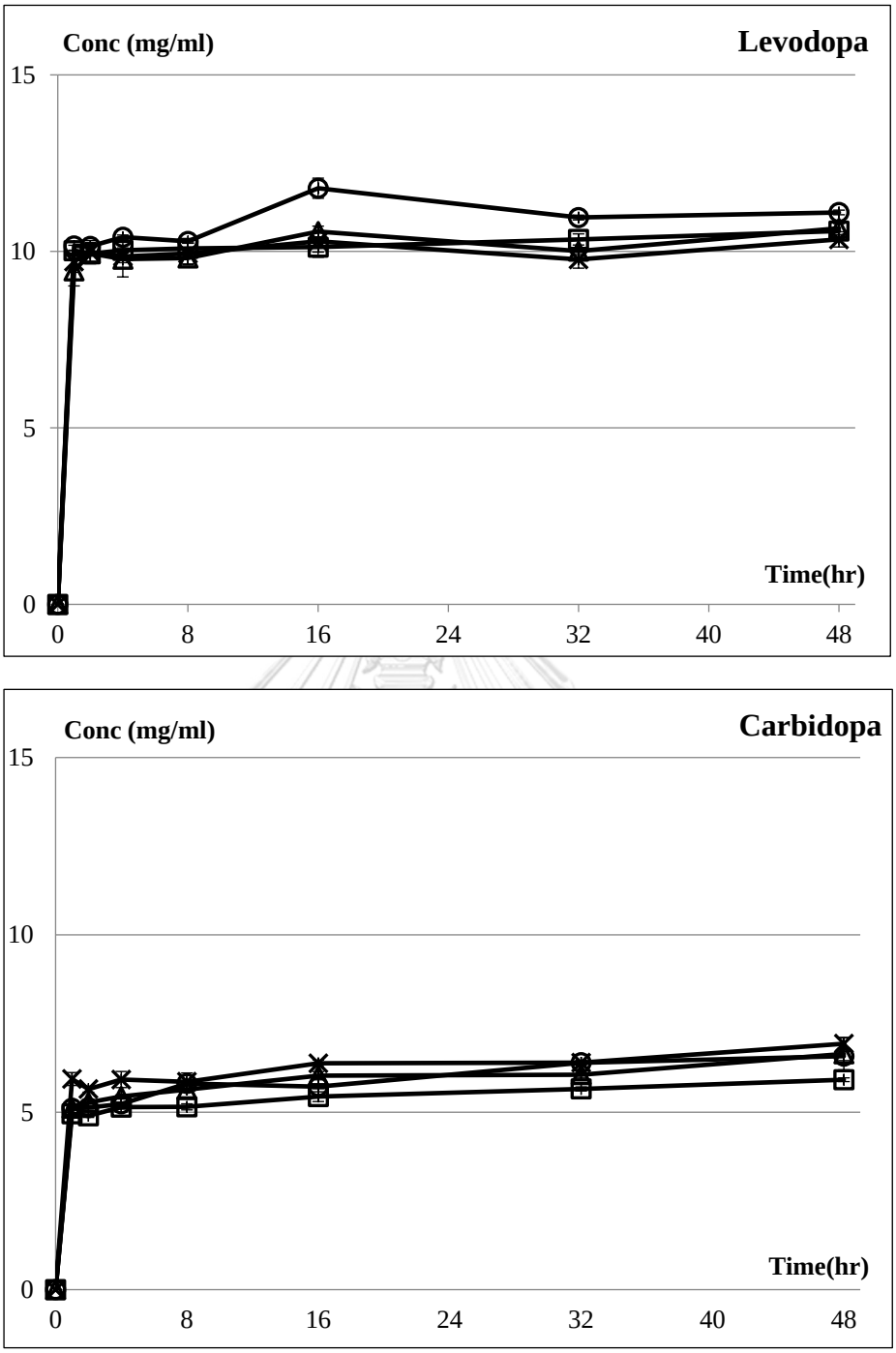


Figure 20 The solubility of LD and CD in acid solvent with and without Tween80; (O) 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5; (□) 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5 with Tween80 5% v/v; (Δ) 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5 with Tween80 10% v/v; (×) 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5 with Tween80 20% v/v

1.2 Effect of pullulan polymer on the solubility of LD and CD

Polymer can commonly interact with drug and solvent in the system which resulting in the solubilizing structures like micelles, colloids and ionic complexes in order to enhance drug solubility (70). It has been found that electrostatic bonds or other types of force such as van der Waals' forces and hydrogen bridges were the main mechanism of the polymer-materials interaction. In this study, the higher amount of pullulan used would be expected to obtain higher interaction with LD and CD and resulting in higher solubilization.

The solubility determination of LD and CD in acid solvents with and without the addition of pullulan at 6, 8 and 10%w/v are shown in Figure 21. The results demonstrated that all solubility determination was not significant difference ($p<0.05$). The higher concentration of both drugs would not properly be occurred from the increasing of pullulan concentration. Therefore, in this study, the solubility of LD and CD did not relate to the increased of polymer content. Loftsson (1996) exhibited that the solubility of drug initially increased upon the increasing of polymer concentration after that the solubility levels either remain unchanged or decreased (37). It was due to the fact that higher polymer concentration more able to form electrostatic bond between themselves that would decrease the ability to form the complexation with drug. From this study, pullulan concentration at 6%w/v or over might be overused and not be a suitable level for improving the solubility of LD and CD.

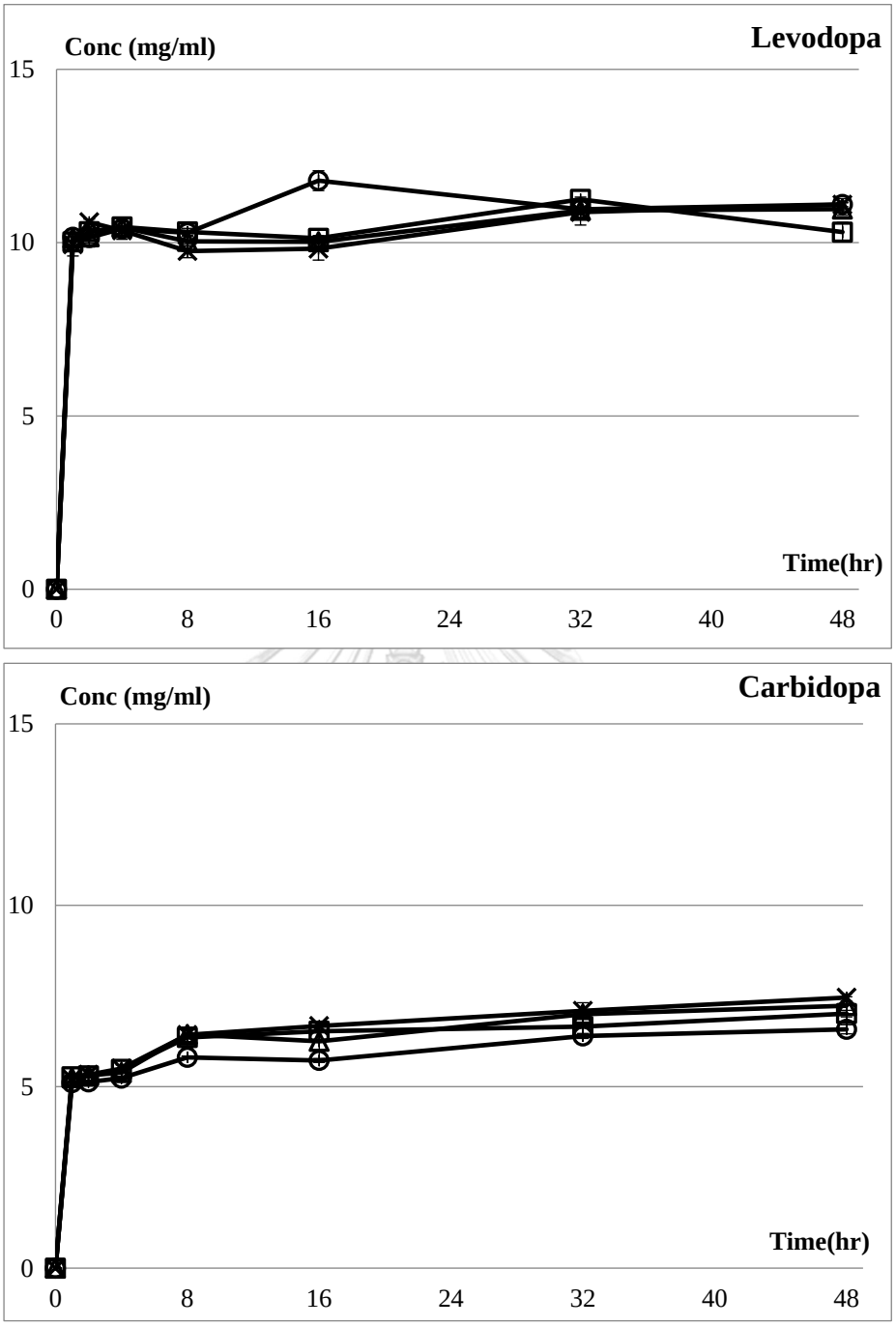


Figure 21 The solubility of LD and CD in acid solvents with and without the addition of pullulan; (O) 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5; (□) 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5 with pullulan 6% w/v; (Δ) 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5 with pullulan 8% w/v; (×) 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5 with pullulan 10% w/v

Part 2 Formulation development of pullulan OTF containing LD and CD

2.1 Effect of additive on OTF containing LD and CD formulation

From the review of the factor affecting pullulan base OTF preparation (31) and pullulan OTF prototype containing LD and CD formulation (Part 1), it was found that the pivotal factors that strongly affected on the properties of OTF were plasticizer and polymer content. That is the reason why, the study of glycerin content was conducted primarily. The content of pullulan was later sequentially performed. The outcome from above designed experiment could answer the question "what is an acceptable OTF containing LD and CD formulation".

2.1.1 Effect of glycerin content on OTF containing LD and CD

The development of OTF formulation by changing glycerin content was carried out. The objectives were related to the improving of film tackiness including the optimization of proper mechanical properties. Pallavi (2014) suggested that plasticizer is able to enhance the mechanical properties of OTF such as tensile stress and strain by reducing the T_g (14). The effect of plasticizer like glycerin was determined by varying its content at 0, 1, 2, 5, 10 %w/w of pullulan dry weight. The product gained using various plasticizer contents were shown in Figure 22 and will further be evaluated (Table 10).

A B C D

Figure 22 Physical appearance of OTF formulation containing LD and CD using pullulan content at 6 %w/w in acid solvents with different glycerin contents: (A-1% w/w, B -2%w/w, C - 5 %w/w and D - 10 %w/w of pullulan dry weight)

Table 10 Evaluation of OTF containing LD and CD formulation with and without glycerin content at 0, 1, 2, 5, 10 %w/w of pullulan dry weight

Characterization	Plasticizer content				
	none	1% w/w	2% w/w	5% w/w	10% w/w
Physical properties					
- Transparency	Clear	Clear	Clear	Clear	Clear
- Smoothness	Smooth	Smooth	Smooth	Smooth	Smooth
- Fragility	breakable	Unbreakable	Unbreakable	Unbreakable	Unbreakable
- Precipitated solid particle	Not presence	Not presence	Not presence	Not presence	Not presence
- Tackiness of film	Non-tacky	Very slightly-tacky	Very slightly-tacky	Tacky	Tacky
- Thickness [mm]	0.093±0.006 (6.208)	0.097±0.012 (12.598)	0.092±0.002 (2.174)	0.108±0.009 (8.027)	0.098±0.004 (4.082)
- Weight [gram]	0.283±0.007* (2.405)	0.315±0.002 (0.550)	0.319±0.006 (1.783)	0.326±0.007 (2.011)	0.316±0.003 (0.837)
- Moisture content [%]	2.587±0.515* (19.915)	3.497±0.608 (17.386)	4.553±0.345 (7.578)	4.283±0.323 (7.545)	4.127±0.102 (2.475)
- Disintegration time [second]	20.100±1.997	18.57±3.250	14.470±1.320*	18.700±0.854	23.467±3.495
Mechanical properties					
- Tensile stress [mPa]	ND	10.232±2.261*	2.506±0.428	1.376±0.324	0.989±0.313
- Strain	ND	0.137±0.044*	1.256±0.211	1.970±0.417	2.130±0.660
- Young's modulus [mPa]	ND	82.667±36.364*	1.999±0.170	0.699±0.054	0.480±0.126

Mean ± SD, %RSD is shown in parentheses

**p<0.05*

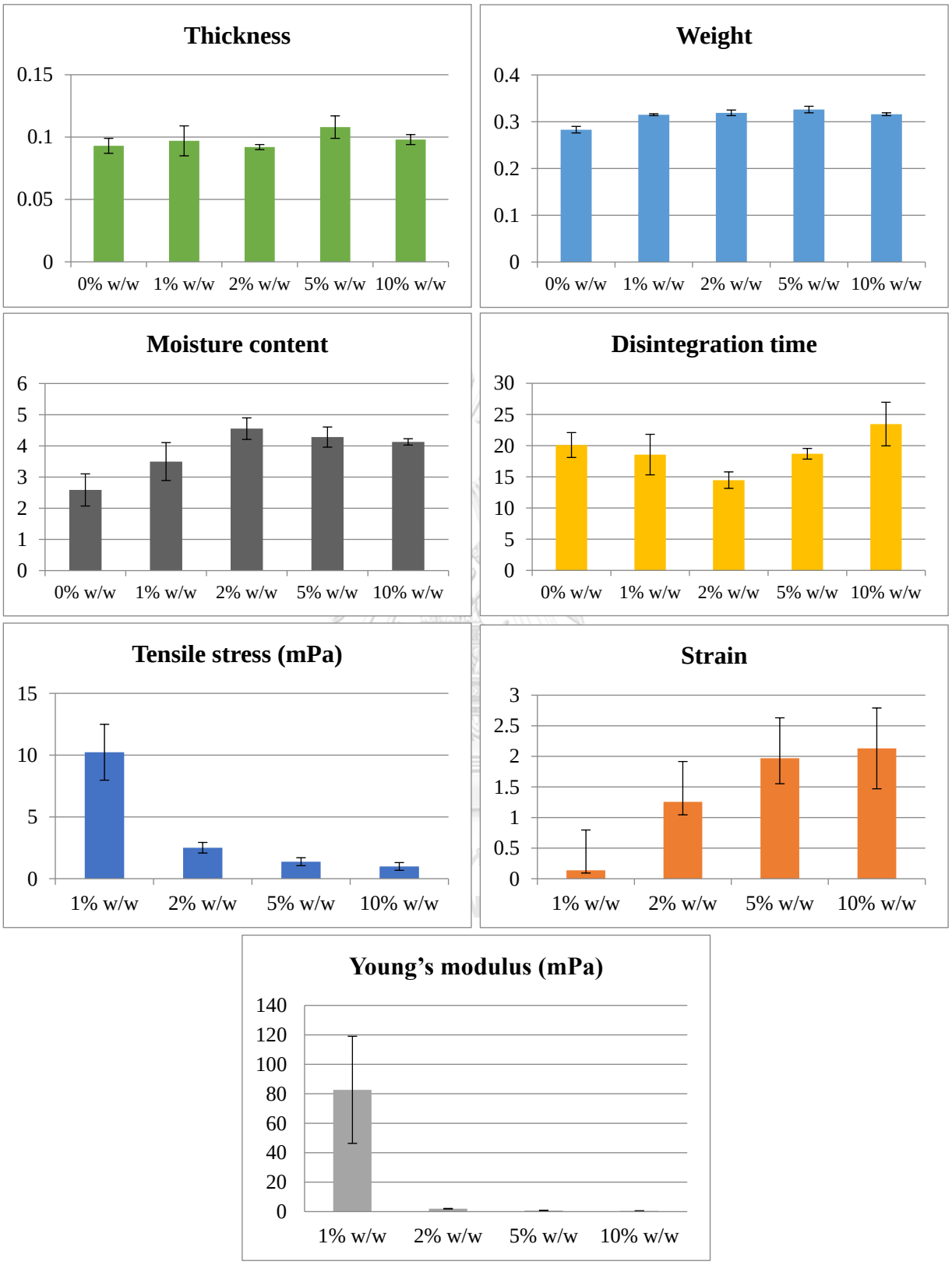


Figure 23 Product evaluation of OTF containing LD and CD formulation using glycerin content at 0, 1, 2, 5, 10 %w/w of pullulan dry weight

As can be seen in physical appearance, all samples were found to be clear and transparent without any precipitated solid crystal under polarized light (Appendix B, Figure 32). It should be concluded that all components in the film formulation were molecularly miscible and eventually resulting in homogeneous OTF. However, they showed some different properties. The OTF without glycerin added could easily break with more fragile and could not be prepared the sample for mechanical properties determination. Furthermore, a comparative evaluation showed two substantial differences of OTF between the group of low glycerin content (1 and 2% w/w) and high level of glycerin (5 and 10 %w/w). OTF contained high level of glycerin showed the more tackiness than those of the low glycerin level.

Multiple group comparison using ANOVA test was conducted and shown in Table 10. The utilization of glycerin in OTF showed certain signs of statistical significance of testing parameter such as weight, moisture content, disintegration time and mechanical properties ($p<0.05$). It appeared that tensile stress and Young's modulus at 1% w/w tended to be clearly higher than others while strain tended to be lower. However, several testing parameters such as thickness, weight, moisture content and disintegration time were no remarkable correlation (Figure 23). It can be proposed that the mechanical properties of OTF were possibly affected by either glycerin or moisture. In general, polyol and retained moisture in polymeric film system were able to act as plasticizer via the lowering of Tg (31). However, the result clearly demonstrated the insignificant of OTF's moisture content. It was thus a scientific sound evidence to indicate that glycerin was a key component on the changing of physico-mechanical attributes of OTF. The increasing of glycerin content could provide more possibility on the reaction among polymer chains. Glycerin, the plasticizer, gets into the space between the polymer chain and occupied more void space by decreasing the inter-molecular force between polymer chains (31). The OTF produced with lower glycerin content tended to be more stiffness or more resistance to elastic deformation. Vuddanda (2017) demonstrated that pullulan was able to has the intermolecular hydrogen bonding and later molecularly miscible with glycerin. Glycerin could decrease Tg of pullulan OTF according to the concentration dependent manner and tensile stress exhibited a tendency to decrease when the plasticizer concentration

increased (32). Prajapati (2018) suggested that if the plasticizer increased, strain also tended to be increase. OTF was thus easy handing at low plasticizer concentration (6).

In conclusion from the varying of glycerin content, the OTF produced without glycerin was breakable. Whereas the others (with 5 and 10% w/w of glycerin) were tackier than others. Their mechanical properties were not also acceptable. They could not be held or carried. Therefore, glycerin content at 1 and 2% w/w of pullulan dry weight were the most appropriate in this study and suitable for further studies.

2.1.2 Effect of pullulan content on OTF containing LD and CD

The development of OTF formulation by varying pullulan content was used as a sequence following the previous study. Irfan (2016) points out the productive development of an OTF are a function of justified selection and concentration of polymers as the mechanical properties of films (10). The comparative evaluation of OTF was done in two different glycerin level. The resulted were tabulated and shown in Table 11.

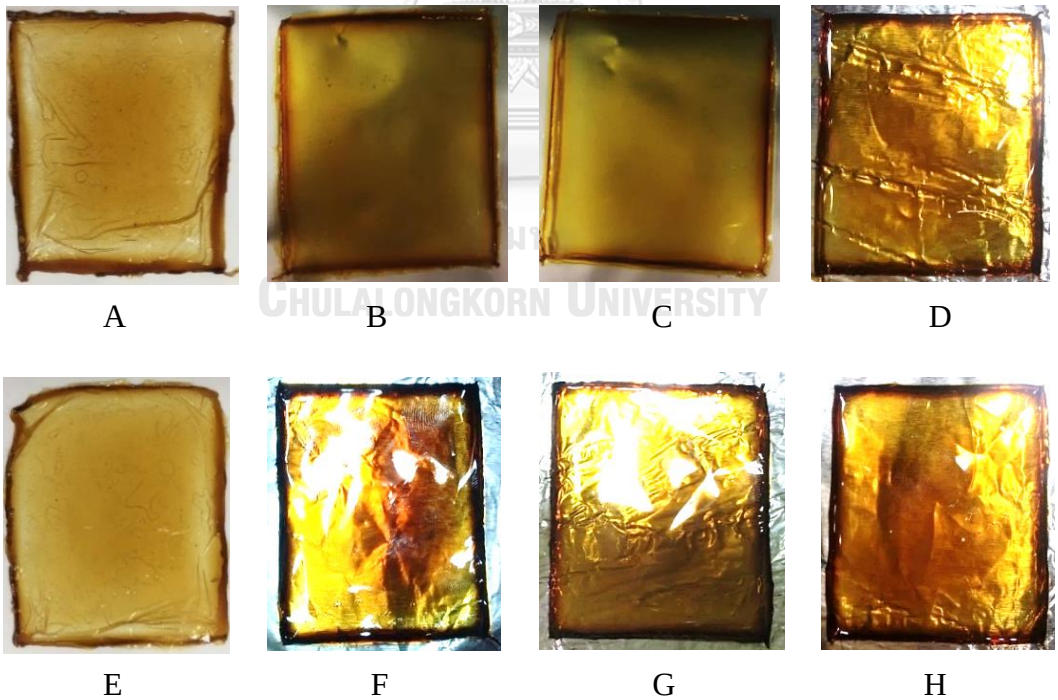


Figure 24 OTFs contained LD and CD using glycerin content at 1 %w/w of pullulan dry weight and pullulan content at 6(A), 8(B), 10(C), 12(D)% w/w in acid solvent system and glycerin content at 2 %w/w of pullulan dry weight and pullulan content at 6(E), 8(F), 10(G), 12(H) %w/w in acid solvent system

Table 11 Product evaluation of OTF containing LD and CD formulation using pullulan at 6, 8, 10, 12 % w/w in acid solvent with constant glycerin concentration at 1 and 2 %w/w of pullulan dry weight

characterization parameters	film former content of glycerin 1% formulation				film former content of glycerin 2% formulation			
	pullulan 6%	pullulan 8%	pullulan 10%	pullulan 12%	pullulan 6%	pullulan 8%	pullulan 10%	pullulan 12%
Physical properties								
- Transparency	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear
- Smoothness	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
- Fragility	Unbreakable	Unbreakable	Unbreakable	Unbreakable	Unbreakable	Unbreakable	Unbreakable	Unbreakable
- Precipitated solid particle	Not presence	Not presence	Not presence	Not presence	Not presence	Not presence	Not presence	Not presence
- Tackiness of film	Very slightly-tacky	Non-tacky	Non-tacky	Non-tacky	Very slightly-tacky	Non-tacky	Non-tacky	Non-tacky
- Thickness [mm]*	0.097±0.016 (16.378)	0.115±0.023 (19.956)	0.112±0.026 (22.938)	0.139±0.017 * (12.032)	0.093±0.011 (11.826)	0.109±0.017 (15.687)	0.125±0.033 (26.510)	0.209±0.038* (18.066)
- Weight [gram]*	0.315±0.002* (0.550)	0.392±0.004 (0.966)	0.399±0.004 (1.003)	0.439±0.005 * (1.169)	0.321±0.004* (1.246)	0.400±0.001 (0.289)	0.406±0.008 (2.051)	0.485±0.006* (1.288)
- Moisture content [%]	3.497±0.608 (17.386)	3.123±0.318 (10.188)	2.717±0.558 (20.526)	3.717±0.464 (12.473)	3.827±0.302* (7.884)	2.927±0.327 (11.188)	2.480±0.269 (10.850)	2.403±0.317 (13.175)

Table 11 Product evaluation of OTF containing LD and CD formulation using pullulan at 6, 8, 10, 12 % w/w in acid solvent with constant glycerin concentration at 1 and 2 %w/w of pullulan dry weight**,** (Continued)

characterization parameters	film former content of glycerin 1% formulation				film former content of glycerin 2% formulation			
	pullulan 6%	pullulan 8%	pullulan 10%	pullulan 12%	pullulan 6%	pullulan 8%	pullulan 10%	pullulan 12%
- Disintegration time [second]*	18.570±3.250*	26.630±2.150*	38.500±3.418	42.867±1.401	15.830±1.222*	27.800±1.758	36.400±6.758	45.233±6.158
Mechanical properties								
- Tensile stress [mPa]*	10.236±2.261*	20.959±4.461	28.692±2.291	29.814±7.950	4.409±0.236*	27.698±1.762	30.574±9.492	29.376±13.123
- Strain*	0.137±0.044	0.094±0.002	0.061±0.022	0.017±0.006*	0.555±0.350*	0.107±0.004	0.080±0.017	0.029±0.009
- Young's modulus [mPa]*	82.597±36.364	221.514 ±41.379	513.513 ±182.030	1934.851 ±954.950*	13.535±13.349	259.008 ±17.761	416.069 ±235.174	1213.141 ±875.251

Mean ± SD, %RSD is shown in parentheses

*$p<0.05$

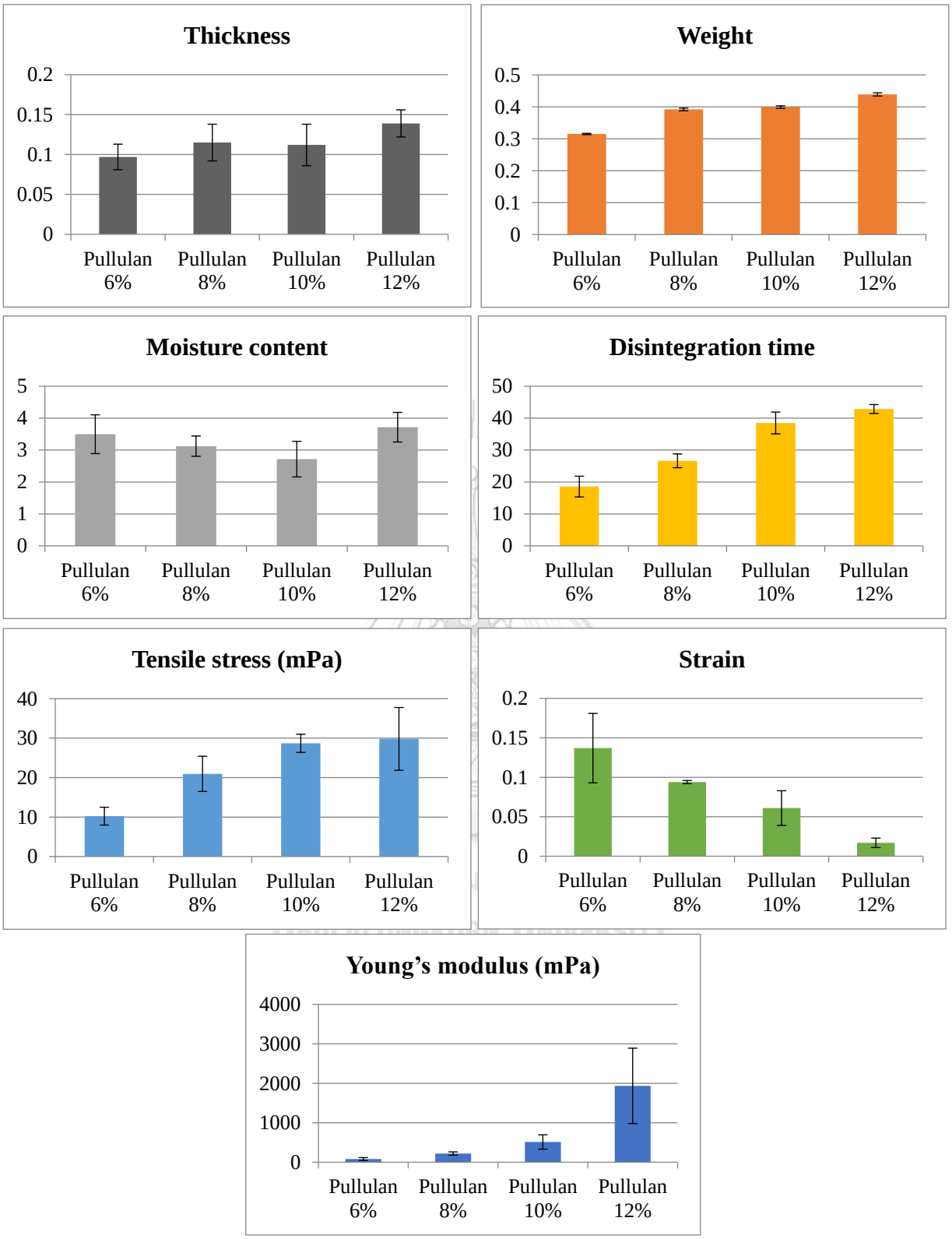


Figure 25 Product evaluation of OTF containing LD and CD formulation using pullulan at 6, 8, 10, 12 % w/w in acid solvent with constant glycerin content at 1 %w/w of pullulan dry weight

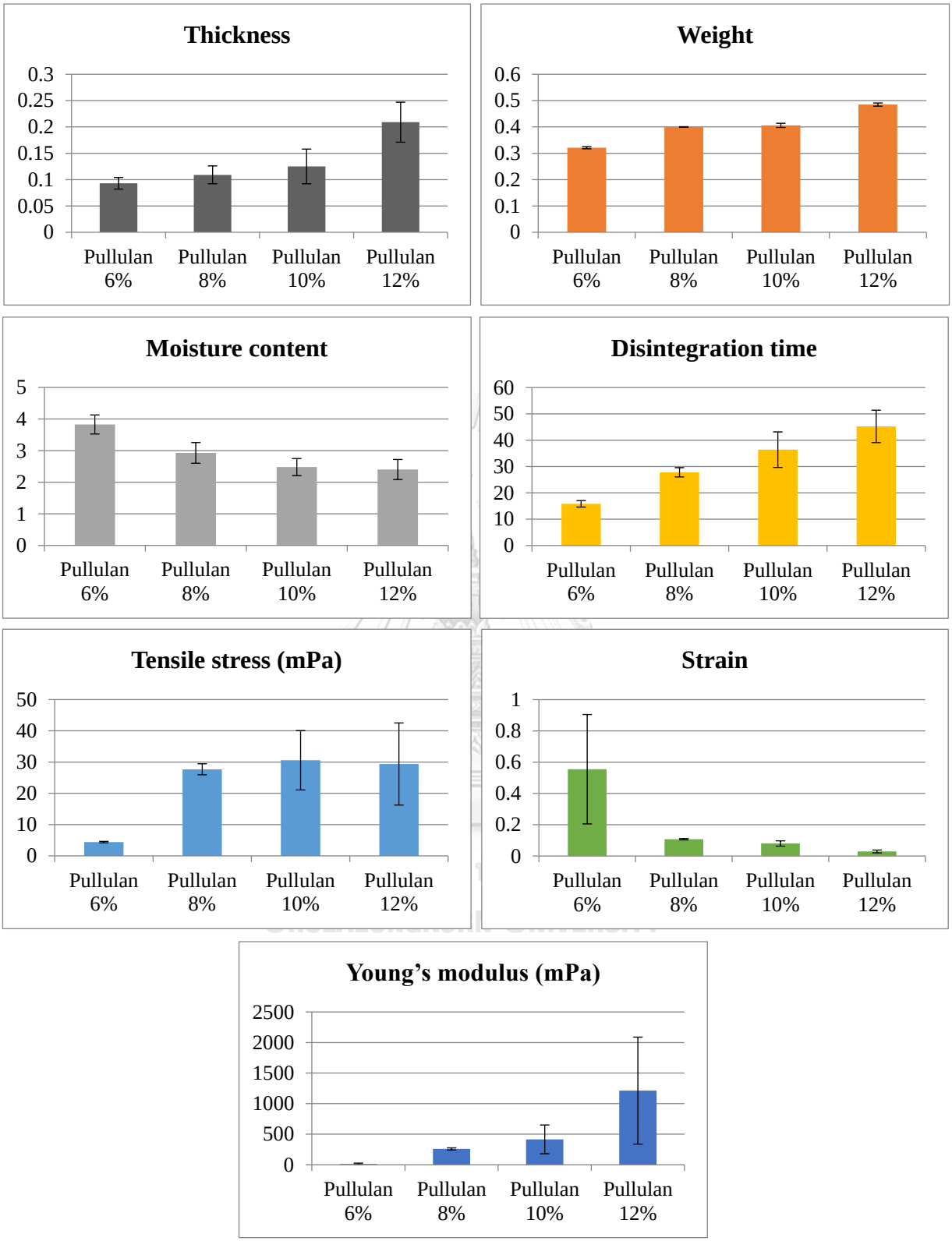


Figure 26 Product evaluation of OTF containing LD and CD formulation using pullulan at 6, 8, 10, 12 % w/w in acid solvent with constant glycerin content at 2 %w/w of pullulan dry weight

The physical appearance of OTFs contained LD and CD using glycerin at the content of 1 and 2 %w/w were clear and transparent without any precipitated solid crystal under polarized light (Appendix B, Figure 31). The non-tacky OTF using pullulan at 8 to 12 %w/w were able to prepare. They were easy to handle and suitable for manufacture. It was due to the fact that the higher of pullulan content provided the more acceptable mechanical properties of OTFs. ANOVA testing of sample produced indicated that thickness, weight, disintegration time and mechanical properties of different pullulan loading were significant different *($p<0.05$).* Weight, disintegration time and tensile stress at 6% w/w tended to be lower than others OTFs (Figure 25 and 26). They were gradually increased as function of pullulan content of 6 to 12%w/w. Theoretically, the increasing of OTF's thickness directly affects on the physico-mechanical properties (31). The highest thickness of OTF containing pullulan (12 %w/w) had significant impact on its mechanical properties *($p<0.05$)* (Table 11). When considering the OTF produced with lower pullulan concentration range (6 to 10 %w/w), it was found that the film thickness was not statistically different. The physico-mechanical properties should not also difference. Nevertheless, the result of this study showed that the tensile stress of 6 %w/w pullulan OTF was significantly lower than others. In another word, the strain of 6 %w/w pullulan OTFs showed the significant higher value and progressively decrease as a function of pullulan content (Table 11). Young's modulus, an attribute related to stress and strain, were found to be higher when pullulan concentrations used were increased. However, the increment of such Young's modulus was not significantly differences. In conclusion, it did not illustrate the directly proportional concentration dependent manner between experimental lower pullulan concentration range and their physico-mechanical properties.

Therefore, the handling and mechanical characteristic were improved since the higher polymer was used. The OTF produced with higher pullulan content showed more stiffness and resistance to the elastic deformation under force applied. It was due to the increasing of polymer concentration resulted in the upward shifted of Tg. Higher polymer concentration or number of polymer chain is able to decelerate the molecular mobility of polymer chain and increase the density or compaction of polymer network (31). This result well complied with the study of Fundo

(2015) and Ganduri (2016). They found that OTF with high load of polymer exhibited a longer disintegration time and thickness while its strain tended to be decrease (71, 72). Nevertheless, the higher pullulan of 10 and 12 %w/w expressed the longer disintegration of over 30 seconds. Indeed, there is no official guideline on the specification of the disintegration time for OTFs but the range of 5 to 30 seconds was suggested by Bala (2013) (25). Therefore, our developed OTFs that met the requirement was found to be composed of 8 %w/w of pullulan with either glycerin added of 1 or 2 %w/w of dry pullulan.

2.2 Short term stability of OTF containing LD and CD

The optimized 8 %w/w of pullulan content and glycerin content at both 1, 2 %w/w of pullulan dry weight formulations were selected for stability studies. The stability of OTF containing LD and CD was performed by determining the physical appearance including the analysis of LD and CD content in OTF by HPLC. Analytical method validation was shown in Appendix C. The results of stability studies was gathered and illustrated in Table 12 and 13.

Table 12 Stability studies of OTFs containing LD and CD fabricating with pullulan 8% w/w and glycerin 1%w/w at different conditions

Storage condition			30±2 °C 75±5 %RH	40±2 °C 75±5 %RH
Test parameter	Requirement	initial	1 month	1 month
Physical appearance				
- Product color	Light yellow	Light yellow	Dark yellow	Dark yellow
- Transparency	Clear	Clear	Clear	Clear
- Smoothness	Smooth	Smooth	Smooth	Smooth
- Fragility	Unbreakable	Unbreakable	Unbreakable	Unbreakable
- Precipitated solid particle	Not presence	Not presence	Not presence	Not presence
- Tackiness of film	Non-tacky	Non-tacky	Non-tacky	Non-tacky
Assay content of				
- LD	90-110 %LA	92.748±1.759 (1.159 mg/cm^2)	97.313±4.575 (1.216 mg/cm^2)	77.656±5.207* (0.971 mg/cm^2)
- CD	90-110 %LA	ND	ND	ND

Mean ± SD

*$p<0.05$

Table 13 Stability studies of OTFs containing LD and CD fabricating with pullulan 8% w/w and glycerin 2%w/w at different conditions

Storage condition			30±2 °C 75±5 %RH	40±2 °C 75±5 %RH
Test parameter	Requirement	initial	1 month	1 month
Physical appearance				
- Product color	Light yellow	Light yellow	Dark yellow	Dark yellow
- Transparency	Clear	Clear	Clear	Clear
- Smoothness	Smooth	Smooth	Smooth	Smooth
- Fragility	Unbreakable	Unbreakable	Unbreakable	Unbreakable
- Precipitated solid particle	Not presence	Not presence	Not presence	Not presence
- Tackiness of film	Non-tacky	Non-tacky	Non-tacky	Non-tacky
Assay content of				
- LD*	90-110 %LA	99.4366±1.880 (1.243 mg/cm^2)	103.420±2.061* (1.293 mg/cm^2)	90.153±5.334 (1.127 mg/cm^2)
- CD*	90-110 %LA	58.937±11.039 (0.184 mg/cm^2)	ND	ND

Mean ± SD

**p<0.05*

Table 12 and 13 express a comparison of pullulan 8% w/w with glycerin 1 and 2 %w/w of pullulan dry weight formulation at ambient condition (30±2 °C, 75±5 %RH) and accelerated condition (40±2 °C, 75±5 %RH), respectively. The physical appearance at initial period was acceptable and retained their original appearance after 1 month of storage. However, the discoloration of product was dramatically investigated from light yellow to dark brown color. Zhou (2012) reviewed that the degradation pathway via oxidation for LD would lead to the formation of darker pigment of melanin as the end product. Temperature was one of the critical

environment factors that affected on the formation rate of melanin from LD precursor (17). It should be a good supporting evidence on the development of darker color of OTFs containing LD upon storage particularly high temperature condition. In term of the remaining content of LD, the complying of LD was found of both OTFs formulation. Meanwhile, lower content of LD (out of specification) was determined under accelerated condition. It should be due to the oxidative degradation with the acceleration of high temperature of storage.

Nevertheless, the instability of both formulations was detected by the absence of CD after freshly prepared. OTFs with 1 %w/w glycerin was completely gone while half of loading CD that out of the requirement was found in glycerin 2 %w/w formulation (Appendix C, Figure 35 and 36). There is no clearly information that relating to ability of glycerin on the prevention of CD degradation. Nevertheless, Jenning (2016) suggested that the aliphatic compound as glycerin could stabilize drug by increasing the hydrophobicity of microclimate environment that eventually slowed down the hydrolysis (73). Then, the formulation that using higher glycerin content might stabilize CD more effectively than that of lower glycerin. However, after 1 month of storage, the CD content of both formulations could not be detected even at ambient condition or accelerated condition. The problem might be affected by several factors such as the ascorbic acid as stabilizer in OTFs, the drying temperature of the preparation including the accelerated condition temperature at 40±2 °C for storage. Generally, Bhatnagar (2015) reported that CD could degrade under certain condition like acid hydrolysis, oxidative degradation and exposure to 40°C and 75 %RH (74). In addition, Remenar (2006) pointed out that the ascorbic acid could be a cause of CD degradation. The high temperature that product exposed could also cause the degradation (9). In summary, the result of this study revealed that CD was likely to oxidized easier than ascorbic acid. It might thus able to protect the autoxidation of ascorbic acid as a redox couple and eventually resulting in the higher disappearing of CD incorporated OTFs. Furthermore, ascorbic acid was expected to suppress the microclimate pH of the polymeric thin film. Therefore, molecularly dispersed CD in polymeric OTFs could then easily hydrolyze under above strong acid condition.

Conclusively, both formulations were not stable for long time storage and showed significantly negative changes in LD and CD content. Therefore, the OTF containing LD and CD in both formulations were still not stable and might not be appropriated for enlarge the scale of manufacturing.

CHAPTER V

CONCLUSION

The significant increasing of proton concentration in the solution was an efficient method for increasing the solubility of LD and CD. The control of bulk pH played an important role on the solubility improvement. Desired pH should be lower than the pKa1 of LD and CD that would make the more dissolving of LD and CD. The improving of LD and CD solubilization was related to the carboxylate group of zwitterion molecule. It behaved as a proton acceptor from enriched hydronium ion of low pH environment. Consequently, the overall structure of both LD and CD finally presented in form of cationic molecule. It was then induced to appear as charge species that easily dissolved in aqueous system. The acid solvent (0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5) was a suitable solvent for loading the higher amount of LD and CD. Nevertheless, the effect of charge protonation was not sufficient to provide required loading quantity of LD and CD compared to the target strength in tablet formulation. Thus, other solubilization approaches should be observed.

The solubility profile of LD and CD in 0.1 M hydrochloric acid/ 0.1 M citric acid pH 1.5 which showed highest solubility of both drugs was then incorporated with a group of solubilizer as ethanol, glycerin, PEG400 and Tween80 at 5, 10 and 20 % v/v, respectively. They were not significantly improved the loading quantity of both drugs in this acidic system. In addition, the increment of pullulan polymer over the range of 6 to 10 % w/v was also not significantly increase the solubility of both drugs. Interestingly, it was found that the addition of cosolvent, surfactant and higher polymer content tended to decrease LD and CD solubility.

Formulation development of OTF containing LD and CD was done by studying the effect of additive that strongly influenced on the properties of OTF. The effect of plasticizer like glycerin was determined by varying its content at 0, 1, 2, 5, 10 %w/w of pullulan dry weight. The OTF without glycerin added was fragile and could not be prepared for mechanical properties determination. Meanwhile, the addition of glycerin at low level (1 and 2 %w/w) in OTFs showed higher stiffness or resistance to elastic deformation due to the plasticization effect. Nevertheless, the higher glycerin content

at 5 and 10% w/w provided the tackier of dry film and not able to handling properly. Therefore, OTFs containing 1 or 2 % of glycerin would be used for observing the effect of higher polymer content later. In term of the pullulan content, the higher the pullulan content the more the acceptable mechanical properties of OTFs. However, the higher pullulan of 10 and 12 %w/w showed the longer disintegration of over 30 seconds that not complied with the requirement of OTF.

Primary conclusion of optimized OTF formulation would be composed of 8% w/w pullulan with glycerin of either 1% or 2% w/w of pullulan dry weight. However, both formulations were not stable and showed significant changes in LD and CD content after freshly prepare including at short term storage of only one month.

Suggestion for further study

The finding of this study indicated that the OTF containing LD and CD in 8% w/w of pullulan with either glycerin 1% or 2% w/w of pullulan content were not suitable due to the potency loss of LD and CD after manufactured. It will be an important issue that future research will be continuously conducted to develop the more stable formulation of OTF containing LD and CD. In order to suggest or design the experiment, several factors affecting on the stability of both drug such as ascorbic acid and drying temperature of the preparation should be determined. In addition, other antioxidant compounds such as sulfite antioxidant and/or tocopherol derivatives should be further investigated.

REFERENCES

1. Clinical practice guideline for diagnosis and management of parkinson's disease [Internet]. Chulalongkorn centre of excellence for Parkinson's disease & related disorders. 2553. Available from: http://www.chulapd.org/uploads/download_list/2/a-02.pdf.
2. Braunwald, Fauci, Kasper, Hauser, Longo, Jameson. Parkinson's disease: McGrawHill; 2008.
3. Prasad KN. Can we prevent parkinson's and alzheimer's disease? Journal of Postgraduate Medicine. 2003;49:236-45.
4. Cause and solution: Intermittent response to levodopa [Internet]. Faculty of pharmaceutical sciences Chulalingkorn university. 2016. Available from: http://ccpe.pharmacycouncil.org/index.php?option=article_detail&subpage=article_detail&id=213.
5. Lai KL, Fang Y, Han H, Li Q, Zhang S, Li HY, et al. Orally-dissolving film for sublingual and buccal delivery of ropinirole. Colloids and Surfaces B: Biointerfaces. 2018;163:9-18.
6. Prajapati VD, Chaudhari AM, Gandhi AK, Maheriya P. Pullulan based oral thin film formulation of zolmitriptan: Development and optimization using factorial design. International Journal of Biological Macromolecules. 2018;107(Pt B):2075-85.
7. Bredenberg S, Nyholm D, Aquilonius S-M, Nyström C. An automatic dose dispenser for microtablets—a new concept for individual dosage of drug in tablet form. International Journal of Pharmaceutics. 2003;261:137-46.
8. Pappert E, Lipton J, Goetz C, Ling Z, Stebbins G, Carvey P. The stability of carbidopa in solution. Movement disorders : official journal of the Movement Disorder Society. 1997;12:608-10.
9. Remenar J, Almarsson O, JR AJM, Zhang Z, inventors; Transform pharmaceutical Inc, assignee. Pharmaceutical compositions and method of using levodopa and carbidopa patent United States patent US 2005/0203185 A1. 2005 Sep 15.
10. Irfan M, Rabel S, Bukhtar Q, Qadir MI, Jabeen F, Khan A. Orally disintegrating films: a modern expansion in drug delivery system. Saudi Pharmaceutical Journal. 2016;24(5):537-46.
11. Sujaritnarakorn N, Suksaard N, Chaiyawuttaparuk L. Formulation development of oral film containing levodopa and carbidopa. Bangkok: Chulalongkorn University; 2016.
12. Levodopa chemical and physical properties [Internet]. 2018 [cited 2018 11 October]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/levodopa#section=Chemical-and-Physical-Properties.
13. Carbidopa chemical and physical properties [Internet]. 2018 [cited 2018 11 October]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/carbidopa#section=Chemical-and-Physical-Properties.
14. Patil P, Shrivastava SK. Fast dissolving oral films: An innovative drug delivery system. International Journal of Science and Research (IJSR). 2014;3(7):2068-93.

15. Nagar P, Chauhan I, Yasir M. Insights into polymers: Film formers in mouth dissolving films. Drug Invention Today. 2011;3(12):280-9.
16. Preis M, Pein M, Breitkreutz J. Development of a taste-masked orodispersible film containing dimenhydrinate. Pharmaceutics. 2012;4(4):551-62.
17. Zhou YZ, Alany RG, Chuang V, Wen J. Studies of the rate constant of L-DOPA oxidation and decarboxylation by HPLC. Chromatographia. 2012;75:597–606.
18. Thakur N, Bansal M, Sharma N, Yadav G, Khare P. Overview "a novel approach of fast dissolving films and their patients". Advances in Biological Research. 2013;7(2):50-8.
19. Kumar D, Saini N, Pandit V, Ali S. An insight to pullulan: A biopolymer in pharmaceutical approaches. International Journal of Basic and Applied Sciences. 2012;1.
20. Ramesan R, Sharma C. Pullulan as a promising biomaterial for biomedical applications: A perspective. Trends in Biomaterials and Artificial Organs. 2007;20:111-6.
21. Cooke B. A taxonomic study in the black yeast. 1963.
22. Leathers TD. Biotechnological production and applications of pullulan. Appl Microbiol Biotechnol. 2003;62(5-6):468-73.
23. Damager I, Engelsen SB, Blennow A, Lindberg Møller B, Motawia MS. First principles insight into the α-glucan structures of starch: Their synthesis, conformation, and hydration. Chemical Reviews. 2010;110(4):2049-80.
24. Frederiksen K, Guy RH, Petersson K. Formulation considerations in the design of topical, polymeric film-forming systems for sustained drug delivery to the skin. Eur J Pharm Biopharm. 2015;91:9-15.
25. Bala R, Pawar P, Khanna S, Arora S. Orally dissolving strips: A new approach to oral drug delivery system. International Journal of Pharmaceutical Investigation. 2013;3(2):67-76.
26. Joshua JM, Hari R, Jyothish FK, Surendran SA. Fast dissolving oral thin films: An effective dosage form for quick releases. International Journal of Pharmaceutical Sciences Review and Research. 2016;38(1):282-9.
27. Panda BP, Dey NS, Rao MEB. Development of innovative orally fast disintegrating film dosage forms: A review. International Journal of Pharmaceutical Sciences and Nanotechnology. 2012;5(2):1666-74.
28. Arya A, Chandra A, Sharma V, Pathak K. Fast dissolving oral films: An innovative drug delivery system and dosage form. International Journal of ChemTech Research. 2010;2(1):576-83.
29. Karki S, Kim H, Na S-J, Shin D, Jo K, Lee J. Thin films as an emerging platform for drug delivery. Asian Journal of Pharmaceutical Sciences. 2016;11(5):559-74.
30. Nair AB, Kumria R, Harsha S, Attimarad M, Al-Dhubiab BE, Alhaider IA. In vitro techniques to evaluate buccal films. Journal of Controlled Release. 2013;166(1):10-21.
31. Jadhav N, Gaikwad V, Nair K, Kadam H. Glass transition temperature: Basics and application in pharmaceutical sector. Asian Journal of Pharmaceutics. 2009;3(2).
32. Vuddanda PR, Montenegro-Nicolini M, Morales JO, Velaga S. Effect of plasticizers on the physico-mechanical properties of pullulan based pharmaceutical oral films. European Journal of Pharmaceutical Sciences. 2017;96:290-8.

33. Seedher N, Kanojia M. Co-solvent solubilization of some poorly-soluble antidiabetic drugs. Pharmaceutical Development and Technology. 2009;14(2):185-92.
34. Tseng H-C, Lee C-Y, Weng W-L, Shiah IM. Solubilities of amino acids in water at various pH values under 298.15K. Fluid Phase Equilibria. 2009;285(1-2):90-5.
35. Huang Y, Dai WG. Fundamental aspects of solid dispersion technology for poorly soluble drugs. Acta Pharmaceutica Sinica B. 2014;4(1):18-25.
36. Marsac PJ, Shamblin SL, Taylor LS. Theoretical and practical approaches for prediction of drug-polymer miscibility and solubility. Pharmaceutical Research. 2006;23(10):2417-26.
37. Loftsson T. The effect of water-soluble polymers on aqueous solubility of drugs. International Journal of Pharmaceutics. 1996;127(2):293-6.
38. Xi K, Tabata Y, Uno K. Liver targeting of interferon through pullulan conjunction. Pharmaceutical Research. 1996;13(12).
39. Singh RS, Saini GK. Biosynthesis of pullulan and its applications in food and pharmaceutical industry. Microorganisms in Sustainable Agriculture and Biotechnology2012. p. 509-53.
40. Li H, Bian S, Huang Y, Liang J, Fan Y, Zhang X. High drug loading pH-sensitive pullulan-DOX conjugate nanoparticles for hepatic targeting. Journal of Biomedical Materials Research. 2014;102(1):150-9.
41. Hasegawa U, Nomura S-i, Kaul S, Hirano T, Akiyoshi K. Nanogel-quantum dot hybrid nanoparticles for live cell imaging. Biochemical and Biophysical Research Communications. 2005;331:917-21.
42. Miyamoto Y, Goto H, Sato H, Okano H, Iijima M, inventors; Zeria Shinyaku Kogyo Kabushiki Kaisha, assignee. Process for sugar-coating solid preparation. Japan1986.
43. Yasuo lzutsu KS, Shizuo Okamoto, Terukazu Tanaka, inventor; Dainippon Pharmaceutical Co., Ltd., assignee. Sugar-coated pharmaceutical composition and method for preparing the same1984.
44. Galgatte UC, Khanchandani SS, Jadhav YG, Chaudhari PD. Investigation Of different polymers plasticizers and super disintegrating agents alone and in combination for use in the formulation of fast dissolving oral films. International Journal of PharmTech Research. 2013;5(4):1465-72.
45. Panchal MS, Patel H, Bagada A, K.R.Vadalia. Formulation and evaluation of mouth dissolving film of ropinirole hydrochloride by using pullulan polymers. International Journal of Pharmaceutical Research & Allied Sciences. 2012;1(3):60-72.
46. Krull SM, Ma Z, Li M, Dave RN, Bilgili E. Preparation and characterization of fast dissolving pullulan films containing BCS class II drug nanoparticles for bioavailability enhancement. Drug Development and Industrial Pharmacy. 2016;42(7):1073-85.
47. Gherman S, Zavastin D, Ochiuz L, Biliuta G, Coseri S. Enalapril maleate loaded pullulan film for mucoadhesive buccal drug delivery applications. The Cellulose Chemistry and Technology journal. 2016;50(5-6):593-600.
48. Wadetwar RN, Ali F, Kanojiya P. Formulation and evaluation of fast dissolving sublingual film of paroxetine hydrochloride for treatment of depression. Asian Journal Pharmaceutical and Clinical Research. 2019;12(10).
49. Esim O, Ozkan CK, Kurbanoglu S, Arslan A, Tas C, Savaser A, et al. Development and in vitro/in vivo evaluation of dihydroergotamine mesylate loaded

maltodextrin-pullulan sublingual films. Drug Development and Industrial Pharmacy. 2019;45(6):914-21.
50. Baka E, Comer JEA, Takacs-Novak K. Study of equilibrium solubility measurement by saturation shake-flask method using hydrochlorothiazide as model compound. Journal of Pharmaceutical and Biomedical Analysis. 2008;46:335-41.
51. Kawakami K, Oda N, Miyoshi K, Funaki T, Ida Y. Solubilization behavior of a poorly soluble drug under combined use of surfactants and cosolvents. European Journal of Pharmaceutical Sciences. 2006;28(1):7-14.
52. Lou H, Liu M, Qu W, Hu Z, Brunson E, Johnson J, et al. Evaluation of chlorpheniramine maleate microparticles in orally disintegrating film and orally disintegrating tablet for pediatrics. Drug Development and Industrial Pharmacy. 2014;40(7):910-8.
53. Marques MRC, Loebenberg R, Almukainzi M. Simulated biological fluids with possible application in dissolution testing. Dissolution Technology. 2011:15-28.
54. Asean guideline on stability study of drug product [Internet]. 2013. Available from: https://asean.org/wp-content/uploads/2018/01/25PPWG-ANNEX-7-iv-Final-ASEAN-Guideline-on-Stability-Study-Drug-Product-R2.pdf.
55. Montes I, Lai C, Sanabria D. Like dissolves like: A guided inquiry experiment for organic chemistry. Journal of Chemical Education. 2003;80.
56. Nakhaie D, Asselin E. The dissolution kinetics and salt film precipitation of Zn and Fe in chloride solutions: Importance of the common-ion effect and diffusivity. Corrosion Science. 2019;146:152-62.
57. de Wit JN, Kessel Tv. Effects of ionic strength on the solubility of whey protein products. A colloid chemical approach. Food Hydrocolloids. 1996;10(2):143-9.
58. Hitchcock DI. The solubility of tyrosine in acid and in alkali. The Journal of General Physiology. 1924:747-57.
59. Nayak AK, Panigrahi PP. Solubility enhancement of etoricoxib by cosolvency approach. ISRN Physical Chemistry. 2012;2012:1-5.
60. Synthesis crystal growth and characterization of bis (L – DOPA) hydrobromide single crystal C9H12NO4+·Br−·C9H11NO4 [Internet]. Available from: http://14.139.116.20:8080/jspui/bitstream/10603/225792/11/11_chapter%205.pdf.
61. Liu Z, Liu W, Lang C, Li Y. Effect of surfactant HLB value on methane hydrate formation in non-ionic surfactant-oil water emulsions systems. Energy Procedia. 2019;158:5275-80.
62. Karjiban RA, Basri M, Rahman MBA, Salleh AB. Structural properties of nonionic Tween80 micelle in water elucidated by molecular dynamics simulation. APCBEE Procedia. 2012;3:287-97.
63. York P, Anwar J, Amani A, Waard H. Molecular dynamics simulation of a polysorbate 80 micelle in water. Soft Matter. 2011;7.
64. Sasahara K, Uedaira H. Solubility of amino acids in aqueous poly(ethylene glycol) solutions. Colloid & Polymer Science. 1993;271(11):1035-41.
65. The solubility of amino acids in various solvent systems [Internet]. 1970. Available from: https://digitalcommons.uri.edu/oa_diss/159.
66. B P Dey SCL. Solubilities of amino acids in different mixed solvents. Indian Journal of Chemistry. 1985;25A:136-40.
67. Gekko K. Mechanism of polyol-induced protien stabilization: Solubility of amino acid and diglycine in aqueous polyol solution. J Biochem. 1981;90:1633-41.

68. Li M, Qiao N, Wang K. Influence of sodium lauryl sulfate and tween 80 on carbamazepine-nicotinamide cocrystal solubility and dissolution behaviour. Pharmaceutics. 2013;5(4):508-24.
69. May Essa Mahmood DAFA-K. Effect of temperature changes on critical micelle concentration for Tween series surfactant. Global Journal of Science Frontier Research Chemistry. 2013;13(4).
70. Lu Y, Zhang E, Yang J, Cao Z. Strategies to improve micelle stability for drug delivery. Nano Res. 2018;11(10):4985-98.
71. Fundo JF, Galvis-Sanchez AC, Delgadillo I, Silva CLM, Quintas MAC. The effect of polymer/ plasticiser ratio in film forming solutions on the properties of chitosan films. Food Biophysics. 2015;10(3):324-33.
72. Ganduri VSRK, Reethika NM, Jayasai M, Sirisha V. Effect of pullulan concentration in fast dissolving films formulation and exploration of film properties. Journal of Pharmacy Research. 2016;10(5):211-5.
73. Jennings CL, Dziubla TD, Puleo DA. Combined effects of drugs and plasticizers on the properties of drug delivery films. J Bioact Compat Polym. 2016;31(4):323-33.
74. P B, D V. Stability indicating HPLC method for simultaneous estimation of entacapone levodopa and carbidopa in pharmaceutical formulation. Journal of Chromatography & Separation Techniques. 2015;06(07).
75. Reddy BJc, Sarada NC. A Simple validated stability indicating RP-HPLC method for the determination of three antiparkinsonism compounds in oral contraceptive tablet Formulations. International Journal of ChemTech Research. 2017;10(3):636-46.
76. Sravanthi D, Anusha M, Madhavi S, Firdose S, Nalluri BN. Simultaneous estimation of levodopa and carbidopa in bulk, pharmaceutical dosage forms and dissolution sample analysis by RP-HPLC-PDA method. Journal of Chemical and Pharmaceutical Research. 2013;5(11):422-8.
77. Validation of chromatographic method [Internet]. Center for drug evaluation and research. 1994. Available from: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/reviewer-guidance-validation-chromatographic-methods.

APPENDIX A

Quantitative determination of LD and CD

UV-spectrophotometry was used for quantitatively determination of solubilized drugs (LD and CD). The relationship between dissolved drug concentrations versus time was constructed.

UV Spectrum of LD and CD

The maximum wavelength of LD and CD were 280 and 279 nm, respectively as shown in Figure 27 and 28.

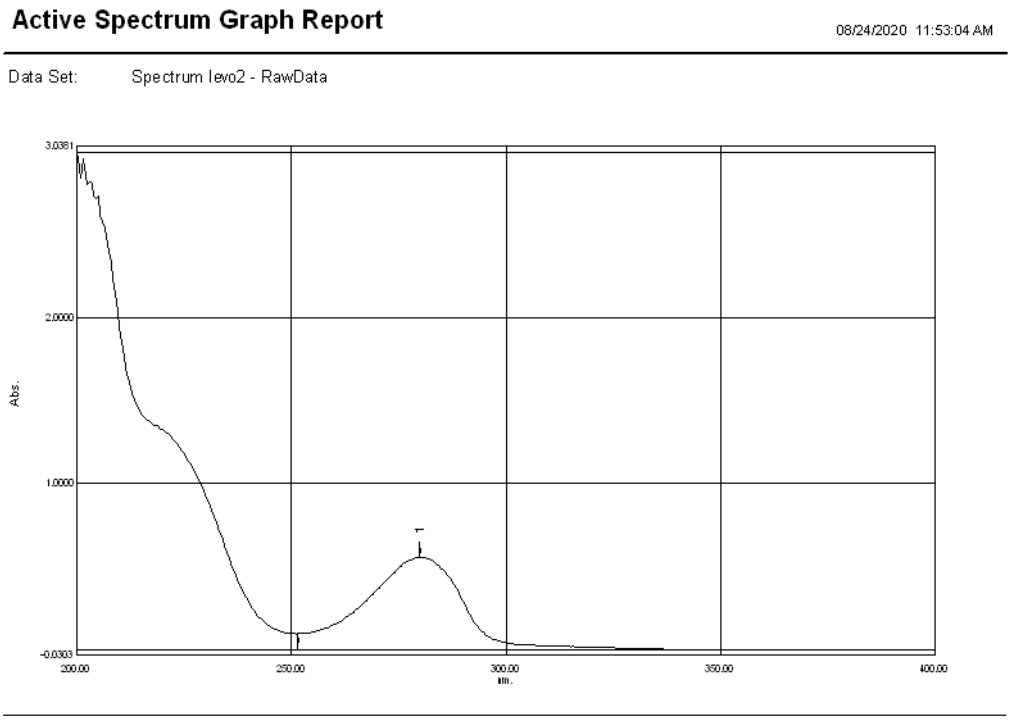


Figure 27 UV spectrum of LD dissolved in DI water

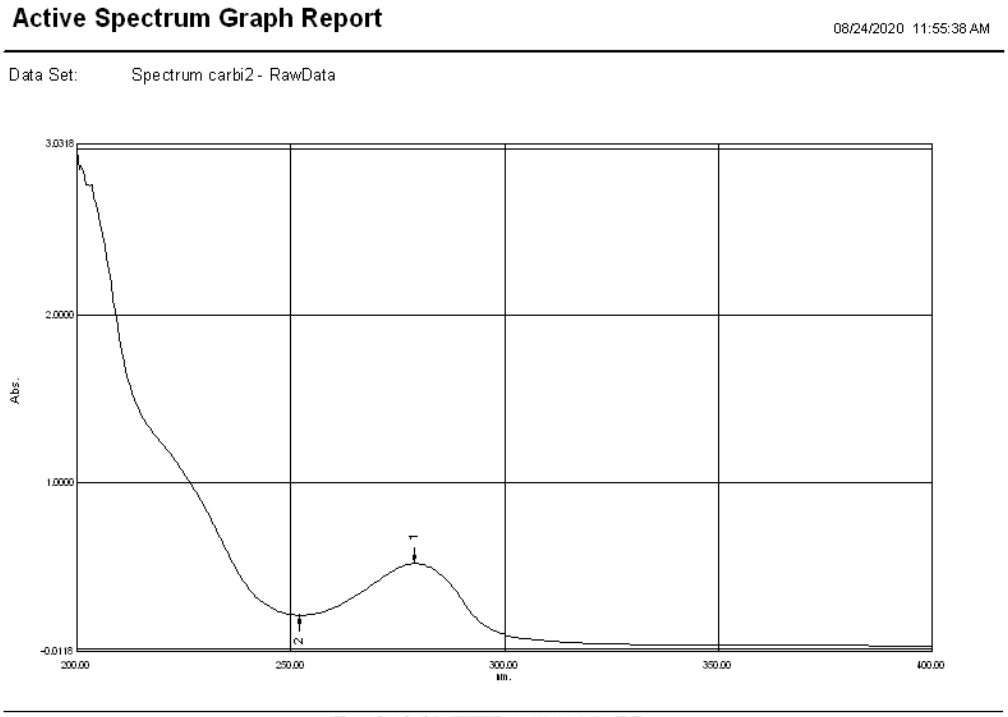


Figure 28 UV spectrum of CD dissolved in DI water

Table 14 Data of calibration curve of LD and CD standard preparation by UV method

drug	concentration	absorbance			
		n 1	n 2	n 3	average
LD	20 µg/ml	0.2846	0.3062	0.2928	0.2945
	30 µg/ml	0.4228	0.4362	0.4262	0.4284
	40 µg/ml	0.5661	0.5664	0.5867	0.5731
	50 µg/ml	0.7099	0.7017	0.6951	0.7022
	60 µg/ml	0.8335	0.8292	0.8395	0.8341
			R^2		0.9997
CD	20 µg/ml	0.2692	0.2873	0.2735	0.2767
	30 µg/ml	0.3993	0.4253	0.4144	0.4130
	40 µg/ml	0.5258	0.5628	0.5428	0.5438
	50 µg/ml	0.6683	0.668	0.6626	0.6663
	60 µg/ml	0.7628	0.7764	0.7743	0.7712
			R^2		0.9976

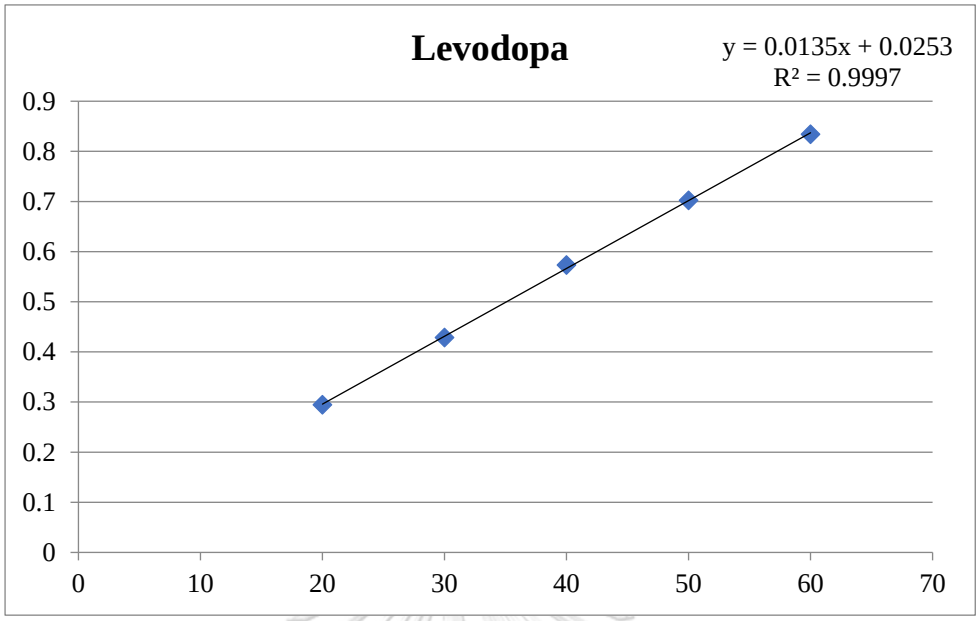


Figure 29 Standard curve of LD in DI water

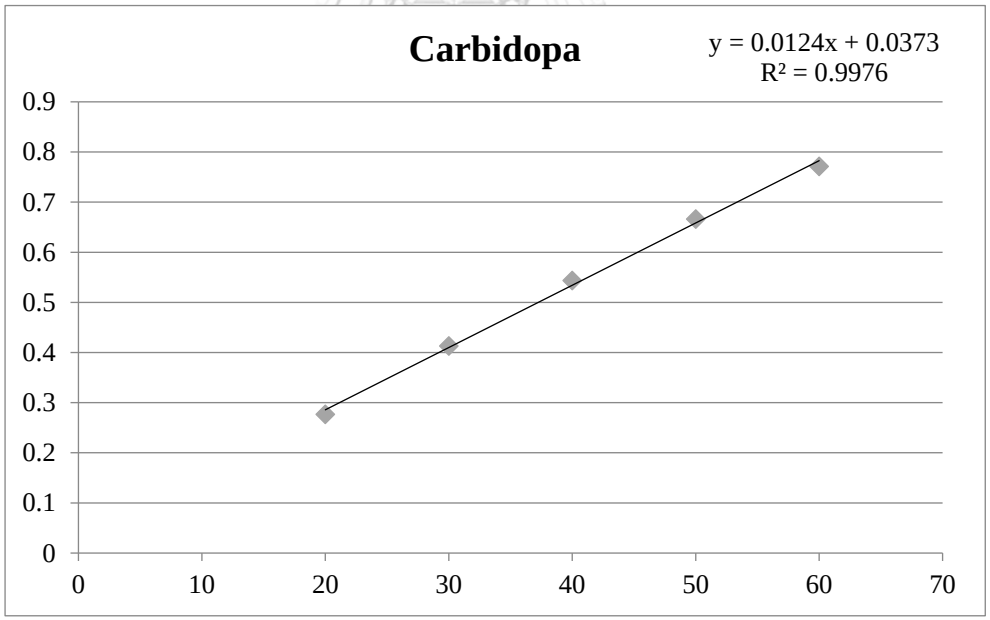


Figure 30 Standard curve of CD in DI water

Solubility determination of LD and CD in various acid solvent systems

Table 15 Solubility determination of LD in various acid solvent systems

Solvent system	Time (hr)	n 1 (mg/ml)	n 2 (mg/ml)	n 3 (mg/ml)	Average (mg/ml)	SD	%CV
0.2 M HCl-0.1 M citric acid pH 1.5	1	11.7452	11.8963	11.8459	11.8291	0.0769	0.6505
	2	11.9615	12.0563	12.0119	12.0099	0.0474	0.3950
	4	12.0682	12.0622	12.1867	12.1057	0.0702	0.5799
	8	12.4711	12.2904	12.4533	12.4049	0.0996	0.8030
	16	13.3304	13.7482	13.7185	13.5990	0.2331	1.7143
	32	13.1852	13.3422	13.4489	13.3254	0.1327	0.9955
	48	12.9422	12.8800	12.9304	12.9175	0.0330	0.2558
0.1 M HCl-0.1 M citric acid pH 1.5	1	10.7082	10.9541	10.8207	10.8277	0.1231	1.1370
	2	11.2444	11.1763	10.8978	11.1062	0.1837	1.6537
	4	12.0919	11.7096	12.1185	11.9733	0.2288	1.9106
	8	11.5496	11.6593	11.8756	11.6948	0.1659	1.4181
	16	11.9763	11.9674	11.9882	11.9773	0.0104	0.0869
	32	12.2578	12.3289	12.4889	12.3585	0.1184	0.9578
	48	11.9556	12.1037	12.9215	12.3269	0.5202	4.2201
0.1 M HCl-0.1M KCl pH 1.5	1	8.6711	8.2578	9.1393	8.6894	0.4410	5.0754
	2	9.3719	9.6074	9.4178	9.4657	0.1249	1.3192
	4	9.1481	9.4163	9.4148	9.3264	0.1544	1.6554
	8	9.3970	9.4504	9.5985	9.4820	0.1044	1.1010
	16	9.5867	9.7556	9.4281	9.5901	0.1637	1.7073
	32	9.5452	9.7793	9.9126	9.7457	0.1860	1.9084
	48	9.5111	9.6074	9.5881	9.5689	0.0510	0.5325

Table 15 Solubility determination of LD in various acid solvent systems**,** (Continued)

Solvent system	Time (hr)	n 1 (mg/ml)	n 2 (mg/ml)	n 3 (mg/ml)	Average (mg/ml)	SD	%CV
0.1 M citric acid	1	7.1807	7.4044	7.1600	7.2484	0.1355	1.8699
pH 2	2	7.1037	6.9541	6.9719	7.0099	0.0817	1.1661
	4	7.7941	7.5956	7.7052	7.6983	0.0994	1.2917
	8	7.5896	7.7837	7.7096	7.6943	0.0979	1.2729
	16	7.8163	7.9778	8.0148	7.9363	0.1056	1.3301
	32	7.6978	8.1185	7.7511	7.8558	0.2291	2.9160
	48	7.6163	7.5467	7.6667	7.6099	0.0603	0.7918
0.1 M citric/citrate	1	5.7170	5.9096	5.7822	5.8030	0.0980	1.6881
buffer pH 2.5	2	6.0904	6.3096	6.1496	6.1832	0.1134	1.8343
	4	5.8519	5.8874	5.9452	5.8948	0.0471	0.7991
	8	6.3215	6.3081	6.1807	6.2701	0.0777	1.2391
	16	6.3956	6.5422	6.6400	6.5259	0.1230	1.8853
	32	6.0341	6.1200	6.1481	6.1007	0.0594	0.9741
	48	6.4533	6.4711	6.3437	6.4227	0.0690	1.0743
0.1 M citric/citrate	1	4.8385	4.8948	4.8370	4.8568	0.0329	0.6782
buffer pH 3	2	5.2400	5.3230	5.3911	5.3180	0.0757	1.4230
	4	5.0844	5.0089	5.1304	5.0746	0.0613	1.2088
	8	5.1748	5.2015	5.2681	5.2148	0.0481	0.9219
	16	5.6400	5.4444	5.4815	5.5220	0.1039	1.8811
	32	5.0859	5.2267	5.1615	5.1580	0.0704	1.3655
	48	5.3896	5.3719	5.5200	5.4272	0.0809	1.4905
0.1 M citric/citrate	1	4.4044	4.3852	4.4074	4.3990	0.0121	0.2743
buffer pH 3.5	2	4.9941	5.1956	5.1748	5.1215	0.1108	2.1639
	4	5.1644	5.2459	5.4311	5.2805	0.1367	2.5879
	8	4.6459	4.6178	4.7600	4.6746	0.0753	1.6111
	16	5.0252	5.0948	5.1289	5.0830	0.0529	1.0399
	32	4.7096	4.7319	4.6652	4.7022	0.0339	0.7219
	48	4.8059	4.8756	4.9319	4.8711	0.0631	1.2950

Table 16 Solubility determination of CD in various acid solvent systems

Solvent system	Time (hr)	n 1 (mg/ml)	n 2 (mg/ml)	n 3 (mg/ml)	Average (mg/ml)	SD	%CV
0.2 M HCl-0.1 M citric acid pH 1.5	1	5.7968	5.9194	6.2129	5.9763	0.2138	3.5781
	2	5.8484	6.0516	6.2226	6.0409	0.1873	3.1010
	4	6.4387	7.3258	7.0113	6.9253	0.4498	6.4945
	8	6.0274	5.9129	6.0597	6.0000	0.0771	1.2856
	16	6.1290	6.3258	6.4355	6.2968	0.1553	2.4659
	32	5.8968	6.2194	6.2484	6.1215	0.1952	3.1882
	48	6.6887	6.7677	6.8774	6.7780	0.0948	1.3982
0.1 M HCl-0.1 M citric acid pH 1.5	1	5.6855	5.6887	5.5581	5.6441	0.0745	1.3202
	2	5.3823	5.5710	5.5081	5.4871	0.0961	1.7511
	4	6.1613	6.6048	6.5677	6.4446	0.2461	3.8183
	8	5.7597	5.7871	5.5694	5.7054	0.1186	2.0786
	16	5.8871	5.9274	5.8645	5.8930	0.0319	0.5407
	32	5.3145	5.6145	5.6968	5.5419	0.2012	3.6305
	48	5.7823	6.0177	5.7468	5.8489	0.1473	2.5179
0.1 M HCl-0.1 M KCl pH 1.5	1	4.6919	4.5290	4.3210	4.5140	0.1859	4.1192
	2	4.6145	4.6065	4.8629	4.6946	0.1458	3.1055
	4	5.0984	5.1855	5.4000	5.2280	0.1552	2.9692
	8	4.6161	4.3742	4.7065	4.5656	0.1718	3.7629
	16	4.6984	4.8177	4.9758	4.8306	0.1392	2.8808
	32	4.4129	5.2355	4.7694	4.8059	0.4125	8.5833
	48	5.0048	5.2145	5.1032	5.1075	0.1049	2.0539
0.1 M citric acid pH 2	1	3.0492	3.1403	3.1710	3.1202	0.0633	2.0301
	2	3.1532	3.1411	3.2444	3.1796	0.0564	1.7748
	4	3.0685	3.1202	3.0726	3.0871	0.0287	0.9299
	8	2.8806	2.8008	2.8105	2.8306	0.0436	1.5393
	16	3.0234	3.1395	3.1879	3.1169	0.0846	2.7126
	32	2.9661	2.9573	2.9605	2.9613	0.0045	0.1516
	48	3.0573	3.2516	3.1694	3.1594	0.0976	3.0879

Table 16 Solubility determination of CD in various acid solvent systems**,** (Continued)

Solvent system	Time (hr)	n 1 (mg/ml)	n 2 (mg/ml)	n 3 (mg/ml)	Average (mg/ml)	SD	%CV
0.1 M citric/citrate buffer pH 2.5	1	1.8839	1.8511	1.9156	1.8835	0.0323	1.7127
	2	1.9247	1.9608	2.0543	1.9799	0.0669	3.3779
	4	2.0688	2.2054	2.0677	2.1140	0.0792	3.7443
	8	1.9430	1.9823	1.9903	1.9719	0.0253	1.2836
	16	2.1946	2.2323	2.2645	2.2305	0.0350	1.5683
	32	2.0097	2.1070	2.0919	2.0695	0.0524	2.5311
	48	2.2263	2.2995	2.2801	2.2687	0.0379	1.6699
0.1 M citric/citrate buffer pH 3	1	1.5446	1.5769	2.0070	1.7095	0.2581	15.1003
	2	1.6032	1.6575	1.6285	1.6297	0.0272	1.6673
	4	1.6968	1.7247	1.6763	1.6993	0.0243	1.4295
	8	1.6930	1.6871	1.6269	1.6690	0.0366	2.1925
	16	1.7957	1.8199	1.8731	1.8296	0.0396	2.1648
	32	1.7602	1.7312	1.7091	1.7335	0.0256	1.4778
	48	1.9355	1.9629	1.9129	1.9371	0.0250	1.2926
0.1 M citric/citrate buffer pH 3.5	1	1.4108	1.4274	1.4263	1.4215	0.0093	0.6562
	2	1.5930	1.4935	1.4957	1.5274	0.0568	3.7196
	4	1.5882	1.6258	1.6194	1.6111	0.0201	1.2492
	8	1.5247	1.5543	1.5758	1.5516	0.0256	1.6527
	16	1.7059	1.7349	1.7075	1.7161	0.0163	0.9508
	32	1.6081	1.6145	1.6188	1.6138	0.0054	0.3354
	48	1.7446	1.7640	1.8032	1.7706	0.0299	1.6863

Effect of cosolvents on the solubility of LD and CD in acid solvent system

Table 17 Effect of PEG400 on the solubility of LD

Solvent system	Time (hr)	n 1 (mg/ml)	n 2 (mg/ml)	n 3 (mg/ml)	Average (mg/ml)	SD	%CV
0.1 M HCl-0.1 M	1	10.1284	10.2735	10.0630	10.1549	0.1077	1.0608
Citric acid pH1.5	2	10.3488	10.0864	9.9988	10.1447	0.1821	1.7952
	4	10.3290	10.4296	10.4475	10.4021	0.0639	0.6142
	8	10.2673	10.3025	10.2796	10.2831	0.0179	0.1736
	16	11.4228	11.9377	12.0074	11.7893	0.3193	2.7082
	32	11.0457	10.9358	10.8963	10.9593	0.0774	0.7063
	48	11.1302	11.1611	11.0179	11.1031	0.0754	0.6788
0.1 M HCl-0.1 M	1	9.7886	9.6020	10.0128	9.8012	0.2057	2.0989
Citric acid pH1.5+	2	10.0909	10.4405	10.4790	10.3368	0.2138	2.0688
PEG400 5%v/v	4	10.6202	9.9960	10.2380	10.2848	0.3147	3.0600
	8	10.3644	10.4040	10.6104	10.4596	0.1321	1.2626
	16	11.6632	12.0573	11.2296	11.6500	0.4140	3.5535
	32	11.3402	11.5615	11.1595	11.3537	0.2013	1.7732
	48	10.7289	10.6380	10.2686	10.5452	0.2438	2.3116
0.1 M HCl-0.1 M	1	9.8874	9.8973	10.0948	9.9598	0.1170	1.1747
Citric acid pH1.5+	2	10.9521	10.5244	10.1926	10.5564	0.3808	3.6069
PEG400 10%v/v	4	10.3615	10.3407	10.3309	10.3444	0.0156	0.1511
	8	10.4277	10.3546	10.5067	10.4296	0.0761	0.7294
	16	11.8054	11.7195	11.2336	11.5862	0.3084	2.6615
	32	11.1852	10.2252	10.9975	10.8026	0.5088	4.7101
	48	10.5689	10.7003	10.5156	10.5949	0.0951	0.8972
0.1 M HCl-0.1 M	1	9.4370	9.4914	8.8444	9.2576	0.3588	3.8762
Citric acid pH1.5+	2	9.6454	9.2701	10.0484	9.6547	0.3892	4.0314
PEG400 20%v/v	4	9.9684	10.0869	9.7314	9.9289	0.1810	1.8234
	8	9.9664	10.2351	10.1442	10.1152	0.1366	1.3509
	16	11.5398	11.6632	11.2612	11.4881	0.2059	1.7924
	32	11.0667	10.6163	10.3072	10.6634	0.3819	3.5818
	48	10.5472	10.6815	9.8923	10.3737	0.4222	4.0700

Table 18 Effect of PEG400 on the solubility of CD

Solvent system	Time (hr)	n 1 (mg/ml)	n 2 (mg/ml)	n 3 (mg/ml)	Average (mg/ml)	SD	%CV
0.1 M HCl-0.1 M	1	5.1108	5.0995	5.1280	5.1127	0.0143	0.2807
Citric acid pH1.5	2	5.1091	5.1032	5.1812	5.1312	0.0434	0.8459
	4	5.2425	5.2091	5.2527	5.2348	0.0228	0.4351
	8	5.7892	5.7935	5.8462	5.8097	0.0317	0.5462
	16	5.7656	5.6570	5.7457	5.7228	0.0578	1.0104
	32	6.4688	6.3183	6.4022	6.3964	0.0754	1.1793
	48	6.4414	6.6118	6.6925	6.5819	0.1282	1.9475
0.1 M HCl-0.1 M	1	5.0613	5.1204	4.5441	4.9086	0.3171	6.4593
Citric acid pH1.5+	2	4.9586	4.9312	5.0731	4.9876	0.0753	1.5095
PEG400 5%v/v	4	5.2081	5.1602	5.3054	5.2246	0.0740	1.4158
	8	5.1559	5.1903	5.1758	5.1740	0.0173	0.3339
	16	5.8435	6.0038	5.9527	5.9333	0.0818	1.3794
	32	6.3505	6.3054	6.1538	6.2699	0.1031	1.6440
	48	5.3403	5.3505	6.2000	5.6303	0.4934	8.7635
0.1 M HCl-0.1 M	1	5.2785	5.2672	5.1398	5.2285	0.0770	1.4733
Citric acid pH1.5+	2	5.2161	5.2118	5.0263	5.1514	0.1084	2.1033
PEG400 10%v/v	4	5.3720	5.2941	5.2833	5.3165	0.0484	0.9106
	8	5.3489	5.3726	5.2629	5.3281	0.0577	1.0833
	16	6.1839	6.1328	6.1699	6.1622	0.0264	0.4283
	32	6.3059	6.2602	6.1823	6.2495	0.0625	1.0005
	48	5.5973	5.6935	5.6382	5.6430	0.0483	0.8559
0.1 M HCl-0.1 M	1	5.2301	5.1849	5.6108	5.3419	0.2339	4.3785
Citric acid pH1.5+	2	5.2796	5.4484	5.4575	5.3952	0.1002	1.8574
PEG400 20%v/v	4	5.4737	5.4780	5.8962	5.6159	0.2427	4.3224
	8	5.6129	5.3753	5.7355	5.5746	0.1831	3.2854
	16	5.9070	6.0403	6.3473	6.0982	0.2258	3.7027
	32	5.9914	6.6414	6.2522	6.2950	0.3271	5.1964
	48	5.4909	5.4731	5.4860	5.4833	0.0092	0.1673

Table 19 Effect of ethanol on the solubility of LD

Solvent system	Time (hr)	n 1 (mg/ml)	n 2 (mg/ml)	n 3 (mg/ml)	Average (mg/ml)	SD	%CV
0.1 M HCl-0.1 M	1	10.1284	10.2735	10.0630	10.1549	0.1077	1.0608
Citric acid pH1.5	2	10.3488	10.0864	9.9988	10.1447	0.1821	1.7952
	4	10.3290	10.4296	10.4475	10.4021	0.0639	0.6142
	8	10.2673	10.3025	10.2796	10.2831	0.0179	0.1736
	16	11.4228	11.9377	12.0074	11.7893	0.3193	2.7082
	32	11.0457	10.9358	10.8963	10.9593	0.0774	0.7063
	48	11.1302	11.1611	11.0179	11.1031	0.0754	0.6788
0.1 M HCl-0.1 M	1	8.7388	8.8395	8.6440	8.7407	0.0978	1.1188
Citric acid pH1.5+	2	9.3007	9.3946	9.4114	9.3689	0.0596	0.6363
ethanol 5%v/v	4	9.4183	9.5625	9.4074	9.4627	0.0866	0.9147
	8	9.3975	9.5091	9.6494	9.5187	0.1262	1.3258
	16	10.9007	11.1872	10.9106	10.9995	0.1626	1.4781
	32	10.5738	10.8504	10.6647	10.6963	0.1410	1.3178
	48	9.3343	9.2543	8.8968	9.1618	0.2330	2.5429
0.1 M HCl-0.1 M	1	8.5748	8.5995	8.3180	8.4974	0.1559	1.8344
Citric acid pH1.5+	2	8.8099	9.4637	8.9798	9.0844	0.3393	3.7344
ethanol 10%v/v	4	8.9709	8.9235	9.1684	9.0209	0.1299	1.4401
	8	9.1635	8.8385	9.0765	9.0262	0.1682	1.8637
	16	10.7763	9.9338	9.2487	9.9863	0.7652	7.6620
	32	9.9368	9.7284	9.3649	9.6767	0.2894	2.9908
	48	8.8770	8.9215	8.9521	8.9169	0.0377	0.4233
0.1 M HCl-0.1 M	1	7.2998	8.7467	7.3827	7.8097	0.8125	10.4035
Citric acid pH1.5+	2	7.8785	8.0889	7.6425	7.8700	0.2233	2.8378
ethanol 20%v/v	4	7.8617	7.9477	7.8795	7.8963	0.0454	0.5744
	8	8.1719	8.2459	8.0514	8.1564	0.0982	1.2040
	16	9.1398	9.2948	8.8059	9.0802	0.2498	2.7514
	32	8.5857	8.5017	8.4879	8.5251	0.0529	0.6207
	48	7.7294	7.2326	7.5575	7.5065	0.2523	3.3610

Table 20 Effect of ethanol on the solubility of CD

Solvent system	Time (hr)	n 1 (mg/ml)	n 2 (mg/ml)	n 3 (mg/ml)	Average (mg/ml)	SD	%CV
0.1 M HCl-0.1 M	1	5.1108	5.0995	5.1280	5.1127	0.0143	0.2807
Citric acid pH1.5	2	5.1091	5.1032	5.1812	5.1312	0.0434	0.8459
	4	5.2425	5.2091	5.2527	5.2348	0.0228	0.4351
	8	5.7892	5.7935	5.8462	5.8097	0.0317	0.5462
	16	5.7656	5.6570	5.7457	5.7228	0.0578	1.0104
	32	6.4688	6.3183	6.4022	6.3964	0.0754	1.1793
	48	6.4414	6.6118	6.6925	6.5819	0.1282	1.9475
0.1 M HCl-0.1 M	1	4.5231	4.5151	4.4667	4.5016	0.0305	0.6782
Citric acid pH1.5+	2	4.5828	4.5774	4.6145	4.5916	0.0200	0.4366
ethanol 5%v/v	4	4.8091	4.7704	4.7145	4.7647	0.0476	0.9984
	8	4.7559	4.7005	4.7597	4.7387	0.0331	0.6987
	16	5.0839	5.1543	5.1306	5.1229	0.0358	0.6996
	32	5.4812	5.7306	5.7489	5.6536	0.1496	2.6458
	48	5.0489	4.9183	4.9382	4.9685	0.0704	1.4168
0.1 M HCl-0.1 M	1	4.4134	4.3059	4.1925	4.3039	0.1105	2.5673
Citric acid pH1.5+	2	4.4720	4.4559	4.4414	4.4565	0.0153	0.3440
ethanol 10%v/v	4	4.5871	4.4871	4.6898	4.5880	0.1013	2.2090
	8	4.8172	4.6962	4.6409	4.7181	0.0902	1.9114
	16	4.9667	5.2914	5.0280	5.0953	0.1725	3.3861
	32	5.7081	5.3651	5.4414	5.5048	0.1801	3.2715
	48	5.1054	4.8323	4.8054	4.9143	0.1660	3.3777
0.1 M HCl-0.1 M	1	4.0651	4.0629	4.1140	4.0806	0.0289	0.7079
Citric acid pH1.5+	2	4.1699	4.1323	4.1581	4.1534	0.0192	0.4634
ethanol 20%v/v	4	4.2274	4.4371	4.2968	4.3204	0.1068	2.4725
	8	4.2527	4.2161	4.3113	4.2600	0.0480	1.1269
	16	4.8323	4.9258	4.8747	4.8776	0.0468	0.9603
	32	5.3376	5.3129	5.3280	5.3262	0.0125	0.2340
	48	4.2801	4.1876	4.1667	4.2115	0.0604	1.4332

Table 21 Effect of glycerin on the solubility of LD

Solvent system	Time (hr)	n 1 (mg/ml)	n 2 (mg/ml)	n 3 (mg/ml)	Average (mg/ml)	SD	%CV
0.1 M HCl-0.1 M	1	10.1284	10.2735	10.0630	10.1549	0.1077	1.0608
Citric acid pH1.5	2	10.3488	10.0864	9.9988	10.1447	0.1821	1.7952
	4	10.3290	10.4296	10.4475	10.4021	0.0639	0.6142
	8	10.2673	10.3025	10.2796	10.2831	0.0179	0.1736
	16	11.4228	11.9377	12.0074	11.7893	0.3193	2.7082
	32	11.0457	10.9358	10.8963	10.9593	0.0774	0.7063
	48	11.1302	11.1611	11.0179	11.1031	0.0754	0.6788
0.1 M HCl-0.1 M	1	9.8311	10.2558	9.7086	9.9319	0.2872	2.8912
Citric acid pH1.5+	2	9.7333	9.7570	9.5733	9.6879	0.0999	1.0314
glycerin 5%v/v	4	9.6810	9.9012	9.8598	9.8140	0.1170	1.1926
	8	10.2153	9.9625	9.7383	9.9720	0.2387	2.3933
	16	9.8765	9.5072	9.3541	9.5793	0.2686	2.8039
	32	10.2973	9.9269	9.7432	9.9891	0.2822	2.8254
	48	10.8504	10.3674	10.4721	10.5633	0.2541	2.4052
0.1 M HCl-0.1 M	1	10.2153	9.2326	9.6879	9.7119	0.4918	5.0639
Citric acid pH1.5+	2	10.0099	9.4440	9.3205	9.5914	0.3676	3.8325
glycerin 10%v/v	4	10.0435	9.6257	9.3096	9.6596	0.3681	3.8106
	8	9.6731	9.5447	9.9891	9.7356	0.2287	2.3494
	16	8.8790	9.3817	9.2593	9.1733	0.2621	2.8577
	32	10.1975	10.2064	9.8746	10.0928	0.1891	1.8734
	48	10.2825	10.1185	10.5768	10.3259	0.2322	2.2488
0.1 M HCl-0.1 M	1	9.0588	8.9728	8.9027	8.9781	0.0782	0.8705
Citric acid pH1.5+	2	9.3402	9.0173	9.3254	9.2277	0.1823	1.9760
glycerin 20%v/v	4	8.9956	9.4864	9.4825	9.3215	0.2823	3.0281
	8	9.1941	9.1960	9.8005	9.3969	0.3495	3.7198
	16	8.9185	9.3867	9.5289	9.2780	0.3194	3.4421
	32	9.7412	9.7254	9.2474	9.5714	0.2807	2.9323
	48	10.2854	9.8696	9.4420	9.8657	0.4217	4.2748

Table 22 Effect of glycerin on the solubility of CD

Solvent system	Time (hr)	n 1 (mg/ml)	n 2 (mg/ml)	n 3 (mg/ml)	Average (mg/ml)	SD	%CV
0.1 M HCl-0.1 M	1	5.1108	5.0995	5.1280	5.1127	0.0143	0.2807
Citric acid pH1.5	2	5.1091	5.1032	5.1812	5.1312	0.0434	0.8459
	4	5.2425	5.2091	5.2527	5.2348	0.0228	0.4351
	8	5.7892	5.7935	5.8462	5.8097	0.0317	0.5462
	16	5.7656	5.6570	5.7457	5.7228	0.0578	1.0104
	32	6.4688	6.3183	6.4022	6.3964	0.0754	1.1793
	48	6.4414	6.6118	6.6925	6.5819	0.1282	1.9475
0.1 M HCl-0.1 M	1	4.8054	4.8554	4.5570	4.7392	0.1598	3.3720
Citric acid pH1.5+	2	4.7575	4.9027	4.7677	4.8093	0.0810	1.6847
glycerin 5%v/v	4	5.0333	5.2962	5.0419	5.1238	0.1494	2.9151
	8	5.2398	5.3065	5.3296	5.2919	0.0466	0.8810
	16	5.7344	5.8425	5.7758	5.7842	0.0545	0.9426
	32	5.8796	5.8323	5.9570	5.8896	0.0630	1.0691
	48	6.1812	6.2124	6.1618	6.1851	0.0255	0.4123
0.1 M HCl-0.1 M	1	4.7274	5.1129	4.7323	4.8575	0.2212	4.5533
Citric acid pH1.5+	2	5.1210	5.0704	4.9199	5.0371	0.1046	2.0766
glycerin 10%v/v	4	5.2048	5.4532	5.2839	5.3140	0.1269	2.3881
	8	5.6022	5.5468	5.5000	5.5496	0.0511	0.9214
	16	6.1763	6.5140	6.3048	6.3317	0.1704	2.6914
	32	6.4220	6.3478	6.2613	6.3437	0.0805	1.2683
	48	6.7500	6.7048	6.7495	6.7348	0.0259	0.3849
0.1 M HCl-0.1 M	1	5.3661	5.6737	5.9134	5.6511	0.2744	4.8549
Citric acid pH1.5+	2	5.5516	5.5941	6.0360	5.7272	0.2683	4.6838
glycerin 20%v/v	4	5.8065	5.8430	6.3930	6.0142	0.3286	5.4639
	8	6.1081	6.2215	6.6199	6.3165	0.2688	4.2557
	16	6.5624	6.6634	6.7398	6.6552	0.0890	1.3372
	32	6.7231	7.1629	7.1554	7.0138	0.2518	3.5896
	48	6.3156	7.5989	6.4817	6.7987	0.6979	10.2657

Table 23 Effect of Tween80 on the solubility of LD

Solvent system	Time (hr)	n 1 (mg/ml)	n 2 (mg/ml)	n 3 (mg/ml)	Average (mg/ml)	SD	%CV
0.1 M HCl-0.1 M	1	10.1284	10.2735	10.0630	10.1549	0.1077	1.0608
Citric acid pH1.5	2	10.3488	10.0864	9.9988	10.1447	0.1821	1.7952
	4	10.3290	10.4296	10.4475	10.4021	0.0639	0.6142
	8	10.2673	10.3025	10.2796	10.2831	0.0179	0.1736
	16	11.4228	11.9377	12.0074	11.7893	0.3193	2.7082
	32	11.0457	10.9358	10.8963	10.9593	0.0774	0.7063
	48	11.1302	11.1611	11.0179	11.1031	0.0754	0.6788
0.1 M HCl-0.1 M	1	9.8272	10.1610	10.0760	10.0214	0.1735	1.7312
Citric acid pH1.5+	2	9.7600	10.3072	9.7333	9.9335	0.3239	3.2604
Tween 5%v/v	4	9.9891	10.0138	10.0879	10.0303	0.0514	0.5124
	8	10.1926	10.1620	9.9012	10.0853	0.1601	1.5876
	16	10.4217	10.1719	9.7689	10.1208	0.3294	3.2547
	32	10.5363	10.2765	10.2015	10.3381	0.1757	1.6995
	48	10.8454	10.3783	10.5047	10.5761	0.2416	2.2847
0.1 M HCl-0.1 M	1	9.8588	9.5872	8.9017	9.4492	0.4932	5.2195
Citric acid pH1.5+	2	10.1383	10.1343	9.5575	9.9434	0.3342	3.3606
Tween 10%v/v	4	10.3486	9.1990	9.7936	9.7804	0.5749	5.8784
	8	9.8775	9.5674	9.9921	9.8123	0.2197	2.2392
	16	10.5264	10.6726	10.4909	10.5633	0.0963	0.9118
	32	9.9309	10.1294	9.9605	10.0069	0.1071	1.0702
	48	10.8780	10.6015	10.4849	10.6548	0.2019	1.8949
0.1 M HCl-0.1 M	1	9.9852	9.3126	9.8440	9.7139	0.3547	3.6510
Citric acid pH1.5+	2	9.6721	10.0138	10.2756	9.9872	0.3026	3.0300
Tween 20%v/v	4	10.0751	9.7620	9.7314	9.8561	0.1902	1.9299
	8	9.9299	10.1946	9.6879	9.9374	0.2534	2.5501
	16	10.6716	10.1363	10.0454	10.2844	0.3384	3.2900
	32	9.4854	10.0602	9.7758	9.7738	0.2874	2.9406
	48	10.0642	10.4178	10.5402	10.3407	0.2472	2.3905

Table 24 Effect of Tween80 on the solubility of CD

Solvent system	Time (hr)	n 1 (mg/ml)	n 2 (mg/ml)	n 3 (mg/ml)	Average (mg/ml)	SD	%CV
0.1 M HCl-0.1 M	1	5.1108	5.0995	5.1280	5.1127	0.0143	0.2807
Citric acid pH1.5	2	5.1091	5.1032	5.1812	5.1312	0.0434	0.8459
	4	5.2425	5.2091	5.2527	5.2348	0.0228	0.4351
	8	5.7892	5.7935	5.8462	5.8097	0.0317	0.5462
	16	5.7656	5.6570	5.7457	5.7228	0.0578	1.0104
	32	6.4688	6.3183	6.4022	6.3964	0.0754	1.1793
	48	6.4414	6.6118	6.6925	6.5819	0.1282	1.9475
0.1 M HCl-0.1 M	1	4.9349	4.9887	4.9495	4.9577	0.0278	0.5610
Citric acid pH1.5+	2	4.8618	4.8855	4.9505	4.8993	0.0459	0.9376
Tween 5%v/v	4	5.1258	5.2118	5.0935	5.1437	0.0611	1.1887
	8	5.1392	5.2473	5.0710	5.1525	0.0889	1.7257
	16	5.4624	5.3763	5.4849	5.4412	0.0573	1.0532
	32	5.6258	5.7263	5.6183	5.6568	0.0603	1.0666
	48	5.9253	5.9742	5.8538	5.9177	0.0606	1.0235
0.1 M HCl-0.1 M	1	5.0930	5.1876	5.0608	5.1138	0.0659	1.2896
Citric acid pH1.5+	2	5.2559	5.2339	5.3462	5.2787	0.0595	1.1279
Tween 10%v/v	4	5.3957	5.4500	5.4919	5.4459	0.0483	0.8860
	8	5.7398	5.5651	5.6086	5.6378	0.0910	1.6133
	16	6.0274	6.1452	5.9199	6.0308	0.1127	1.8683
	32	6.1672	5.9823	6.0301	6.0599	0.0960	1.5841
	48	6.3871	6.3065	7.2419	6.6452	0.5184	7.8010
0.1 M HCl-0.1 M	1	5.7731	5.8672	6.1731	5.9378	0.2091	3.5222
Citric acid pH1.5+	2	5.6914	5.5435	5.7226	5.6525	0.0956	1.6920
Tween 20%v/v	4	5.6226	6.0989	6.0468	5.9228	0.2613	4.4112
	8	5.8452	5.5801	6.1527	5.8593	0.2866	4.8905
	16	6.4065	6.2656	6.4710	6.3810	0.1050	1.6459
	32	6.4065	6.4419	6.3473	6.3986	0.0478	0.7471
	48	6.7075	6.9785	7.1113	6.9324	0.2058	2.9684

Table 25 Effect of pullulan polymer on the solubility of LD

Solvent system	Time (hr)	n 1 (mg/ml)	n 2 (mg/ml)	n 3 (mg/ml)	Average (mg/ml)	SD	%CV
0.1 M HCl-0.1 M Citric acid pH1.5	1	10.1284	10.2735	10.0630	10.1549	0.1077	1.0608
	2	10.3488	10.0864	9.9988	10.1447	0.1821	1.7952
	4	10.3290	10.4296	10.4475	10.4021	0.0639	0.6142
	8	10.2673	10.3025	10.2796	10.2831	0.0179	0.1736
	16	11.4228	11.9377	12.0074	11.7893	0.3193	2.7082
	32	11.0457	10.9358	10.8963	10.9593	0.0774	0.7063
	48	11.1302	11.1611	11.0179	11.1031	0.0754	0.6788
0.1 M HCl-0.1 M Citric acid pH1.5+ pullulan 6%w/v	1	9.8284	10.0235	10.2099	10.0206	0.1908	1.9037
	2	10.2914	10.3679	10.2858	10.3150	0.0459	0.4448
	4	10.2784	10.6932	10.3870	10.4529	0.2151	2.0578
	8	10.3944	10.3599	10.1802	10.3115	0.1150	1.1152
	16	10.0512	10.2247	10.1031	10.1263	0.0890	0.8792
	32	11.1765	11.2981	11.2735	11.2494	0.0643	0.5714
	48	11.3667	10.9525	11.5932	11.3041	0.3249	2.8743
0.1 M HCl-0.1 M Citric acid pH1.5+ pullulan 8%w/v	1	10.0938	10.0488	10.2605	10.1344	0.1115	1.1005
	2	9.7975	10.2494	10.4611	10.1693	0.3390	3.3331
	4	10.2130	10.5117	10.5901	10.4383	0.1990	1.9066
	8	10.1667	9.8593	10.0846	10.0368	0.1592	1.5858
	16	9.9488	10.0173	10.1105	10.0255	0.0812	0.8097
	32	10.8920	10.9296	10.9710	10.9309	0.0395	0.3616
	48	10.7562	10.9735	11.1691	10.9663	0.2066	1.8837
0.1 M HCl-0.1 M Citric acid pH1.5+ pullulan 10%w/v	1	9.5037	10.1772	10.1759	9.9523	0.3885	3.9033
	2	10.5148	10.6395	10.6204	10.5916	0.0672	0.6340
	4	10.0519	10.6333	10.3710	10.3521	0.2912	2.8130
	8	9.5574	9.7204	9.9926	9.7568	0.2199	2.2535
	16	9.7636	10.2333	9.4815	9.8261	0.3798	3.8653
	32	11.1056	11.1593	10.3827	10.8825	0.4337	3.9850
	48	10.9537	11.3210	11.0130	11.0959	0.1972	1.7771

Table 26 Effect of pullulan polymer on the solubility of CD

Solvent system	Time (hr)	n 1 (mg/ml)	n 2 (mg/ml)	n 3 (mg/ml)	Average (mg/ml)	SD	%CV
0.1 M HCl-0.1 M	1	5.1108	5.0995	5.1280	5.1127	0.0143	0.2807
Citric acid pH1.5	2	5.1091	5.1032	5.1812	5.1312	0.0434	0.8459
	4	5.2425	5.2091	5.2527	5.2348	0.0228	0.4351
	8	5.7892	5.7935	5.8462	5.8097	0.0317	0.5462
	16	5.7656	5.6570	5.7457	5.7228	0.0578	1.0104
	32	6.4688	6.3183	6.4022	6.3964	0.0754	1.1793
	48	6.4414	6.6118	6.6925	6.5819	0.1282	1.9475
0.1 M HCl-0.1 M	1	5.1946	5.3108	5.3140	5.2731	0.0680	1.2895
Citric acid pH1.5+	2	5.2554	5.3285	5.3054	5.2964	0.0374	0.7056
pullulan 6%w/v	4	5.4489	5.1909	5.7914	5.4771	0.3013	5.5003
	8	6.2753	6.0801	6.7177	6.3577	0.3267	5.1388
	16	6.2747	6.4941	6.8194	6.5294	0.2740	4.1968
	32	6.4070	6.8118	6.7554	6.6581	0.2193	3.2932
	48	6.9242	6.9984	7.1210	7.0145	0.0994	1.4167
0.1 M HCl-0.1 M	1	5.2290	5.2608	5.2570	5.2489	0.0173	0.3302
Citric acid pH1.5+	2	5.2726	5.3188	5.3344	5.3086	0.0322	0.6057
pullulan 8%w/v	4	5.3839	5.3828	5.3995	5.3887	0.0093	0.1731
	8	6.3247	6.4505	6.5500	6.4418	0.1129	1.7525
	16	6.4247	6.7263	5.5962	6.2491	0.5852	9.3640
	32	7.0468	6.9151	7.0349	6.9989	0.0729	1.0412
	48	7.4108	6.9097	7.3758	7.2321	0.2798	3.8682
0.1 M HCl-0.1 M	1	5.2097	5.1027	5.3409	5.2177	0.1193	2.2863
Citric acid pH1.5+	2	5.4785	5.1344	5.3780	5.3303	0.1769	3.3193
pullulan 10%w/v	4	5.3747	5.5656	5.5667	5.5023	0.1105	2.0083
	8	6.2328	6.4242	6.6301	6.4290	0.1987	3.0907
	16	6.5505	6.6457	6.8269	6.6744	0.1404	2.1034
	32	6.8048	7.1839	7.2855	7.0914	0.2533	3.5721
	48	7.3661	7.4183	7.5866	7.4570	0.1152	1.5449

APPENDIX B

Physical appearance of OTF contained LD and CD under polarized light

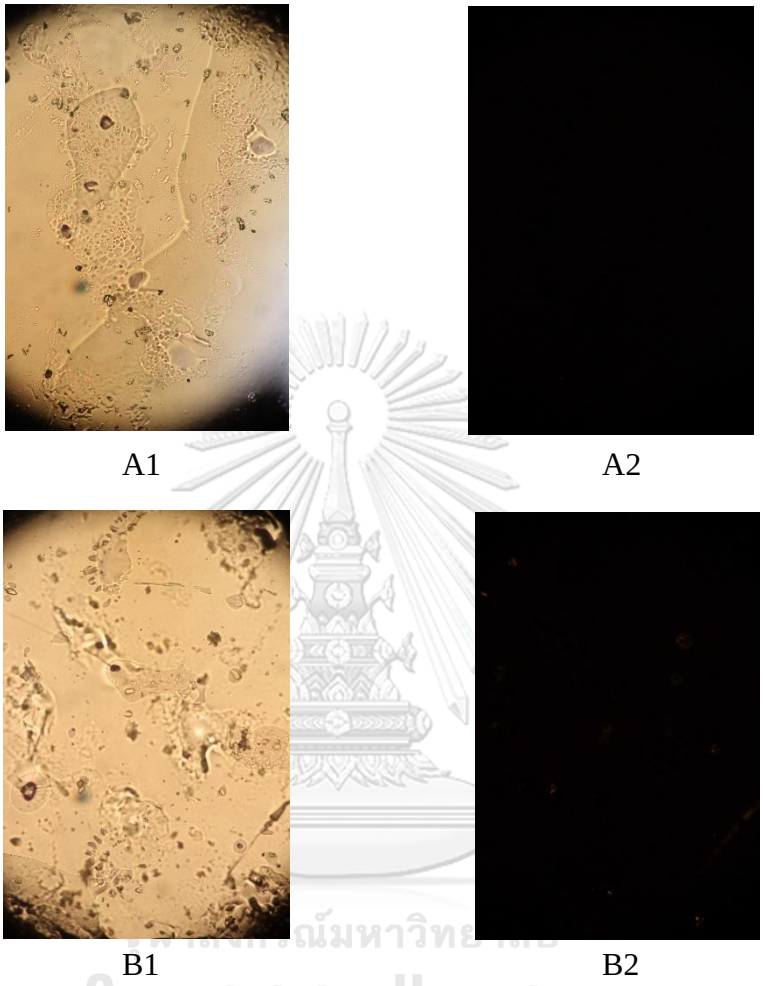
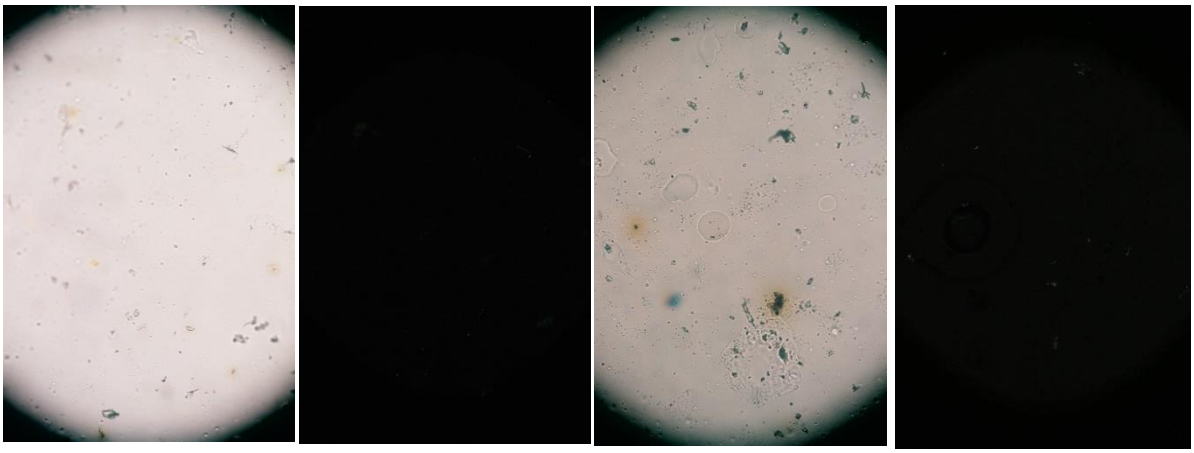
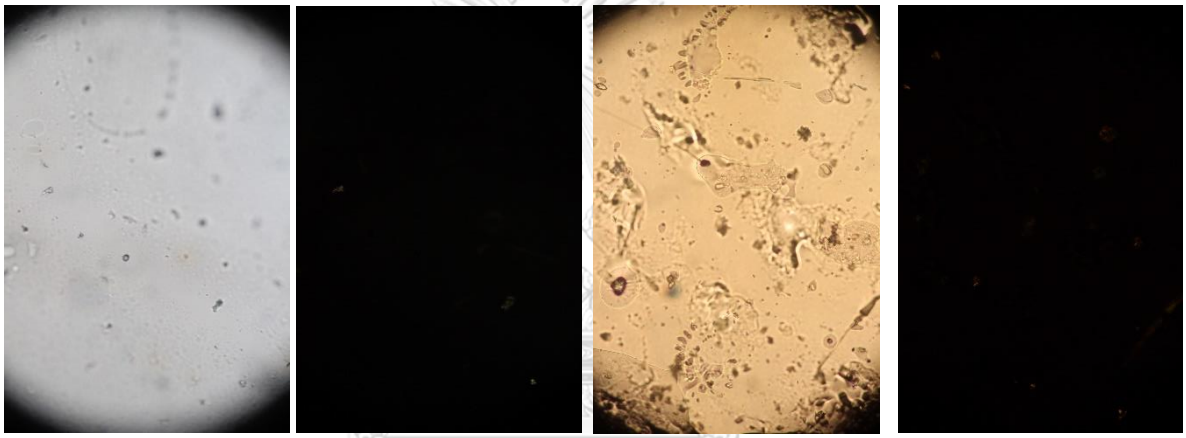


Figure 31 The photomicrograph of OTF contained LD and CD using 0.2 M hydrochloric acid/0.1 M citric acid pH 1.5 (A1- under visible light, A2- under polarized light) and 0.1 M Hydrochloric acid/0.1 M citric acid pH 1.5 (B1- under visible light, B2- under polarized light), magnification x100



A1 A2 B1 B2



C1 C2 D1 D2

Figure 32 The photomicrograph of OTF contained LD and CD using pullulan content at 6 %w/w and modify glycerin content: 1%w/w of pullulan dry weight (A1- under visible light, A2- under polarized light), 2%w/w of pullulan dry weight (B1- under visible light, B2- under polarized light), 5%w/w of pullulan dry weight (C1- under visible light, C2- under polarized light) and 10%w/w of pullulan dry weight (D1- under visible light, D2- under polarized light), magnification x100

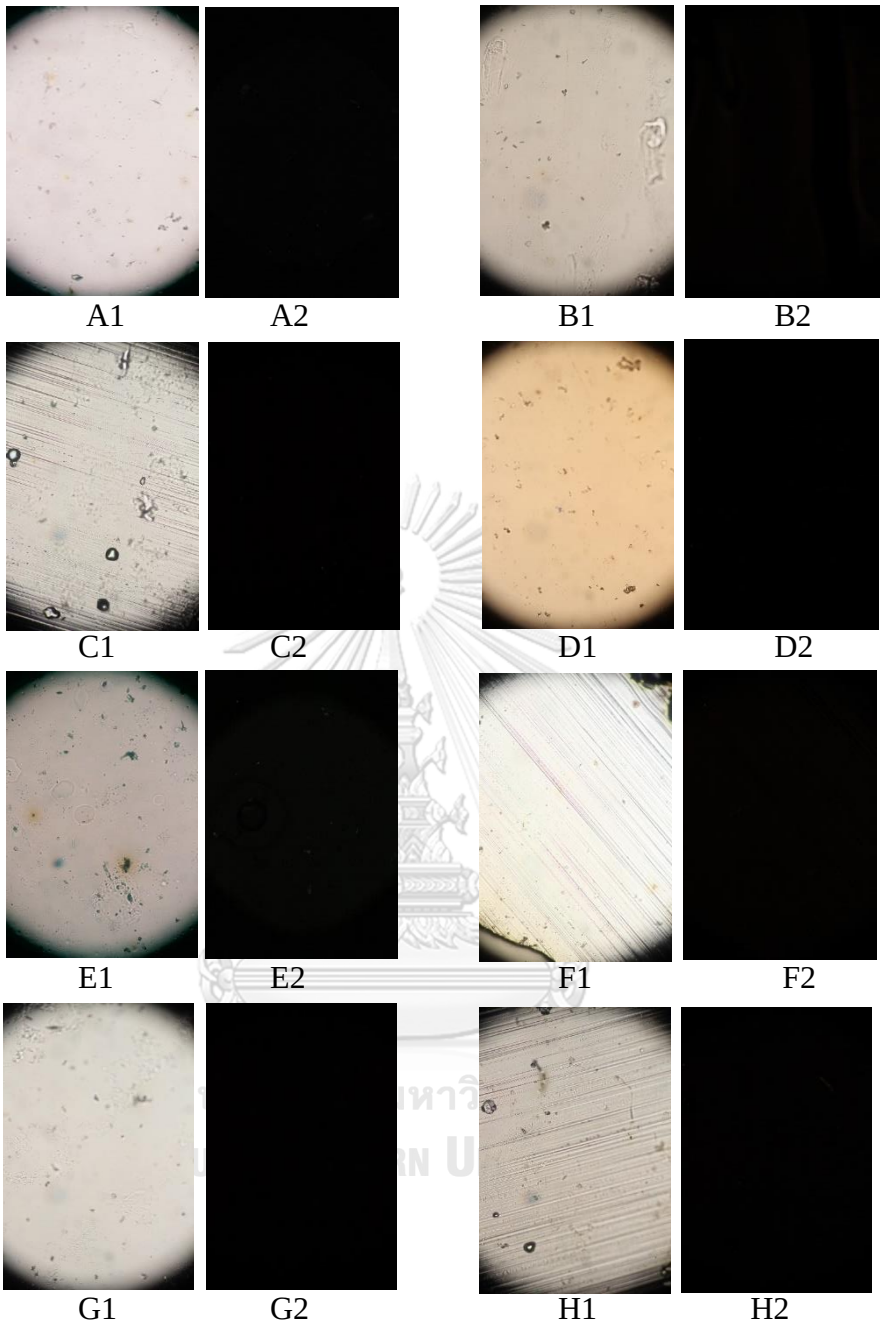


Figure 33 The photomicrograph of OTF contained LD and CD using glycerin content at 1% w/w of dry pullulan weight with pullulan content at 6% w/w (A1- under visible light, A2- under polarized light), 8% w/w (B1- under visible light, B2- under polarized light), 10% w/w (C1- under visible light, C2- under polarized light), 12% w/w (D1- under visible light, D2- under polarized light) and glycerin content at 2% w/w of dry pullulan weight with pullulan content at 6% w/w (E1- under visible light, E2- under polarized light), 8% w/w (F1- under visible light, F2- under polarized light), 10% w/w (G1- under visible light, G2- under polarized light), 12% w/w (H1- under visible light, H2- under polarized light), magnification x100

APPENDIX C

Chemical analysis of LD and CD content in OTF using validated HPLC

HPLC method was used for the determination of LD and CD content in OTF. It was modified from Jala Chandra Reddy (2013) and Sravanthi (2013) (75), (76). The analytical method validation was performed. HPLC condition was assigned as following;

Column:	4.6 mm * 150 mm * 5 µm C18
Detector:	UV detector at 282 nm
Injection volume:	20 µl
Mobile phase:	0.1 M Orthophosphoric acid/Acetonitrile (92.5:7.5 volume ratio)
Flow rate:	0.8 ml/min

Mobile phase was filtered through a cellulose acetate membrane 0.45 µm and degassed at least 30 minutes prior to use.

Part 1 Analytical validation procedure

The parameters to be considered for analytical method validation of HPLC are specificity, linearity and range, accuracy and precision.

1.1 Standard preparation

The standard stock solution was used for analytical validation test.

Standard preparation of LD: 120 mg of LD were accurately weighed in a 100 ml volumetric flask and diluted with 0.1 M Orthophosphoric acid / Acetonitrile (92.5:7.5 volume ratio) to volume at 1.20 mg/ml. Further dilutions were carried out to achieve seven serial concentrations (48, 75, 96, 120, 144, 168 and 192µg/ml).

Standard preparation of CD: 60 mg of CD were accurately weighed in a 100 ml volumetric flask and diluted with 0.1 M Orthophosphoric acid / Acetonitrile (92.5:7.5 volume ratio) to volume at 0.60 mg/ml. Further dilutions were carried out to achieve seven serial concentrations (12, 18, 24, 30, 36, 42 and 48µg/ml).

Placebo of OTF was used as spike placebo method: Ascorbic acid 0.048 g and glycerin 0.036 g were introduced together with vigorous agitation at 500 rpm for

30 minutes in beaker. 0.720 g of pullulan was later dispersed with continuous stirring until clear and homogeneous solution without any bubbles was obtained. Deionize water was added and brought to 6.000 g. The placebo should be kept at 4-8 ˚C for not more than 2 weeks.

1.2 Specificity

The specificity of each active constituent peak was determined by the retention time, tailing factor and resolution. The tailing factor and resolution indicated the system suitability of this analytical method. 120 µg/ml of LD standard preparation and 30 µg/ml of standard CD preparation and OTF placebo was used for the determining of specificity. All components should be not interfered in analytical test. The acceptance criteria should be complied with the requirement of validation of chromatographic method, USFDA (77).

1.3 Linearity

The specified seven concentrations of LD and CD standard preparation were prepared and analyzed in triplicate. Responses of peak area as a function of defined concentrations should be followed or obeyed Beer's Law. Linear regression was then determined from the coefficient of determination (R^2). R^2 should be higher than 0.999.

1.4 Accuracy

The accuracy test is the quantity of "how close the experimental value is to the true value". This test was performed at 50, 100 and 150% level of label claim. The recovery of LD and CD was assessed by spike placebo (placebo of OTF) with the amount of both drugs at three levels in triplicate. The average of %recovery, SD and percentage of coefficient of variation (%CV) were calculated to estimate the accuracy.

1.5 Precision

The precision was tested by analyzing five replicate injections of LD and CD. %CV was calculated for determine the precision. %CV that is lower than 1% is acceptable (77).

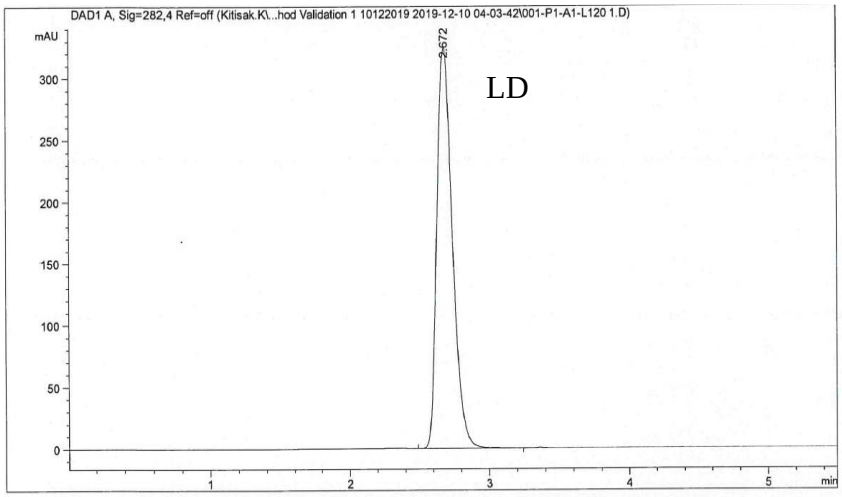
Part 2 Result of analytical validation

1.1 Specificity

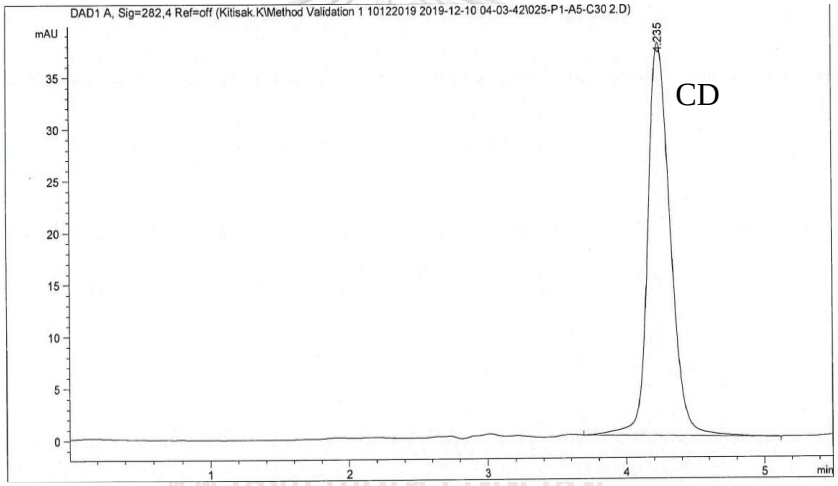
The chromatograms of LD, CD standard preparation and spike placebo were studied and shown in Figure 34. LD and CD were expressed at the retention time around 2.6 and 4.2 minutes and the spike placebo was found at 2.3 minutes. In addition, the tailing factor was reported that LD and CD peak were symmetry. The resolution between peak LD and CD showed that the column separated both drug for individual peaks (Table 27).

Table 27 Data of specificity of LD, CD and OTF analyzed by HPLC method

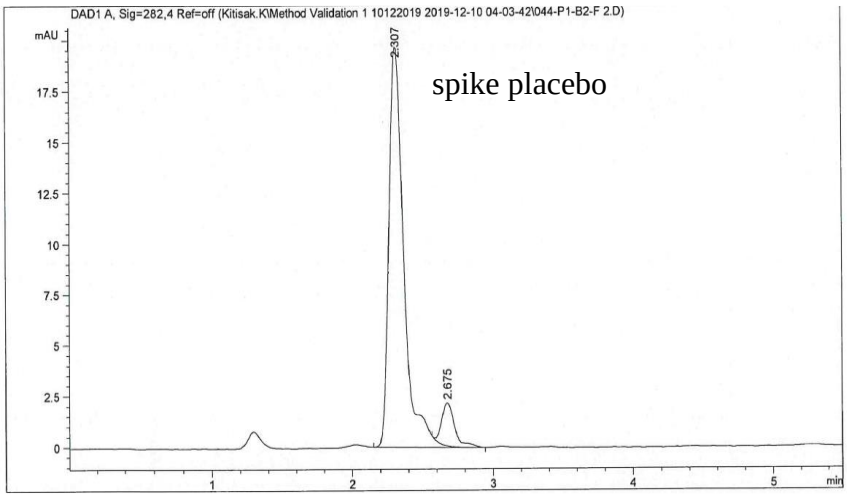
Parameter	Acceptance criteria	Result
Retention time	-	LD, 2.657
		CD, 4.370
		OTF base, 2.305
Tailing factor	Not more than 2	LD, 0.741
		CD, 0.814
Resolution	Not less than 2	12.545



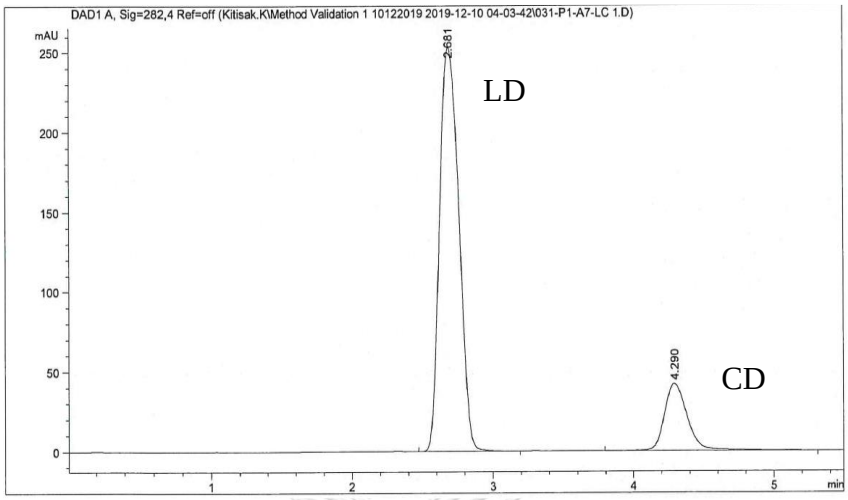
A: LD standard preparation



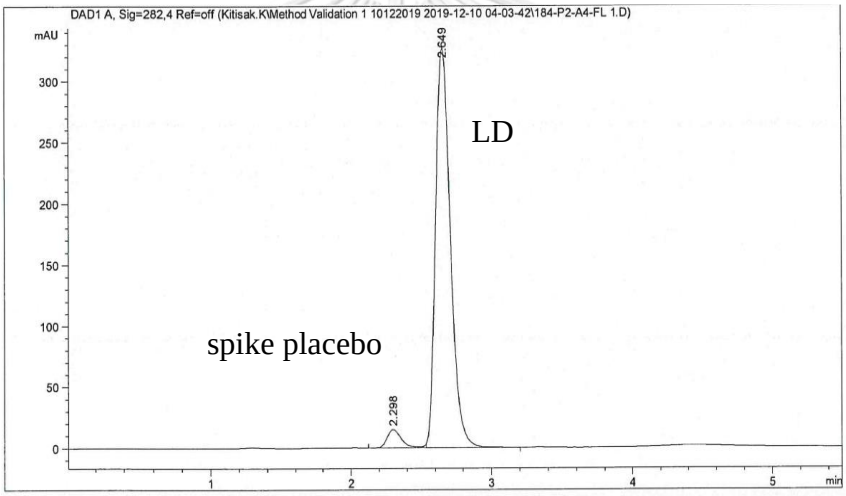
B: CD standard preparation



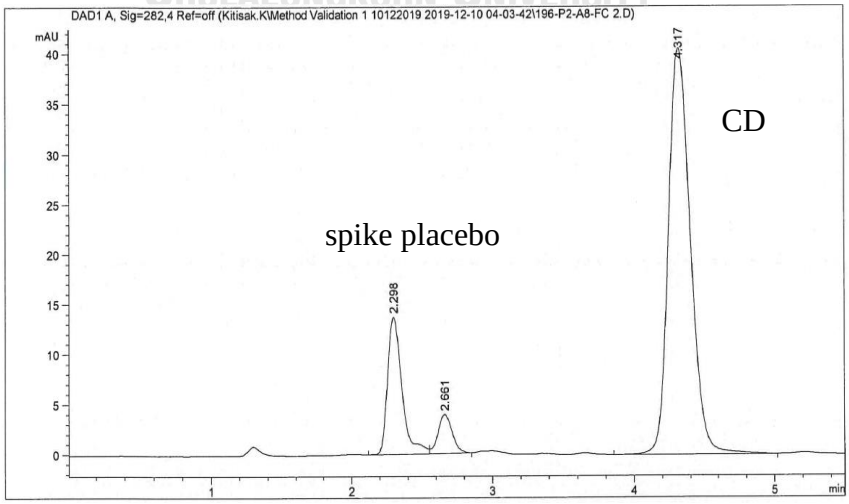
C: spike placebo



D: mixture of LD and CD standard preparation



E: LD standard preparation in spike placebo



F: CD standard preparation in spike placebo

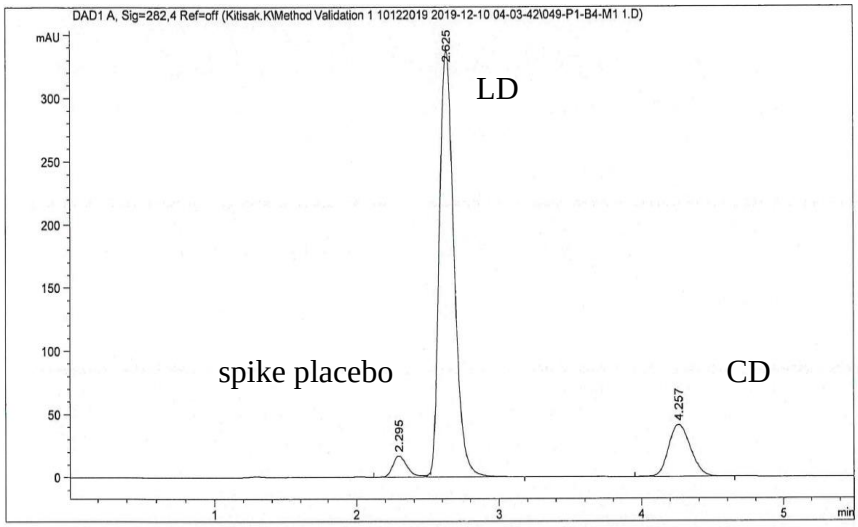
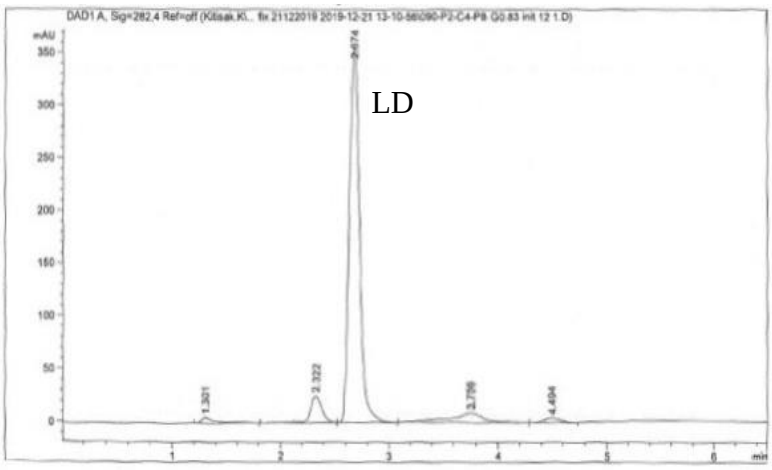
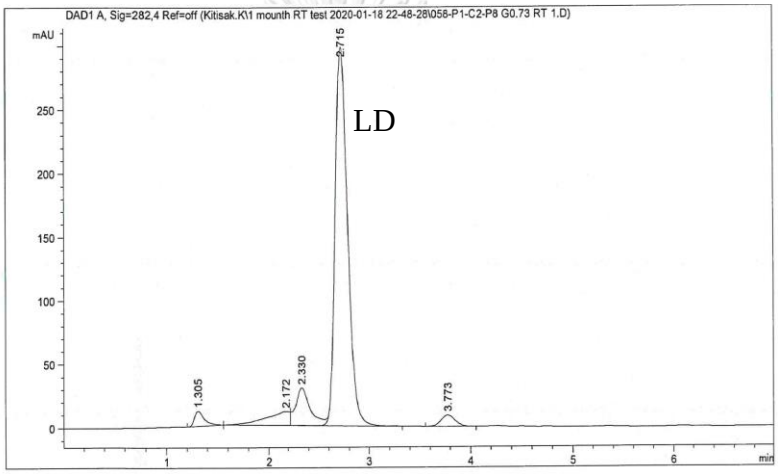


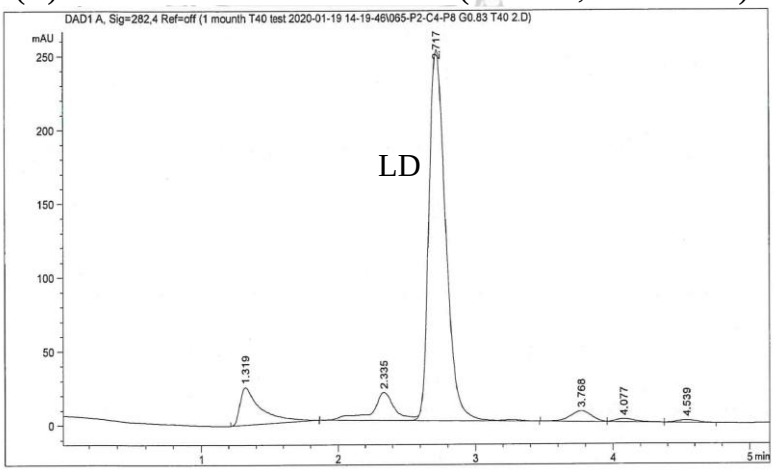
Figure 34 The HPLC chromatogram: (A) LD standard preparation, (B) CD standard preparation, (C) spike placebo, (D) mixture of LD and CD standard preparation, (E) LD standard preparation in spike placebo, (F) CD standard preparation in spike placebo, (G) LD and CD standard preparation in spike placebo



(A) initial period

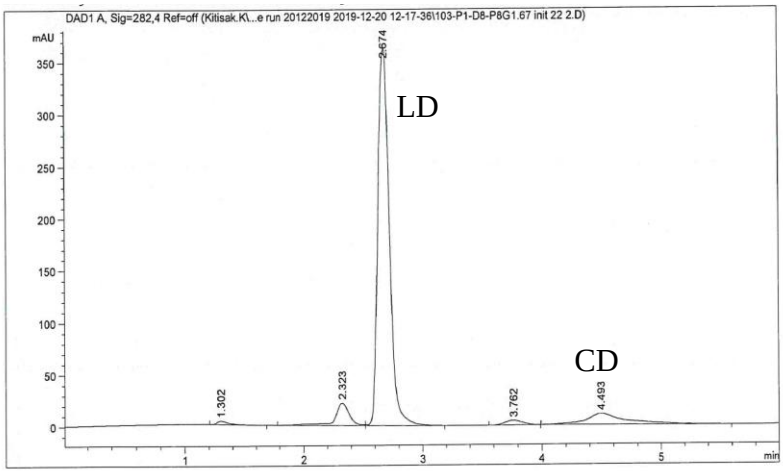


(B) 1 month at ambient condition (30±2 °C, 75±5 %RH)

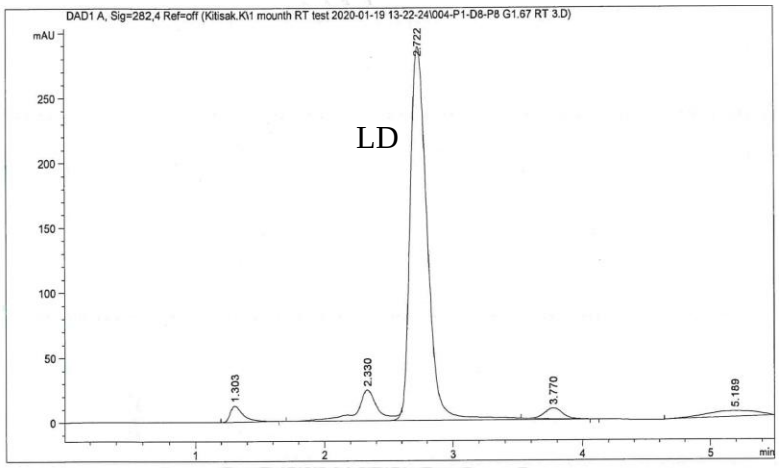


(C) 1 month at accelerated condition (40±2 °C, 75±5 %RH)

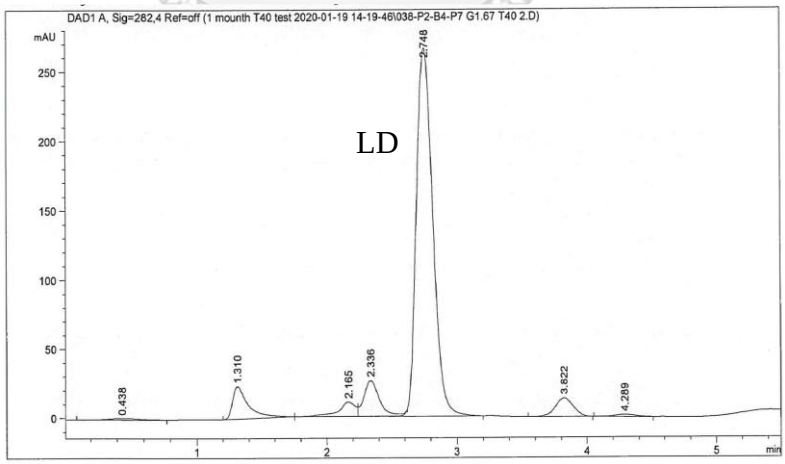
Figure 35 The HPLC chromatogram of OTF containing LD and CD fabricating with pullulan 8% w/w and glycerin 1%w/w: (A) initial period, (B) 1 month at ambient condition (30±2 °C, 75±5 %RH), (C) 1 month at accelerated condition (40±2 °C, 75±5 %RH)



(A) initial period



(B) 1 month at ambient condition (30±2 °C, 75±5 %RH)



(C) 1 month at accelerated condition (40±2 °C, 75±5 %RH)

Figure 36 The HPLC chromatogram of OTF containing LD and CD fabricating with pullulan 8%w/w and glycerin 2%w/w: (A) initial period, (B) 1 month at ambient condition (30±2 °C, 75±5 %RH), (C) 1 month at accelerated condition (40±2 °C, 75±5 %RH)

2.3 Linearity

The calibration curve was plotted between the peak area and the specified seven concentrations of LD and CD standard preparation. The results are shown in Table 29 and Figure 37 and 38. The linear regression was then determined from the R^2 and the result show that both LD and CD were higher than 0.999 (LD 0.9994, CD 0.9995). It indicated that this HPLC method was acceptable to the quantitative determination of LD and CD in the range of 48 to 192µg/ml and of 12 to 48µg/ml, respectively.

Table 28 Data of calibration curve of LD and CD standard preparation by HPLC method

drug	concentration	peak area			
		n 1	n 2	n 3	average
LD	48.32 µg/ml	1015.27	968.63	980.97	988.29
	72.47 µg/ml	1448.50	1464.40	1461.07	1457.99
	96.63 µg/ml	1911.20	1901.23	1891.63	1901.36
	120.79 µg/ml	2421.00	2421.02	2418.12	2420.05
	144.95 µg/ml	2906.07	2913.37	2913.20	2910.88
	169.11 µg/ml	3415.57	3399.70	3428.20	3414.49
	193.26 µg/ml	3839.50	3823.90	3805.57	3822.99
		R^2			0.9994
CD	12.01 µg/ml	173.10	183.17	180.93	179.07
	18.02 µg/ml	260.73	272.97	275.90	269.87
	24.03 µg/ml	355.33	373.53	357.63	362.17
	30.04 µg/ml	457.96	459.12	451.88	456.32
	36.04 µg/ml	545.50	546.57	552.30	548.12
	42.05 µg/ml	651.60	653.07	653.00	652.56
	48.06 µg/ml	743.17	720.37	730.23	731.26
		R^2			0.9995

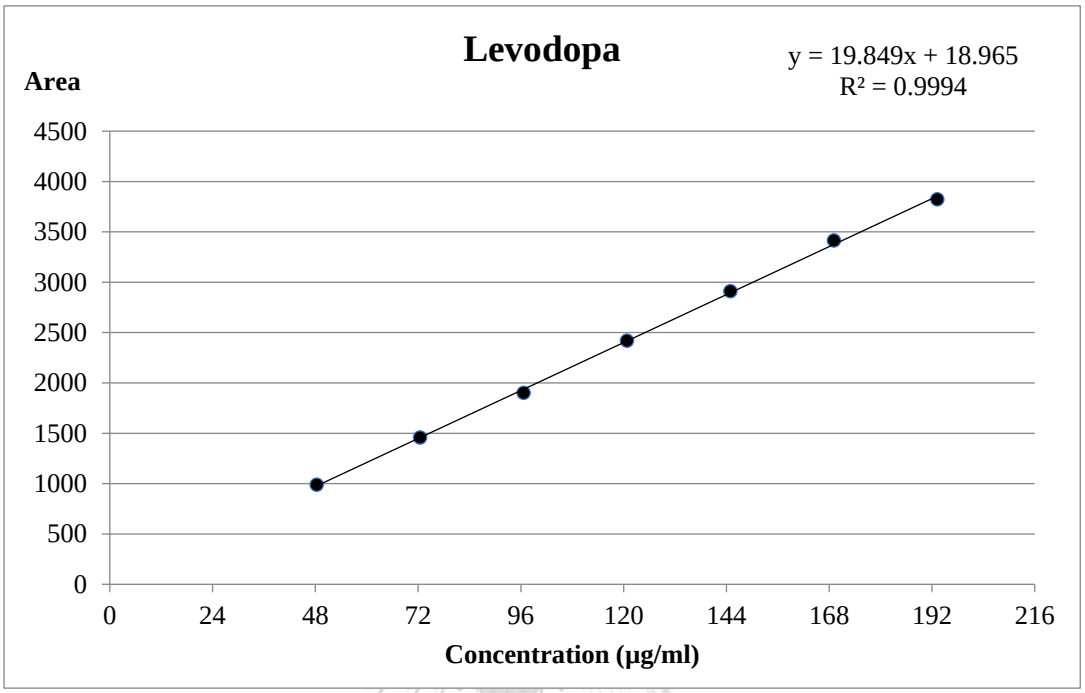


Figure 37 Standard curve of LD standard preparation by HPLC method

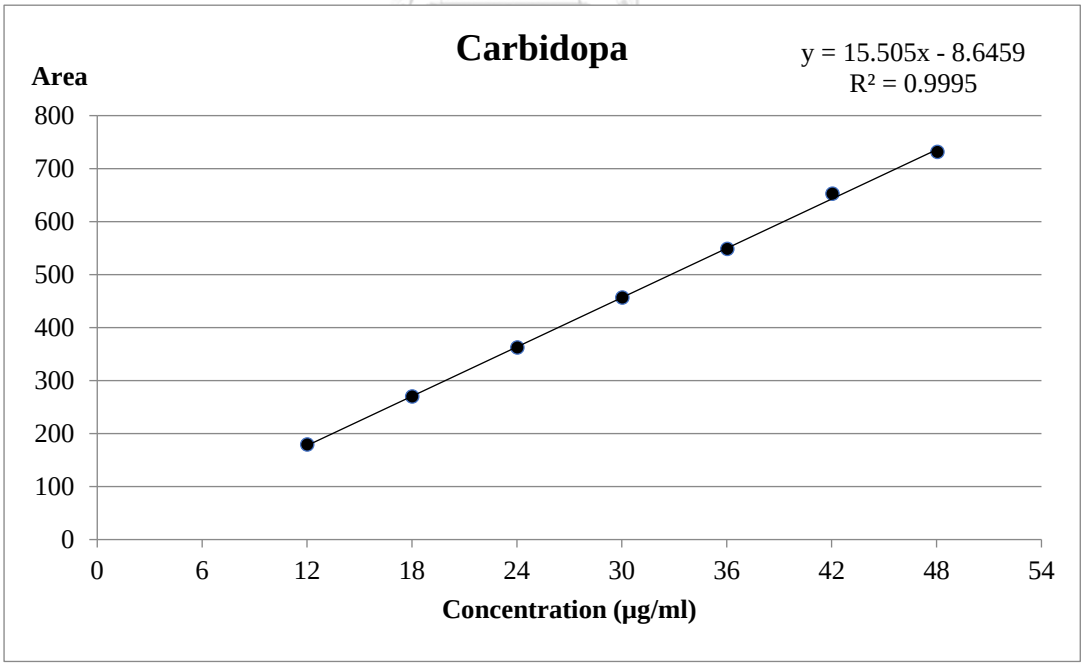


Figure 38 Standard curve of CD standard preparation by HPLC method

2.4 Accuracy

The percentage of analytical recovery of LD and CD at 50, 100 and 150% level of label claim were shown in Table 30. The average of % recovery of LD and CD were 100.829% and 99.591%, respectively. The %CV value of LD and CD were 0.86 and 0.71 %, respectively. It could be concluded that this chromatographic condition was accurate for the determination of LD and CD.

Table 29 Data of accuracy of LD and CD analyzed by HPLC method

Drug	%Concentration	Amount Added	Amount Found	%Recovery	%Mean Recovery	SD	%CV
LD	50%	6.04	6.15	101.82			
	100%	12.08	12.14	100.50	100.83	0.87	0.86
	150%	18.12	18.15	100.17			
CD	50%	1.50	1.45	96.67			
	100%	3.00	2.93	97.66	97.44	0.69	0.71
	150%	4.51	4.42	98.00			

2.5 Precision

The precision studies were carried out in term of repeatability of a homogeneous sample in 5 injections. %CV of LD and CD were 0.01% and 0.40%, respectively (Table 31). The low %CV of both drugs indicates that method is good precision.

Table 30 Data of precision of LD and CD analyzed by HPLC method

Injection	LD	CD
1	120.96	29.96
2	120.98	29.90
3	120.98	30.10
4	120.96	29.85
5	120.96	30.13
Average	120.97	29.99
SD	0.01	0.12
%CV	0.01	0.40

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