The Study of Water Solubility, Physicochemical Properties, Tyrosinase Inhibitory Activity of Glabridin-cyclodextrin Inclusion Complexes and Its Solution-type Lotion

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Sciences Program in Cosmetic Sciences (International Program)

Prince of Songkla University

2012

Thesis Title	The Study of Water Solubility, Physicochemical Properties, Tyrosinase Inhibitory Activity of Glabridin-cyclodextrin Inclusion Complexes and Its Solution-type Lotion
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ACKNOWLEDGEMENTS

This thesis could have been successfully completed with the help of many individuals. The first one, I would like to very sincerely thank to my advisor, Assoc. Prof. Nattha Kaewnopparat for kind meaningful supervision, valuable suggestions throughout the time of my study and giving me the opportunity and the knowledge in this thesis. Besides my advisor, I am very grateful to all the members of my graduated committee for their valuable advices and helpful comments.

My sincere thanks are expressed to Assoc. Prof. Dr. Sanae Kaewnopparat for his kindness and providing HPLC column equipment throughout the time of my study and I wish to extend my warmest thanks to Asst. Prof. Dr. Sarunyoo Songkro for providing the equipments used for release study.

I am extremely owed to Faculty of Pharmaceutical Sciences, Prince of Songkla University for financial support during the master degree program. I would like to give a special thanks to Department of Pharmaceutical Technology, Department of Pharmaceutical Chemistry and Pharmaceutical Laboratory Service Center for permission of laboratory facilities. I am deeply thankful to Asst. Prof. Dr. Chitchamai Ovatlarnporn and Dr. Sukanya Dej-adisai for their kindness and valuable suggestion. I also wish to thank Mrs. Thitima Chuchom, senior scientist, for her guidance in using the Fourier-transform infrared spectroscopy and all help.

I wish to thank to my dear parents and family for their love, spirits and support and thank to my friends for their aids.

Last but not the least, I would like to thank many persons who have not been mentioned by name here, I wish to thank all of making the attainment of this work.

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Academic Year	2011

ABSTRACT

Glabridin is one of the potent tyrosinase inhibitors that used as skin lightening or whitening active ingredient in cosmetic products. It is poorly soluble in water. Therefore, this research aimed to enhance the solubility of glabridin by inclusion complex with three types of cyclodextrins (CDs); beta-cyclodextrin (β-CD), gamma-cyclodextrin (γ-CD) and hydroxypropyl beta-cyclodextrin (HP-β-CD). Phase solubility of glabridin in each cyclodextrin at 32±1°C was investigated. A_L-type phase solubility diagrams were obtained in β-CD and γ-CD systems and B_L-type phase solubility diagram was obtained in HP-β-CD system indicated the formation of 1:1 stoichiometric inclusion complex. The glabridin-CDs inclusion complexes, in molar ratio of 1:1, were prepared by kneading and co-evaporation methods. The obtained inclusion complexes were characterized by differential scanning calorimetry (DSC), Fourier-transform infrared spectroscopy (FT-IR) and powder X-ray diffractometry (PXRD). The FTIR studies demonstrated that the aromatic ring (C=C) of glabridin might be inside the hydrophobic cavity of CDs. The results from the FT-IR, DSC and PXRD studies confirmed that the glabridin-HP-β-CD coevaporated sample exhibited

complete glabridin amorphilization and formed a true inclusion complex. The rank order of water solubility of inclusion complex was glabridin-HP-β-CD co-evaporated sample > glabridin-HP-β-CD kneaded sample > glabridin-β-CD kneaded sample > glabridin-γ-CD kneaded sample. The glabridin-HP-β-CD co-evaporated which gave the highest water solubility was selected to prepare solution-type lotion. This lotion showed physicochemical stability. Its tyrosinase inhibitory activity was slightly decreased after a storage at room temperature (30°C) and 45°C for 3 months. On the other hand, the precipitation of glabridin was observed when the lotion was kept at 8°C for longer than 60 days. The *in-vitro* release rate (K_H) through cellulose acetate membrane of glabridin from lotion containing glabridin-HP-β-CD co-evaporated sample was higher than that of lotions containing glabridin-HP-β-CD physical mixture and pure glabridin.

ชื่อวิทยานิพนธ์	การศึกษาความสามารถในการละลายน้ำ คุณสมบัติทางเคมีกายภาพ การยับยั้งเอนไซม์ไทโรซิเนสของสารประกอบเชิงซ้อนกลาบริดิน-ไซโคลเด็กซ์ตรินและตำรับในรูปสารละลาย
ผู้เขียน	นาย พิภพ พิทักษ์ศักดิ์เสรี
สาขาวิชา	วิทยาศาสตร์เครื่องสำอาง
ปีการศึกษา	2554

บทคัดย่อ

กลาบริดิน (glabridin) เป็นสารที่มีฤทธิ์ในการยับยั้งเอนไซม์ไทโรซิเนสที่สูง จึงนิยมนำมาใช้เป็นสารสำคัญในผลิตภัณฑ์เครื่องสำอางเพื่อทำให้ผิวขาว glabridin ละลายในน้ำไม่ดี ดังนั้นงานวิจัยนี้มีวัตถุประสงค์เพื่อเพิ่มการละลายของ glabridin ในน้ำโดยการเกิดสารเชิงซ้อนกับไซโคลเด็กซ์ตริน (cyclodextrin) 3 ชนิด ได้แก่ เบต้า-ไซโคลเด็กซ์ตริน (β-CD) แกมมา-ไซโคลเด็กซ์ตริน (γ-CD) และไฮดรอกซีโพรพิล-เบต้า-ไซโคลเด็กซ์ตริน (HP-β-CD) จากการศึกษา phase solubility ของ glabridin ใน cyclodextrin แต่ละชนิดที่อุณหภูมิ 32±1°C พบว่า phase solubility ของ glabridin ใน β-CD และ γ-CD เป็น A_L-type ส่วน phase solubility ของ glabridin ใน HP-β-CD เป็น B_L-type และการเกิด inclusion complexes ระหว่าง glabridin และ cyclodextrin ทั้งสามชนิดเป็นแบบ 1:1 โดยโมล เมื่อเตรียม glabridin-cyclodextrin inclusion complexes อัตราส่วน 1:1 โดยโมล โดยวิธี kneading และ co-evaporation และทำการศึกษาค่าการละลายและคุณลักษณะทางเคมีกายภาพของ inclusion complexes ที่ได้ โดยวิธี differential scanning calorimetry (DSC), Fourier-transform infrared spectroscopy (FT-IR) และ powder X-ray diffractometry (PXRD) จากผล

การศึกษา FT-TR บ่งบอกว่า inclusion complexes ที่ได้ระหว่าง glabridin และ cyclodextrins ทั้ง 3 ชนิด เกิดอันตรกิริยาโดยวงแหวนอะโรมาติกของ glabridin อาจจะเข้าไปอยู่ในช่องว่างของ cyclodextrin จากผลการศึกษา FT-TR, PXRD และ DSC สรุปได้ว่า glabridin-HP-β-CD co-evaporated sample อยู่ในรูปอสัณฐานและเกิด true inclusion complex จากการศึกษาค่าการละลายพบว่าลำดับการละลายน้ำของ glabridin-HP-β-CD co-evaporoated sample > glabridin-HP-β-CD kneaded sample > glabridin-β-CD kneaded sample > glabridin-γ-CD kneaded sample เมื่อนำ glabridin-HP-β-CD co-evaporated sample ซึ่งให้ค่าการละลายน้ำสูงสุดมาเตรียมรูปแบบโลชันชนิดสารละลาย (solution-type lotion) พบว่าโลชันที่ได้มีความคงตัวทางกายภาพ ทางเคมี ฤทธิ์ต้านเอนไซม์ไทโรซิเนส ลดลงเล็กน้อยเมื่อเก็บไว้ที่อุณหภูมิห้อง (30°C) และ 45°C เป็นเวลา 3 เดือน ส่วนโลชันที่เก็บไว้ที่อุณหภูมิ 8°C จะไม่คงตัวโดยมีการตกตะกอนของ glabridin เมื่อเก็บไว้เป็นเวลานานกว่า 60 วัน จากการศึกษาอัตราการปลดปล่อย (K_H) ของ glabridin ที่เตรียมในรูปโลชัน ผ่านเยื่อกั้นเซลลูโลสอะซีเตต พบว่าโลชันที่ประกอบด้วย glabridin-HP-β-CD co-evaporoated sample ให้อัตราการปลดปล่อยของ glabridin ที่สูงกว่าโลชันที่ประกอบด้วย glabridin-HP-β-CD physical mixture และโลชันที่ประกอบด้วย glabridin อย่างเดียว

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LISTS OF ABBREVIATIONS AND SYMBOLS

β	=	beta
β-CD	=	beta cyclodextrin
°C	=	degree Celsius
CD	=	cyclodextrin
CDs	=	cyclodextrins
cm^2	=	square centimeter
cm^3	=	cubic centimeter
cP	=	centi poise
Da	=	dalton
DMSO	=	dimethyl sulfoxide
DSC	=	differential scanning calorimetry
et al.	=	and others
etc.	=	and other things
FDA	=	food and drug administration
FT-IR	=	Fourier-transform infrared spectroscopy
G_2-β-CD	=	maltosyl β-cyclodextrin
γ-CD	=	gamma cyclodextrin
γ	=	gamma
HP-β-CD	=	hydroxypropyl beta cyclodextrin
HP-γ-CD	=	hydroxypropyl gamma cyclodextrin
IC_{50}	=	half maximal inhibitory concentration value
INCI	=	the international nomenclature of cosmetic ingredients

IR	=	infrared
KBr	=	potassium bromide
K_s	=	stability constant
LDL	=	low-density lipoprotein
L*value	=	measure the lightness (100 is white)
M	=	molar
mg	=	milligram
mJ	=	millijoule
ml	=	milliliter
mm^2	=	square milimeter
mM	=	millimolar
MW	=	molecular weight
µg/ml	=	microgram per milliliter
µg	=	microgram
n	=	number of sample
N	=	normality
nm	=	nanometer
PXRD	=	powder X-ray diffractometry
PVP	=	polyvinylpyrrolidone
%	=	percentage
q.s.	=	a sufficient quantity
R^2	=	correlation coefficient
RM-β-CD	=	random methyl beta cyclodextrin

rpm	=	round per minute
RSD	=	relative standard deviation
SBE-β-CD	=	sulfobutyl ether beta cyclodextrin
Θ	=	theta
UV	=	ultraviolet
v	=	volume
w	=	weight
w/v	=	weight by volume
w/w	=	weight by weight

CHAPTER 1

INTRODUCTION

1.1 Background and rationale

Hyperpigmentation is a common, usually harmless condition in which patches of skin become darker in color than the normal surrounding skin. This darkening occurs when an excess of melanin, the brown pigment that produces normal skin color, deposits in the skin. Hyperpigmentation can affect the skin color of people of any race. Hyperpigmentation is one of the problems of person's skin that results in dermatological disorders such as melasma, freckles, sunspots, age spots and site of actinic damage. Hyperpigmentation in skin is caused by an increase in melanin or accumulation of an excessive level of melanin, the substance in the body that is responsible for color. Certain conditions such as pregnancy or Addison's disease (decreased function of adrenal gland), may cause a greater production of melanin and hyperpigmentation. Exposure to sunlight is a major cause of hyperpigmentaion and will darken already hyperpigmented areas. Hyperpigmentation can also be caused by various drugs including some antibiotics, antiarrhythmics and antimalaria drugs.

One of the methods that frequently used to prevent or reduce the hyperpigmentation is the inhibition of melanin biosynthesis. Melanin is formed through a series of oxidative reactions revolving the amino acid tyrosine in the presence of the enzyme tyrosinase. Human tyrosinase is an essential enzyme which regulates the production of melanin. Melanin biosynthesis can be inhibited by avoiding the UV exposure, reducing melanocyte metabolism and proliferation, exfoliating the corneal layer of epidermis and using tyrosinase inhibitors. In cosmetic

products, the use of tyrosinase inhibitors obtain popular because they inhibit initially the melanin biosynthesis. There are many active ingredients having tyrosinase inhibitory activity that have been used in cosmetic products such as kojic acid, arbutin, tretinoin (retinoic acid), niacinamide (vitamin B3), magnesium L-ascorbyl-2 phosphate (vitamin C derivative), resveratrol, linoleic acid (Petit and Piérard, 2003), and glabridin (Yokota *et al.*, 1998). Among these tyrosinase inhibitors, glabridin is one of natural origin and considered one of the most effective skin lightening agents. Therefore, glabridin is interesting in this thesis.

Glycyrrhiza glabra Linn., commonly call licorice, is a perennial herb of family Fabaceae. The generic name "glycyrrhiza" stems from ancient Greek, meaning "sweet root". The *Glycyrrhiza* genus contains about 30 species and it is widely distributed all over the world. Licorice has been extensively researched for its medicinal and food uses because of its useful properties. The roots and rhizomes of licorice containing triterpene saponins and flavonoids show a variety of pharmacological activities including antiulceric, antioxidative, antiinflammatory, antispasmodic, antiallergic, antimicrobial, antiviral, antidiabetic, anticancer, antidepressive, hepatoprotective, expectorant, memory enhancing activities and tyrosinase inhibitory activity (Zhang and Ye, 2009). Most tyrosinase inhibitory activity is attributed to the major flavonoids, like liquiritigenin, liquiritin, isoliquiritigenin, isoliquiritin, glabrene, licochalcone A, licochalcone B and glabridin (Fu *et al.*, 2005).

Glabridin is a natural compound found in licorice root in amount of 0.4% w/w. It was first reported to inhibit the tyrosinase activity of melanocytes by Yokota *et al.* (1998). They investigated inhibitory effect of glabridin on

melanogenesis by using cultured B16 murine melanoma cells. The result indicated that glabridin inhibited tyrosinase activity of these cells at concentrations of 0.1 to 10 μg/ml. The inhibitory effect of glabridin in the concentration of 0.1 μg/ml, 1 μg/ml and 10 μg/ml was 17.5%, 39.1%, and 49%, respectively over a control. Moreover, they reported that UVB-induced skin pigmentations (250 mJ/cm^2 UVB/day for 4 successive days) caused in the backs of the brownish guinea pigs were reduced by topical application of 0.5% glabridin solution for 3 weeks, started after the last UVB irradiation. The darkness-lightness (0 = black, 100 = white) was represented by the symbol "L*". The L* value of glabridin-treated skins of the three guinea pigs was significantly higher than that of the control (base solution-treated skins) indicating that the skin color of glabridin-treated skins became lighter than that of the control. Base on these data, they concluded that glabridin has inhibitory effects on melanogenesis both *in vitro* and *in vivo*. Another research studied by Nerya *et al.* (2003) reported that the half maximal inhibitory concentration (IC_{50}) value of glabridin on monophenolase activity and on diphenolase activity are 0.09 μM and 3.94 μM, respectively. In addition, they concluded that glabridin was a very effective agent with an IC_{50} value of inhibition of cultured G361 human melanocyte pigmentation due to melanin synthesis of about 7.4 μM.

The glabridin solution-type lotion prepared by dissolving glabridin in water which contain no oily substances is suitable for people who have oily skin or acne prone skin. However, the main problem of using glabridin in aqueous cosmetic preparations is its poor solubility in water. Although glabridin is soluble in ethanol and polyols such as propylene glycol, butylene glycol, the large amounts of ethanol or polyols used in the formulation cause the problem of skin irritation (Richard and

Garzouzi, 2005). If glabridin is not completely dissolved, the cosmetic formulation will become turbid or lose its transparency and thus reducing its commercial value (Matsuda *et al.*, 1995). Therefore, increasing glabridin solubility in water is necessary in development of glabridin solution-type lotion.

There are various techniques available to improve the water solubility of poorly soluble compounds. These include particle size reduction, solid dispersions (Zhu *et al.*, 2007), solubilization by using surfactants (Edris and El-Galeel, 2010), cosolvency and inclusion complexation (Shinde, 2007).

There are several previous studies about enhancing the solubility of cosmetic ingredients and drugs by using inclusion complex with cyclodextrin and other methods. For examples:

- Zhu *et al.* (2007) found that the solubility of quercetin was enhanced by solid dispersion using polyvinylpyrrolidone (PVP) as carrier.

- Edris and El-Galeel (2010) found that Tween 20 was able to solubilize peppermint oil in a surfactant/water in ratios of 1:1, 2:1 and 3:1 approximately 17%, 38% and 45% w/w, respectively.

- Lu *et al.* (2009) found that resveratrol inclusion complex with β-cyclodextrin or hydroxypropyl-β-cyclodextrin showed a linear phase solubility diagram (A_L type system) indicating that resveratrol solubility was increased by the cyclodextrins concentration.

- Liu and Zhu (2006) found that inclusion complexes of prazosin hydrochloride with β-cyclodextrin or hydroxypropyl-β-cyclodextrin prepared by grinding method, ultrasonic method and co-precipitating method could enhance the drug solubility.

- Nalawade *et al.* (2010) found that inclusion complexes of valsartan with β-cyclodextrin or hydroxypropyl-β-cyclodextrin prepared by kneading method and co-evaporation method could enhance the drug solubility.

Among these methods, inclusion complex with cyclodextrin is widely used to enhance the solubility of many drugs and cosmetic compounds.

In this study, enhancement the solubility of glabridin by inclusion complex with cyclodextrins (β-cyclodextrin, γ-cyclodextrin and hydroxypropyl-β-cyclodextrin) was examined. The inclusion complex that exhibited the highest solubility of glabridin was selected to prepare glabridin solution-type lotion. The obtained lotion was evaluated both tyrosinase inhibitory activity and *in vitro* release through synthetic membrane compared with pure glabridin. The stability of the prepared lotion was also determined.

1.2 Review of literature

1.2.1 Pigment theory of human skin

Skin color results from the presence and ratio of several chromophores in the skin. Oxyhaemoglobin (bright red), reduced haemoglobin (bluish red) and bilirubin (yellow) are found in the small blood vessels of the dermis (Petit and Piérard, 2003). Additionally, pigments from melanin processes are eumelanin (dark brown to black) and phaeomelanin (red to yellow) present in epidermis layer (Schlossman, 2000). Skin color is determined by the ratio of eumelanin to pheomelanin, where the more eumelanin present, the darker tone of an individual, the absolute amount and concentration of melanin in the skin and the size of the packets

that melanin travels in (melanosomes), the number of melanosomes, and how well spread the melanosomes are.

1.2.2 Melanogenesis pathway

It is known that melanins are synthesized at the bottom of the epidermis (top layer of the skin), in a region termed the basal layer. Special cells located in the basal layer, termed melanocytes, produce melanin containing packets called melanosomes. This process is termed melanogenesis.

Melanins, the end-products of complex multistep transformations of L-tyrosine, are polymorphous and multifunctional biopolymers, represented by eumelanin, pheomelanin, neuromelanin, and mixed melanin. Melanin biosynthesis or melanogenesis can be initiated from either the hydroxylation of L-phenylalanine to L-tyrosine or directly from L-tyrosine (Slominski *et al.*, 2004). The simplified melanogenic pathway and the biosynthetic pathway of melanin are shown in Figure 1.1 and Figure 1.2, respectively. Tyrosinase, a copper containing oxidase enzyme, converts tyrosine by hydroxylation to L-dihydroxyphenylalanine (L-DOPA). L-DOPA serves as a precursor to both melanins and catecholamines, acting along separate pathways. The next step, oxidation of L-DOPA to dopaquinone (DOPA quinone) is common to both eumelanogenic and pheomelanogenic pathways. Eumelanogenesis involves the further transformation of dopaquinone to leukodopachrome (DOPA chrome) followed by a series of oxidoreduction reactions with production of the intermediates 5, 6 dihydroxyindole (DHI). The intermediates 5, 6 dihydroxyindole is converted to indole-5,6-quinone (IQ) and polymerized to form DHI melanins (eumelanins). The other pathway of eumelanogenesis starts with the interconversion

of DOPA chrome to 5,6 dihydroxyindole-2-carboxylic acid (DHICA) with the presence of DOPA chrome tautomerase (also known as tyrosinase-related protein 2; TRP-2). Then DHICA oxidase (also known as TRP-1) promotes further oxidation and polymerization of DHICA to DHICA melanins (eumelanins). Pheomelanogenesis also starts with DOPA quinone which conjugated to cysteine or glutathione to yield cysteinyldopa and glutathionyldopa, respectively. Then further transformation by oxidation, cyclization and polymerization into pheomelanins. Eumelanins are polymorphous nitrogenous biopolymers (predominantly copolymers of DHI and DHICA) which exhibit black (DHI melanin) to brown (DHICA melanin) in color and insoluble in most solvents. In contrast to eumelanins, pheomelanins have backbone of benzothiazine units and exhibit a yellow to reddish-brown color and are alkali soluble.

The synthesis of melanin is only a part of the melanogenic process. Melanogenesis also involves the development of melanosomes, the organelle of the melanocyte in which melanin is synthesized, and the subsequent transfer of the melanosomes into the keratinocytes.

1.2.3 Melanosome development

Dermal melanin is produced by melanocytes, specialized skin cells located in the basal layer of the epidermis. Melanocytes are branched structures consisting of a central cell body and numerous branches or dendrites. Melanocytes insert granules of melanin into specialized cellular vesicles or organelles called melanosomes. Melanosomes are produced inside the melanocytes and they pass through several developmental stages, starting in the middle of the melanocyte cell and migrating to the outer edge of the cell through the dendrites. Melanin biosynthesis

is taking place inside the melanosomes. The process of melanin biosynthesis leads to the mature melanin grains that are gradually transported to the keratinocytes of the upper epidermis layers via the dendrites.

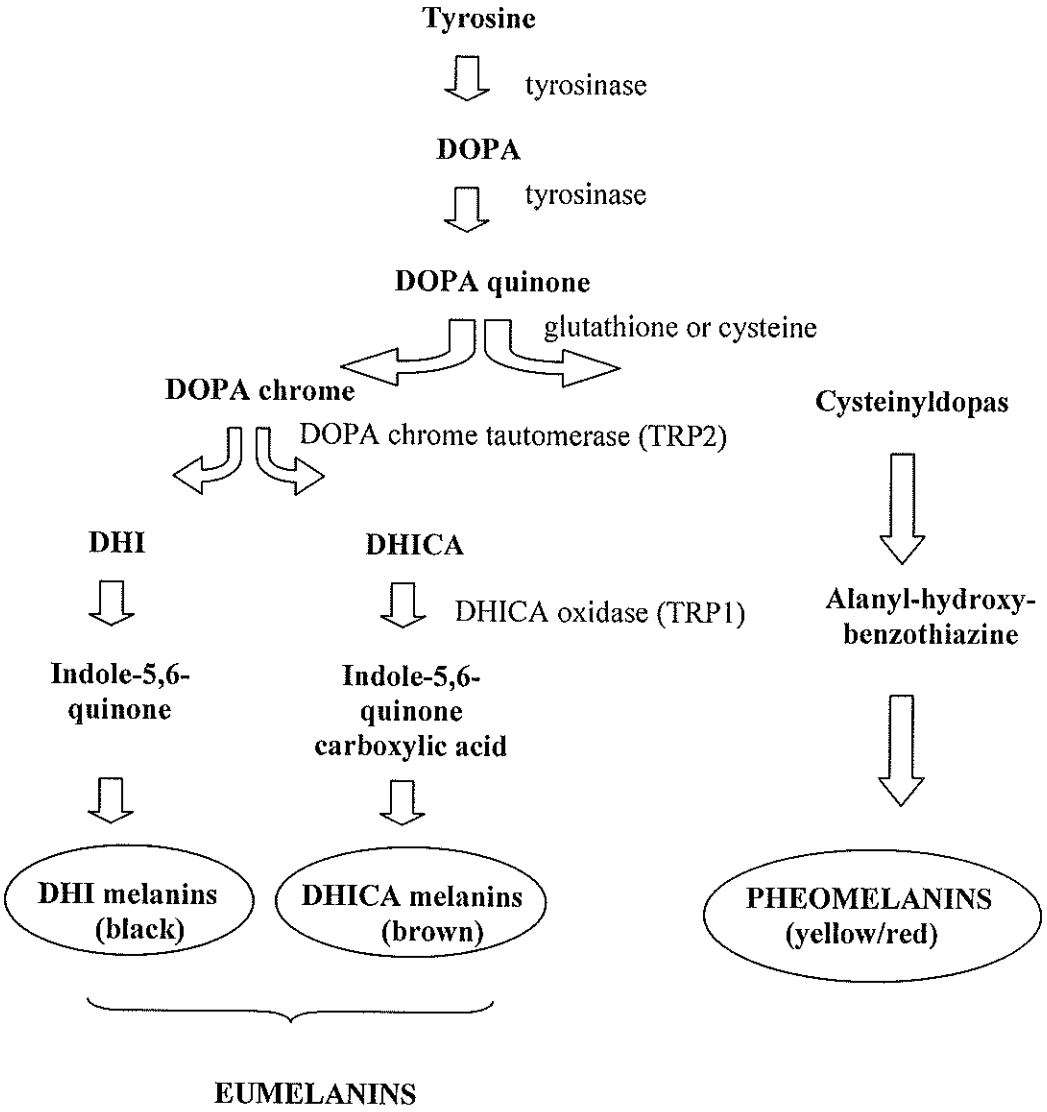

Figure 1.1 The simplified melanogenic pathway (Petit and Piérard, 2003).

DOPA: dihydroxyphenylalanine;

DHI: 5,6 dihydroxyindole

DHICA: 5,6 dihydroxyindole-2-carboxylic acid

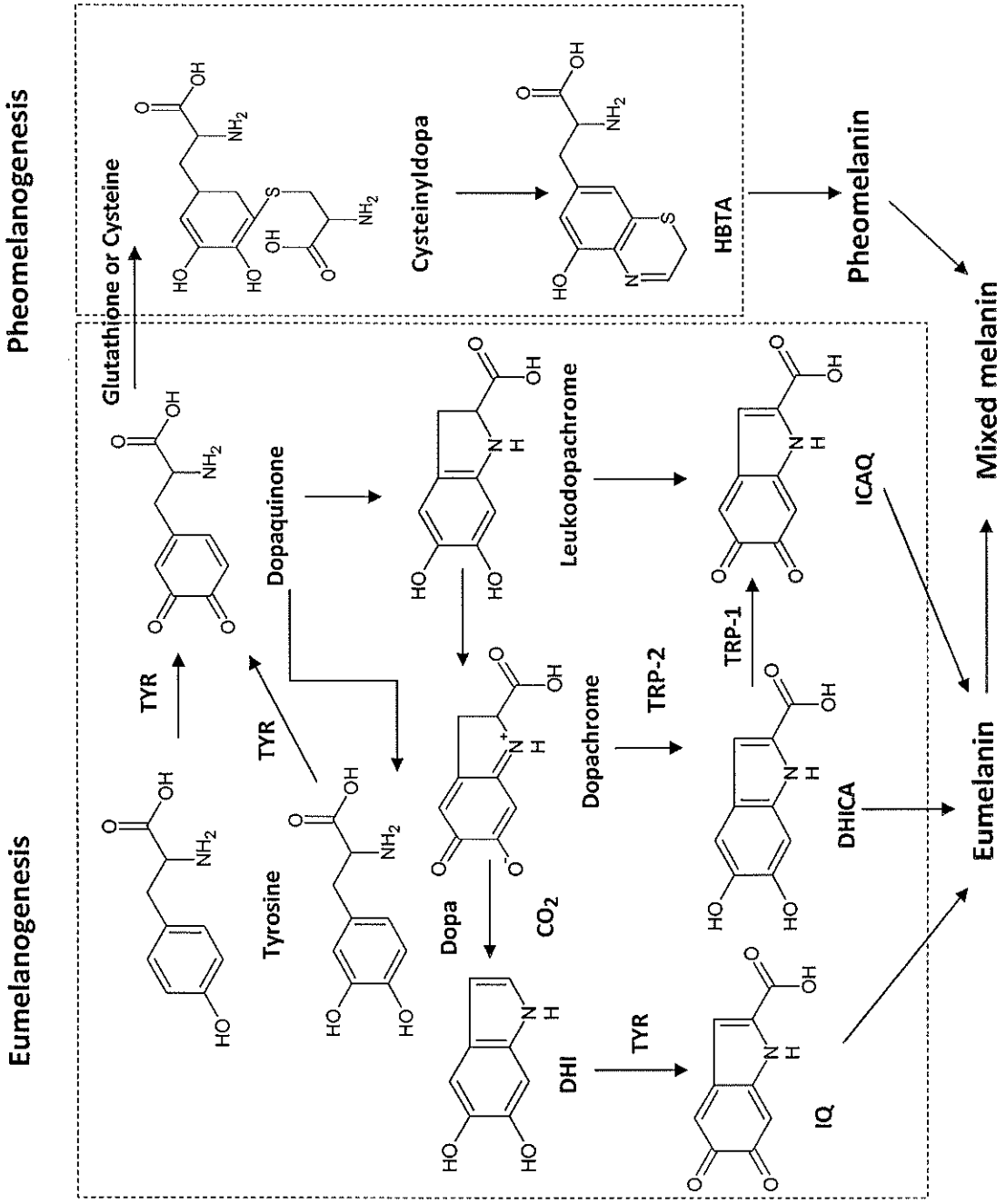

Figure 1.2 Biosynthetic pathway of melanin (Chang, 2009).

TYR: tyrosinase; TRP: tyrosinase related protein;

Dopa: 3,4-dihydroxyphenylalanine; DHI: 5,6-dihydroxyindole;

DHICA: 5,6-dihydroxyindole-2-carboxylic acid;IQ: indole- 5,6-quinone;

ICAQ: indole-2-carboxylic acid-5, 6-quinone;

HBTA: 5-hydroxy-1,4-benzothiazinylalanine

Melanosome development process is shown in Figure 1.3. This process is generally divided into four phases. Phase 1, the melanosomes contain tyrosinase but remain clear. Phase 2, the melanosome assuming an oblong shape as microtubes and microfilaments begin to expand. Melanin now begins to be formed. Phase 3, melanosomes darken and develop a higher density; they also begin to migrate towards the dendrites of the melanocytes. Phase 4, melanosomes are opaque and ready for transfer from the dendrites into keratinocytes which found in epidermis layer of the skin (Schlossman, 2000). Melanosome transfer is a cytophagic ("cell engulfing") process during which a portion of a melanocyte dendrite is pinched off by the epidermal cell so that melanosomes and melanocyte cytoplasm are incorporated into the keratinocytes. Keratinocytes are the cells that produce the protein keratin, the key structural component of hair and feathers. The melanin containing melanosomes spread out above the nucleus, where they stay, protecting the DNA inside the organelle from harmful UV radiation. The skin cells eventually rise to the top of the epidermis where they die and are desquamated (shed away).

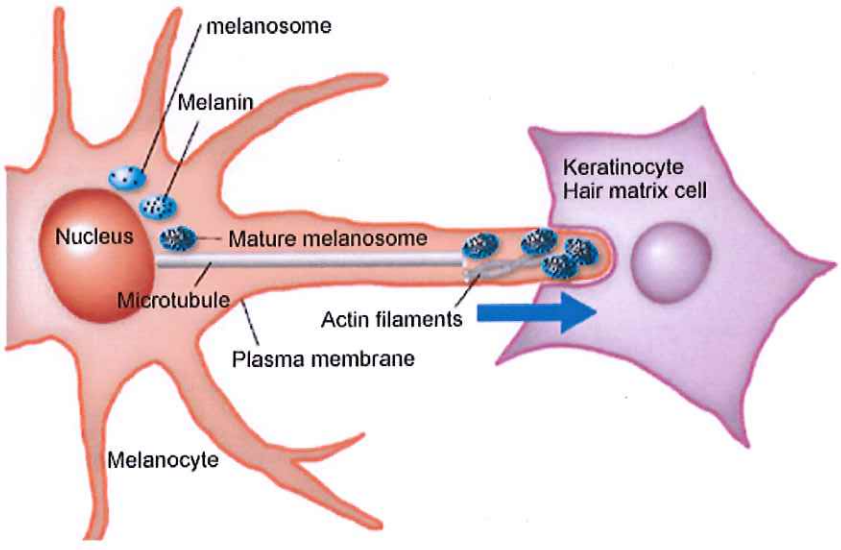

Figure 1.3 Melanosome development process (Fukuda, 2008).

1.2.4 Function of melanin

Melanin plays a protective role in healthy skin. It functions as an absorptive pigment. In humans, the two types of melanin, eumelanins and pheomelanins, presented in the skin are responsible for different skin tone (which is induced by ultraviolet radiation). Eumelanin is a black or brown pigment and is mainly concerned with the protection of the skin. Its role is to protect the skin from ultraviolet (UV) damage by absorbing the UV sunlight and removing reactive oxygen species. This protective ability warrants melanin to be termed a photoprotectant (a substance capable of providing protection against radiation from the sun). Pheomelanin is a reddish pigment, a very weak absorptive of UV radiation, further it also acts as a photosensitizer (makes skin sensitive to light), where it increases sun sensitivity and skin ageing. Before global migration, most humans with the darkest skin lived closest to the equator. Melanin absorbs UV light, thus protecting humans from harmful UV radiation. UV radiation damages skin cells and promotes melanoma, cancer of the skin. European Americans have a 10 times higher risk of melanoma than African Americans. Even though melanin is protective, complete shielding would be harmful, because shortwave UV radiation needs to penetrate the skin to produce vitamin D. Approximately 90 percent of vitamin D is synthesized in the skin.

Melanin is able to protect the skin from UV radiation by several methods. It is able to scatter incoming UV radiation, absorb UV and can also absorb compounds produced by photochemical action which would be toxic or carcinogenic. The amount of UV radiation that is absorbed or scattered is determined by a number of factors. These include size, shape, and distribution of melanosomes as well as the

wavelength of the incident ray. The absorption spectrum for melanin at different wavelengths is shown in Figure 1.4. Melanin is best suited to absorb radiation in the range of 320-340 nm, which is in the UVB/UVA regions.

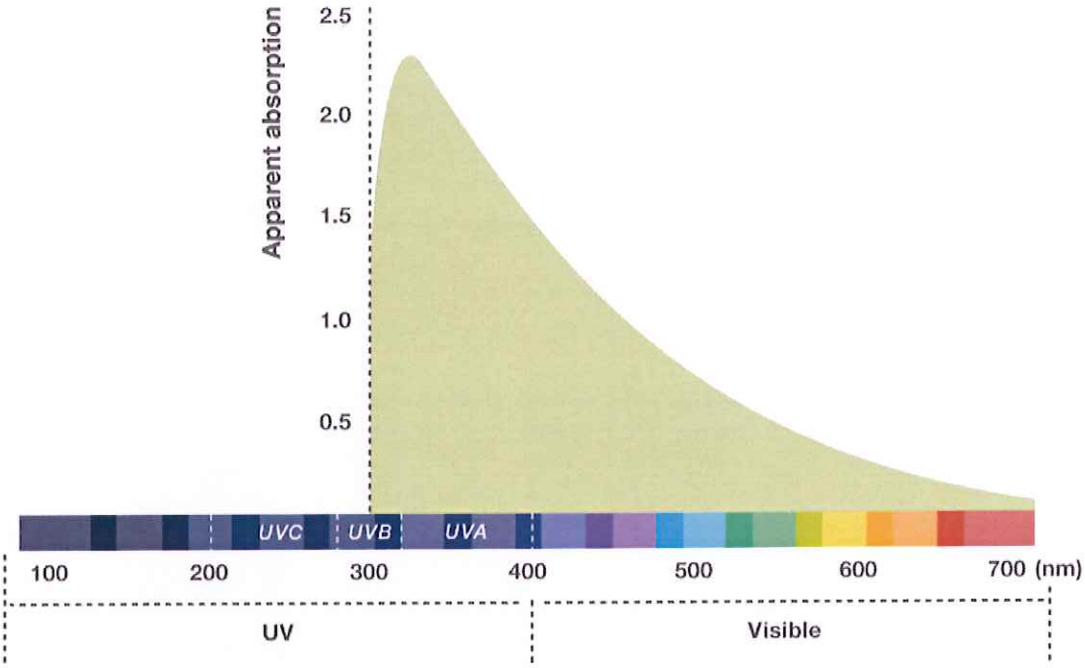

Figure 1.4 Absorption spectrum of melanin against specific wavelengths of incident radiation (Clinuvel Pharmaceuticals, 2011).

1.2.5 Melanin and skin pigmentation problems

Because the important role of melanin plays in the skin, its production or lack of production can also be directly connected to several skin disorders. For example, the rare condition known as albinism is caused when the enzyme tyrosinase activity is blocked in the cells. Without this activity, the melanocytes cannot produce melanin. As a result, the skin and other parts of the body have little pigmentation thus causing the very pale skin and eye color associated with the condition. On the other

hand, if melanin is overproduced, the accumulation of an excessive level of melanin will result in various dermatological disorders such as melasma, age spots, freckles, sunspots and site of actinic damage which causes of skin hyperpigmentation (Nerya *et al.*, 2003). Melasma, a patchy brown discoloration of the skin, is one of the examples of skin hyperpigmentation which is common developed during pregnancy. The cells produce too much melanin and end up creating dark patches over parts of the body including the face, hands, and other regions. It typically occurs on the face, particularly the forehead, cheeks and above the upper lip. The dark patches often appear on both sides of the face in a nearly identical pattern. The darker-colored patches of skin can be any shade, from tan to deep brown. Melasma, freckles, age spots, and other darkened skin patches can become darker or more pronounced when skin is exposed to the sunlight. This happens because melanin absorbs the energy of the sun's harmful ultraviolet rays in order to protect the skin from overexposure. The usual result of this process is skin tanning which tends to darken areas that are already hyperpigmented. Although melanin has mainly a photoprotective function in human skin, the accumulation of an abnormal amount of melanin in different specific parts of the skin resulting in more pigmented skin patches might become a skin problem. Therefore, prevention of melanin accumulation or treatment of skin hyperpigmentation is necessary.

1.2.6 Method for slowing melanogenesis

There are several methods which can slow down melanogenesis.

- Reduce UV exposure
- Use sunscreen products
- Reduce melanocyte activity
- Use antioxidants
- Reduce tyrosinase production

Reducing exposure to UV sunlight reaching the skin works best for slowing melanogenesis. Therefore, the use of sunscreens is advocated. UV radiation directly stimulates the generation of tyrosinase and generates inflammation that might increase pigmentation.

Reducing pigmentation is to reduce the activity of the melanocytes. Melanocytes activity can be reduced through the use of selectively cytotoxic materials such as hydroquinone and cortisone. Alternatively, materials like melatonin, which are α-MSH antagonists, can be used to prevent the activation of melanocytes.

Another popular common approach for slowing melanogenesis is to take advantage of the fact that melanogenesis is an oxidative process. Highly reactive antioxidants such as ascorbic acid can be used to compete with melanin precursors to reduce the oxidative stress on the system. This approach not only inhibits the production of melanin but also reduces the amount of melanin already present to the skin.

The best way of minimizing the amount of adaptive pigmentation is by reducing tyrosinase production. This can be accomplished by blocking the production

of tyrosinase, inhibiting the enzymatic activity of tyrosinase or preventing the uptake of tyrosinase by the melanosome. So, using tyrosinase inhibitors are interesting in cosmetic field.

1.2.7 Tyrosinase inhibitors in cosmetic products

In cosmetic formulations, hydroquinone, in the past, was widely used as an effective skin lightening agent because it was thought to be the safest and the most effective treatment for hyperpigmentation including age spots, melasma, sun damage and other discolorations. However, many researches have reported that there are serious side effects associated with long term use of hydroquinone. In addition, its use has been connected with mutagenicity and the increased incidence of ochronosis in African countries. Therefore, the FDA announced to remove hydroquinone based products from store shelves and limited its use to only prescription based medications (Smit *et al.*, 2009). Consequently, many manufacturers have begun to produce natural alternatives which mimic the skin lightening properties of hydroquinone. Ingredients such as arbutin (alpha-arbutin, beta-arbutin), kojic acid, mulberry extracts, and licorice extract have become quite popular. When combined, these ingredients can often produce results that even surpass hydroquinone but without the associated risks.

The process of lightening the skin occurs in several stages. Most of the current skin lightening ingredients on the market work at different stages of the process and typically provide the best results when combined together into one product. Listed below are a few of the more popular skin lightening ingredients used by manufacturers of skin lightening products (Meladerm, 2011).

Beta-arbutin (Bearberry Extract)

Beta-arbutin, a glycosylated form of hydroquinone, is often referred to arbutin. It is a natural extract found in bearberry (*Uva Ursi*) plants. Though arbutin is a natural derivative of hydroquinone, it does not possess the same risks or side effects. Arbutin has been shown to be a very safe ingredient and does not break down into hydroquinone very readily. Though it is cheaper to manufacture than alpha-arbutin, the skin lightening effect is much less than that of alpha-arbutin. For this reason, many new skin lightening products now use alpha-arbutin as opposed to only beta-arbutin (Meladerm, 2011). *In vitro* studies of human melanocytes exposed to beta-arbutin showed that beta-arbutin at concentrations below 300 μg/ml decreased tyrosinase activity and melanin content with little evidence of cytotoxicity (Integrated laboratory systems, 2006).

Alpha-arbutin

Alpha-arbutin is a biosynthetic active ingredient that synthesized from hydroquinone by glucosidation. It is the epimer of arbutin and research has proven that it has a stronger inhibitory action (up to 10 times more effective) than that of beta-arbutin because the alpha-glucosidic bond found in alpha-arbutin offers higher stability and efficiency than the beta form found in the related beta-arbutin (Meladerm, 2011). Sugimoto *et al.* (2004) reported that treatment of the human skin model with 250-500 μg/tissue of α-arbutin did not inhibit cell viability and melanin synthesis was reduced to 40% when compared with non-treated cells (at 250 μg of α-arbutin). These results indicated that α-arbutin is an effective and safe ingredient for skin-lightening.

Kojic acid

Kojic acid is a by-product in the fermentation process of malting rice used in the manufacturing of sake, the Japanese rice wine. It is discovered in 1989 and is used extensively as a natural alternative to hydroquinone. Studies have shown that it is effective as a lightening agent by inhibiting the production of melanin (Meladerm, 2011). Wikipedia (2012) reported that kojic acid is not carcinogenic but it can cause allergic contact dermatitis and skin irritation.

Licorice extract

The licorice plant serves many purposes in skin care. The ingredient that is responsible for the skin lightening aspect of the plant is known as glabridin. Glabridin inhibits pigmentation by preventing tyrosinase activation. Studies have shown that it can provide a considerable skin lightening effect while remaining non-toxic to the melanin forming cells. In addition, glabridin has anti-inflammatory activity due to inhibition of superoxide anion production and cyclooxygenase activity. Therefore, it is a very popular ingredient in the skin care industry (Meladerm, 2011). Yokota *et al.* (1998) reported that glabridin inhibits tyrosinase activity of B16 melanoma cells at concentrations of 0.1 to 1 μg/ml and had no detectable effect on their DNA synthesis.

Niacinamide

Niacinamide is commonly known as Vitamin B3. It is an effective skin lightening compound that works by inhibiting melanosome transfer from melanocytes to keratinocytes. This ingredient works best when combined with other

skin lightening treatments. Niacinamide (Vitamin B3) is also known to be effective in reducing acne (Meladerm, 2011). Navarrete-Solis *et al.* (2011) reported that 4% niacinamide may cause side effects such as erythema, pruritus, and burning approximately 7% (2/27 of patients).

Mulberry extract

Paper mulberry extract is obtained from the bark and the root of *Broussonetia kazinoki*, Siebold. or *B. papyrifera*, L., Vent., family Moraceae. Extract of the root is potent inhibitor of tyrosinase enzyme. The active constituents present in the extract are prenylated, polyhydroxylated mono-and bis-phenylderivatives. A 0.4% concentration of paper mulberry extract inhibits tyrosinase by 50% compared to 5.5% for hydroquinone and 10.0% for kojic acid. The paper mulberry extract in the concentration of 1% is not significant irritant (Meladerm, 2011).

Vitamin C

Vitamin C is a natural antioxidant that occurs in many different forms (some stable and others unstable). Several of these forms have been shown to reduce melanin formation and provide a skin lightening effect. These include l-ascorbic acid, magnesium ascorbyl phosphate and sodium ascorbyl phosphate. These forms when used individually or together can assist in slowing down hyperactive melanocytes and thus resulting in lighter skin (Meladerm, 2011). Espinal-Perez *et al.* (2004) reported that ascorbic acid at 6.25% may cause skin irritation.

Among these tyrosinase inhibitors, Nerya *et al.* (2003) reported that glabridin is a very potent inhibitor of monophenolase activity and diphenolase

activity. The IC_{50} value of glabridin, as shown in Table 1.1, is stronger than that of other tyrosinase inhibitors, such as isoliquitigenin, glabrene, kojic acid, arbutin, quercitin and resveratrol. Therefore, glabridin is chosen to formulate in solution-type lotion in this experiment.

Table 1.1 IC_{50} of tyrosinase inhibitors.

Inhibitor	substrate	(IC_{50}, μM)	References
1. Glabridin	L-Tyrosine	0.09	Nerya *et al.*, 2003
	L-DOPA	3.94	
2. Isoliquitigenin	L-Tyrosine	3.5	Nerya *et al.*, 2003
	L-DOPA	47	
3. Glabrene	L-Tyrosine	8.1	Nerya *et al.*, 2003
	L-DOPA	7,600	
4. Kojic Acid	L-Tyrosine	8.66	Ley and Bertram, 2001
	L-DOPA	22	
5. 1, 4-hydroquinone	L-DOPA	0.654	Curto *et al.*, 1999
6. Resveratrol	L-DOPA	155	Shin *et al.*, 1998
7. Oxyresveratrol	L-DOPA	1	Shin *et al.*, 1998
8. Arbutin	L-Tyrosine	146	Zheng *et al.*, 2008
9. Quercetin	L-Tyrosine	57.8	Zheng *et al.*, 2008

1.2.8 Glabridin

Glabridin is a major isoflavan found in lipophilic extract of licorice roots which scientific name is *Glycyrrhiza glabra* L., family Fabaceae. *Glycyrrhiza glabra* L. is a perennial plant native to Southern Europe and parts of Asia. The photo of this plant is shown in Figure 1.5. The molecular structure of glabridin is shown in Figure 1.6 (Tian *et al*., 2008). The molecular formula of glabridin is $C_{20}H_{20}O_4$ and the molecular weight is 324.37 g/mol. It is off-white powder, soluble in ethanol (less than 1 part), propylene glycol (1 to 64 parts), butylene glycol (1 to 17 parts) but insoluble in water. It is known for its beneficial effects on skin and widely used as cosmetic ingredient due to its skin lightening, anti-inflammatory (Yokota *et al.,* 1998), antioxidant (Chin *et al*., 2007) and radical scavenging properties (Belinky *et al.,* 1998).

Figure 1.5 *Glycyrrhiza glabra* Linn (Taylor, 2011).

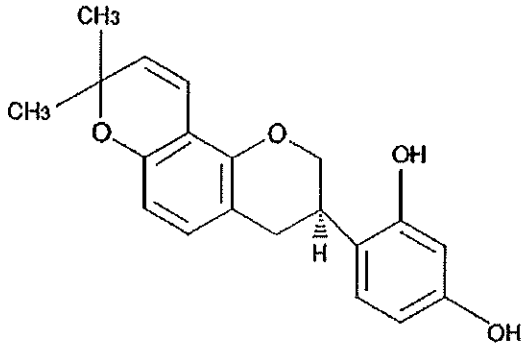

Figure 1.6 Molecular structure of glabridin (Tian *et al.*, 2008).

Activities of glabridin

Skin whitening effect (Inhibition of melanogenesis)

Glabridin may inhibit melanogenesis by one of two mechanisms:

1. Inhibition of the production of active oxygen species: (O_2)

2. Inhibition of tyrosinase enzyme

It is known that a number of reactions (e.g. inflammatory) are induced when human skin is exposed to UV radiation. The membrane phospholipids of the skin tissue are damaged by UV-induced active oxygen. Histological changes occur in the skin that manifest as erythemas and skin pigmentation. Active oxygen is one of the species that induces skin pigmentation. Thus, prevention of its production is linked to inhibition of melanogenesis. To test this, an assay was performed to study the inhibitory effect of glabridin on superoxide anion production. As shown in Figure 1.7, glabridin inhibited superoxide (active oxygen) formation at concentrations from 0.33 μg/ml to 33.3 μg/ml. Thus, glabridin may be useful for treating conditions like melasma or pigmentation of skin due to sun-exposure.

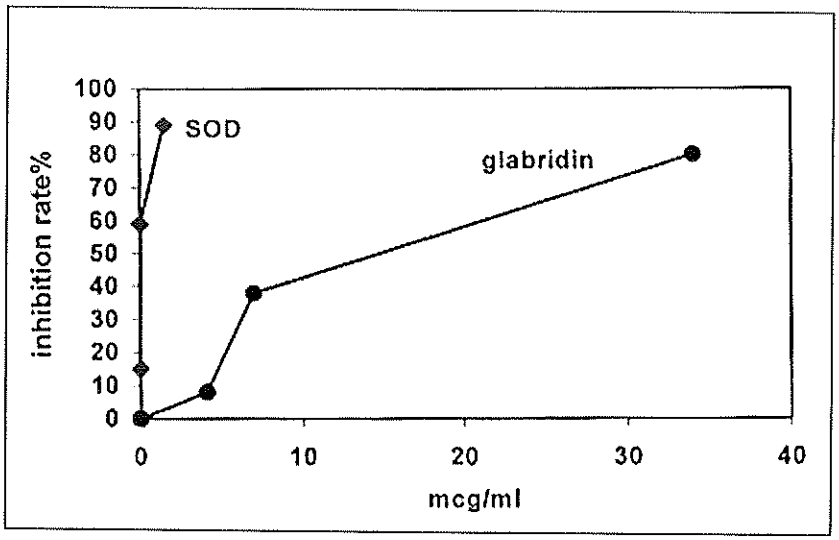

Figure 1.7 Inhibitory effect of glabridin on superoxide anion production (Yokota *et al.*, 1998). SOD: superoxide dismutase (the positive control inhibiting superoxide anion production).

In a comprehensive study carried out by Yokota *et al.* (1998), the inhibitory effect of glabridin on melanogenesis was examined. The structure-function relationship of glabridin was also studied. Topical skin-depigmentation activities of the active component, glabridin, were examined using UVB-induced pigmented skins of brownish guinea pigs (250 mJ/cm^2 UVB/day for 4 successive days). A 0.5% glabridin solution was topically applied for 3 weeks to the skin. Topical application of glabridin significantly reduced pigmentation induced by UVB radiation on the backs of the brownish guinea pigs. Skin samples were also taken from each of the glabridin treated areas for histological studies. The treated tissue was stained with 0.1% DOPA and the inhibition of melanogenesis was evaluated by counting the number of DOPA-positive melanocytes/mm^2 under an optical microscope. Epidermal histological studies showed that DOPA-positive melanocytes reduced in number on the skin

treated with glabridin. Treatment with glabridin also lightened the skin color due to inhibition of melanogenesis. Yokota *et al.* (1998) concluded that the glabridin presented in licorice roots inhibited melanin synthesis. They also observed that the property of glabridin was related to its structure, both of the hydroxyl groups of glabridin.

Anti-inflammatory effect

Yokota *et al.* (1998) evaluated the anti-inflammatory activity of glabridin when used for topical application. UVB-induced pigmented skins of guinea pigs were treated with 0.5% glabridin solution. It was observed that glabridin decreased the inflammation induced by UVB irradiation on the skin. The erythema manifested as redness in skin color is indicated by "a" value. The extent by which the inflammation decreased was calculated by recording the "a" value by using colorimeter before irradiation, after irradiation and after the topical application of glabridin. The "a" value increases with the appearance of erythema. As shown in Figure 1.8, the "a" value of the skin treated with glabridin was lower than that of the control, indicating a decrease in the inflammation.

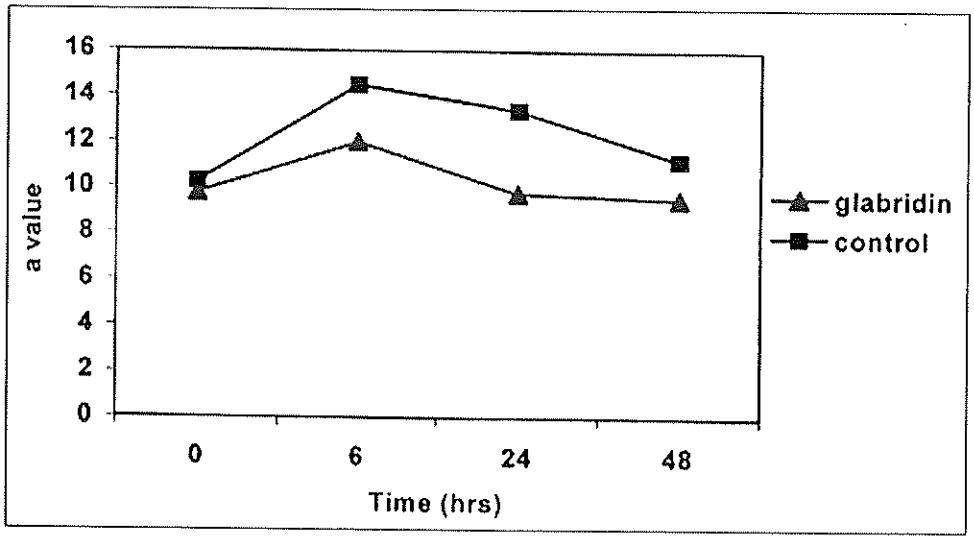

Figure 1.8 Anti-inflammatory effect of glabridin (Yokota *et al.*, 1998).

Yokota *et al.* (1998) determined the inhibitory effect of glabridin on cyclooxygenase activity. Cyclooxygenase is an enzyme that metabolizes arachidonic acid into prostaglandins, which are mediators that initiate the inflammatory cascade reaction. It was observed that addition of 6.25 μg/ml of glabridin inhibited the cyclooxygenase activity with respect to the control. The positive control in this experiment was indomethacin, a known cyclooxygenase inhibitor. It is believed that glabridin has the anti-inflammatory effect through the arachidonic acid cascade by inhibition to cyclooxygenase as shown in Figure 1.9.

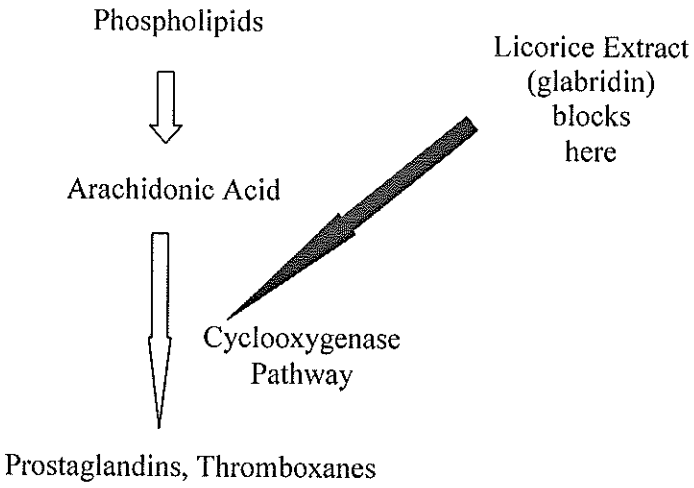

Figure 1.9 Inhibition of cyclooxygenase synthesis by licorice extract (Sami Labs, 2008).

Antioxidant effect

Berlinky *et al.* (1998) determined the inhibitory effect of glabridin on low-density lipoprotein (LDL) oxidation. LDL is one of the five major groups of lipoproteins that promote health problems and cardiovascular disease. It was often informally called the bad cholesterol particles. It was observed that the position of the hydroxyl group of glabridin at B ring (Figure 1.10) significantly affected the inhibitory efficiency on LDL oxidation. Two hydroxyl groups at position 2′ and 4′ or one hydroxyl at position 2′ on ring B successfully inhibited the formation of conjugated dienes, thiobarbituric acid reactive substances and lipid peroxides, and inhibited the electrophoretic mobility of LDL under oxidation.

It can be concluded that glabridin has an antioxidant effect in addition to its skin-lightening (anti-melanogenetic) and anti-inflammatory properties.

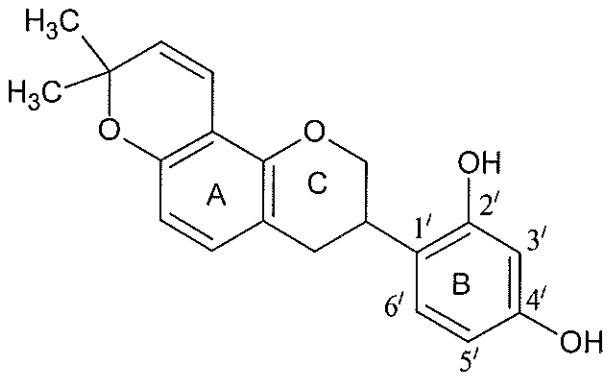

Figure 1.10 Position of hydroxyl groups of glabridin molecule (Berlinky *et al.*, 1998).

Nerya *et al.* (2003) reported that glabridin was very potent inhibitor of monophenolase activity and diphenolase activity which IC_{50} was 0.09 μM (0.03 μg/ml) and 3.94 μM (1.28 μg/ml), respectively which stronger than that of other tyrosinase inhibitors such as isoliquitigenin, glabrene, kojic acid, arbutin, quercitin and resveratrol. They continuously reported that IC_{50} value of glabridin in inhibition of cultured G361 human melanocytes pigmentation was 2.4 μg/ml and glabridin, at the concentration of 10 μM (3.24 μg/ml) showed low cytotoxicity with 92.2% survival. Cao *et al.* (2007) reported that glabridin at 0.1 to 100 μM did not show significant cytotoxicity (<10%) to Caco-2 cells.

According to Curto *et al.* (1999), a potentially efficacious skin depigmentation agent is the substance that inhibits tyrosinase with $IC_{50} < 25$ μg/ml, inhibits melanocyte cell pigmentation with $IC_{50} < 100$ μg/ml and is noncytotoxic to cells with $IC_{50} > 100$ μg/ml. Therefore, glabridin is one of the potent substances and safe to use as skin lightening agent. Nowadays, many famous cosmetic companies such as L'OREAL, Shiseido, Avon, Kose, MaryKay, Christian Dior, Chanel, Nivea, Godiva use glabridin as a skin lightening agent in many cosmetic products such as

creams, lotions and solutions. The effective concentration of glabridin for skin-lightening is 0.025-0.05%w/v.

1.2.9 Cyclodextrin constitutes

Cyclodextrin is cyclic oligosaccharides consisted of a variable number of d-(+) glucopyranose units attached by α-(1,4) glucosidic bonds. The structure of glucopyranose units attached by glucosidic bond is shown in Figure 1.11. The natural cyclodextrins are α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin, which consist of six, seven and eight d-(+) -glucopyranose units, respectively. Different number of glucopyranose units lead to different cavity sizes. The molecular structure of natural cyclodextrins is shown in Figure 1.12. The cavity diameter of the natural cyclodextrins is shown in Figure 1.13. The molecular weight and water solubility of natural cyclodextrins are shown in Table 1.2.

Cyclodextrins are not perfectly cylindrical molecules, but are somewhat cone shaped because of the chair conformation of the glucopyranose units. The conical shape of the cyclodextrin molecule is shown in Figure 1.14. The hydroxyl functions are orientated to the cone exterior with the primary hydroxyl groups at the narrow edge of the cone and the secondary hydroxyl groups at the wider edge. The central cavity of the cyclodextrin molecule is lined with skeletal carbons and ethereal oxygens, which gives a lipophilic character. In aqueous solutions, the hydroxyl groups form hydrogen bonds with the water molecules resulting in a hydration shell around the dissolved cyclodextrin molecule, so that the inclusion complex can enhanced the solubility of poorly soluble compounds.

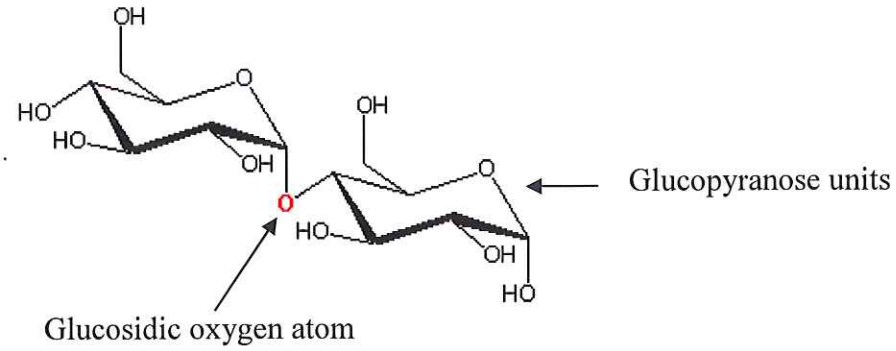

Figure 1.11 Structure of glucopyranose units attached by glucosidic bond (The Moyna Group, 2000).

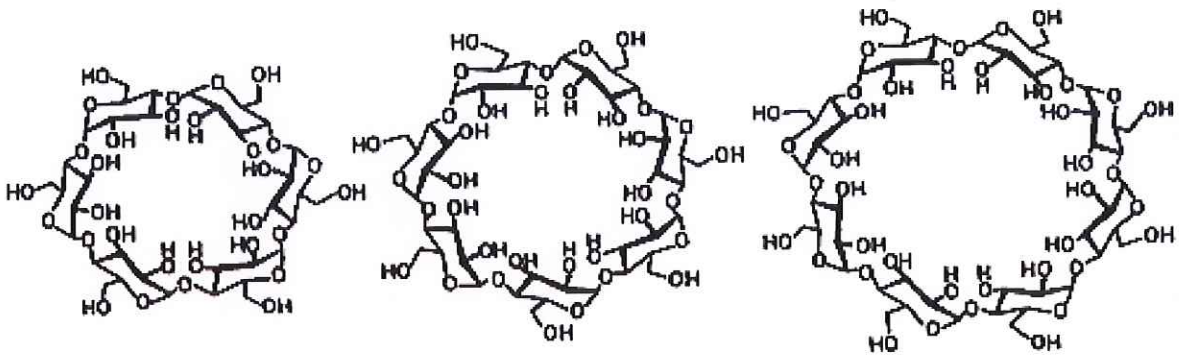

Figure 1.12 Molecular structure of natural cyclodextrins (Szejtli, 2004).

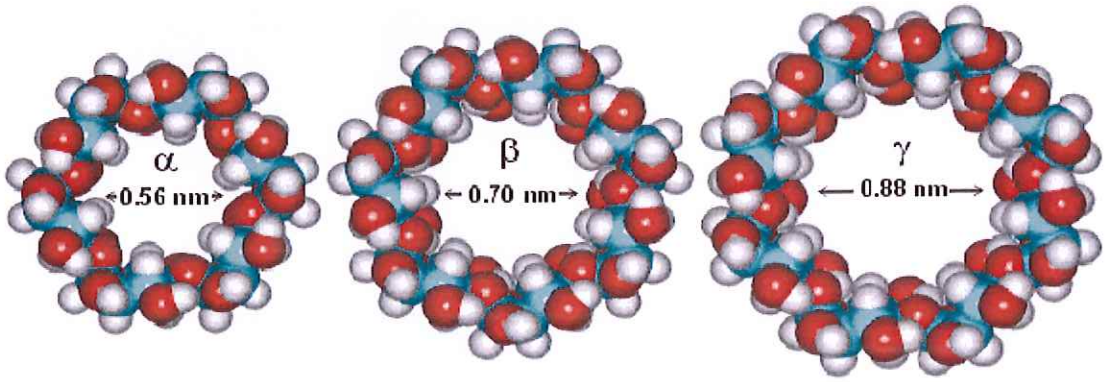

Figure 1.13 Cavity diameter of the natural cyclodextrins (Chaplin, 2011).

Table 1.2 The molecular weight and water solubility of natural cyclodextrins (Brewster and Loftsson, 2007).

Natural cyclodextrins	MW (Da)	Solubility in water (mg/ml)
α-cyclodextrin	972	145
β-cyclodextrin	1135	18.5
γ-cyclodextrin	1297	232

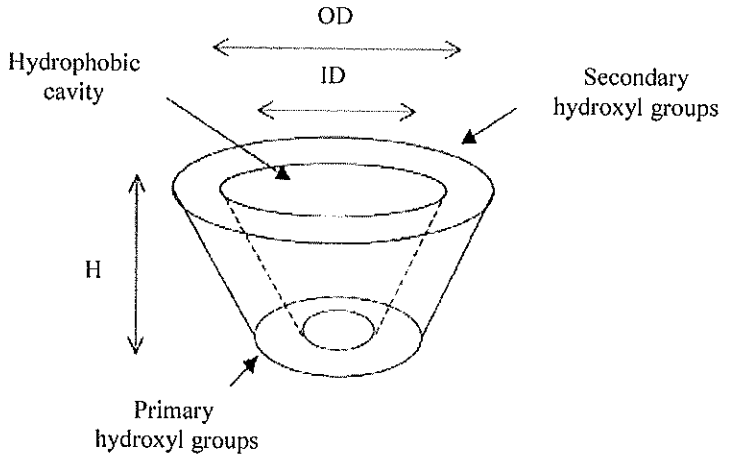

Figure 1.14 The conical shape of the cyclodextrin molecule (Brewster and Loftsson, 2007).

1.2.10 Cyclodextrin derivatives

The natural cyclodextrins have limited application in solubilizing guest molecules owing to their limited aqueous solubility, especially β-cyclodextrin, because of occurring strong intramolecular hydrogen bonding (Figure 1.15). In the natural cyclodextrins, every glucopyranose unit has three free hydroxyl groups. In β-cyclodextrin, 21 hydroxyl groups can be modified by substituting the hydrogen atom or the hydroxyl group by a large variety of substituting groups like alkyl-, hydroxyalkyl-, carboxyalkyl-, amino-, thio-, tosyl-, glucosyl-, maltosyl-, etc. groups.

Thousands of ethers, esters, anhydro- deoxy-, acidic, basic, etc. derivatives can be prepared by chemical or enzymatic reactions. The examples of cyclodextrin derivatives are hydroxypropyl derivatives of β-cyclodextrin and γ-cyclodextrin (i.e. HP-β-CD and HP-γ-CD), the randomly methylated β-cyclodextrin (RM-β-CD), sulfobutylether β-cyclodextrin (SBE-β-CD), and the so-called branched cyclodextrins such as maltosyl β-cyclodextrin (G_2-β-CD). The molecular structure and the solubility of some cyclodextrin derivatives are illustrated in Figure 1.16 and Table 1.3, respectively. Among industrially produced, standardized, available β-cyclodextrin derivatives, the most important ones are the heterogeneous, amorphous, highly water-soluble methylated β-cyclodextrin and 2-hydroxypropylated β-cyclodextrin. Hydroxypropyl-β-cyclodextrin is a chemically modified β-cyclodextrin obtained by treating a base-solubilized solution of β-cyclodextrin with propylene oxide. This cyclodextrin derivative has a much greater solubility than unfunctionalized β-cyclodextrin. The molecular structure of hydroxypropyl-β-cyclodextrin is shown in Figure 1.16.

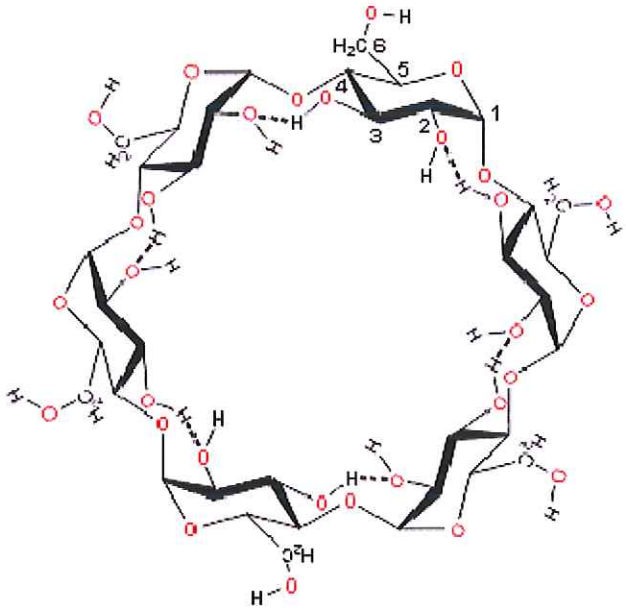

Figure 1.15 Intramolecular hydrogen bonding in the crystal lattice of cyclodextrin (Chaplin, 2011).

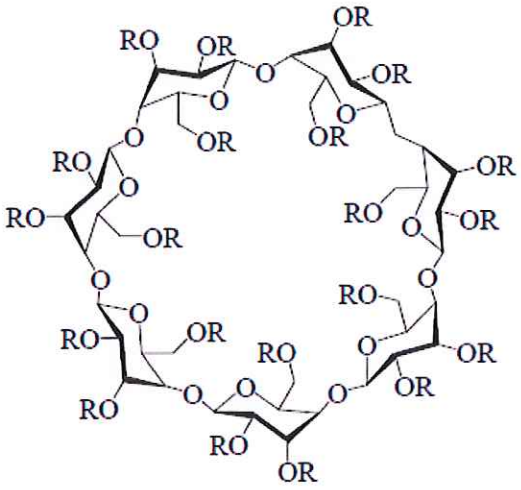

Figure 1.16 The molecular structure of some cyclodextrin derivatives (Magnúsdóttir *et al.*, 2002).

Randomly methylated β-cyclodextrin (R = CH_3 or H)

2-Hydroxypropyl-β-cyclodextrin (R = $CH_2CHOHCH_3$ or H)

Sulfobutylether β-cyclodextrin sodium salt (R = $(CH_2)_4SO_3Na$ or H)

Table 1.3 The molecular weight and water solubility of cyclodextrin derivatives (Brewster and Loftsson, 2007).

Cyclodextrin	Substitution[a]	MW (Da)	Solubility in water (mg/ml)
Hydroxypropyl-β-cyclodextrin	0.65	1400	>600
Hydroxypropyl-γ-cyclodextrin	0.6	1576	>500
Randomly methylated-β-cyclodextrin	1.8	1312	>500
Sulfobutylether-β-cyclodextrin	0.9	2163	>500

[a] Substitution: average number of substituent per glucopyranose unit.

1.2.11 Mechanism of complex formation in an aqueous solution

Cyclodextrin shows a hydrophilic outer surface and a lipophilic cavity to which a hydrophobic guest molecule can complex. With lipophilic inner cavity and hydrophilic outer surface, cyclodextrins are capable of interacting with a large variety of guest molecules to form non-covalent inclusion complexes.

The formation of cyclodextrin inclusion complex in an aqueous solution is shown in Figure 1.17. The slightly apolar cyclodextrin cavity is occupied by water molecules that are energetically unflavored (polar–apolar interaction), and therefore can be readily substituted by appropriate "guest molecules", which are less polar than water. One, two, or three cyclodextrin molecules contain one or more entrapped "guest" molecules. Most frequently, the host:guest ratio is 1:1 (Szejtli, 2004).

Inclusion complexes are formed by the insertion of the nonpolar molecule or the nonpolar region of guest molecule into the cavity of host molecule. The major structural requirement for inclusion complexation is a fit of the guest into the cavity of host molecule. The cavity of host must be large enough to suit the guest

and small enough to eliminate water. No covalent bonds are formed or broken during the complex formation and guest molecules in the complex are in rapid equilibrium with free molecules in the solution. The driving forces for the complex formation include release of enthalpy-rich water molecules from the cavity, electrostatic interactions, van der Waals interactions, hydrophobic interactions, hydrogen bonding and charge-transfer interactions (Brewster and Loftsson, 2007). The physicochemical properties of free guest molecules are different from those bound to the cyclodextrin molecules. Likewise, the physicochemical properties of free cyclodextrin molecules are different from those in the complex.

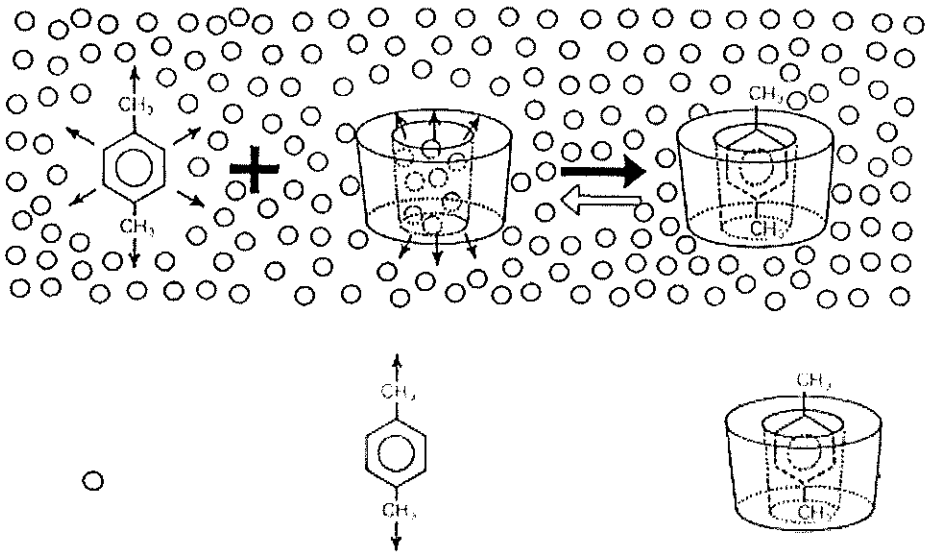

Figure 1.17 The formation of inclusion complex of the cyclodextrin and guest molecule (Szejtli, 2004).

1.2.12 Methods of preparing inclusion complex

The inclusion complex of guest molecule and cyclodextrin can be prepared by several methods such as slurry complexation, kneading, co-evaporation, co-precipitation, ultrasonic, spray drying and freeze-drying method and etc. The effectiveness or suitable method depends on the nature or properties of guest and cyclodextrin (Shinde, 2007). The physicochemical properties of inclusion complexes are influenced not only by the constituents but also by the preparation method.

Slurry complexation method

A slurry is a thick suspension of solid in a liquid. It is not necessary to dissolve the cyclodextrin completely to form a complex. Cyclodextrin can be added to water and stirred. The aqueous phase will be saturated with cyclodextrin in solution. Guest molecules will complex with the cyclodextrin in solution and the complex will precipitate out of the aqueous phase. The inclusion complex is separated and dried to give the product. The examples of inclusion complexes prepared by slurry complexation method were piliosanol-α-cyclodextrin, piliosanol-β-cyclodextrin and piliosanol-γ-cyclodextrin (Madhavi and Kagan, 2011).

Advantages:

- Generally, slurry complexation is performed at ambient temperature.
- The reduction of the amount of water needed.

Disadvantages:

- The amount of time required to complete the complexation is variable, and depends on type and molecular size of guest. Assays must be done to determine the amount of time required.

- With many guests, some heat may be applied to increase the rate of complexation, but care must be applied since too much heat can destabilise the complex and the complexation reaction may not be able to take place completely.

Kneading method

The kneading method is similar to the slurry complexation method. The cyclodextrin is dissolved with a minimal amount of water to form paste. The guest is added and mixed thoroughly. The resulting complex is dried and milled to obtain a powder form. The examples of inclusion complex prepared by this method were prazosin hydrochloride-β-cyclodextrin and prazosin hydrochloride-hydroxypropyl-β-cyclodextrin (Liu and Zhu, 2006).

Advantages:

- Simplicity and not high cost.
- The resulting complex can be dried directly.
- Not using of organic solvents.

Disadvantages:

- Sometimes, the dried paste forms a hard mass instead of a fine powder. Generally, the hard mass can be dried and milled to obtain a powdered form of complex.
- The amount of time required is depended on the guest.

Co-evaporation or evaporation method

For preparation of complexes using the co-evaporation method, the required quantities of guest molecules and cyclodextrin are dissolved in the solvent. Both solutions are mixed and the solvent is evaporated by heating. The resulting complex is pulverized and sieved. The example of inclusion complex prepared by this method was norflurazon-β-cyclodextrin (Villaverde *et al.*, 2004).

Advantages:

- Degree of mixing, amount of heating and time can be controlled.
- Not all guests are readily solubilised in water, making complexation either very slow or impossible. The use of an organic solvent to dissolve the guest is desirable.
- The guest and cyclodextrin are completely and easily dissolved.

Disadvantages:

- Hazard from solvent evaporated.
- The solvent must be selected to suitable for the guest and cyclodextrin.

Co-precipitation method

A solution of cyclodextrin is prepared by dissolving cyclodextrin in water and the guest compound is added slowly to the cyclodextrin solution. The solution of cyclodextrin and guest are cooled while stirring before a precipitate is formed. The precipitate is separated by centrifugation or filtration. The precipitate may be washed with cold water. Then the precipitate is dried. The examples of

inclusion complexes prepared by co-precipitation method were reported by Sapkal *et al.* (2007) and Farcas *et al.* (2006).

Advantages:

- Widely used method in the laboratory. It is performed with readily available equipment, a beaker and stirrer.

- Can easily see the complex forming and see that guest has been included in the complex by disappearance of the guest (Hedges, 1998).

Disadvantage:

- For scale-up, the limited solubility of the cyclodextrin, large volumes of water have to be used. Tank capacity, time and energy for heating and cooling may become important cost factors (Martin Del Valle, 2004).

Spray drying method

The cyclodextrin is dissolved in aqueous solution. The guest is dissolved in solvent and added to cyclodextrin solution under stirring. The clear solution is spray dried. A solid complex can be obtained by removal the water by evaporation. The example of inclusion complex prepared by this method was norflurazon-β-cyclodextrin (Villaverde *et al.*, 2004).

Advantage:

- This method can produce high yield.

Disadvantages:

- Precipitation must be controlled in order to avoid the particles becoming too large and blocking the spray nozzle.

- With volatile guests, some optimization of drying conditions is required in order to reduce the losses.

- Spray drying is not suitable means for drying highly volatile and heat-labile guests.

Freeze-drying method

The cyclodextrin is dissolved in aqueous solution. The guest is dissolved in solvent and added to cyclodextrin solution under stirring. The clear solution is frozen and then frozen solution is lyophilized in a freeze dryer. The examples of inclusion complexes prepared by this method were fixolide-β-cyclodextrin, fixolide-methyl-β-cyclodextrin and fixolide-hydroxypropyl-β-cyclodextrin (Chittiteeranon *et al.*, 2007).

Advantages:

- Low temperature reduces the loss of extremely volatile guests.
- This method is especially useful for heat labile guests.

Disadvantage:

- This method is time consuming.

Supercritical antisolvent technique

A supercritical fluid is a fluid used at temperatures and pressures above its critical values. In the supercritical antisolvent technique, carbon dioxide is used as antisolvent for the solute. The use of supercritical carbon dioxide is advantageous as its low critical temperature and pressure makes it attractive for processing heat-labile pharmaceuticals. It is also non-toxic, nonflammable, inexpensive, usable in relative mild process with the supercritical points of 73.8 bars at 31.1°C. and is much easier to

remove from the polymeric materials when the process is complete, even through small amount of carbon dioxide remains trapped inside the polymer, it poses no danger to the consumer. In this technique, guest compound and cyclodextrin are dissolved in a good solvent then the solution is fed into a pressure vessel under supercritical conditions, through a nozzle into supercritical fluid antisolvent. When the solution is sprayed into supercritical fluid antisolvent, the antisolvent rapidly diffuses into that liquid solvent as the carrier liquid solvent counter diffuses into the antisolvent. Because the supercritical fluid expanded solvent has lower solvent power than the pure solvent, the mixture becomes supersaturated resulting in the precipitation of the solute and the solvent is carried away with the supercritical fluid flow. The example of inclusion complex prepared by this method was benzocaine-cyclodextrin inclusion complex (Al-Marzougui *et al.*, 2007)

Advantages:

- Suitable for thermolabile guest compound.
- This is a non-toxic method as it is not utilizing any organic solvent.
- Reduce the time of preparation.

Microwave irradiation method

Microwave irradiation method makes it possible to obtain rapidly high temperatures inside irradiated products. For the preparation of inclusion complex, a mixture of guest compound and cyclodextrin with a minimum amount of solvents is subjected to microwave, most often for 90 seconds at 60°C (150 Watts). It also seems possible to subject the physical mixture itself to microwave treatment at 500-700 Watts for 5-10 minutes. The examples of inclusion complexes prepared by this

method were inclusion complexes of ziprasidone-β-CD and ziprasidone-HP-β-CD. In this method, molar (1:1) quantities of drug and cyclodextrins were weighed and transferred to a round bottom flask. Minimum amount of solvent mixture (methanol:water, 1:1 v/v) was then added to it. The mixture was reacted at 60°C for 2 minutes in the microwave oven. After reaction was complete, adequate amount of solvent mixture was added to remove the residual free drug and β-CD or HP-β-CD. Precipitate obtained was filtered through Whatman filter paper and dried in vacuum oven at 40°C for 48 hours (Deshmukh *et al.*, 2007)

Advantage:

- Reduce the time of preparation.

Disadvantage:

- This method is not suitable for heat labile guests.

1.2.13 Aplications of cyclodextrins in cosmetics

Since each guest molecule is individually surrounded by a cyclodextrin, this can lead to advantage changes in the chemical and physical properties of the guest molecule. The most important advantages for the use of cyclodextrins in cosmetics are protection of the guest molecules, solubility enhancement of the guest molecules in water, elimination of undesired odors and side effects, improvement of handling and establishment of controlled release.

Protection of the guest molecules

The formation of inclusion complex of poorly stable guests with cyclodextrins results in an improved stability of the guests due to protection by the cyclodextrin molecule. The guest molecules are protected from decomposition reactions induced by light or heat, oxidation or hydrolysis, chemical reactions with other organic compounds and loss by evaporation. Many examples of stabilization are known, for example reduced oxygen uptake of essential oils, vitamins or omega 3 oils; improved light or UV stability of vitamins and other compounds such as tea tree oil. Inclusion complex of tea tree oil with cyclodextrin demonstrates that the antimicrobial and antiinflammatory properties of tea tree oil are not affected by the complex formation and the complex protect the unsaturated bonds of the compounds in tea tree oil against damage by UV irradiation in simulated sunlight (UVA & UVB) at elevated temperatures. The cyclodextrin complex of tea tree oil showed no loss of content of the tea tree oil after 2 hours of irradiation at 45°C while physical mixture of tea tree oil and cyclodextrin loses 50% of the tea tree oil content after 30 minutes, and reaches 70% degraded tea tree oil after 1 hour (Wacker Chemical Corporation, 2011). The commercial product of tea tree oil-cyclodextrin inclusion complex is Epicutin-TT (CLR Chemisches Laboratorium Dr. Kurt Richter GmbH) that designed for application to problem skin.

Volatile compounds like perfumes or components of them are stabilized against evaporation as cyclodextrin complexes. Regiert (2007) reported that the preparation of linoleic acid inclusion complex with α-cyclodextrin showed highly stable to light. A cream with 4:1 α-cyclodextrin/linoleic acid complex did not smell rancid even after storage for 12 months. Other findings from Numanoğlu *et al.* (2007)

revealed that the volatility of linalool or benzyl acetate, fragrance materials, was decreased when inclusion complex with 2-hydroxypropyl-β-cyclodextrin at 1:1 molar ratio which performed in gel formulation for 6 months. The result showed that the decrease in linalool concentration of gel formulation containing uncomplexed linalool was 98%, while linalool complexed was 55%. The formation of thymol-β-cyclodextrin and geraniol-β-cyclodextrin inclusion complexes protected these volatile substances from oxidation (Mourtzinos *et al.*, 2008).

Solubility enhancement of the guest molecules in water

The solubility of guest molecule can be changed when complex with a cyclodextrin. The guest, in the cavity of the cyclodextrin, is essentially surrounded by the molecular of cyclodextrin. The hydrophobic groups of the guest that would be in contact with the water in the free state interact with the atoms of the cavity of the cyclodextrin instead. The outer surface of cyclodextrin interacts with the water resulting in increasing the solubility of the guest. The viscosity of cyclodextrin solution will be changed by the increasing of the concentration of cyclodextrin.

A large number of cosmetic components such as vegetable oils, hydrocarbons, higher fatty acids and their esters, vitamins, hormones, and preservatives are poorly soluble in water. All these chemical substances are able to form inclusion complexes with cyclodextrins. As a result, these complexes are more soluble compared to the pure compounds. This effect can be generally used for the formulation of cosmetic. The examples of cosmetic compounds that the water solubility is increased by complexing formation of cyclodextrin are salicylic acid, triclosan and menthol.

Salicylic acid is mainly used as antibacterial and keratolytic. The solubility of this acid in aqueous solution is low. The complex with cyclodextrin is much more soluble. The example of a commercial salicylic-hydroxypropyl cyclodextrin is Lipo® CD-SA (Lipo, 2011).

In many personal care products triclosan acts as a topical antiseptic and disinfectant. It is poorly soluble in water, moderately soluble in alkaline solutions and quite soluble in organic solvents. The Lipo® CD-TC (Lipo Chemicals, Inc) is a commercial triclosan-hydroxypropyl cyclodextrin complex which is soluble in water.

Menthol has an effect as a cooling agent in different cosmetic products. Menthol is slightly soluble in water. To increase the solubility, alcohol is normally added. The menthol-cyclodextrin complex is freely soluble in water, giving a clear solution without the presence of alcohols. The example of a commercial menthol-cyclodextrin inclusion complex is Lipo® CD-Menthol (Lipo Chemicals, Inc. USA).

Elimination of undesired odors and side effects

Cyclodextrins can protect the skin against the undesired side effects of guest molecule. Elimination of undesired and bad odors can also be achieved through complexation with cyclodextrins. When a guest included in a molecule of cyclodextrin, it is isolated and prevented from coming into contact with surface of the body where it could cause unwanted side effects such as odors, tastes and irritation. For examples, menthol is used as a cooling agent in various preparations. Due to the low water solubility of menthol, alcohol is usually added to the formulation as the solvent. Inclusion complex of menthol and cyclodextrin can enhanced the solubility of menthol in water and forms a clear solution without using alcohol. In addition, the

bitter taste and characteristic smell of menthol were resolved with the preparation of complexes with cyclodextrins. Mercapto compounds are widely used in waving lotions but they are the cause of bad odor that arises during application of the preparation. This odor is sometimes suppressed with perfume. The elimination of undesired odor can be improved by the formation of inclusion complex of these compounds with cyclodextrins.

Hara *et al.* (2002) found that the preparation of unsaturated aldehyde inclusion complex with cyclodextrins was demonstrated to reduce "aging odor". The methyl-β-cyclodextrin was the most effective type of cyclodextrin for the deodoration of "aging odor".

Improvement of handling

Inclusion complexes of guests with cyclodextrin can change the physical property of these compounds. As the guest is liquid or oily substance, the guest molecule which entrapped in the cavity of the cyclodextrin molecule may change from liquid form to powder form. For example, Koontz *et al.* (2009) found that the preparation of oily α-tocopherol inclusion complex with β-cyclodextrin results in a solid or dry powder. This inclusion complex is used as antioxidant in many skin care products in order to protect the stratum corneum surface from damage caused by free radicals. It should be used as a powder or suspended in lotions and creams. The formation of complex with cyclodextrin serves to enhance the stability of tocopherol.

Octyl methoxycinnamate is used as an oil-soluble sunscreen. As a cyclodextrin complex, it is not soluble in water or oil. The solid complex can be used in formulations as a powder or suspended in appropriate cream and lotion vehicles.

Peroxyacetic acid is a liquid that is handled as an aqueous solution. It forms solid complexes with cyclodextrins. These stable powders are easy to handle and they can be used in cosmetic formulations as skin lightening substance. These complexes also act as mild oxidants with disinfectant properties.

Establishment of controlled release

Inclusion complex with cyclodextrin is used in the cosmetic preparations to control the delivery of cosmetic ingredients as well as to protect those agents from environmental degradation. Considering volatile nature of fragrances, the fragrance molecules have to be formed as inclusion compounds with cyclodextrin molecules in order to retain fragrances for a long time. The inclusion complex trap fragrances for controlled or sustained release, protect particularly volatile components or suppress fragrance volatility and provide release at a delayed time. Complexation of the fragrances with cyclodextrins is intensively used in cosmetics. The inclusion complex of fragrance with cyclodextrin makes possible the establishment of controlled release since only the free fragrance section, which is in balance with the complex-forming section, is released. The examples of using inclusion complexes to control the release of fragrances include the use of cyclodextrin-fragrance complex in skin preparations such as talcum powder stabilizes the fragrance against the loss by evaporation and oxidation over a long period. Numanoğlu *et al.* (2007) reported that the volatility of linalool or benzyl acetate, fragrance materials, was decreased when

inclusion complex with 2-hydroxypropyl-β-cyclodextrin at 1:1 molar ratio. Wacker Chemical Corporation (2012) reported that 75% of rosemary oil evaporated in 2 hours at 40°C in water. Under the same conditions, the inclusion complex with HP-β-CD (CAVASOL® W7 HP) took 6 hours to evaporate. In the inclusion complex with γ-CD (CAVAMAX® W8) 80 % of rosemary oil was still present after 16 hours.

1.2.14 Examples of commercial cosmetic active ingredient-cyclodextrins inclusion complexes

Cyclodextrins used in cosmetic applications benefit from the same advantages as cyclodextrins used in food or pharmaceuticals. Many ingredients in cosmetics are unstable and break down quickly when exposed to atmospheric oxygen and light. These active ingredients are hosted inside complexes cyclodextrin molecules to protect them from air and light. After the products have been applied to the skin, the moisture in the skin triggers the release of the active ingredients at a controlled rate. Nowadays, there are many commercially available cosmetic active ingredients-cyclodextrins inclusion complexes.

- CAVAMAX®W7/menthol complex (Wacker fine chemicals) is an inclusion complex of menthol with pharmaceutical grade β-cyclodextrin. Menthol is one of the most important flavors and used in pharmaceuticals, cosmetics or personal care. Menthol stimulates cold receptors independent of any change in temperature and possesses analgesic properties by reducing the sensation of pain in topical application. Menthol is an efficient cooling ingredient. Major inconveniences like volatility, water solubility, irritant potential by contact with mucous membranes will be improved by complexation with CAVAMAX®W7.

- CAVAMAX®W8/retinol (Wacker fine chemicals) complex is an inclusion complex of retinol with pharmaceutical grade γ-cyclodextrin. Retinol is well known to reduce wrinkles and helps restore UV-damaged tissue. Retinol, the parent compound of vitamin A, can interrupt this vicious cycle. The fibroblasts absorb the retinol and convert it into retinoic acid. This active form of the vitamin stimulates collagen synthesis and inhibits the production of collagen-degrading enzymes. However, retinol itself is inherently unstable. Retinol can irritate the skin and is very quickly degraded by exposure to light and air. In this inclusion complex, the critical positions of the retinol molecule are protected by the cavity of gamma-cyclodextrin. The controlled release of retinol has a further advantage in addition to protect it from the effects of oxygen and light. An anti-wrinkle cream containing 0.1% retinol can cause skin irritation. But when formulated with cyclodextrin as an inclusion complex, much higher concentrations of the active ingredient can be added to cosmetics without causing skin irritation, since the retinol is released gradually. This significantly increases the effectiveness of the anti-aging or anti-wrinkle products. CAVAMAX® W8/retinol complex is designed for use in skin care products and can be formulated in emulsion products.

- CAVAMAX®W8/d-alpha-tocopherol complex (Wacker fine chemicals) is an inclusion complex of natural d-alpha-tocopherol (vitamin E) with pharmaceutical grade γ-cyclodextrin. Tocopherol is naturally present in vegetable oils and is nature's best lipophilic antioxidant. It is used to prevent premature aging and reduces UV-induced skin and tissue damage. Tocopherol is sensitive to light and unstable against oxidants. It is another important anti-aging substance. Like retinol, it is ideal for complexation with cyclodextrins. This is because the lipophilic substances

can be readily accommodated by the hydrophobic cavity of the cyclodextrin molecules. Improving the stability of sensitive vitamin E in the host-guest complex has come so far that it is now possible to apply a coat containing vitamin E to metallic surfaces at temperatures of 220°C. A razor blade treated in this way released skin-conditioning tocopherol during shaving (Tausch, 2007). CAVAMAX®W8/d-alpha-tocopherol complex is designed for use in foods, nutraceuticals and skin care products, and can be formulated in emulsion products.

- CAVAMAX®W6/linoleic acid complex (Wacker fine chemicals) is an inclusion complex of linoleic acid (vitamin F) with pharmaceutical grade α-cyclodextrin. Linoleic acid is an essential fatty acid for humans and is a regular component of human skin. Its skin-care and restorative properties can counteract skin irritation, chronic light damage and age spots. It reduces the transepidermal water loss of the skin and is particularly effective when applied externally. However, its sensitivity to oxidation and tendency to go rancid prevent its use in cosmetic preparations. Complexing the linoleic acid with cyclodextrins stabilizes the active ingredient trapped in the cavity and prevents odors by protecting it against oxidation (Regiert, 2006). Complexation opens up the possibility of using linoleic acid in various cosmetic formulations and personal care products.

- CAVAMAX®W7/citral complex (Wacker fine chemicals) is an inclusion complex of citral with pharmaceutical grade β-cyclodextrin. Citral is a well-known terpene with a powerful lemon note and a wide range of uses in fragrances and flavors. This β-cyclodextrin inclusion complex of citral provides controlled release, stabilisation against temperature, light and oxidation and a reduced volatility of citral.

- CAVAMAX®W7/tea tree oil complex (Wacker fine chemicals) is an inclusion complex of tea tree oil with pharmaceutical grade β-cyclodextrin. Tea tree oil is a well known and efficient broad spectrum natural antimicrobial agent. This β-cyclodextrin inclusion complex of tea tree oil provides improved stability and low odor, controlled release, stabilisation against temperature, light and oxidation and a reduced volatility of tea tree oil.

Natural Odors & Polymers Pvt. Ltd launched the novel line of cosmetic delivery system based on cyclodextrin that permit more formulation flexibility of using cosmetic active ingredients. Cyclodextrin can isolate materials within its structure and hence oil can be delivered in powder form and non alcohol based formulation can be made. The commercial cyclodextrin-containing cosmetic active ingredients of this company are listed in Table 1.4.

Table 1.4 The commercial cyclodextrin-containing cosmetic active ingredients of the Natural Odors & Polymers Private Limited (Aneja, 2009).

Product Name	INCI Name
Sphera™ CD - M	Cyclodextrin, Menthol
Sphera™ CD - Vit - E	Cyclodextrin, Tocopheryl Acetate
Sphera™ CD - T	Cyclodextrin, Triclosan
Sphera™ CD - C	Cyclodextrin, Camphor
Sphera™ CD - EMC	Cyclodextrin, Ethylhexyl Methoxycinnamate
Sphera™ CD - SA	Hydroxypropyl Cyclodextrin, Salicylic Acid
Sphera™ CD - MS	Cyclodextrin, Methyl Salicylate

Lipo Chemicals, Inc., a worldwide supplier of chemical ingredients to the personal care, food, industrial and pharmaceutical industries launched the commercial cyclodextrin-containing cosmetic active ingredients as shown in Table 1.5.

Table 1.5 The commercial cyclodextrin-containing cosmetic active ingredients of the Lipo Chemicals Incorporation (Lipo, 2011).

Product Name	INCI Name
Lipo® CD-SA	Hydroxypropyl Cyclodextrin (and) Salicylic Acid
Lipo® CD-Menthol	Cyclodextrin (and) Menthol
Lipo® CD-TC	Hydroxypropyl Cyclodextrin (and) Triclosan
Lipo® CD-OMC	Cyclodextrin (and) Ethylhexyl Methoxycinnamate
Lipo® CD-E	Cyclodextrin (and) Tocopherol

1.2.15 Classification of phase solubility

Phase solubility method was described by Higuchi and Connors (1965). Phase solubility diagrams are frequently used to calculate stoichiometry of guest-cyclodextrin complexes. Experimentally, the drug or substrate (S) of interest is added to several vials that it is always in excess. To the drug or substrate, a constant volume of water containing successively larger concentrations of the cyclodextrin or ligand (L) is added. The vials are mixed at constant temperature until equilibrium is established. The solid drug is then removed and the solution assayed for the total concentration of S. A Phase solubility diagram is constructed by plotting the total molar concentration of S on the *y*-axis and the total molar concentration of L added on the *x*-axis (Figure 1.18). Phase solubility diagrams prepared in this way fall into two

major classes, A- and B-types. A-type curves are indicative for the formation of soluble inclusion complexes. A-curves are subdivided into A_L (linear increases of drug solubility as a function of cyclodextrin concentration), A_P (positively deviating isotherm) and A_N (negatively deviating isotherms) subtypes. A linear diagram (A_L-type systems) indicates that the complexes are first order with respect to cyclodextrin and first order with respect to drug that is the complex usually contains a 1:1 mole ratio of drug to cyclodextrin. Positive deviation from linearity (A_P-type systems) indicates formation of complexes that are first order with respect to the drug but second or higher order with respect to cyclodextrin that is the complex formed may have a drug-cyclodextrin mole ratio greater than one. Negative deviation from linearity (A_N-type systems) may be associated with ligand-induced changes in the dielectric constant of the solvent or self-association of the ligands at high cyclodextrin concentrations.

Sometimes stoichiometry of drug-cyclodextrin complexes cannot be derived from simple phase-solubility studies and drug-cyclodextrin complexes can self-associate to form water-soluble aggregates which then can further soluble the drug through non inclusion complex (Loftsson *et al.*, 2005). The second major class of phase solubility diagram is labeled type B diagram. B-type curves are suggestive of the formation of inclusion complexes of poor solubility. B-type curves are subdivided into B_S, B_L and B_I. B_S diagram shows the initial straight line portion is similar to that observed in type A diagrams, where the apparent solubility of drug is increased owing to soluble complex formation and through plateau region representing a maximum value of concentration of drug that additional quantities of cyclodextrins do not alter. The apparent solubility of the drug remains constant owing to precipitation of the

complex. B_L diagram shows not highly soluble and the break in the curve can be caused by saturation. B_I diagram shows that no initial rise in the apparent solubility of the drug is observed and indicative of insoluble complexes.

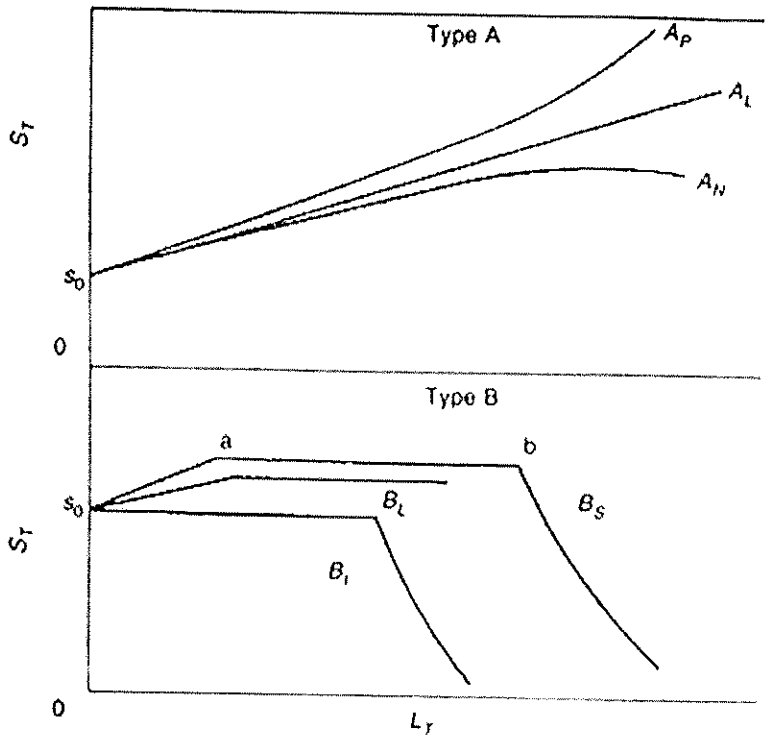

Figure 1.18 Phase solubility diagrams of type A and type B systems (Liu, 2008). L_T : total concentration of cyclodextrin; S_T : total concentration of drug

Measurements of stability or equilibrium constants (K_S)

Measurement of stability or equilibrium constants (K_S) of the drug-cyclodextrin complexes is important since this is an index of changes in physicochemical properties of a compound upon inclusion. The stability constant is

better expressed as $K_{m:n}$ to indicate the stoichiometric ratio of the complex. It can be carried out as follows:

$$\underset{[a-mx]}{m\text{CD}} + \underset{[b-nx]}{n\text{D}} \rightleftharpoons \underset{[x]}{\text{CD}m \bullet \text{D}n} \qquad (1.1)$$

$$K_{m:n} = \frac{[x]}{[a-mx]^{m}\,[b-nx]^{n}} \qquad (1.2)$$

Where a is the total concentration of the cyclodextrin and b is the total concentration of the drug (the sum of the complex and un-complex forms), D is drug or substrate and CD is cyclodextrin or ligand. To this point, intrinsic drug solubility is given as D_o and a formed complex is represented by D•CD.

The phase solubility diagrams not only allows a qualitative assessment of the complexes formed but may also be used to derive equilibrium constants. The equilibrium constants (K_C) for the formation of $[D_mCD_n]$ can be derived from A-type phase and represented by:

$$K_C = \frac{[D_m \bullet CD_n]}{[D]^m[CD]^n} \qquad (1.3)$$

where

$$[D] = D_o \qquad (1.4)$$

$$[D]_t = D_o + m[D_m \bullet CD_n] \qquad (1.5)$$

$$[CD]_t = [CD] + n[D_m \bullet CD_n] \qquad (1.6)$$

Then the values of $[D_m \cdot CD_n]$, [D] and [CD] can be derived as:

$$[D_m \cdot CD_n] = \frac{[D]_t - D_o}{m} \quad (1.7)$$

$$[CD] = [CD]_t - n[D_m \cdot CD_n] \quad (1.8)$$

where D_o is the equilibrium solubility of the drug in the absence of the CD, $[D]_t$ is the total concentration of the drug (i.e. the sum of the complexed and uncomplexed forms) and $[CD]_t$ is the total concentration of the cyclodextrin.

For phase solubility systems that are first order with respect to the cyclodextrin (n=1), the following equation may be derived:

$$D_t = \frac{mKD_o^{\,m}\, CD_t}{1 + KD_o^{\,m}} + D_o \quad (1.9)$$

A plot of $[D]_t$ versus $[CD]_t$ for formation of $D_m \cdot CD$ should give a straight line with the y-intercept representing D_O and the slope defined as:

$$\text{Slope} = \frac{mK\, D_o^{\,m}}{1 + KD_o^{\,m}} \quad (1.10)$$

Therefore, if m is known, K can be calculated. If $m = 1$ (i.e. a 1:1 drug:cyclodextrin complex forms), the following equation can be applied:

$$K_{1:1} = \frac{\text{slope}}{D_o\,(1\text{-slope})} \quad (1.11)$$

1.2.16 Characterization of inclusion complex

Inclusion complexes formed between the guest and cyclodextrin molecules can be characterized in order to check the existence of a true complex or just a mixture of guest and cyclodextrin, evaluate its stoichiometry, calculate its stability constant and discover its structure. Difference types of studies can be carried out both in the solid and solution states by the various methods including thermal analysis method, X-ray diffraction method, spectroscopic method, scanning electron microscopy and solubility study.

Thermal analysis method

Thermal analysis method includes all that examine a characteristic of the system as a function of temperature. Of these, differential scanning calorimetry (DSC) is the most highly regarded method. DSC enables the quantitative detection of all processes in which energy is required or produced (endothermic and exothermic phase transformations). The usual method of measurement is to heat the reference and test samples in such a way that the temperature of the two is kept identical. If an energy-requiring phase transition occurs in the test sample, extra heat is applied to this sample so that its temperature climbs at the same rate as in the reference. The additional heat required is recorded and used to quantitate the energy of the phase transition. Exothermic transitions, such as conversion of one polymorph to a more stable polymorph, can also be detected. Broad of a melting peak in the DSC of an inclusion complex indicates that the guest is present in an amorphous rather than a crystalline form. Since the method is quantitative in nature, the degree of crystallinity can also be calculated for systems in which the drug is partly amorphous and partly

crystalline. However, crystallinity of less than 2% cannot generally be detected with DSC (Leuner and Dressman, 2000).

X-ray diffraction method

The principle of X-ray diffraction is that when an X-ray beam is applied to the sample, interference bands can be detected. The angle at which the interference bands can be detected depends on the wavelength applied and the geometry of the sample with respect to periodicities in the structure. Crystallinity in the sample is reflected by a characteristic fingerprint region in the diffraction pattern. Owing to the specificity of the fingerprint, crystallinity in the guest can be separately identified from crystallinity in the carrier. However, crystallinity of under 5-10% cannot generally be detected with X-ray diffraction.

Spectroscopic method

Infrared (IR) spectroscopy is one of these methods that analyzed the interaction between cyclodextrin and the guest molecules in the solid state. Cyclodextrin bands often change only slightly upon complex formation and if the fraction of the guest molecules encapsulated in the complex is less than 25%, bands which could be assigned to the included part of the guest molecules are easily masked by the bands of the spectrum of cyclodextrin (Singh *et al.,* 2010). Structural changes and lack of a crystal structure can lead to change in bonding between functional groups which can be detected by infrared spectroscopy.

Scanning electron microscopy

The scanning electron microscope is a microscope that uses electrons instead of light to form an image. The instrument has many advantages over traditional microscopes. It has a large depth of field, which allows more of a specimen to be in focus at one time. It also has much higher resolution, so closely spaced specimens can be magnified at much higher levels. In this study, samples are fixed on a brass stub and made electrically conductive by coating with a thin layer of copper, gold or gold-palladium alloy. For a good comparison, samples should be observed at the same magnification. However, it is difficult to conclude as to the formation of an inclusion complexation because of the morphological change that occurs between the physical mixture and the product obtained. The preparation process generally has a great influence on the characteristic of the product. The products obtained from spray-drying process very often leads to small spheroids, the co-evaporated samples or kneaded samples shows large fragments, and the freeze-dried samples usually thin and more or less crystalline particles.

Solubility study

In solubility study, changes in solubility of the guest are plotted as a function of the cyclodextrin concentration. If the solubility of a potential guest increases with increasing cyclodextrin concentration, therefore, complex formation in solution is indicated.

1.2.17 Advantage and disadvantage of complexation

Solubilization by complexation is achieved through specific reactions rather than changes in bulk solvent properties as in other solubilizing systems such as cosolvents, emulsions, and pH adjustment. The dissociation is very rapid, quantitative and therefore predictable. Another significant advantage of the complexation technique is that some commonly used complexing agents such as hydroxypropyl-β-cyclodextrin and sulfobutylether-β-cyclodextrin are less toxic compared to other solubilizing agents such as surfactants and cosolvents. Since most complexes form are 1:1 complexes of the A_L-type, dilution of the complex will not result in a solution which is supersaturated with respect to drug. This can be rather important for very insoluble compounds that may precipitate on injection when solubilized by other systems such as cosolvents.

Despite all the attractive advantages of complexation, there are several disadvantages. First, the compound has to be able to form complexes with a selected ligand. For compound with very limited solubility to start with, the solubility enhancement can be very limited. Second, limitation is that for the complexes of A_P type, dilution of a system may still result in precipitation. This is also true for solubilization through combined techniques such as complexation with pH adjustment. Third, the potential toxicity issue, regulatory, and quality control issues related to the presence of the ligand may add to complication and cost of the development process. Finally, the complexation efficiency is often rather low thus relatively large amount of cyclodextrins are typically required to achieve desirable solubilization effect.

1.2.18 Lotions

Dosage forms are first classified as liquid or semisolid. Solutions, suspensions and lotions fall under the liquid category. Creams, gels, pastes and ointments belong to the semisolid category. The current Food and Drug Administration, Center for Drug Evaluation and Research Data Standards Manual defines lotions as "topical suspensions, solutions and emulsions intended for application to the skin". Solutions are clear, homogeneous liquids, while suspensions are two-phase (i.e., solid-in-liquid) liquids. The term lotions can then be reserved for those formulations that are liquid emulsions. Lotions have low- to medium-viscosity, below 50,000 cP. By contrast, creams and gels have higher viscosity. Lotions are liquid that viscosities display newtonian or pseudoplastic flow behavior with little or no force necessary to initiate flow (Buhse *et al.*, 2004).

1.3 Objectives of this study

- To study the effect of β-cyclodextrin, γ-cyclodextrin and hydroxypropyl-β-cyclodextrin on glabridin water solubility.
- To study the effect of preparation methods of inclusion complexes on glabridin water solubility.
- To characterize the physicochemical properties of glabridin inclusion complexes compared with pure glabridin and glabridin-cyclodextrins physical mixtures.
- To evaluate the tyrosinase inhibitory activity of glabridin-cyclodextrins inclusion complexes compared with pure glabridin and glabridin-cyclodextrins physical mixtures.

- To prepare glabridin solution-type lotion and evaluate the physical, chemical stability and tyrosinase inhibitory activity of glabridin solution-type lotion after storage for 3 months.
- To study *in vitro* release of glabridin lotions through cellulose acetate membrane.

CHAPTER 2

MATERIALS AND METHODS

Materials

1. Absolute alcohol (Lot no. 023600, Merck ®, Darmstadt, Germany)
2. Acetic acid (P.C. Drug Center Co., Ltd., Thailand)
3. Acetronitrile (Batch number 09050235, HPLC grade, Lab Scan, Thailand)
4. β-cyclodextrin (CAVAMAX® W7 Pharma, Wacker-Chemie GmbH, Germany)
5. Cellulose acetate membrane (Spectra/ Por®3 Dialysis Membrane, MWCO 3,500 Dalton, USA)
6. Dimethyl sulfoxide (Guangdong Guanghua, China)
7. Disodium EDTA (Namsiang Co., Ltd., Thailand)
8. Disodium phosphate (Na_2HPO_4) (Fluka, Switzerland)
9. Distilled water (Faculty of Pharmaceutical Sciences, Prince of Songkhla University, Thailand)
10. Gamma-cyclodextrin (CAVAMAX® W8 Pharma, Wacker-Chemie GmbH, Germany)
11. Glabridin (Vital-Chem Zhuhai Co., Ltd., China)
12. Hydroxypropyl cellulose, low viscosity (P.C. Drug Center Co., Ltd., Thailand)
13. Hydroxypropyl-β-cyclodextrin (CAVASOL® W7 HP Pharma, Wacker-Chemie GmbH, Germany)
14. Kojic acid (Lot no. 0805001, Namsiang Co., Ltd., Thailand)

15. L-DOPA (Lot no. 1394779, Sigma-Aldrich, China)
16. Propylene glycol (P.C. Drug Center Co., Ltd., Thailand)
17. Sodium metabisulfite (P.C. Drug Center Co., Ltd., Thailand)
18. Sodium phosphate monobasic dihydrate ($NaH_2PO_4.2H_2O$) (Fluka, Switzerland)
19. Tyrosinase (Lot no. 1179697, 2033 units/mg, Fluka, Switzerland)
20. Uniphen P-23 (Namsiang Co., Ltd., Thailand)

Equipments

1. Analytical balance (Mettler Toledo, Switzerland)
2. Differential scanning calorimeter (Perkin Elmer DSC7, USA)
3. FT-IR spectrophotometer (Spectrum One, Perkin Elmer Ltd., United Kingdom)
4. High performance liquid chromatography, model 10 AVP (Shimadzu, Japan)
5. HPLC column (Agilent, Zorbax 300 SB-C18, 4.6 mm ID x 150 mm column, particle size 5 μm, USA)
6. Incubator (Memmert, Germany)
7. Microplate reader, Power Wave X (Bio-TEX Instruments, USA)
8. Modified Franz diffusion cell (Hanson® 57-6M, Hanson Research Corporation, USA)
9. pH meter, PHM 82 (Radiometer, Denmark)
10. Powder X-ray diffractometer (Philips X'Pert MPD, Netherland)
11. Rotary evaporator (Eyela, Tokyo, Japan)
12. Syringe filter (Vertical Chromatography Co., Ltd., Thailand)
13. Ultrasonicator (Crest, New York, USA)
14. UV-Spectrophotometer (Genesys® 5 Miltonroy, USA)
15. Viscometer (DV-III ultra, Brookfield, USA)
16. Water bath shaker (WB-14, Memmert, Germany)

Methods

2.1 Standard curve of glabridin

The stock solution I of glabridin was prepared by dissolving accurate weight of glabridin (18.6 mg) with absolute alcohol and adjusting to 50 ml with absolute alcohol. The stock solution II of glabridin was prepared by pipetting 10 ml of stock solution I, transferring to 50 ml volumetric flask and adjusting to volume with absolute alcohol.

A serial of standard solutions was prepared by diluting stock solution II with distilled water to obtain concentrations of 7.443, 8.931, 14.885, 17.862 and 20.839 μg/ml. The absorbance of each standard solution of glabridin was analyzed by using a UV-spectrophotometer at 280 nm without the interference of CDs. The standard curve was constructed by plotting the absorbance value of glabridin against the concentration of glabridin. The equation with linear regression of the line was used to determine the concentration of glabridin from the samples.

2.2 Phase solubility study

The preliminary studied demonstrated that the solubility equilibrium was achieved within 3 days (the absorbance of glabridin on 3rd day did not differ from on 4th day). An excess amount of glabridin was added to 10 ml of β-CD solutions (0, 2.5, 5.0, 7.5, 10.0 and 15.0 mM) or γ-CD solutions (0, 2.5, 5.0, 7.5, 10.0 and 15.0 mM) or HP-β-CD solutions (0, 2.5, 7.5, 10.0, 15.0 and 20.0 mM). The reason for using β-CD or γ-CD concentrations from 0 to15 mM because of the limit solubility of β-CD, furthermore using HP-β-CD concentration from 0 to 20 mM because of HP-β-CD has

higher solubility than β-CD and γ-CD. The suspensions were shaken at 32±1°C until equilibrium (3 days). Then, the samples were withdrawn, filtered through a 0.45 μm membrane filter and suitably diluted with distilled water. Glabridin concentration was determined by using a UV spectrophotometer at 280 nm without the interference of CDs. Each experiment was carried out in triplicate. The apparent stability constant (K_S) was calculated from the phase-solubility diagram with the assumption of 1:1 stoichiometry according to the following equation:

$$K_S = \text{slope} / \text{Intercept (1-slope)} \qquad (2.1)$$

Slope is obtained from the initial straight-line portion of the plot of glabridin concentrations against CDs concentrations.

Intercept is the intrinsic solubility of glabridin in the absence of CDs (Brewster and Loftsson, 2007).

2.3 Preparation of glabridin-CDs inclusion complexes and glabridin-CDs physical mixtures

Inclusion complexes of glabridin with each of CDs (β-CD or γ-CD or HP-β-CD) in the molar ratio of 1:1 were prepared by kneading method and co-evaporation method.

Preparation of inclusion complex by kneading method

The β-CD (1.748 g or 1.541 mmol) or γ-CD (1.998 g or 1.541 mmol) or HP-β-CD (2.161 g or 1.541 mmol) was dissolved in 5 ml of distilled water. Glabridin (0.5 g or 1.541 mmol) was slowly added. The mixture was ground for 1 hour. The paste was dried at 45°C for 12 hours. The dried complex was pulverized and kept in a desiccator, protected from light.

Preparation of inclusion complex by co-evaporation method

The glabridin (0.5 g or 1.541 mmol) and β-CD (1.748 g or 1.541 mmol) or γ-CD (1.998 g or 1.541 mmol) or HP-β-CD (2.161 g or 1.541 mmol) were dissolved in 40 ml of absolute alcohol. The solvent was evaporated by using a rotary evaporator at 45°C. The dried complex was pulverized and kept in a desiccator, protected from light.

Preparation of glabridin-CDs physical mixtures

Physical mixtures of glabridin (0.5 g or 1.541 mmol) and β-CD (1.748 g or 1.541 mmol) or γ-CD (1.998 g or 1.541 mmol) or HP-β-CD (2.161 g or 1.541 mmol) were prepared by simply mixing until homogeneous (Liu and Zhu, 2006) and kept in a desiccator, protected from light.

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2.4 Determination of glabridin content from glabridin-CDs inclusion complexes and glabridin-CDs physical mixtures

The glabridin content from glabridin-CDs (β-CD or γ-CD or HP-β-CD) inclusion complexes and physical mixtures was determined by dissolving accurate weight (equivalent to glabridin 0.01g) of sample with absolute alcohol and transfering

to a 50 ml volumetric flask and adjusting to 50 ml with absolute alcohol. Then, the solution was suitable diluted with distilled water. The glabridin content was analyzed by using a UV-spectrophotometer at 280 nm.

2.5 Water solubility study of pure glabridin, glabridin-CDs inclusion complexes and glabridin-CDs physical mixtures

The solubility of glabridin-CDs (β-CD or γ-CD or HP-β-CD) inclusion complexes compared with glabridin-CDs physical mixtures and pure glabridin was determined.

The excess amount of sample (25 mg) was added to a test tube containing 10 ml of distilled water. The mixture was shaken at 32±1°C until equilibrium (3 days). Then, the sample was withdrawn, filtered through 0.45 μm membrane filter and suitable diluted with distilled water. Glabridin concentration was determined by using a UV-spectrophotometer at 280 nm. Each experiment was carried out in triplicate.

2.6 Physicochemical characterization

Physicochemical properties of glabridin-CDs (β-CD or γ-CD or HP-β-CD) inclusion complexes compared with glabridin-CDs physical mixtures and pure glabridin were studied.

Fourier transform infrared (FT-IR) spectroscopy

FT-IR spectra of glabridin, β-CD, γ-CD, HP-β-CD, glabridin-CDs inclusion complexes and glabridin-CDs physical mixtures were analyzed by FT-IR

spectroscopy. Each spectrum was recorded in the frequency range of 4,000-400 cm^{-1} and 16 scans were obtained at 4 cm^{-1} resolution. Potassium bromide pellet method was used for determination. The sample (10 mg) was mixed with potassium bromide (100 mg). The KBr disk was prepared by compressing the powder under force in a hydraulic press (Lui and Zhu, 2006).

Powder X-ray diffractometry (PXRD)

Powder X-ray diffraction patterns of glabridin, β-CD, γ-CD, HP-β-CD, inclusion complexes and physical mixtures were analyzed by using a Powder X-ray diffractometer with Ni filtered Cu K_α (λ =1.54 °A) line as the source of radiation. This instrument was operated at the voltage of 40 kV and the current of 45 mA. Each sample was weighed in a cavity of an aluminum sample holder, smoothed with a glass slide and inserted into the sample holder. The sample and detector were moved in a circular path to determine the angles of scatted radiation. The sample was analyzed in the 2θ angle range between 0° and 60° with a scan speed of 3θ/min and step size of 0.01°.

Differential scanning calorimetry (DSC)

Differential scanning calorimeter was used to determine melting point characterization of the glabridin, β-CD, γ-CD, HP-β-CD, glabridin-CDs inclusion complexes and glabridin-CDs physical mixtures. The sample was accurately weighed in aluminum pans and crimped (sealed). A sealed empty pan was used as reference and heated from 50-500°C at scan rate of 20°C/minute under the nitrogen stream with the flow rate of 20 cm^3/minute.

2.7 Preparation of lotions containing pure glabridin, glabridin-HP-β-CD physical mixture and glabridin-HP-β-CD coevaporated sample as cosmetic active ingredient

Three formulated lotions containing pure glabridin, glabridin-HP-β-CD physical mixture and glabridin-HP-β-CD coevaporated sample as cosmetic active ingredient were prepared. The concentration of glabridin in each formulation was 0.025%w/v (250 μg/ml) or in case of glabridin-HP-β-CD physical mixture and glabridin-HP-β-CD coevaporated were equivalent to glabridin 0.025%w/v.

The glabridin-HP-β-CD co-evaporated sample which exhibited the highest solubility of glabridin was selected to prepare glabridin solution-type lotion. The composition of each ingredient used in these lotions is listed in Table 2.1. The lotion was prepared by simple mixing of all ingredients in distilled water. The blank lotion (vehicle without glabridin) was consisted of the same amount of ingredients listed in Table 2.1 without glabridin.

Table 2.1 The composition of each ingredient used in lotions.

Suspension-type lotion containing glabridin		
Function	**Ingredients**	**Amount**
1. Active ingredient	Glabridin	0.025 g
2. Humectant	Propylene glycol	5.0 ml
3. Viscosity modifier	Hydroxypropyl cellulose (low viscosity)	0.01 g
4. Antioxidant	Sodium metabisulfite	0.1 g
5. Chelating agent	Disodium EDTA	0.02 g
6. Preservative	Methyl, ethyl, propyl, butyl parabens and 2-phenoxyethanol (Uniphen P-23)	0.3 ml
7. Solvent	Purified water	q.s. to 100 ml
Suspension-type lotion containing glabridin-HP-β-CD physical mixture		
Function	**Ingredients**	**Amount**
1. Active ingredient	Glabridin-HP-β-CD physical mixture	Equivalent to glabridin 0.025 g [a]
2. Humectant	Propylene glycol	5.0 ml
3. Viscosity modifier	Hydroxypropyl cellulose (low viscosity)	0.01 g
4. Antioxidant	Sodium metabisulfite	0.1 g
5. Chelating agent	Disodium EDTA	0.02 g
6. Preservative	Methyl, ethyl, propyl, butyl parabens and 2-phenoxyethanol (Uniphen P-23)	0.3 ml
7. Solvent	Purified water	q.s. to 100 ml
Solution-type lotion containing glabridin-HP-β-CD co-evaporated sample		
Function	**Ingredients**	**Amount**
1. Active ingredient	Glabridin-HP-β-CD co-evaporated sample	Equivalent to glabridin 0.025 g [a]
2. Humectant	Propylene glycol	5.0 ml
3. Viscosity modifier	Hydroxypropyl cellulose (low viscosity)	0.01 g
4. Antioxidant	Sodium metabisulfite	0.1 g
5. Chelating agent	Disodium EDTA	0.02 g
6. Preservative	Methyl, ethyl, propyl, butyl parabens and 2-phenoxyethanol (Uniphen P-23)	0.3 ml
7. Solvent	Purified water	q.s. to 100 ml

[a] 0.135 g of glabridin-HP-β-CD co-evaporated sample or glabridin-HP-β-CD physical mixture

2.8 Tyrosinase inhibitory activity of glabridin, β-CD, γ-CD, HP-β-CD, glabridin-CDs physical mixtures, glabridin-CDs inclusion complexes and glabridin solution-type lotion

Tyrosinase inhibition of glabridin, β-CD, γ-CD, HP-β-CD, glabridin-CDs physical mixtures, glabridin-CDs inclusion complexes and glabridin solution-type lotion was analyzed by using the modified dopachrome method with L-DOPA as substrate (Masuda *et al.,* 2005). Assays were conducted in a 96-well plate. Kojic acid was used as positive control. Test sample was dissolved with dimethyl sulfoxide. The tyrosinase reaction was prepared by adding 120 µl of phosphate buffer (20 mM, pH 6.8), 30 µl of tyrosinase (203.3 units/ml) or 30 µl of phosphate buffer (in case of sample blank or control blank) and 20 µl of DMSO with or without an added sample into the well and incubated at 25°C for 10 minutes. The absorbance of the sample was measured by using a micro plate reader at 492 nm. Then, 30 µl of L-DOPA (0.85 mM) was added and incubated at 25°C for 20 minutes. Afterwards, the absorbance was determined by micro plate reader at 492 nm. The final volume of tyrosinase reaction is 200 µl per well. The percentage tyrosinase inhibition was calculated by the following equation:

$$\%\ inhibition = 1 - \frac{A - B}{C - D} \times 100 \qquad (2.2)$$

Where

A: The difference of optical density before and after incubation at 492 nm with test sample (sample)

B: The difference of optical density before and after incubation at 492 nm with test sample, but without enzyme (sample blank)

C: The difference of optical density before and after incubation at 492 nm without test sample (control)

D: The difference of optical density before and after incubation at 492 nm without test sample and enzyme (control blank)

2.9 Stability study

2.9.1 Stability study of glabridin, glabridin-CDs physical mixtures and glabridin-CDs inclusion complexes

Glabridin, glabridin-CDs physical mixtures and glabridin-CDs inclusion complexes were kept at three temperatures (8°C, room temperature (30°C) and 45°C) and protected from light. The content of glabridin was determined immediately after preparation and after storage for 3 months.

2.9.2 Stability study of glabridin solution-type lotions

The glabridin solution-type lotion was stored in 30-ml glass bottles, and protected from light at three temperature (8°C, room temperature (30°C) and 45°C)

2.9.2.1 Physical stability

The general appearances such as clarify (visual precipitation), odor and color were observed. The viscosity was determined by using a viscometer at 250 rpm, 25°C. The pH of samples was determined by using a pH meter at 25°C.

hydrated cellulose acetate membrane was mounted between the donor and receptor compartment of the diffusion cell (diffusion area, 1.77 cm^2). The cell was clamped and the receiver medium was stirred continuously at the speed of 200 rpm with magnetic bar and magnetic stirrer. The circulating water bath was maintained at 37°C in order to ensure the surface cellulose acetate temperature of 32°C. After equilibrium for 30 minutes, 1 ml of each sample (equivalent to glabridin 250 µg/ml) was loaded into the donor chamber. During the study, the donor chamber and the sampling port of receiver chamber were covered by parafilm in order to prevent evaporation. One ml of sample was withdrawn from the receiver chamber at predetermined time intervals (0.5, 1, 2, 3, 6, 9, 12 hours) and replaced immediately after sampling with the same volume of fresh medium maintained at 32°C. The glabridin content was determined by HPLC. Each experiment was performed in quadruplet. The cumulative amount of glabridin released per unit area was plotted against time. The release rate was calculated from the slope of release profile using linear regression analysis.

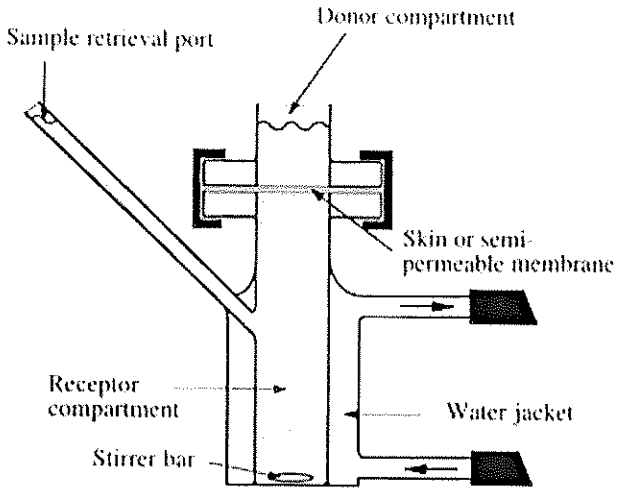

Figure 2.1 The modified Franz diffusion cell (Masson *et al.*, 1999).

The cumulative amount of glabridin released can be calculated according to the following equation (Thakker and Chern, 2003):

$$Qt = \frac{CmVo + \sum_{i=1}^{m-1} CiV}{A} \qquad (2.3)$$

Where

Q*t*: cumulative amount of glabridin at sampling m time per area (μg/cm^2)

C*m*: glabridin concentration at sampling m time (μg/ml)

C*i*: glabridin concentration at sampling i time (μg/ml)

V*o*: volume of receptor chamber (ml)

V: volume of sampling (ml)

A: effective diffusion surface area (cm^2)

The release profiles were further evaluated using three different models as follows: Zero order equation; $Q = Q_0 + k_0t$, First order equation; $\ln Q = \ln Q_0 + k_1t$, Higuchi equation; $Q = k_Ht^{1/2}$, K_0, K_1 and K_H are release rate constants of zero order, first order and Higuchi model, respectively.

2.11 Statistical analysis

The data (solubility, %tyrosinase inhibitory activity and the amount of glabridin released) were expressed as means ± standard deviation (SD). The initial concentration of glabridin was defined as 100%. The means concentrations of glabridin for each time were determined and converted to percentage of initial concentration. The percentage initial glabridin remaining in each preparation was compared to its initial concentration. The stability was defined as the remaining not less than 90% of the original concentration of the active ingredient. The statistical comparison was performed using Student's t-test or one-way analysis of variance (ANOVA). Differences were considered statistically significant at $p<0.05$.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Standard curve of glabridin

UV absorbance of various concentrations of glabridin in distilled water is shown in Table 3.1. A standard curve of glabridin solution is shown in Figure 3.1. A standard curve of glabridin is linear over the range between 7.443 and 20.839 μg/ml with coefficient of determination (R^2) of 0.9999.

Table 3.1 UV absorbance of various concentrations of glabridin in distilled water.

Concentration of glabridin (μg/ml)	Absorbance [a]
7.443	0.264 (0.007)
8.931	0.312 (0.005)
14.885	0.520 (0.004)
17.862	0.628 (0.008)
20.839	0.731 (0.006)

[a] mean ± SD (n=3)

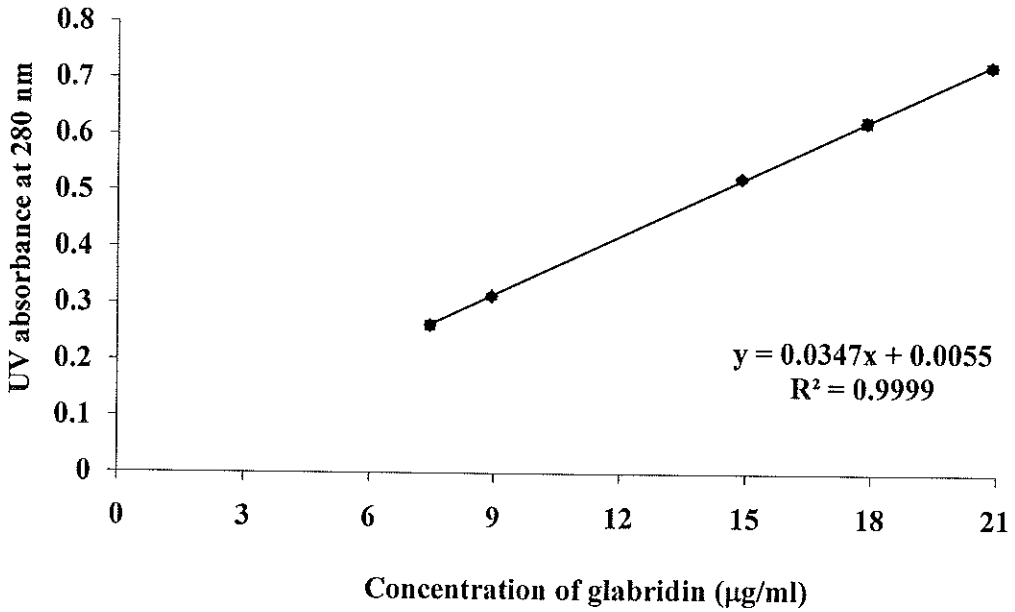

Figure 3.1 A standard curve of glabridin solutions. The line is a linear regression fit to the data. The plotted data are mean ± SD (n=3).

3.2 Phase solubility study

Phase solubility study of glabridin in various concentrations of β-CD

The solubility of glabridin in the presence of various β-CD concentrations (0-15 mM) at 32±1°C is shown in Table 3.2. The phase solubility diagram of glabridin-β-CD is shown in Figure 3.2. The phase solubility diagram of glabridin-β-CD could be classified as the A_L type, slope value of 0.2756 and coefficient of determination (R^2) of 0.9988. The solubility of the glabridin is increased linearly as the function of β-CD concentrations in the range of 0-15 mM. The diagram is a straight line with slope less than 1 and it may be ascribed to the formation of a 1:1 complex stoichiometry.

Table 3.2 Solubility of glabridin in various concentrations of β-CD at 32±1°C.

Concentration of β-CD (mM)	Concentration of glabridin (mM) [a]
0	0.004 ± 0.001
2.5	0.688 ± 0.022
5.0	1.372 ± 0.034
7.5	2.055 ± 0.035
10.0	2.847 ± 0.037
15.0	4.214 ± 0.049

[a] mean ± SD (n=3)

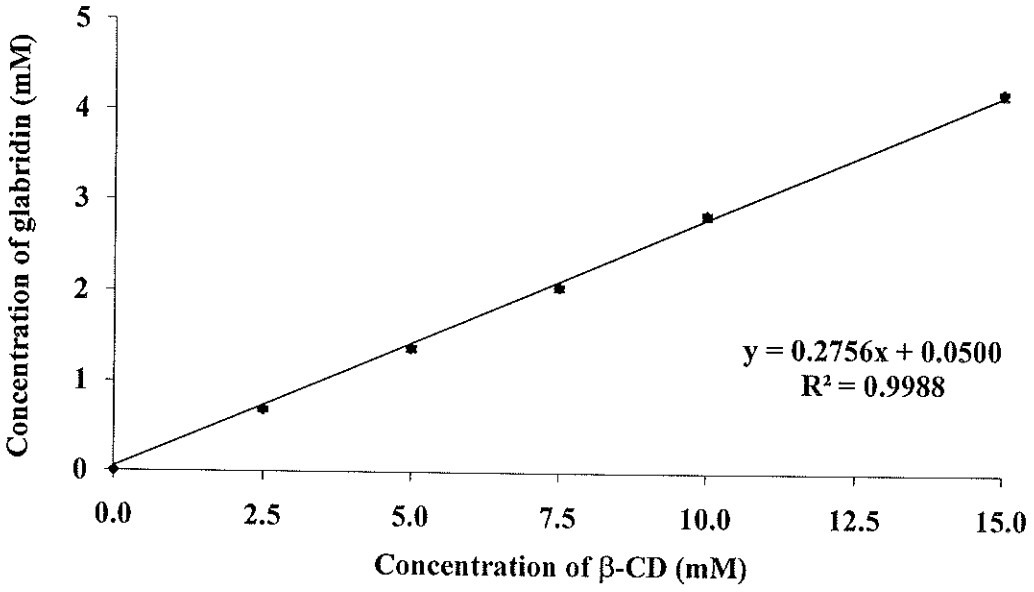

Figure 3.2 Phase-solubility diagram of glabridin-β-CD system at 32±1°C.

The plotted data are mean ± SD (n=3).

The apparent stability constant (K_s) is calculated according to equation 2.1. A value of K_s = 7,609 M^{-1} is obtained. The solubility of glabridin is increased with β-CD concentration at 15 mM to about 1,019 times when compared with pure glabridin.

Phase solubility study of glabridin in various concentrations of γ-CD

The solubility of glabridin in the presence of various γ-CD concentrations (0-15 mM) at 32±1°C is shown in Table 3.3. The phase solubility diagram of glabridin-γ-CD is shown in Figure 3.3.

Table 3.3 Solubility of glabridin in various concentrations of γ-CD at 32±1°C.

Concentration of γ-CD (mM)	Concentration of glabridin (mM) [a]
0	0.004 ± 0.001
2.5	0.030 ± 0.010
5.0	0.064 ± 0.005
7.5	0.095 ± 0.006
10.0	0.137 ± 0.010
15.0	0.195 ± 0.018

[a] mean ± SD (n=3)

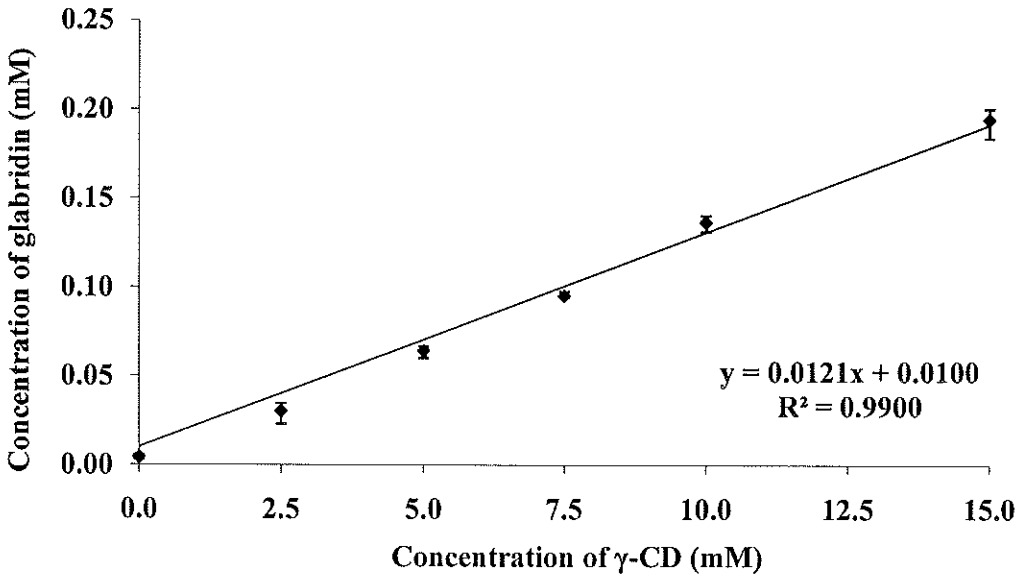

Figure 3.3 Phase-solubility diagram of the glabridin-γ-CD system at 32±1°C. The plotted data are mean ± SD (n=3).

The phase solubility diagram of glabridin-γ-CD could be classified as the A_L type with slope value of 0.0121 and coefficient of determination (R^2) of 0.9900. The solubility of the glabridin is increased linearly as the function of γ-CD concentrations in the range of 0-15 mM. The diagram is a straight line with slope less than 1 and it may be ascribed to the formation of a 1:1 complex stoichiometry. The apparent stability constant (K_s) is calculated according to equation 2.1. A value of K_s = 1,224 M^{-1} is obtained. The solubility of glabridin is increased with γ-CD concentration at 15 mM to about 48 times when compared with pure glabridin.

Phase solubility study of glabridin in various concentrations of HP-β-CD

The solubility of glabridin in the presence of various HP-β-CD concentrations (0-20 mM) at 32±1°C is shown in Table 3.4. The phase solubility diagram of glabridin-HP-β-CD is shown in Figure 3.4. The phase solubility diagram of glabridin-HP-β-CD could be classified as the B_L type. The solubility of the glabridin is increased linearly as the function of HP-β-CD concentrations in the range of 0-15 mM. When the concentration of HP-β-CD is more than 15 mM, the curve passes the plateau region and tends towards to the maximum value of concentration of glabridin owing to the limitation of solubility. The molar ratio of the inclusion complex was achieved from the initial ascending part of the curve, slope value of 0.4460.

Table 3.4 Solubility of glabridin in various concentrations of HP-β-CD at 32±1°C.

Concentration of HP-β-CD (mM)	Concentration of glabridin (mM) [a]
0	0.004 ± 0.001
2.5	1.447 ± 0.038
7.5	4.155 ± 0.033
10.0	5.198 ± 0.072
12.5	5.582 ± 0.106
15.0	6.363 ± 0.291
20.0	6.616 ± 0.128

[a] mean ± SD (n=3)

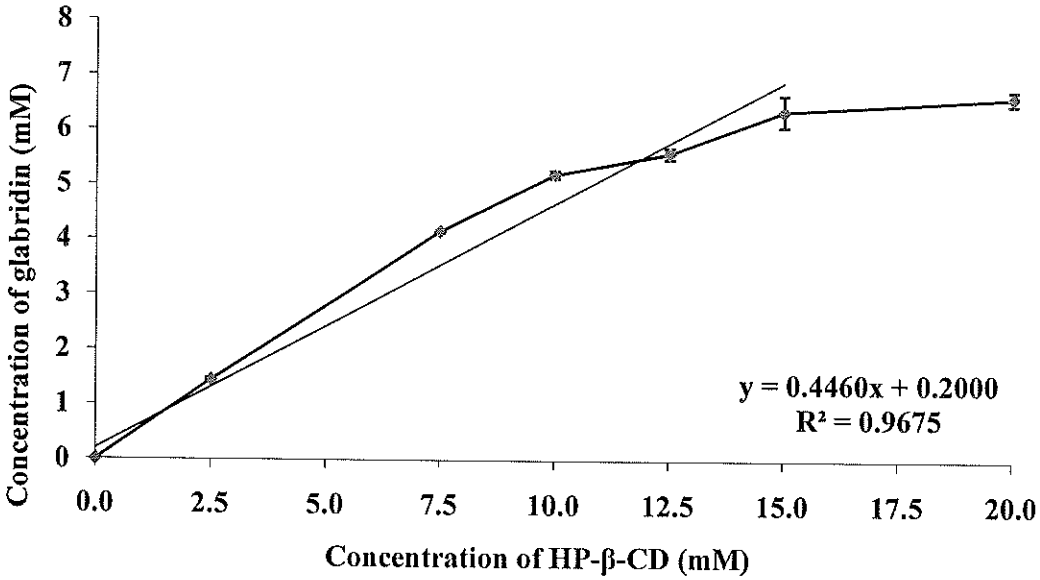

Figure 3.4 Phase-solubility diagram of the glabridin-HP-β-CD system at 32±1°C. The plotted data are mean ± SD (n=3).

The solubility of glabridin was increased with HP-β-CD concentrations. Solubility of glabridin was increased by 1,560-fold and 1,654-fold at 15 mM and 20 mM concentrations of HP-β-CD, respectively when compared with pure glabridin. The straight line of initial ascending part of the phase solubility diagram with slope less than 1 is obtained. This may be ascribed to the formation of a 1:1 complex stoichiometry. The apparent stability constant (K_s) calculated according to equation 2.1 is 4,025 M^{-1}.

Phase solubility analysis is used for evaluation of the affinity between cyclodextrins and guest molecule in water. According to the values of stability constants (K_s) of glabridin-β-CD, glabridin-γ-CD and glabridin-HP-β-CD, it is concluded that glabridin is stronger complex with β-CD than γ-CD. This result may

be owing to the cavity size of β-CD seems to be optimal for entrapment of glabridin molecule and consequently provides the greater stability constant (Naidu *et al.*, 2004). In addition, glabridin is stronger complex with β-CD than HP-β-CD. This result is probably due to the steric hindrance of the substitutive hydroxypropyl group of cyclodextrin (Ferreira *et al.*, 2004).

3.3 Preparation of glabridin-CDs inclusion complexes and glabridin-CDs physical mixrtures

Product of glabridin-β-CD inclusion complex

The kneaded sample was obtained as off-white, odorless and free flowing powder and shown in Figure 3.5(a). The inclusion complex prepared by co-evaporation method was unsuccessful because β-CD was slightly soluble in absolute alcohol, using a large amount of absolute alcohol to dissolve β-CD, indicated that this method was not suitable.

Product of glabridin-β-CD physical mixture

The physical mixture was obtained as off-white, odorless and free flowing powder.

Product of glabridin-γ-CD inclusion complex

The kneaded sample was obtained as off-white, odorless and free flowing powder and shown in Figure 3.5(b). The inclusion complex prepared by co-evaporation method was unsuccessful because γ-CD very slightly soluble in

absolute alcohol, using a large amount of absolute alcohol to dissolve γ-CD, indicated that this method was not suitable.

Product of glabridin-γ-CD physical mixture

The physical mixture was obtained as off-white, odorless and free flowing powder.

Product of glabridin-HP-β-CD inclusion complexes

The kneaded and co-evaporated samples were obtained as off-white, odorless and free flowing powder and shown in Figure 3.5(c) and Figure 3.5(d), respectively.

Product of glabridin-HP-β-CD physical mixture

The physical mixture was obtained as off-white, odorless and free flowing powder.

3.4 Determination of glabridin contents from glabridin-CDs inclusion complexes and glabridin-CDs physical mixtures

Table 3.5 shows glabridin contents from glabridin-CDs physical mixtures and glabridin-CDs inclusion complexes prepared by kneading and co-evaporation methods. These results showed that the glabridin contents were in the range between 95.01 and 105.91 %. This indicates the stability of glabridin during preparation process.

Table 3.5 Glabridin contents from glabridin-CDs physical mixtures and glabridin-CDs inclusion complexes prepared by kneading and co-evaporation methods.

	Samples	Glabridin content (%)[a]
1	Glabridin-β-CD, physical mixture	98.94 ± 0.486
2	Glabridin-β-CD, kneaded sample	98.59 ± 0.356
3	Glabridin-γ-CD, physical mixture	97.99 ± 0.687
4	Glabridin-γ-CD, kneaded sample	95.01 ± 0.552
5	Glabridin-HP-β-CD, physical mixture	101.19 ± 0.774
6	Glabridin-HP-β-CD, kneaded sample	98.69 ± 0.322
7	Glabridin-HP-β-CD, co-evaporated sample	105.91 ± 0.207

The amount of glabridin 0.01 g was defined as 100%

[a] mean ± SD (n=3)

3.5 Water solubility study of pure glabridin, glabridin-CDs inclusion complexes and glabridin-CDs physical mixtures

Water solubility of pure glabridin, glabridin-CDs physical mixtures and glabridin-CDs inclusion complexes prepared by kneading and co-evaporation methods at 32±1°C is shown in Table 3.6. The solubility of pure glabridin was found to be 1.33±0.44 μg/ml. The solubility of glabridin from physical mixtures and inclusion complexes was significantly ($p<0.05$) higher than that of pure glabridin. The increase in glabridin solubility from physical mixtures may be due to the surface tension lowering effect of cyclodextrin, resulting in wetting of hydrophobic glabridin surface and formation of water soluble inclusion complex (Dua *et al.*, 2007). Both glabridin-β-CD and glabridin-HP-β-CD kneaded samples gave higher glabridin solubility than that of their physical mixtures and pure glabridin. These results may be due to the increasing in glabridin wettability, hydrophilicity and reduction in glabridin crystallinity (Cunha-Filho *et al.*, 2007) as described in PXRD section. However, the slightly increased in glabridin solubility obtained from glabridin-γ-CD physical mixture and glabridin-γ-CD kneaded sample when compared with that of pure glabridin may be due to the lower K_s value (1,224 M^{-1}) of glabridin-γ-CD complex when compared with glabridin-β-CD complex (7,609 M^{-1}) and glabridin-HP-β-CD complex (4,025 M^{-1}). The lower value of stability constant of glabridin-γ-CD than that of glabridin-β-CD and glabridin-HP-β-CD indicates less affinity of γ-CD towards glabridin (Aleem *et al.*, 2008). Martin Del Valle (2004) reported that γ-CD can accommodate larger molecules such as macrocycles and steroids. The solubility of glabridin-HP-β-CD co-evaporated sample was 1,779.71± 7.56 μg/ml and 1,300-fold

increased in glabridin solubility compared with that of pure glabridin. The highest solubility of glabridin from glabridin-HP-β-CD co-evaporated sample may be due to the improvement of wettability, hydrophilicity, the lowering of interfacial tension between glabridin and water and the formation of higher energetic amorphous state of glabridin which confirmed by PXRD study.

Table 3.6 Water solubility of pure glabridin, glabridin-CDs physical mixtures and glabridin-CDs inclusion complexes prepared by kneading and co-evaporation methods at 32±1°C.

	Samples	Solubility of glabridin (μg/ml)[a]
1	Glabridin	1.33 ± 0.44
2	Glabridin-β-CD, physical mixture	582.57 ± 2.86
3	Glabridin-γ-CD, physical mixture	27.20 ± 0.33
4	Glabridin-HP-β-CD, physical mixture	978.76 ± 18.15
5	Glabridin-β-CD, kneaded sample	916.86 ± 7.60
6	Glabridin-γ-CD, kneaded sample	8.89 ± 0.30
7	Glabridin-HP-β-CD, kneaded sample	1,308.29 ± 20.60
8	Glabridin-HP-β-CD, co-evaporated sample	1,799.71 ± 7.56

[a] mean ± SD (n=3)

3.6 Physicochemical Characterization

Fourier-transform infrared spectroscopy (FT-IR)

The FT-IR study is a useful technique to analyze the complex formation between guest molecules and cyclodextrins in the solid state. Shifts or intensity changes in the characteristic bands of pure substance are considered as evidence of the complex existence. However, some of the changes are very subtle, requiring careful interpretation of the spectrum (Cunha-Filho *et al.*, 2007).

The FT-IR spectra of glabridin, β-CD, physical mixture and kneaded sample are shown in Figure 3.5. The FT-IR spectrum of glabridin showed two characteristic bands of the OH group which were found at 3528.39 cm^{-1} and 3350.41 cm^{-1}. The C-H stretching vibration appeared at 2965.46 cm^{-1}. Other characteristic bands were found at 1637.44 cm^{-1}, 1620.89 cm^{-1} and 1519.67 cm^{-1} for C=C stretching vibration of aromatic ring. The FT-IR spectrum of β-CD showed the vibration of free OH (3392.89 cm^{-1}), C-H stretching vibration (2925.35 cm^{-1}), H-O-H bending (1647.42 cm^{-1}). The FT-IR spectrum of glabridin-β-CD physical mixture gave similar pattern to the FT-IR spectrum of glabridin than the spectrum of β-CD. This result indicated that there was no interaction between glabridin and β-CD. The similar results were reported by Bongiorno *et al.* (2005) which revealed that the FT-IR spectrum of melatonin-β-CD physical mixture did not change in wave number of both species. However, the kneading system, the intense band of glabridin located at 3528.39 cm^{-1} and 3350.41 cm^{-1} was markedly reduced and shifted to 3529.41 cm^{-1} and 3351.45 cm^{-1}, respectively when compared with physical mixture. These results indicated that in the kneaded sample intermolecular hydrogen bonds between

glabridin and β-CD was formed. Furthermore, the intense band of glabridin in kneaded sample which located at 1637.44 cm^{-1} was markedly reduced when compared with physical mixture. This change was attributed to the aromatic ring (C=C) of glabridin (ring B, the molecular structure is on page 26) that might be inside the hydrophobic cavity of β-CD.

The FT-IR spectra of glabridin, γ-CD, physical mixture and kneaded sample are shown in Figure 3.6. The FT-IR spectrum of γ-CD showed the vibration of free OH (3401.42 cm^{-1}), C-H stretch (2929.41 cm^{-1}), H-O-H bending (1642.95 cm^{-1}). The FT-IR spectrum of glabridin-γ-CD physical mixture gave similar pattern to the FT-IR spectrum of glabridin than the spectrum of γ-CD. This result indicated that there was no interaction between glabridin and γ-CD. However, in the kneading system, not only the intense band of glabridin located at 3528.39 cm^{-1} was shifted to the lower frequency at 3523.80 cm^{-1} but also the intense band of glabridin located at 3350.41 cm^{-1} was shifted to the higher frequency at 3352.18 cm^{-1}. These results indicated that intermolecular hydrogen bonds between glabridin and γ-CD were formed. In addition, the intense bands of glabridin which located at 1620.89 and 1637.44 cm^{-1} were markedly reduced when compared with that of glabridin and physical mixture. These changes were attributed to the aromatic ring (C=C) of glabridin (ring B) that might be inside the hydrophobic cavity of γ-CD.

The FT-IR spectra of glabridin, HP-β-CD, physical mixture, kneaded sample and co-evaporated sample are presented in Figure 3.7. The FT-IR spectrum of HP-β-CD showed the vibration of free OH (3401.97 cm^{-1}), C-H stretching vibration (2930.08 cm^{-1}), H-O-H bending (1645.95 cm^{-1}). The FT-IR spectrum of

glabridin-HP-β-CD physical mixture was the summation of glabridin and HP-β-CD spectra, and the intense band of glabridin located at 3528.39 cm^{-1} was shifted to the lower frequency, 3518.20 cm^{-1}. These results indicated that intermolecular hydrogen bonds between glabridin and HP-β-CD were formed. In kneaded sample, the intense bands of glabridin located at 3528.39 cm^{-1} and 3350.41 cm^{-1} were shifted to 3523.80 cm^{-1} and 3353.55 cm^{-1}, respectively and markedly reduced when compared with those of glabridin and physical mixture. These results indicated that intermolecular hydrogen bonds between glabridin and HP-β-CD were formed. The intense bands of glabridin which located at 1620.89 cm^{-1} and 1637.44 cm^{-1} were markedly reduced when compared with physical mixture. These changes were attributed to the aromatic ring (C=C) of glabridin (ring B) that might be inside the hydrophobic cavity of HP-β-CD. However, in the co-evaporation system, the intense bands of glabridin located at 3528.39 cm^{-1} and 3350.41 cm^{-1} were disappeared completely and presented together with an absorption bands at 3391.90 cm^{-1}. This result indicated that intermolecular hydrogen bonds between glabridin and HP-β-CD of co-evaporated sample was formed and was stronger than that of physical mixture and kneaded sample. Furthermore, the intense bands of glabridin which located at 1620.89 $cm^{-1}$and 1637.44 cm^{-1} were markedly reduced and the intense band at 1519.67 cm^{-1} was shifted to 1524.26 cm^{-1} when compared with physical mixture. These changes were attributed to the aromatic ring (C=C) of glabridin (ring B) that might be inside the hydrophobic cavity of HP-β-CD. The similar results were reported by Wu *et al.* (2010) who revealed that the FT-IR spectrum of sulforaphane-HP-β-CD inclusion complex at 1452 cm^{-1} and 1350 cm^{-1} almost disappeared indicating that the group-CH_2-sulforaphane were entrapped into the host cavities.

In short, the co-evaporated sample exhibited a strong interaction between glabridin and HP-β-CD because of disappearance of intense bands of glabridin located at 3528.39 cm^{-1} and 3350.41 cm^{-1}, corresponding to OH group, and intense bands of glabridin located at 1620.89 cm^{-1} and 1637.44 cm^{-1} (aromatic ring (C=C) of glabridin) were markedly reduced when compared with physical mixture, indicating that aromatic ring (C=C) of glabridin (ring B) might be inside the cavity of HP-β-CD.

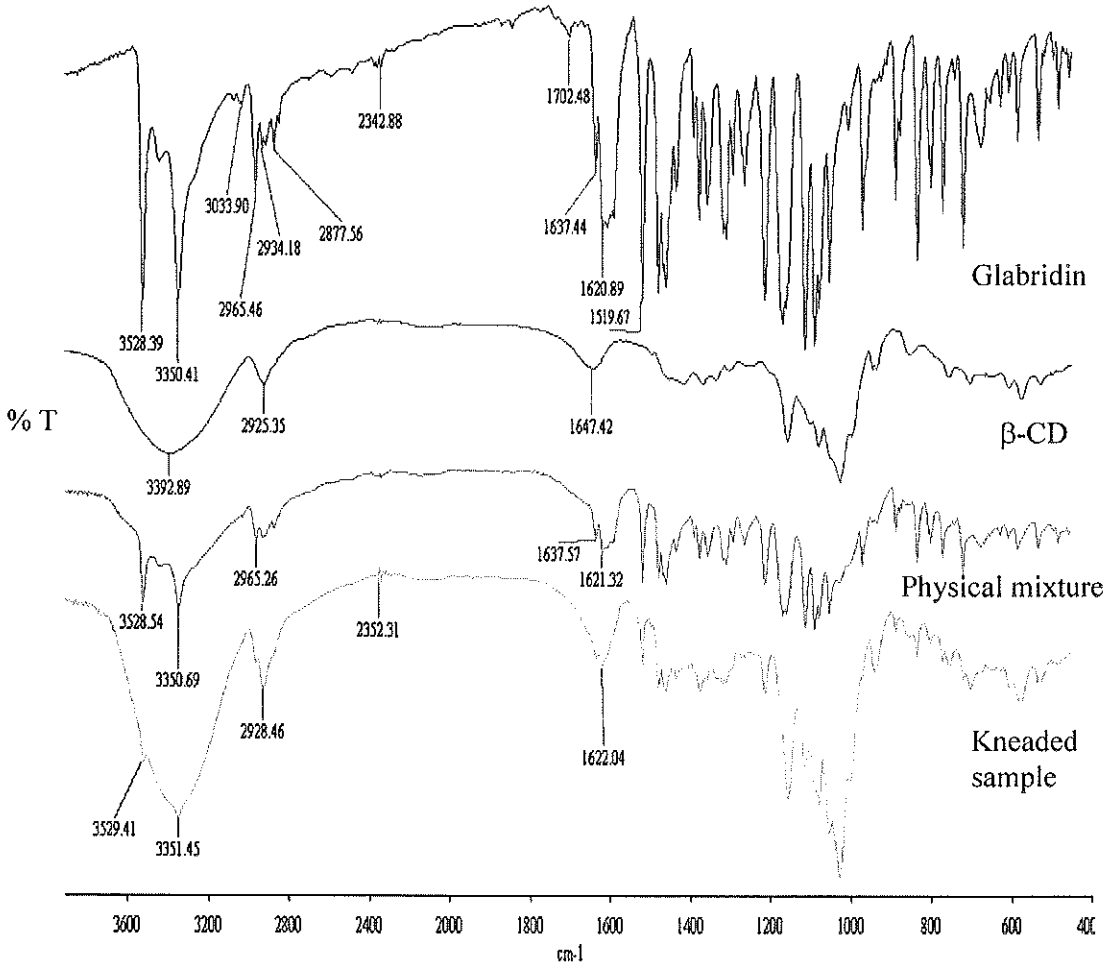

Figure 3.5 FT-IR spectra of glabridin, β-CD, physical mixture and kneaded sample.

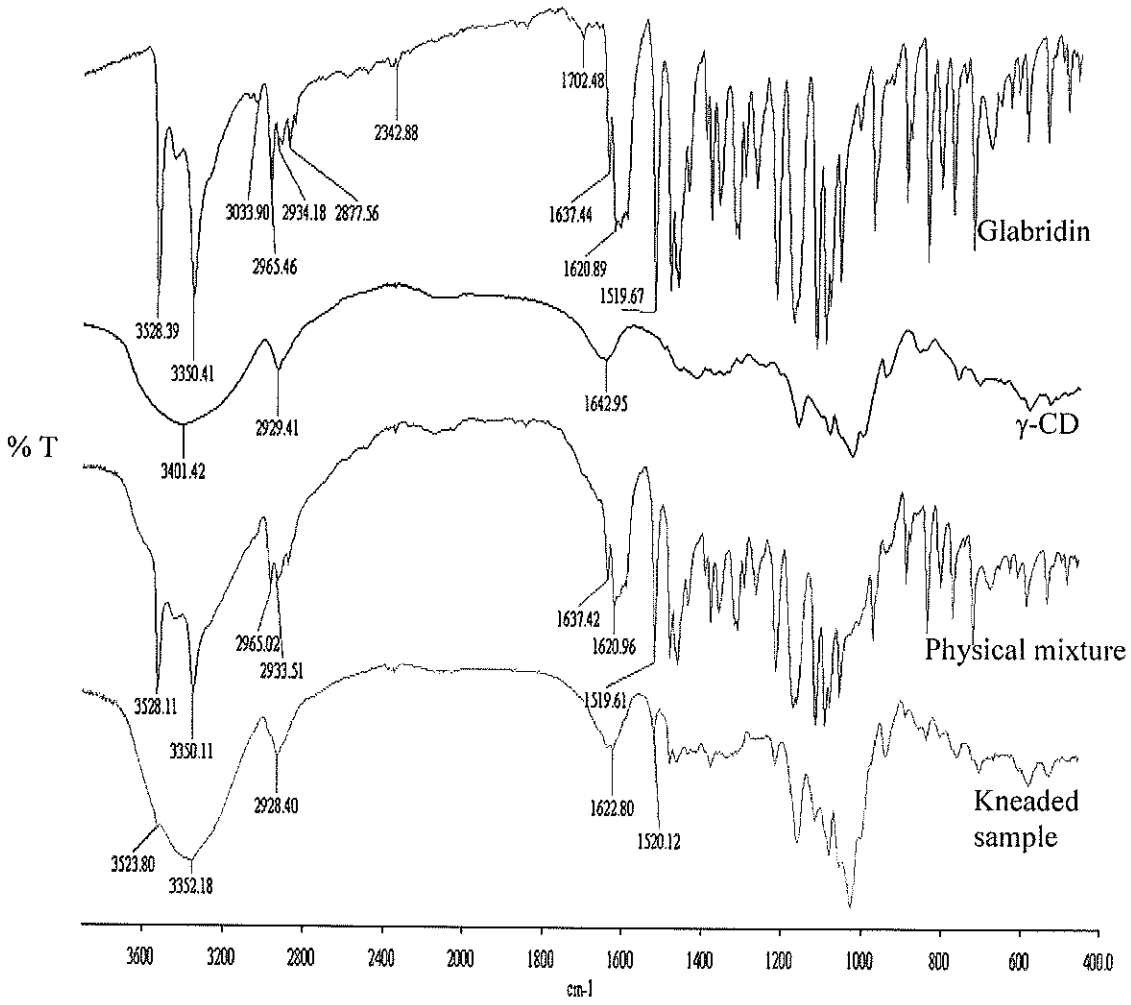

Figure 3.6 FT-IR spectra of glabridin, γ-CD, physical mixture and kneaded sample.

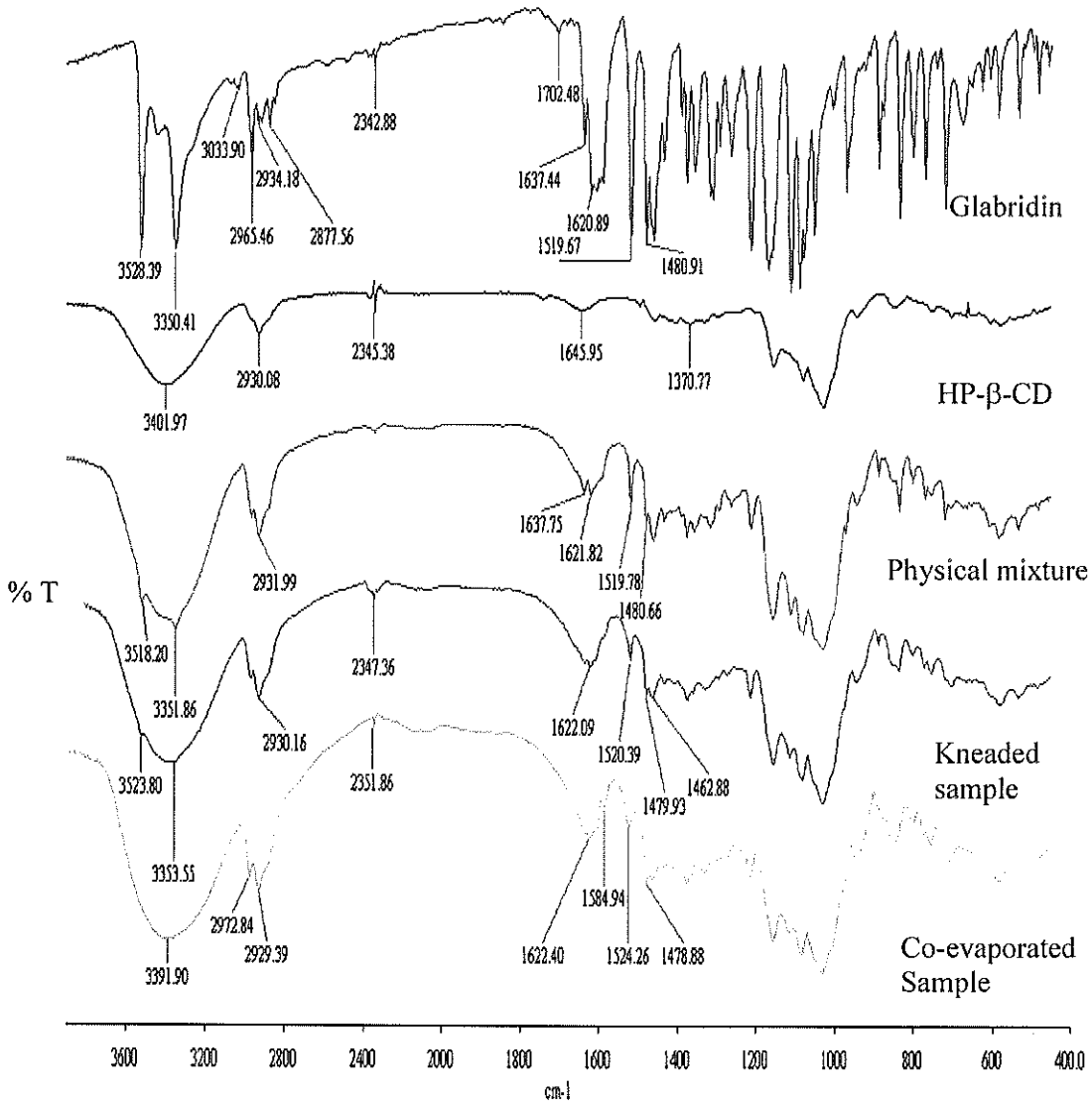

Figure 3.7 FT-IR spectra of glabridin, HP-β-CD, physical mixture, kneaded sample and co-evaporated sample.

Powder X-ray Diffractometry (PXRD)

Powder X-ray diffraction patterns of glabridin, β-CD, physical mixture and kneaded sample are presented in Figure 3.8. The presence of several sharp peaks at 8.31, 14.51, 16.10, 17.68, 19.33, 20.02, 20.56, 22.34, 22.97, 27.41, 29.05, 31.17 and 33.72 (2θ) of glabridin suggested that glabridin was in a crystalline form. The diffraction pattern of β-CD displayed several sharp peaks indicating that β-CD was in a crystalline form. The diffraction pattern of glabridin-β-CD physical mixture was corresponded to the superposition of those of the pure components. Some glabridin crystallinity peaks were still detectable in the physical mixture such as the peaks at 14.51, 16.10, 17.68, 19.33, 20.02, 20.56, 22.34 and 22.97 (2θ). This result indicated that the glabridin was still in a crystalline form. Kneaded sample showed less lower intense peaks compared with that of physical mixture, indicating the reduction in crystallinity of glabridin. The similar result was reported by Sapkal *et al.* (2007) who revealed that gliclazide-β-CD kneaded sample displayed lower crystallinity than that of physical mixture.

Powder X-ray diffraction patterns of glabridin, γ-CD, physical mixture and kneaded sample are presented in Figure 3.9. The diffraction pattern of γ-CD displayed several sharp peaks indicating that γ-CD was in a crystalline form. The diffraction pattern of glabridin-γ-CD physical mixture corresponded to the superposition of those of the pure components. Some glabridin crystalline peaks were still detectable in the physical mixture such as the peaks at 14.51, 16.10, 17.68, 19.33, 20.02, 20.56, 22.34 and 29.05 (2θ). This result indicated that the glabridin was still in a crystalline form. Kneaded sample showed some lower intense peaks, disappeared of the peak at 10.44 (2θ) and expressed the new peak at 7.48 (2θ) compared with that of

physical mixture. These result indicated that the reduction in crystallinity of glabridin and the formation of new crystallinity due to the interaction between glabridin and γ-CD. The similar result was reported by Anselmi *et al.* (2006) who revealed that ferulic acid-γ-CD inclusion complex displayed lower crystallinity than physical mixture and formation of new crystalline.

Powder X-ray diffraction patterns of glabridin, HP-β-CD, physical mixture, kneaded sample and co-evaporated sample are presented in Figure 3.10. The absence of any peaks in HP-β-CD diffractogram revealed the amorphous nature of this compound. The diffraction pattern of glabridin-HP-β-CD physical mixture corresponded to the superposition of those of the pure components. Some glabridin crystallinity peaks were still detectable in the physical mixture such as the peaks at 14.51, 16.10, 17.68, 19.33, 20.02, 20.56, 22.97, 27.41, 29.05, and 33.72 (2θ). This result indicated that the glabridin was still in a crystalline form. Kneaded sample exhibited a large decrease in the number of diffraction peaks, indicating the extreme reduction in crystallinity of glabridin. On the other hand, no diffraction peaks of glabridin in co-evaporated sample were observed. This result indicated that glabridin existed in the amorphous state. The similar result was reported by Jun *et al.* (2007) who revealed that simvastatin-HP-β-CD inclusion complex displayed the complete absence of any diffraction peak corresponding to simvastatin.

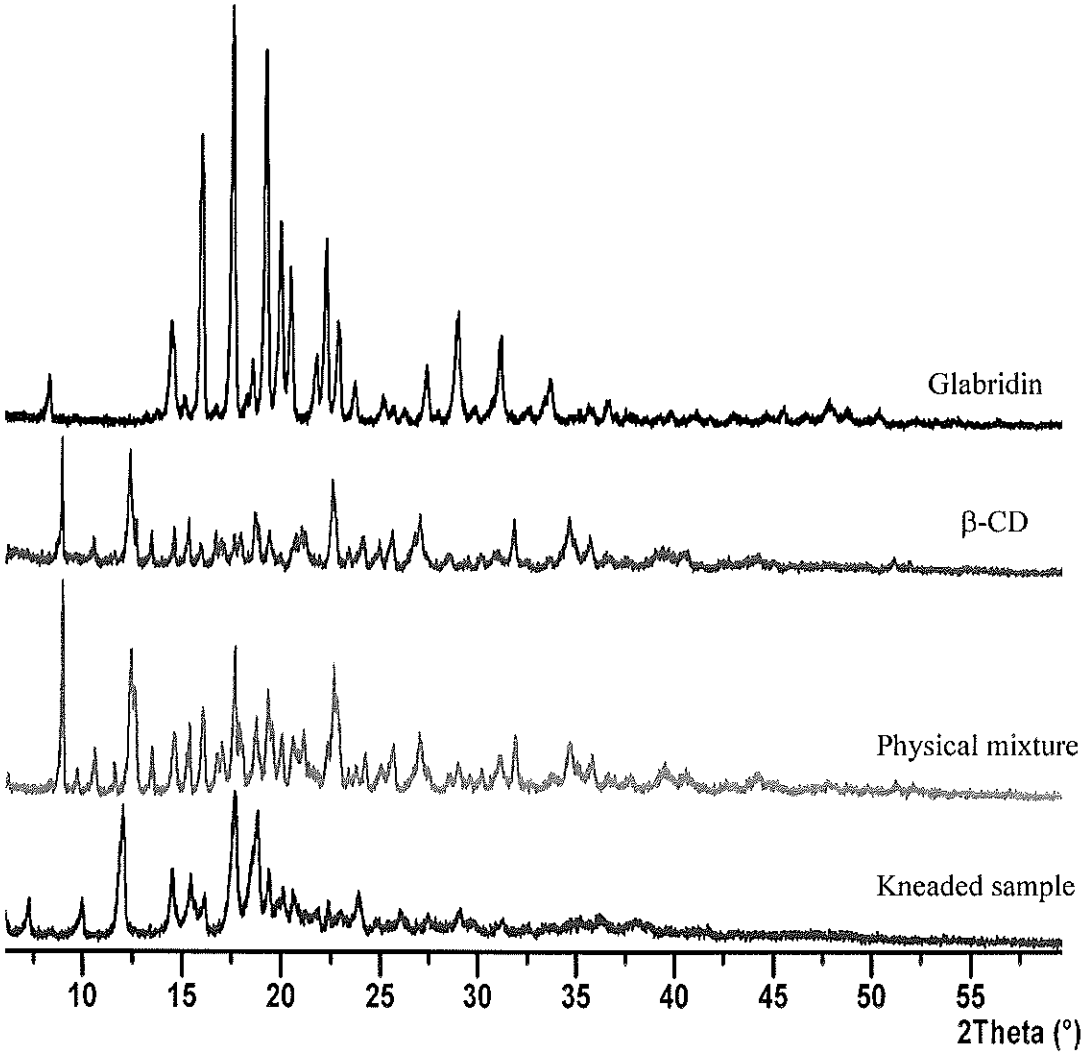

Figure 3.8 Powder X-ray diffraction patterns of glabridin, β-CD, physical mixture and kneaded sample.

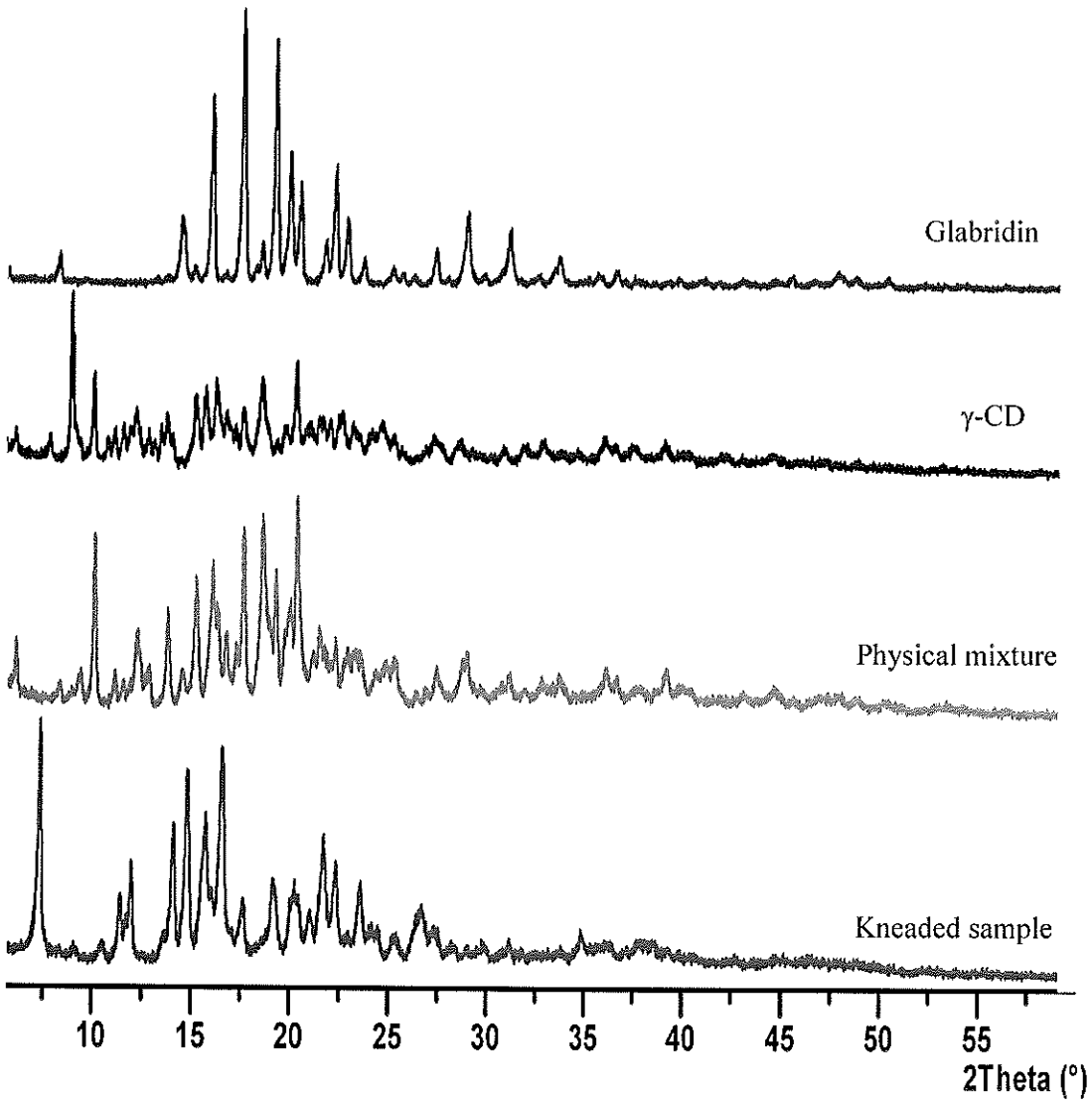

Figure 3.9 Powder X-ray diffraction patterns of glabridin, γ-CD, physical mixture and kneaded sample.

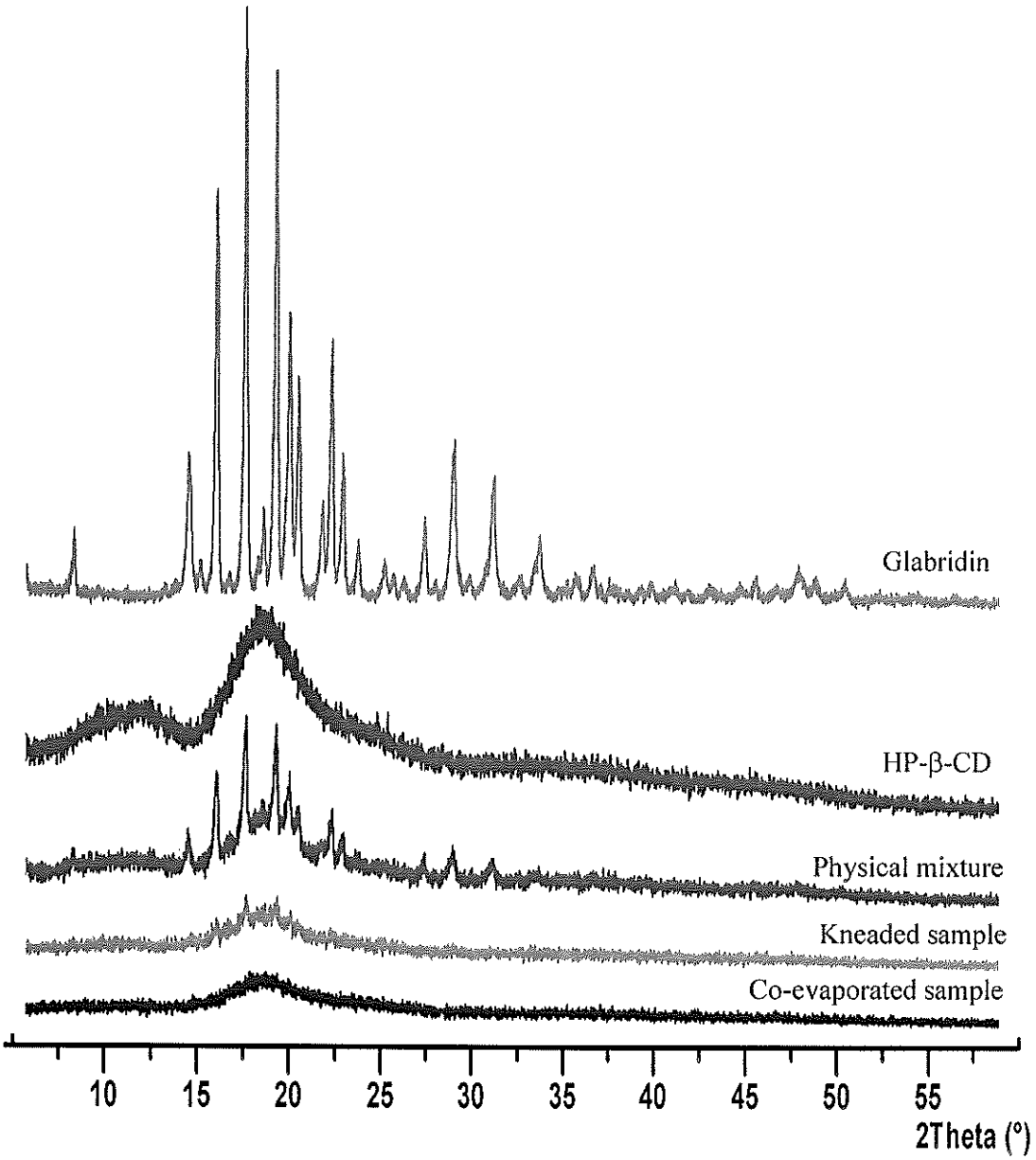

Figure 3.10 Powder X-ray diffraction patterns of glabridin, HP-β-CD, kneaded sample and co-evaporated sample.

Differential Scanning Calorimetry (DSC)

The DSC study is a useful technique to determine the interaction and the complex formation between guest molecules and cyclodextrins in form of endothermic or exothermic reaction. If the DSC curves of complex represent a sum of DSC thermograms of guest and cyclodextrin, this suggests that no interaction occur between guest and host molecules (cyclodextrin).

The DSC thermograms of glabridin, β-CD, physical mixture and kneaded sample are shown in Figure 3.11. The DSC thermogram of glabridin showed a sharp endothermic peak at 231°C corresponding to its melting point. The DSC thermogram of β-CD showed a broad endothermic peak in the range of 90-150°C corresponding to the loss of the water content of β-CD (Villaverde *et al.*, 2004). The DSC thermogram of glabridin-β-CD physical mixture was only the summation of glabridin and β-CD thremograms. The physical mixture thermogram was nearly identical to that of pure glabridin and the endothermic peak was observed at approximately 231°C. This result indicated that there was no interaction between glabridin and β-CD. However, the disappearance of glabridin-melting peak of kneaded sample compared with physical mixture, indicating that the interaction between glabridin and β-CD and a partial inclusion of glabridin in β-CD cavity may be occurred. The similar result was reported by Naidu *et al.* (2004) who revealed that meloxicam-β-CD kneaded sample displayed marked reduction in intensity and/or broadening and shifted to a lower temperature of the meloxicam endotherm. This result indicated a partial inclusion of meloxicam in the cavity of β-CD.

The DSC thermograms of glabridin, γ-CD, physical mixture and kneaded sample are shown in Figure 3.12. The DSC thermogram of γ-CD showed a

broad endothermic peak in the range of 90-150°C corresponding to the loss of the water content of γ-CD (Gil *et al.*, 2004). The DSC thermogram of glabridin-γ-CD physical mixture was only the summation of glabridin and γ-CD thermograms. The physical mixture thermogram was nearly identical to that of pure glabridin, and the endothermic peak was observed at approximately 231°C. This result indicated that there was no interaction between glabridin and γ-CD. However, in the kneaded sample, a marked reduction in the endothermic peak compared with physical mixture was observed, indicating that the interaction between glabridin and γ-CD and a partial inclusion of glabridin in γ-CD cavity may be occurred.

The DSC thermograms of glabridin, HP-β-CD, physical mixture, kneaded sample and co-evaporated sample are shown in Figure 3.13. The DSC thermogram of HP-β-CD showed a broad endothermic peak in the range of 75-120°C corresponding to the loss of the water content of HP-β-CD (Liu *et al.*, 2006). The disappearance of the glabridin-melting peak of physical mixture and kneaded sample may be due to the glabridin amorphization during the DSC run in the presence of amorphous HP-β-CD. These results indicated that glabridin was no longer present as a crystalline material but it was converted into the amorphous state. This similar result was reported by Liu *et al.* (2006) who revealed that the disappearance of baicalein-melting peak of baicalein-HP-β-CD physical mixture might be due to the baicalein amorphization during the DSC run in the presence of amorphous carrier. The complete disappearance of endothermic peak corresponding to glabridin melting point was observed in co-evaporated sample. This result indicated the formation of an amorphous inclusion complex and/or trapping of glabridin inside the HP-β-CD cavity (Patel and Patel, 2007). This similar result was reported by Jun *et al.* (2007) who

revealed that the endothermic peak of simvastatin-HP-β-CD inclusion complex was disappeared, indicating the formation of an amorphous inclusion complex, the molecular encapsulation of the simvastatin inside the HP-β-CD cavity.

According to the results from FT-IR spectra, powder X-ray diffractograms and DSC thermograms, the interaction between glabridin and cyclodextrin from glabridin-β-CD physical mixture and glabridin-γ-CD physical mixture did not occur. On the other hand, the interaction between glabridin and cyclodextrin from glabridin-HP-β-CD physical mixture, glabridin-β-CD kneaded sample and glabridin-γ-CD kneaded sample occurred through hydrogen bonds. In addition, the reduction in crystallinity of glabridin-β-CD kneaded sample and glabridin-γ-CD kneaded sample or the formation of new crystallinity of glabridin-γ-CD kneaded sample, the disappearance of glabridin-melting peak of glabridin-β-CD kneaded sample and marked reduction of glabridin melting peak of glabridin-γ-CD kneaded sample, indicated that the inclusion complex were formed. This similar result was reported by Li *et al.* (2005) who revealed that the reduction in crystallinity of trimethoprim-β-CD inclusion complex and the disappearance of trimethoprim-melting peak indicated the formation of a true inclusion complex. However, in the glabridin-HP-β-CD co-evaporated sample, no endothermic and characteristic diffraction peaks corresponding to glabridin was observed in DSC and PXRD studies. FT-IR study demonstrated the presence of intermolecular hydrogen bonds between glabridin and HP-β-CD in co-evaporated sample, resulting in the formation of amorphous form. The same phenomenon can be observed in the FT-IR spectra reported by Jun *et al.* (2007), who studied inclusion complex of simvastatin-HP-β-CD and Zhou *et al.* (2011) who

studied resveratrol-HP-β-CD inclusion complex prepared by supercritical antisolvent process.

Since, the amorphization of glabridin may be result from the co-evaporation process, it is possible that only the X-ray diffraction data cannot discriminate whether the glabridin-HP-β-CD co-evaporated system obtained are true inclusion complex (amorphous inclusion complex of glabridin with an amorphous HP-β-CD) and/or homogeneous dispersed mixture of the amorphous components (amorphous free glabridin associated with amorphous HP-β-CD as a non-inclusion complex) (Badr-Eldin *et al.*, 2008). However, these results can be interpreted on the basis of the formation of amorphous phase, possibly an amorphous inclusion complex formation or true inclusion complex which confirmed by FT-IR and PXRD analysis.

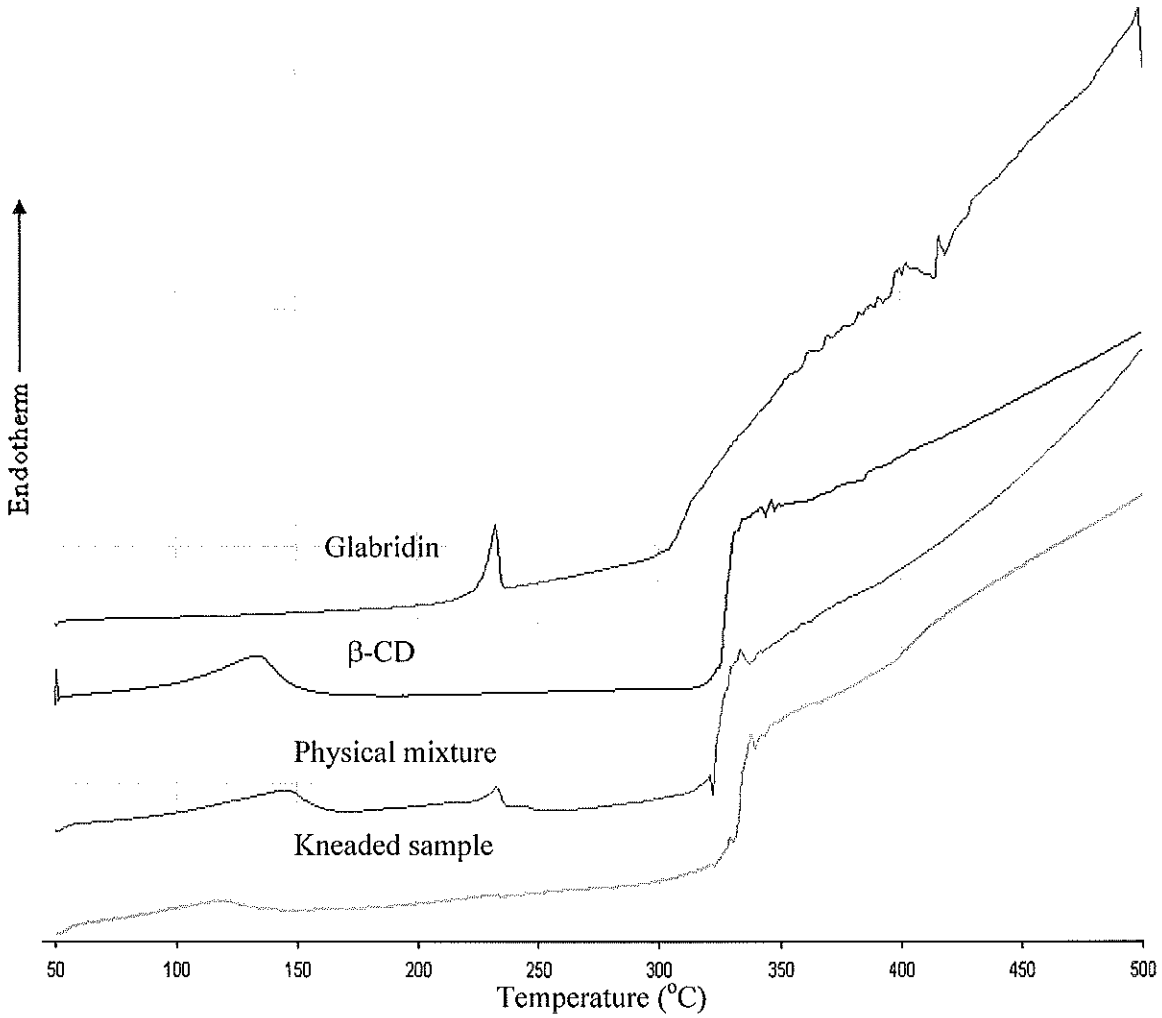

Figure 3.11 DSC thermograms of glabridin, β-CD, physical mixture and kneaded sample.

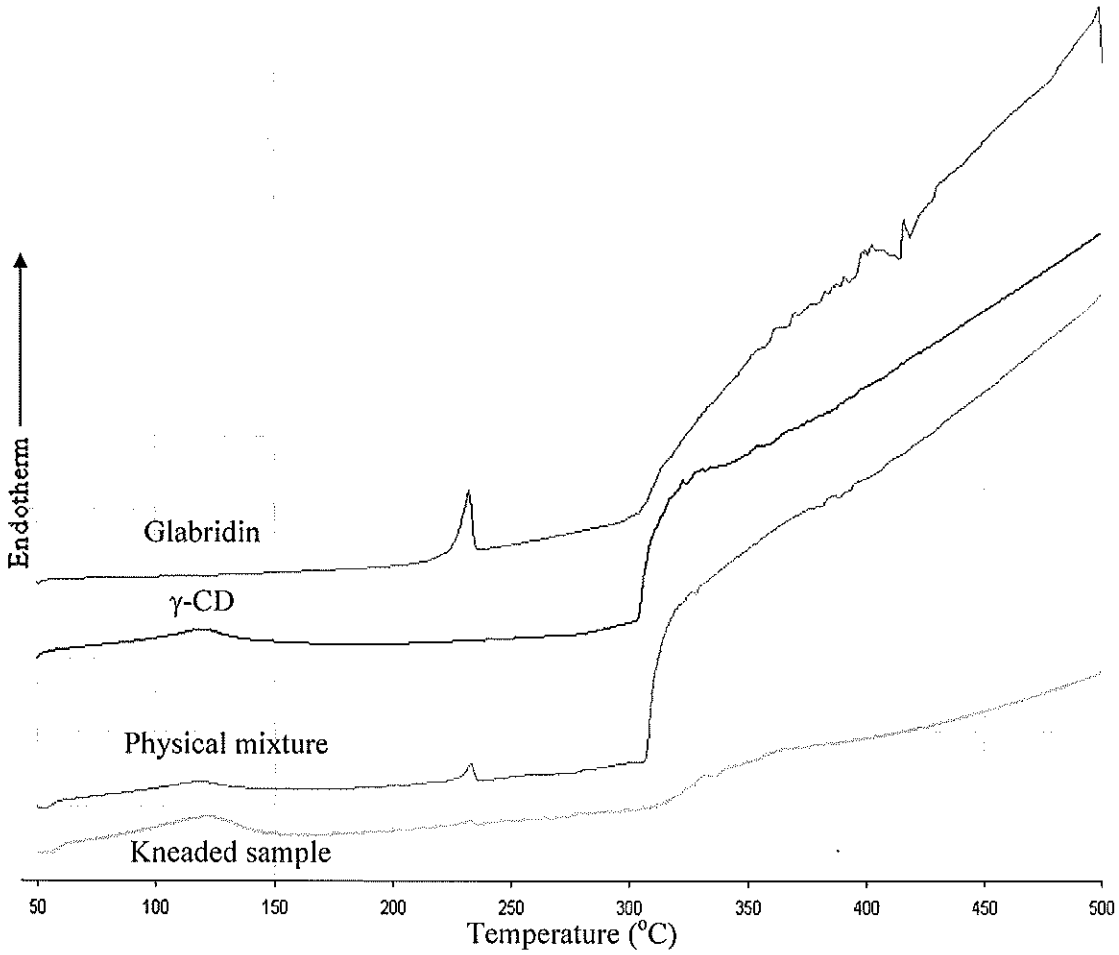

Figure 3.12 DSC thermograms of glabridin, γ-CD, physical mixture and kneaded sample.

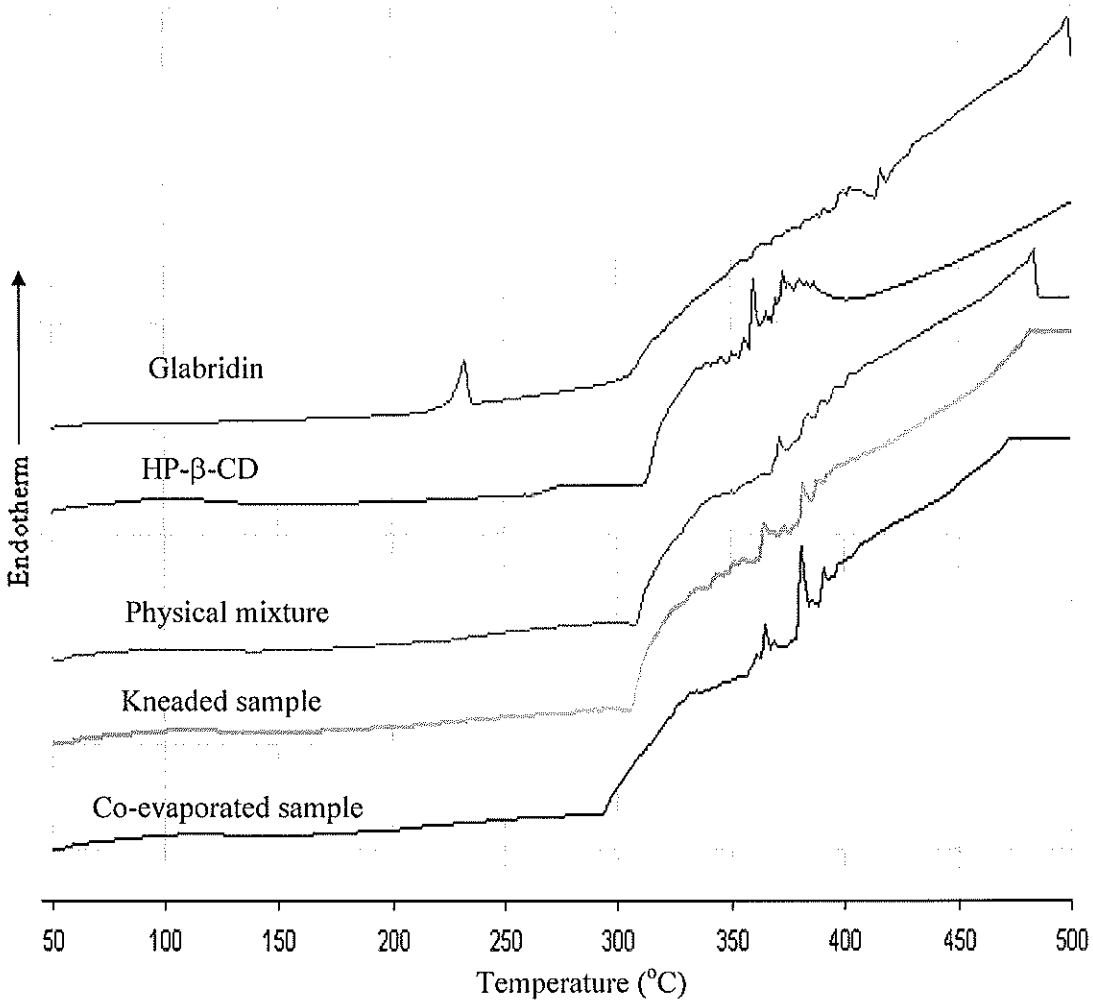

Figure 3.13 DSC thermograms of glabridin, HP-β-CD, physical mixture, kneaded sample and co-evaporated sample.

3.7 Preparation of lotions containing pure glabridin, glabridin-HP-β-CD physical mixture and glabridin-HP-β-CD co-evaporated sample as cosmetic active ingredient

The glabridin-HP-β-CD co-evaporated sample was selected to formulate the solution-type lotion because of the highest solubility of glabridin (1,799 μg/ml) when compared with other inclusion complexes. Three lotions were prepared by using pure glabridin or glabridin-HP-β-CD co-evaporated sample or glabridin-HP-β-CD physical mixture equivalent to glabridin in the concentration of 250 μg/ml (or 0.025% w/v). Figure 3.14(a) and Figure 3.14(b) demonstrate the main drawback of lotion containing pure glabridin and glabridin-HP-β-CD physical mixture, respectively. Both glabridin and glabridin-HP-β-CD physical mixture did not dissolve completely in aqueous vehicle. The obtained suspension-type lotions, became turbid or lose their transparency and thus reducing their commercial value. Figure 3.14(c) shows a clear solution type-lotion using glabridin-HP-β-CD co-evaporated sample as active ingredient. Therefore, glabridin-HP-β-CD co-evaporated sample can solve the main drawback of glabridin in formulating the solution-type lotion by increasing the solubility of glabridin.

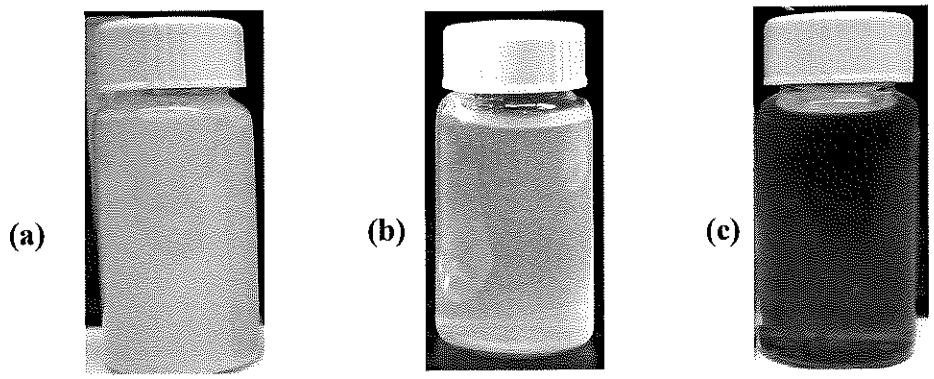

Figure 3.14 Appearance of glabridin lotion containing pure glabridin (a); glabridin-HP-β-CD physical mixture (b) and glabridin-HP-β-CD co-evaporated sample (c).

3.8 Tyrosinase inhibitory activity of glabridin, β-CD, γ-CD, HP-β-CD, glabridin-CDs physical mixtures, glabridin-CDs inclusion complexes and glabridin solution-type lotion

Percent of tyrosinase inhibitory activity of pure glabridin, β-CD, γ-CD, HP-β-CD, glabridin-CDs physical mixtures, glabridin-CDs inclusion complexes, glabridin solution-type lotion, blank lotion and kojic acid are shown in Table 3.7. Kojic acid was used as positive control. The percent tyrosinase inhibitory activity of pure glabridin was 91.85±2.34%. The percent tyrosinase inhibitory activity of glabridin-CDs physical mixtures and inclusion complexes did not significantly different ($p>0.05$) from that of pure glabridin. This result indicated that inclusion complexes and physical mixtures of glabridin with CDs did not effect on the tyrosinase inhibitory activity of glabridin. However, the percent tyrosinase inhibitory activity of solution type-lotion containing glabridin-HP-β-CD co-evaporated sample (99.50 ± 0.36%) was significantly ($p<0.05$) higher than that of the pure glabridin and glabridin-HP-β-CD co-evaporated sample. This result indicated that the components of blank lotion may enhance the tyrosinase inhibitory activity of glabridin-HP-β-CD co-evaporated sample.

Table 3.7 Percent tyrosinase inhibitory activity of glabridin, CDs, glabridin-CDs physical mixtures, glabridin-CDs inclusion complexes, lotion containing co-evaporated sample, blank vehicle and kojic acid.

	Samples	%Tyrosinase inhibitory activity [a]
1	Kojic acid (20 μg/ml per well)	83.75 ± 4.91
2	Glabridin*	91.85 ± 2.34
3	Glabridin-β-CD physical mixture*	89.89 ± 3.02
4	Glabridin-β-CD kneaded sample*	91.37 ± 2.99
5	Glabridin-γ-CD physical mixture*	90.99 ± 3.42
6	Glabridin-γ-CD kneaded sample*	90.40 ± 2.82
7	Glabridin-HP-β-CD, physical mixture*	90.19 ± 3.02
8	Glabridin-HP-β-CD, kneaded sample*	89.59 ± 0.94
9	Glabridin-HP-β-CD, co-evaporated sample*	91.13 ± 3.39
10	Solution type-lotion containing glabridin-HP-β-CD co-evaporated sample*	99.50 ± 0.36
11	Blank lotion	38.41±3.99
12	β-CD (69.92 μg/ml per well)	26.29±2.02
13	γ-CD (79.90 μg/ml per well)	38.79±0.02
14	HP-β-CD (86.44 μg/ml per well)	26.97 ± 5.25

[a] mean ± SD (n=3)

* glabridin concentration per well = 20 μg/ml

3.9 Stability study

3.9.1 Stability study of glabridin, glabridin-CDs physical mixtures and glabridin-CDs inclusion complexes

The percents initial glabridin remaining from glabridin-CDs physical mixtures and glabridin-CDs inclusion complexes after storage at 8°C, room temperature (30°C) and 45°C for 3 months are shown in Table 3.8.

Chemical stability was defined as not less than 90% of initial concentration remaining in the preparation. The percents initial glabridin remaining from physical mixtures and inclusion complexes after storage at 8°C, room temperature and 45°C for 3 months were in the range of 94.26-100.17%. These results indicated that all inclusion complexes and physical mixtures samples were chemical stable during storage at 8°C, room temperature and 45°C for 3 months.

Table 3.8 Percent initial glabridin remaining from glabridin-CDs physical mixtures and glabridin-CDs inclusion complexes after storage at 8°C, room temperature and 45°C for 3 months.

Temperature	Samples	% Initial glabridin remaining[a]
8°C	Glabridin-β-CD, physical mixture	97.12 ± 0.05
	Glabridin-β-CD, kneaded sample	98.52 ± 0.12
	Glabridin-γ-CD, physical mixture	97.07 ± 0.40
	Glabridin-γ-CD, kneaded sample	100.17 ± 0.32
	Glabridin-HP-β-CD physical mixture	99.31 ± 0.38
	Glabridin-HP-β-CD, kneaded sample	98.88 ± 0.22
	Glabridin-HP-β-CD, co-evaporated sample	97.52 ± 0.49
Room temperature	Glabridin-β-CD, physical mixture	97.54 ± 0.20
	Glabridin-β-CD, kneaded sample	99.67 ± 0.18
	Glabridin-γ-CD, physical mixture	97.49 ± 0.53
	Glabridin-γ-CD, kneaded sample	97.52 ± 0.24
	Glabridin-HP-β-CD physical mixture	97.08 ± 0.18
	Glabridin-HP-β-CD, kneaded sample	98.62 ± 0.21
	Glabridin-HP-β-CD, co-evaporated sample	97.97 ± 0.55
45°C	Glabridin-β-CD, physical mixture	94.79 ± 0.32
	Glabridin-β-CD, kneaded sample	95.72 ± 0.11
	Glabridin-γ-CD, physical mixture	95.01 ± 0.52
	Glabridin-γ-CD, kneaded sample	94.93 ± 0.37
	Glabridin-HP-β-CD physical mixture	94.26 ± 0.34
	Glabridin-HP-β-CD, kneaded sample	96.84 ± 0.24
	Glabridin-HP-β-CD, co-evaporated sample	97.97 ± 0.32

[a] mean ± SD (n=3)

Initial glabridin remaining = 100%

3.9.2 Stability study of glabridin solution-type lotions

3.9.2.1. Physical stability

Physical properties of solution-type lotion containing glabridin-HP-β-CD co-evaporated sample after storage at 8°C, room temperature and 45°C for 3 months are shown in Table 3.9. The initial pH value of this solution-type lotion was 6.38 ± 0.25. The final pH values of this solution-type lotion after storage at 8°C, room temperature and 45°C for 90 days were 6.42 ± 0.24, 6.43 ± 0.23 and 6.45 ± 0.24, respectively. The pH values were increased in 0.04-0.07 units. The initial viscosity value was 4.15 ± 0.04 cP. The final viscosity values after storage at 8°C, room temperature and 45°C for 90 days were 3.92 ± 0.13, 3.82 ± 0.12 and 4.05 ± 0.14 cP, respectively. There were no appreciable changes in odor for all samples during the study period. After storage at 8°C for 30 days and at room temperature and 45°C for 90 days, the lotion was still transparent. In contrast, the precipitation occurred after storage at 8°C for 90 days. This precipitation may be due to the fact that the solubility of glabridin-HP-β-CD co-evaporated sample was reduced in the lower temperature. These results indicated that solution-type lotion containing glabridin-HP-β-CD co-evaporated sample had physical stability throughout 3 months of storage at room temperature and 45°C.

Table 3.9 Physical properties of solution-type lotion containing glabridin-HP-β-CD co-evaporated sample after storage at 8°C, room temperature and 45°C for 3 months.

Time (day)	Temperature (°C)	Appearance	pH [a]	Odor	Viscosity [a] (cP) at 250 rpm
0	room temperature	Transparence	6.38 ± 0.25	good	4.15 ± 0.04
		Transparence		good	
		Transparence		good	
30	8	Transparence	6.39 ± 0.22	good	not determined
		Transparence		good	
		Transparence		good	
	room temperature	Transparence	6.41 ± 0.23	good	
		Transparence		good	
		Transparence		good	
	45	Transparence	6.41 ± 0.24	good	
		Transparence		good	
		Transparence		good	
60	8	Precipitation	6.42 ± 0.22	good	
		Precipitation		good	
		Precipitation		good	
	room temperature	Transparence	6.43 ± 0.22	good	
		Transparence		good	
		Transparence		good	
	45	Transparence	6.43 ± 0.23	good	
		Transparence		good	
		Transparence		good	
90	8	Precipitation	6.42 ± 0.24	good	3.92 ± 0.13
		Precipitation		good	
		Precipitation		good	
	room temperature	Transparence	6.43 ± 0.23	good	3.82 ± 0.12
		Transparence		good	
		Transparence		good	
	45	Transparence	6.45 ± 0.24	good	4.05 ± 0.14
		Transparence		good	
		Transparence		good	

[a] mean ± SD (n=3)

3.9.2.2. Chemical stability

The percents initial glabridin remaining from solution-type lotion containing glabridin-HP-β-CD co-evaporated sample after storage at 8°C, room temperature and 45°C for 3 months are shown in Table 3.10. The percents initial glabridin remaining from solution-type lotion after storage at 8°C, room temperature and 45°C for 30 days were 97.47 ± 5.31, 99.94 ± 3.85 and 100.99 ± 3.04 %, respectively. The percents initial glabridin remaining after storage at room temperature and 45°C for 60 days were 97.14 ± 1.59 and 99.32 ± 5.22 %, respectively. The percents initial glabridin remaining after storage for 90 days at room temperature and 45°C was 95.46 ± 4.05 and 98.87 ± 5.01 %, respectively. These results indicated that solution-type lotion containing glabridin-HP-β-CD co-evaporated sample was chemical stable for 3 months when stored in glass bottle and protected from light at room temperature and at 45°C. In addition, this lotion was still chemical stable for 1 month when stored in glass bottle protected from light at 8°C. Nevertheless, the percent initial glabridin remaining after storage at 8°C for 60 days and 90 days was not determined because of the precipitation of glabridin as previously mentioned in the topic of physical stability (section 3.9.2).

Table 3.10 Percent initial glabridin remaining of solution-type lotion containing glabridin-HP-β-CD co-evaporated sample after storage at 8°C, room temperature and 45°C for 3 months.

Temperature (°C)	% Initial glabridin remaining[a]			
	Time (day)			
	0 day	30 days	60 days	90 days
8	100	97.47 ± 5.31	Precipitation	Precipitation
Room temperature	100	99.94 ± 3.85	97.14 ±1.59	95.46 ± 4.05
45	100	100.99 ± 3.04	99.32 ± 5.22	98.87 ± 5.01

Actual initial glabridin remaining was 0.02435%w/v and defined as 100%

[a] mean ± SD (n=3)

3.9.2.3. Tyrosinase inhibitory activity

Tyrosinase inhibitory activity of solution-type lotion containing glabridin-HP-β-CD co-evaporated sample after storage at 8°C for 30 days, room temperature and 45°C for 90 days is summarized in Table 3.11, shown initial tyrosinase inhibitory activity of glabridin-HP-β-CD co-evaporated sample was 99.50 ± 0.36. Percents tyrosinase inhibitory activity of the lotion after storage at 8°C, room temperature and 45°C for 30 days were 97.69 ± 0.50, 98.35 ± 0.54 and 98.17 ± 0.97%, respectively. Percents tyrosinase inhibitory activity of the lotion after storage at room temperature and 45°C for 30 days were not significantly different ($p>0.05$) from the initial value but percent tyrosinase inhibitory activity of the lotion storage at 8°C for 30 days was significantly ($p<0.05$) lower than the initial value.

Percents tyrosinase inhibitory activity of the lotion storage at room temperature and 45°C for 60 days were 96.83 ± 0.54 and 97.14 ± 0.69%, respectively.

Percent tyrosinase inhibitory activity of the lotion storage at room temperature and 45°C for 90 days were 96.30 ± 0.29 and 95.69 ± 0.33%, respectively. Although the percents tyrosinase inhibitory activity after storage for 60 and 90 days were slightly decreased compared with that of freshly prepared lotion, these values were significantly ($p<0.05$) lower than the initial value. The reason for the decreased tyrosinase inhibitory activity could be probably due to the decrease in percent initial glabridin remaining (Table 3.10). However, these results indicated that the lotion still gave high tyrosinase inhibitory activity. In addition, the lotion was still stable in terms of tyrosinase inhibitory activity when stored at room temperature and 45°C for 90 days and at 8°C for 30 days.

Nevertheless, percent tyrosinase inhibitory activity of the lotion storage at 8°C for 60 days and 90 days was not determined because the precipitation of glabridin was observed as mentioned-above in the topic of physical stability.

Table 3.11 Tyrosinase inhibitory activity of solution-type lotion containing glabridin-HP-β-CD co-evaporated sample after storage at 8°C, room temperature and 45°C for 3 months.

Temperature (°C)	%Tyrosinase inhibitory activity [a]			
	Time (day)			
	0 day	30 days	60 days	90 days
8	99.50 ± 0.36	97.69 ± 0.50	Precipitation	Precipitation
Room temperature	99.50 ± 0.36	98.35 ± 0.54	96.83 ± 0.54	96.30 ± 0.29
45	99.50 ± 0.36	98.17 ± 0.97	97.14 ± 0.69	95.69 ± 0.33

[a] mean ± SD (n=3)

3.10 *In vitro* glabridin release studies

The cumulative amount of glabridin released through cellulose acetate membrane per unit area versus time from three formulations (solution-type lotion containing glabridin-HP-β-CD co-evaporated sample, suspension-type lotions containing pure glabridin and glabridin-HP-β-CD physical mixture) is shown in Table 3.12 and Figure 3.15. It can be seen that complexation with HP-β-CD increased the amount of glabridin released through cellulose acetate membrane. After 12 hours, the amount of glabridin released from lotion containing pure glabridin was 3.30 $\mu g/cm^2$. However, when glabridin was simply mixed with HP-β-CD as physical mixture and when complexed with HP-β-CD, the amounts of glabridin released were 9.21 $\mu g/cm^2$ and 14.17 $\mu g/cm^2$, respectively. The increase in glabridin released from lotion containing glabridin-HP-β-CD co-evaporated sample compared with that of lotions containing pure glabridin and glabridin-HP-β-CD physical mixture was probably due to the higher solubility of glabridin obtained from complexing with HP-β-CD.

Table 3.12 Cumulative amount of glabridin released per unit area of lotions containing glabridin, glabridin-HP-β-CD physical mixture and glabridin-HP-β-CD co-evaporated sample.

Time (hours)	Cumulative glabridin released per unit area ($\mu g/cm^2$) of lotion containing		
	glabridin	glabridin-HP-β-CD physical mixture	glabridin-HP-β-CD co-evaporated sample
0	0	0	0
0.5	0.718 ± 0.076	2.478 ± 0.281	3.815 ± 0.632
1	1.127 ± 0.245	3.518 ± 0.742	5.667 ± 1.746
2	1.656 ± 0.511	4.736 ± 0.734	7.787 ± 1.378
3	1.981 ± 0.410	5.469 ± 1.466	8.561 ± 0.971
6	2.543 ± 0.877	7.578 ± 3.391	11.060 ± 0.798
9	3.109 ± 0.961	8.728 ± 3.825	12.476 ± 2.719
12	3.303 ± 0.792	9.210 ± 3.845	14.179 ± 3.529

[a] mean ± SD (n=4)

The *in vitro* release profiles of glabridin from three prepared lotions containing pure glabridin, glabridin-HP-β-CD physical mixture and glabridin-HP-β-CD co-evaporated sample with three different mathematical models, i.e., zero order model, first order model and Higuchi model are shown in Figure 3.16, 3.17 and 3.18, respectively. The cumulative amount of glabridin from all prepared lotions that released through the cellulose acetate membrane into the receptor fluid was plotted against time or square root of time in order to characterize the release kinetics of glabridin. The obtained correlations of determination are summarized in Table 3.13. It was found that the release profiles of glabridin from all prepared lotions were fitted best with Higuchi model (R-squared values closed to 1) rather than zero order and first order model.

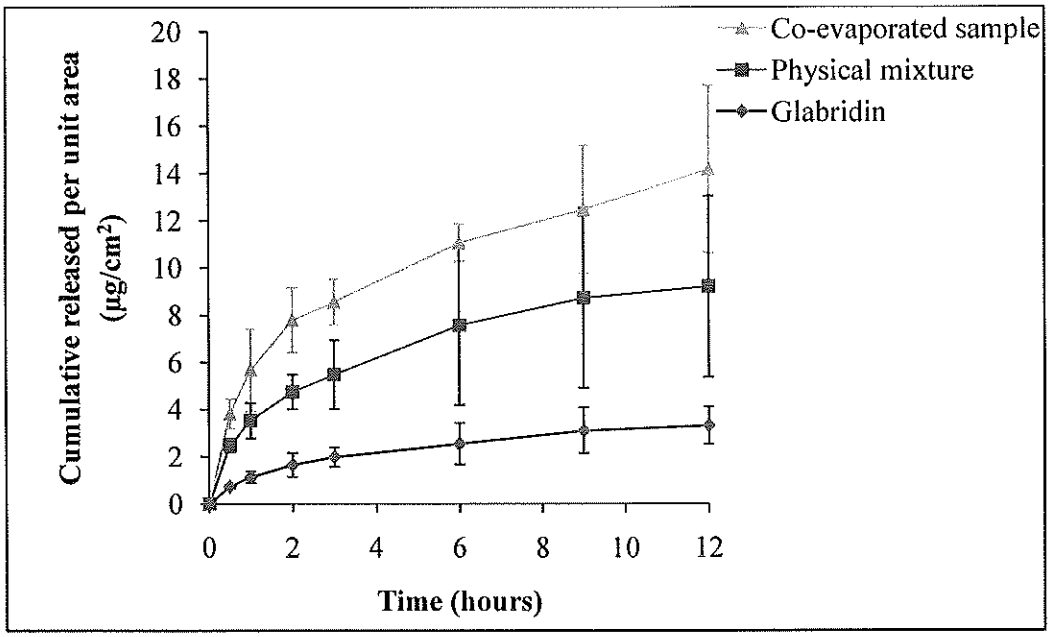

Figure 3.15 *In vitro* release profiles of glabridin from lotions containing pure glabridin, glabridin-HP-β-CD physical mixture and glabridin-HP-β-CD co-evaporated sample. Each point represents mean ± SD, n=4.

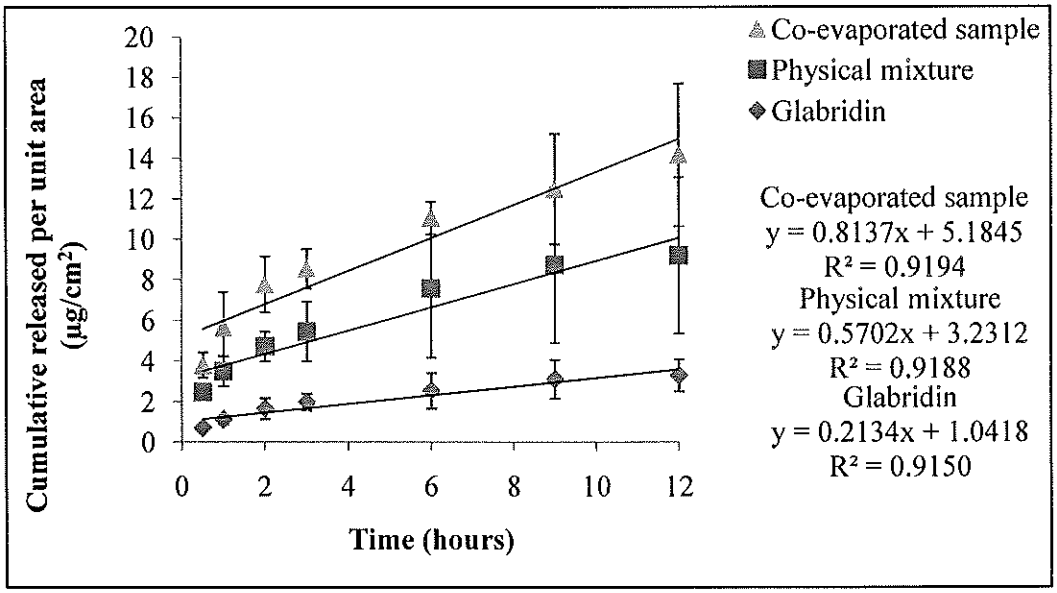

Figure 3.16 Zero order release model of glabridin from lotions containing pure glabridin, glabridin-HP-β-CD physical mixture and glabridin-HP-β-CD co-evaporated sample. Each point represents mean ± SD, n=4.

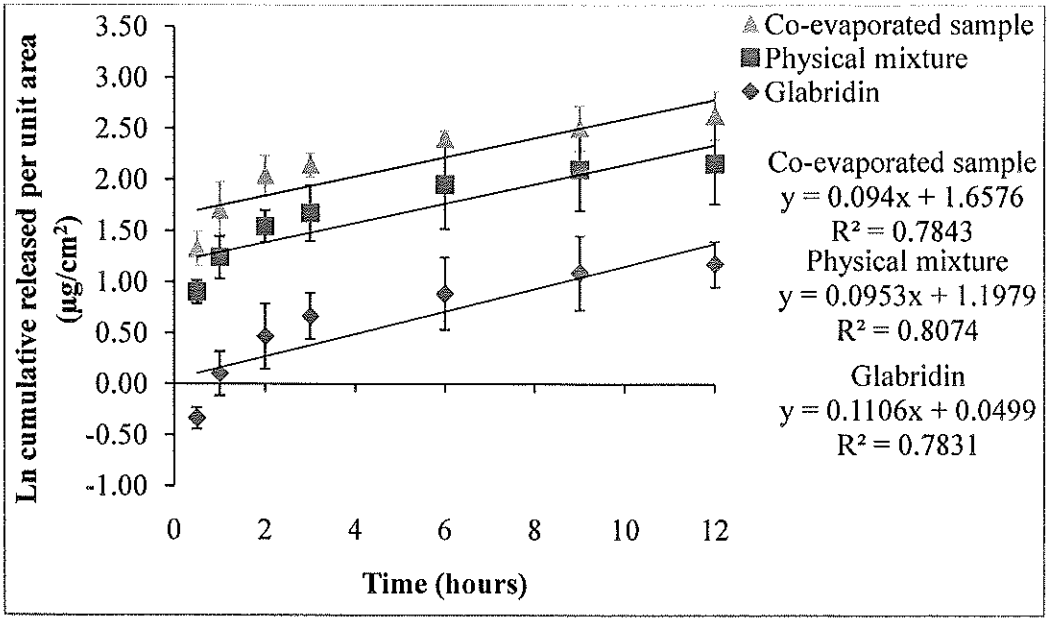

Figure 3.17 First order release model of glabridin from lotions containing pure glabridin, glabridin-HP-β-CD physical mixture and glabridin-HP-β-CD co-evaporated sample. Each point represents mean ± SD, n=4.

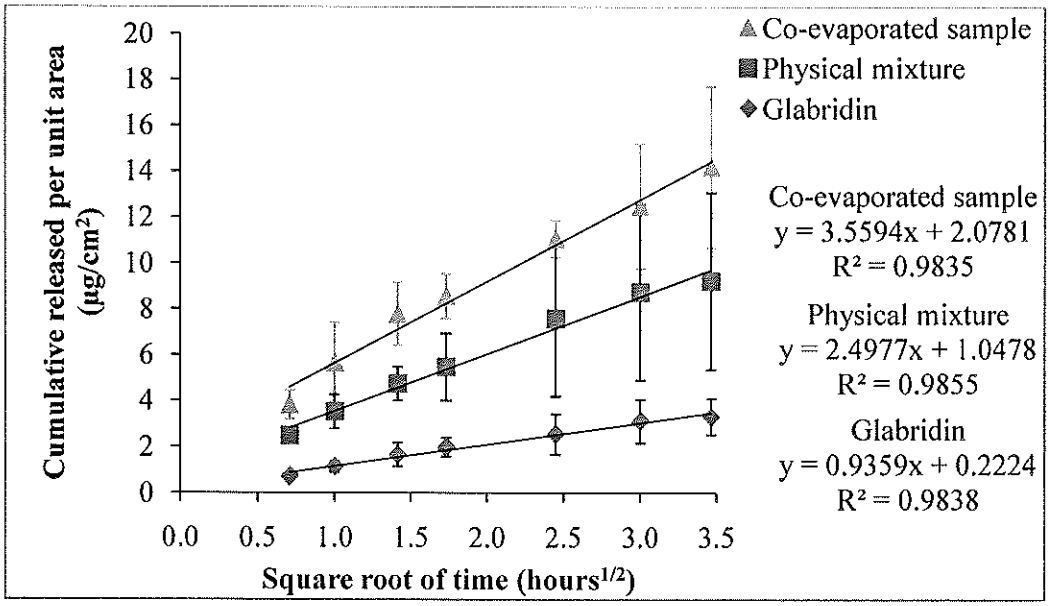

Figure 3.18 Higuchi release model of glabridin from lotions containing pure glabridin, glabridin-HP-β-CD physical mixture and glabridin-HP-β-CD co-evaporated sample. Each point represents mean ± SD, n=4.

Table 3.13 Coefficient of determination of different mathematical model.

Prepared lotions						
Samples	Zero order[a]		First order[b]		Higuchi[c]	
	r^2	K_0* ($\mu g/cm^2/h$)	r^2	K_1* ($\mu g/cm^2/h$)	r^2	K_H* ($\mu g/cm^2/h^{1/2}$)
Glabridin	0.9150	0.2127±0.069	0.7831	0.0812±0.049	0.9838	0.9352±0.310
Physical mixture	0.9188	0.5695±0.412	0.8074	0.0950±0.042	0.9855	2.4972±1.745
Co-evaporated sample	0.9194	0.8133±0.222	0.7843	0.0933±0.007	0.9835	3.5595±0.850

[a]Zero order equation; $Q = Q_0 + k_0t$

[b]First order equation; $\ln Q = \ln Q_0 + k_1t$

[c] Higuchi equation; $Q = k_Ht^{1/2}$

K_0, K_1 and K_H are release rate constants of zero order, first order and Higuchi model, respectively.

*The K_0, K_1 and K_H obtained from the slope of the release models (Figure 4.8 - 4.16)

The linear relationship between cumulative amount of glabridin versus square root of time suggested that the transport of glabridin through cellulose acetate membrane from all prepared lotions were controlled by diffusion. This means that the membrane had no significant effects in the release determinations, and properties of the formulation controlled the release of glabridin (Santoyo *et al.*, 1996). The release rates of glabridin from lotions containing pure glabridin, glabridin-HP-β-CD physical mixture and glabridin-HP-β-CD co-evaporated sample were 0.9352, 2.4972 and 3.5595 $\mu g/cm^2/h^{1/2}$, respectively. In comparison, the release rates of glabridin from lotions containing glabridin-HP-β-CD physical mixture and glabridin-HP-β-CD co-evaporated were approximately 2.6 and 3.8 times greater than that of lotion

containing pure glabridin, respectively. The enhanced release rate (K_H) of glabridin from lotion containing glabridin-HP-β-CD physical mixture compared with that of lotion containing pure glabridin may be due to the surface tension lowering effect of HP-β-CD, resulting in wetting of hydrophobic glabridin surface, the inclusion complex and/or non-inclusion complex obtained during the preparation of lotion and the increased solubility of the glabridin. The glabridin-HP-β-CD co-evaporated sample gave the highest release rate (K_H) compared with that of pure glabridin and physical mixture. This result might be explained on the basis of amorphous state which mentioned above in the topic of PXRD and it has the highest solubility (1,799 μg/ml) which previously described in the topic of water solubility of glabridin-CDs inclusion complex. The similar result was reported by Manosroi *et al.* (2005) who revealed that the higher release rate of azelaic acid observed in the co-evaporation system through three types of synthetic membrane might be attributed to the higher percentages of drug available in solution in donor chamber. In addition, they reported that the enhanced release rate of azelaic acid via the formation of an inclusion complex with HP-β-CD might be due to the increased dissolution rate of azelaic acid in water. Furthermore, Montassier *et al.* (1998) reported that the release rate of tretinoin was significantly improved by complexation with HP-β-CD. This improvement may be attributed to the better solubility of the complexes in aqueous phase and to the dissociation of dissolved inclusion compounds leading to free molecules of tretinoin able to diffuse through the membrane. Finally, The release rate of glabridin from lotion containing glabridin-HP-β-CD co-evaporated sample was significantly ($p<0.05$) higher than the release rate of glabridin from lotion containing glabridin. The release rate of glabridin from lotion containing glabridin-HP-β-CD

physical mixture did not significantly different ($p>0.05$) from lotion containing glabridin-HP-β-CD co-evaporated sample and lotion containing glabridin.

CHAPTER 4

CONCLUSIONS

In this study, the 1:1 molar ratio of inclusion complexes of glabridin with each of cyclodextrins, β-CD, γ-CD and HP-β-CD, were prepared by kneading and co-evaporation methods in order to increase glabridin solubility. The solubility of glabridin was increased linearly with each of the concentrations of β-CD or γ-CD and it could be classified as A_L-type phase solubility diagram with the stability constant ($K_{1:1}$) of 7,609 M^{-1} and 1,224 M^{-1} at 32±1°C, respectively. The solubility of glabridin was increased linearly with the concentrations of HP-β-CD in the range of 0-15 mM. After that the limitation of solubility was obtained. Consequently, it could be classified as B_L-type phase solubility diagram with the stability constant ($K_{1:1}$) of 4,025 M^{-1} at 32±1°C. According to the results of stability constant, glabridin was stronger complex with β-CD than γ-CD and HP-β-CD because the cavity size of β-CD seemed optimal for entrapment of glabridin molecule.

The rank order of water solubility of inclusion complex was glabridin-HP-β-CD co-evaporated sample > glabridin-HP-β-CD kneaded sample > glabridin-β-CD kneaded sample > glabridin-γ-CD kneaded sample, respectively. The co-evaporated sample showed the highest solubility of glabridin (1,779.71 ± 7.56 μg/ml), so it was selected to formulate the solution-type lotion. These observations are

probably due to the fact that the amorphous inclusion complex was obtained between glabridin and HP-β-CD during co-evaporation process which confirmed by PXRD.

The FT-IR demonstrated the aromatic ring (C=C) of glabridin might be inside the hydrophobic cavity of CDs. The results from DSC, and PXRD indicated that the glabridin-HP-β-CD co-evaporated sample was in amorphous state. All glabridin-CDs inclusion complexes still gave high tyrosinase inhibitory activity and were not significantly different ($p>0.05$) from pure glabridin. The glabridin-CDs physical mixtures and glabridin-CDs inclusion complexes showed chemical stability when stored at 8°C, room temperature and 45°C for 3 months and percents initial glabridin remaining were in the range of 94.26-100.17%.

The solution-type lotion containing co-evaporated sample exhibited good appearance, clear solution and no precipitation when stored at room temperature and 45°C for 90 days. In addition, tyrosinase inhibitory activity of the lotion was slightly decreased when stored at room temperature and 45°C for 90 days.

The *in-vitro* release profiles of glabridin from lotions containing pure glabridin, glabridin-HP-β-CD physical mixture and glabridin-HP-β-CD co-evaporated sample were fitted to Higuchi model, indicating that the release of glabridin across cellulose acetate membrane was controlled by diffusion. The *in-vitro* release rate (K_H) rank order of glabridin was found to be lotion containing glabridin-HP-β-CD co-evaporated sample > glabridin-HP-β-CD physical mixture > pure glabridin. This result might be explained on the basis of a change from crystalline to amorphous form in glabridin-HP-β-CD co-evaporated sample and the better solubility of glabridin.

The overall data demonstrated that the glabridin-HP-β-CD inclusion complex in 1 :1 molar ratio, prepared by co-evaporation method exhibited the most efficacious to obtain complex formation. This inclusion complex can be applied for the development of glabridin in aqueous preparations for lightening cosmetic products.

SUGGESTIONS FOR THE FUTURE WORK

From this study, the inclusion complex of glabridin-β-CD and glabridin-γ-CD prepared by co-evaporation method were unsuccessful because β-CD and γ-CD were slightly and very slightly soluble in absolute alcohol, respectively. Therefore, the use of other organic solvents should try for further study.

Besides the physicochemical characterization such as FT-IR, DSC and PXRD in this experimental, the use of scanning electron microscopy (SEM) was useful technique to study the microscopic aspects of guest substances, cyclodextrin and their combinations, respectively.

The viscosity of solution-type lotion may be increased by increasing amount of hydroxypropyl cellulose.

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APPENDIX

Method of the quantitative determination of glabridin by HPLC

The high-pressure liquid chromatographic technique was used for analysis of glabridin. The technique was based on the method of Shanker *et al.* (2007).

HPLC conditions

Analytical column	:	Agilent Zorbax 300 SB C18 4.6x150 mm 5 µm USA.
Mobile phase	:	2% acetic acid : acetonitrile (55 : 45)
Detector wavelength	:	280 nm
Flow rate	:	1.0 ml/minute
Injection volume	:	10 µl

Validation of the method

Validation of HPLC method was analyzed in term of qualitative analysis (identification of a compound out of a sample mixture, retention time and spiking) and quantitative analysis (peak area).

UV spectrometry of glabridin

The maximum absorbance (λ_{max}) of glabridin solution (10 µg/ml) was determined the by using a UV-spectrophotometer in the range of 200 nm to 400 nm. The maximum absorbance (λ_{max}) was 280 nm.

Standard curve of glabridin

The stock solution of glabridin was prepared by dissolving accurate weight (2 mg) of glabridin with absolute ethanol and adjusting to 10 ml with absolute alcohol.

A serial of standard solutions were prepared by diluting stock solution of glabridin with distilled water to obtain concentrations of 1, 10, 20, 25, and 40 μg/ml. The solutions were injected in triplicate into the HPLC column and the absorbance of each standard solution of glabridin was determined by using a detector at 280 nm. The standard curve was constructed by plotting the peak area of glabridin against the concentration of glabridin. The equation with linear regression of the line was used to determine the concentration of glabridin from the samples.

Specificity

Under the chromatographic conditions selected, the peak of other ingredients in the solution-type lotion must not interfere with the peak of glabridin. Chromatogram of the standard solution of glabridin in solution-type lotion was compared with chromatogram of the vehicle.

Forced degradation studies

The stability-indicating capability of the glabridin was determined by oxidation, acid hydrolysis and basic hydrolysis. Degradation of an aqueous solution of standard glabridin was forced by oxidation with hydrogen peroxide, by adjusting the pH of standard glabridin solution to pH 2 with 1 N hydrochloric acid and to pH 12 with 1 N sodium hydroxide. Then, the solution was heated to 100°C for 3 hours. The pH was corrected to pH 5-6 and the samples were assayed after suitable dilution.

Linearity

Glabridin standard solutions in the concentration range of 1, 10, 20, 25 and 40 μg/ml were prepared and analyzed for three days (n=3). Each of standard solution was injected three times. Linear regression analysis of the means peak area versus their concentrations was performed. The linear regression coefficient (r^2) ≥ 0.999 is the acceptable criteria.

Precision

Within run precision (Repeatability, intra-day) and accuracy

Three sets of three standard solutions of glabridin (5, 15 and 30 μg/ml) were analyzed by HPLC within one day. Each solution was injected three times. The standard deviation and percent relative standard deviation of peak area were calculated. The percentage of relative standard deviation (%RSD) should not more than 2%.

Between run precision (Intermediate precision, inter-day) and accuracy

Three sets of three standard solution of glabridin (5, 15 and 30 μg/ml) were analyzed by HPLC on different days. Each sample was injected three times. The data used to calculate standard deviation and percent relative standard deviation of peak area. The percentage of relative standard deviation (%RSD) should not more than 2%.

Limit of detection (LOD) and Quantification (LOQ)

The limit of detection (LOD) and Quantification (LOQ) did not determine because the response of test sample was in the range of standard curve.

Accuracy

a) Analysis of standard solution of glabridin

Three sets of standard solutions of glabridin in the concentration of 5, 15 and 30 μg/ml were prepared and injected for three days. Each individual sample was analyzed three times by HPLC. Percent analytical recovery of each sample was calculated by comparing amounts of drug found and amount of drug added. According to ICH Q2A and Q2B guidelines recommendation, the average percentage recovery should be 95-105% (Walfish, 2006).

b) Analysis of glabridin in solution-type lotion

Standard glabridin was spiked into solution-type lotion at a level of 200, 250 and 300 μg/ml. Then standard glabridin solution-type lotion was pipetted, suitable diluted with mobile phase to the concentrations of 20, 25 and 30 μg/ml. Each individual sample was analyzed three times by HPLC. Percent analytical recovery of each sample was calculated by comparing amounts of glabridin found and amount of glabridin added. The percentage of recovery needs to be 95-105%.

A validated stability-indicating HPLC method for analysis of glabridin

Quantitative analysis of glabridin by using HPLC

The HPLC method for quantitative determination of glabridin in our study was based on the method of Shanker *et al.* (2007) with minor modifications. Figure 4.1 shows chromatogram of glabridin standard solution with the retention time of glabridin approximately 8.12 minutes

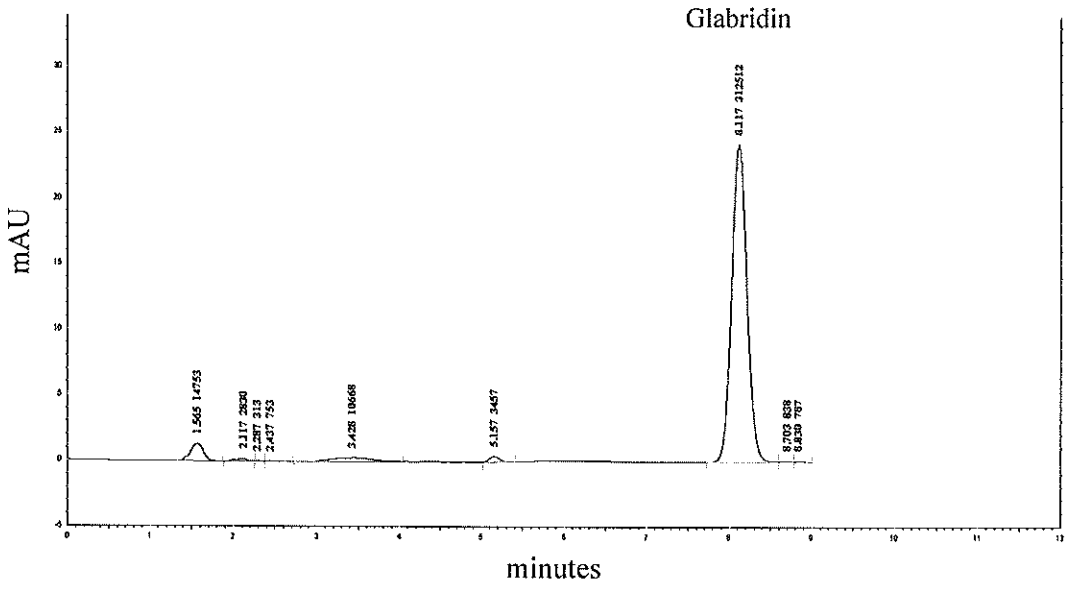

Figure 4.1 Chromatogram of glabridin standard solution.

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its degradation products. To establish that there was no interference from the components of lotion vehicle or degradation products in the stability-indicating ability of the procedure, the lotion vehicle was injected into the HPLC. Figure 4.2 and Figure 4.3 depict typical chromatograms of the assay preparation of glabridin-HP-β-CD co-evaporated sample in solution-type lotion and solution-type lotion vehicle, respectively. The glabridin peak was well resolved from the lotion vehicle. The results of specificity studies demonstrated that the peak of glabridin in solution-type lotion was not interfered by the peak of any excipients from solution-type lotion vehicle.

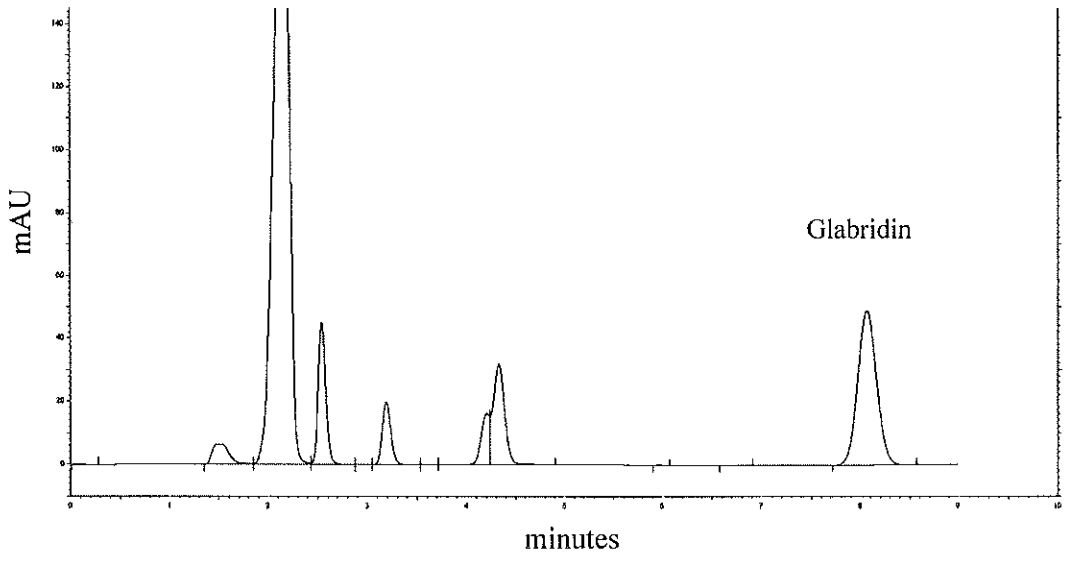

Figure 4.2 Chromatogram of glabridin-HP-β-CD co-evaporated sample in solution-type lotion.

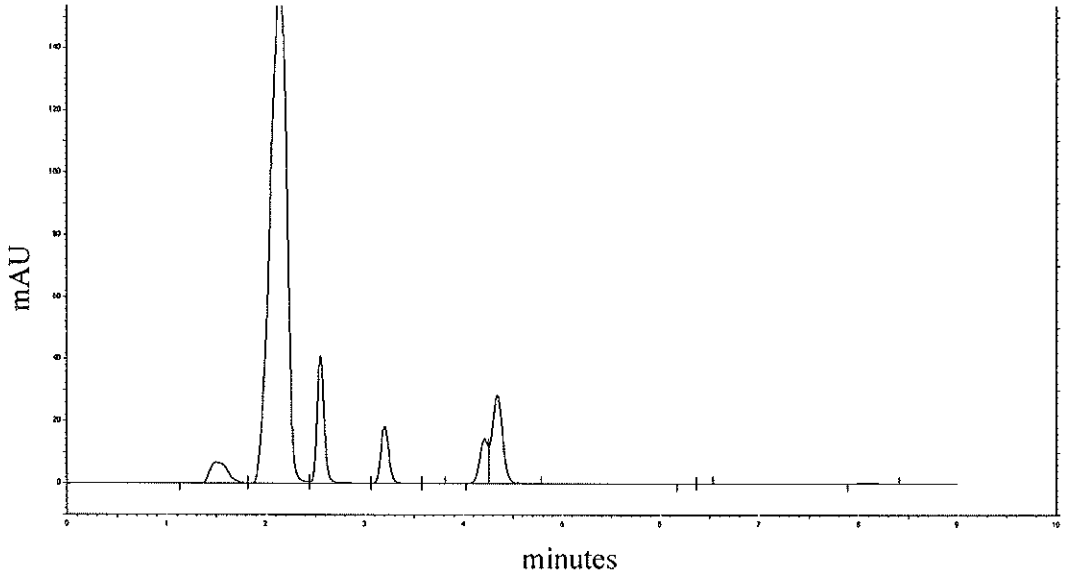

Figure 4.3 Chromatogram of vehicle in solution-type lotion.

Forced degradation studies

The degradation of glabridin was evaluated from forced degradation of glabridin under three conditions; oxidation, acid hydrolysis and basic hydrolysis.

Figure 4.4 shows chromatogram of glabridin from forced degradation under oxidation. The peak of glabridin obtained from degradation by oxidation was similar to peak of glabridin standard solutions. This result indicated that glabridin was not degraded by oxidation.

Figure 4.5 shows chromatogram of glabridin from forced degradation under acid hydrolysis. The peak of glabridin obtained from degradation by acid hydrolysis was similar to peak of glabridin standard solutions. This result indicated that glabridin was not degraded by acid hydrolysis.

Figure 4.6 shows chromatogram of glabridin from forced degradation under basic hydrolysis. The degradation of glabridin was observed by basic hydrolysis.

The peak purity of glabridin was found satisfactory under different stress conditions. There was no interference of any peak of degradation products observed at the retention time of the glabridin peak. The forced-degradation experiment showed that this assay was stability-indicating.

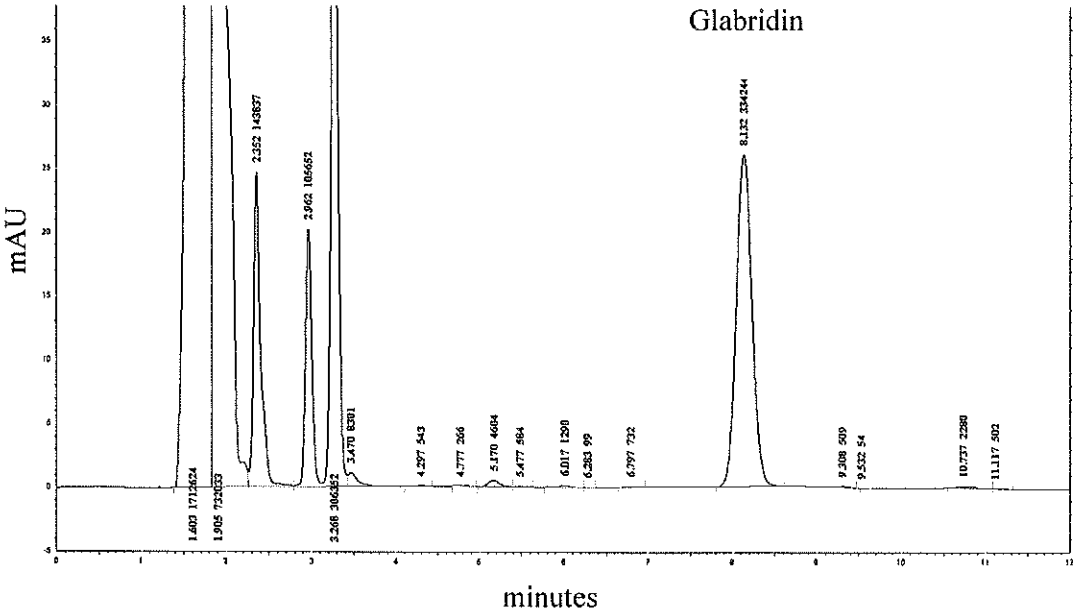

Figure 4.4 Chromatogram of glabridin was forced degradation by oxidation.

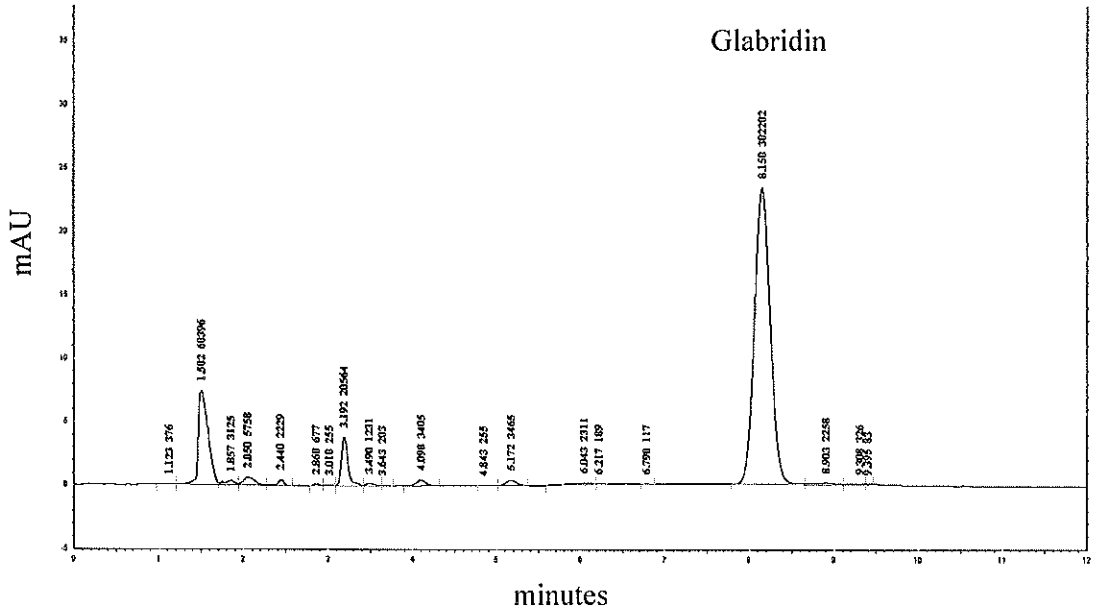

Figure 4.5 Chromatogram of glabridin was forced degradation by acid hydrolysis.

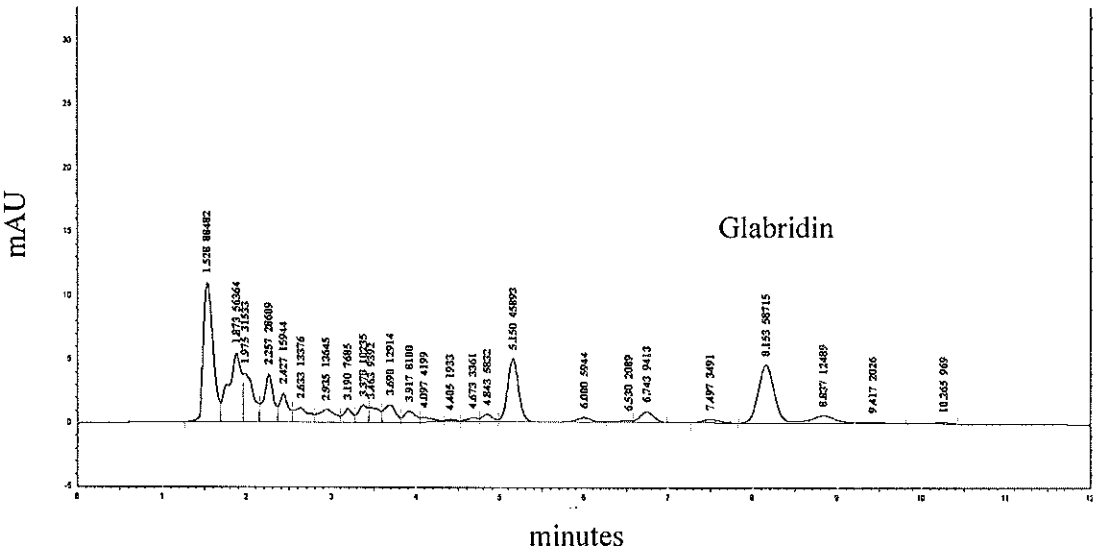

Figure 4.6 Chromatogram of glabridin was forced degradation by basic hydrolysis.

Linearity

The linearity of the assay was determined by plotting the peak area against standard glabridin solutions. Five points calibration curve, Figure 4.7, was obtained in a concentration range from 1-40 μg/ml of glabridin. The response of the glabridin was found to be linear in the investigated concentration range and the linear regression equation was y = 44548x + 27179 with the correlation coefficient of 0.9993 (desired correlation coefficient: ≥0.999). High values of correlation coefficient indicated good linearity.

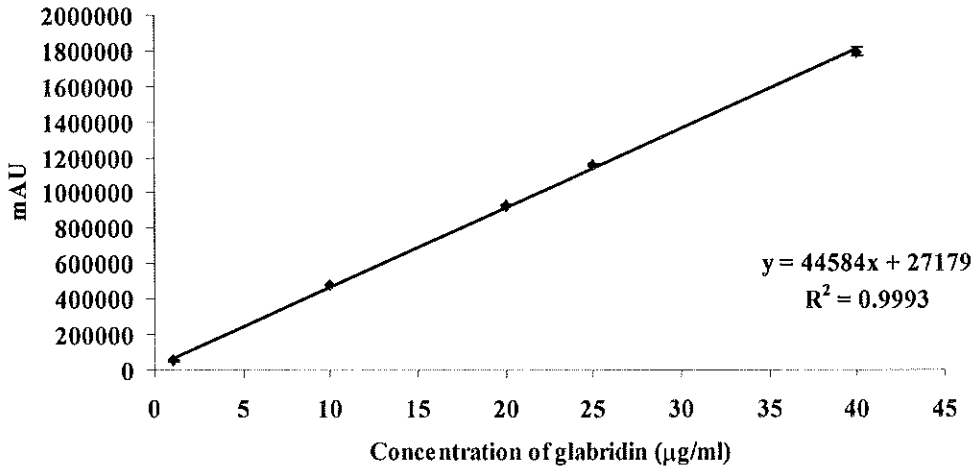

Figure 4.7 A standard calibration curve of glabridin. The plotted data are mean ± SD (n=3). Solid line is a linear regression fit to the data.

Intra-day and inter-day precision and accuracy

Intra-day precision and accuracy were evaluated by preparing and analyzing three low (5 μg/ml), three middle (15 μg/ml) and three high (30 μg/ml) replicates within one day. Inter-day precision and accuracy were evaluated by

preparing and analyzing three low (5 µg/ml), three middle (15 µg/ml) and three high (30 µg/ml) replicates for three consecutive days.

Within run precision (Repeatability, intra-day) and accuracy

Three sets of three standard solutions of glabridin in the concentrations of 5, 15 and 30 µg/ml were prepared and analyzed within one day. The data obtained from precision experiments is given in Table 4.1 for intra-day precision study. The precision was evaluated in the terms of the relative standard deviation (%RSD). The %RSD value was less than 2%, confirming that the method was sufficiently precise. The mean recoveries of glabridin were found to be in the range of 98.86-101.23% indicating high degree of accuracy of the HPLC method.

Table 4.1 Intra-day variability of glabridin analysis.

Glabridin standard solution added (µg/ml)	Set	Measured Concentration (µg/ml)	Mean measured Concentration (µg/ml)±SD	Average recovery (%)	Average % RSD
5	1	4.93	4.94 ± 0.05	98.80	1.01
	2	5.00			
	3	4.90			
15	1	15.34	15.26 ± 0.23	101.73	1.51
	2	15.44			
	3	15.00			
30	1	30.00	30.37 ± 0.38	101.23	1.25
	2	30.75			
	3	30.35			

Between run precision (intermediate precision, inter-day) and accuracy

Three sets of three standard solutions of glabridin in the concentrations of 5, 15 and 30 µg/ml were prepared and analyzed on three consecutive days. The data obtained from precision experiments is given in Table 4.2 for inter-day precision

study. The %RSD value was less than 2%, confirming that the method was sufficiently precise. The mean recoveries of glabridin were found to be in the range of 98.20-101.60% indicating high degree of accuracy of the HPLC method.

Table 4.2 Inter-day variability of glabridin analysis.

Glabridin standard solution added (µg/ml)	Day	Measured Concentration (µg/ml)	Mean measured Concentration (µg/ml)±SD	Average recovery (%)	Average % RSD
5	1	4.93	4.93 ± 0.02	98.60	0.41
	2	4.94			
	3	4.91			
15	1	15.34	15.05 ± 0.28	100.33	1.86
	2	14.82			
	3	14.92			
30	1	30.00	29.83 ± 0.36	99.43	1.21
	2	29.41			
	3	30.07			
5	1	5.00	4.96 ± 0.04	99.20	0.81
	2	4.95			
	3	4.92			
15	1	15.44	15.24 ± 0.18	101.60	1.18
	2	15.10			
	3	15.17			
30	1	30.75	30.19 ± 0.62	100.63	2.05
	2	29.53			
	3	30.28			
5	1	4.90	4.91 ± 0.02	98.20	0.41
	2	4.90			
	3	4.93			
15	1	15.01	15.16 ± 0.14	101.07	0.92
	2	15.27			
	3	15.21			
30	1	30.35	30.06 ± 0.57	101.20	1.90
	2	29.41			
	3	30.43			

Accuracy

The accuracy of the assay was demonstrated by spiking in triplicate freshly prepared lotion vehicle with known amounts of glabridin at 80%, 100% and 120% of the analyte nominal concentration. The concentrations of spiked glabridin were 200, 250 and 300 μg/ml. Individual and average recoveries were calculated and reported in Table 4.3. Percents of recovery of glabridin standard solutions at the concentration of 200, 250, 300 μg/ml were 97.53±1.43, 97.41±0.98 and 97.23±1.34, respectively. The average percent glabridin recovery was 97.39. The procedure precision expressed as % RSD was evaluated for each spiking level and did not exceed 2.0%. According to ICH Q2A and Q2B guidelines recommendation, the average percentage recovery should be 95-105%. A average percentage recovery of glabridin of 97.37 % with % RSD less than 2% was obtained. Therefore, this method was accurate.

Table 4.3 Percent recovery of spike glabridin in solution-type lotion.

Glabridin added (μg/ml)	Set	Glabridin found (μg/ml)	% Recovery	Average recovery (%) ± SD	Average % RSD
199.95	1	196.38	98.21	97.53 ± 1.43	1.47
	2	196.93	98.49		
	3	191.71	95.88		
249.94	1	244.07	97.65	97.41 ± 0.98	1.00
	2	245.54	98.24		
	3	240.77	96.33		
299.93	1	295.66	98.58	97.23 ± 1.34	1.38
	2	291.53	97.20		
	3	287.62	95.90		

A validated stability-indicating HPLC analytical method has been developed for the determination of glabridin. The above results reveal that the method

is selective and stability-indicating. The proposed HPLC method is simple, accurate, precise, specific, and has the ability to separate the glabridin from degradation products and excipients found in lotion vehicle.

Phosphate buffer solution pH 7.4 (20 mM)

20 mM $NaH_2PO_4.2H_2O$ (312.02 mg/100 ml) and 20 mM Na_2HPO_4 (355.98 mg/100 ml) were prepared separately. Then, 40 ml of 20 mM Na_2HPO_4 was mixed with 10 ml of 20 mM $NaH_2PO_4.2H_2O$ until the pH reaches 7.4 (the pH of 20 mM $NaH_2PO_4.2H_2O$ = 4.67 and the pH of 20 mM Na_2HPO_4 = 8.9).

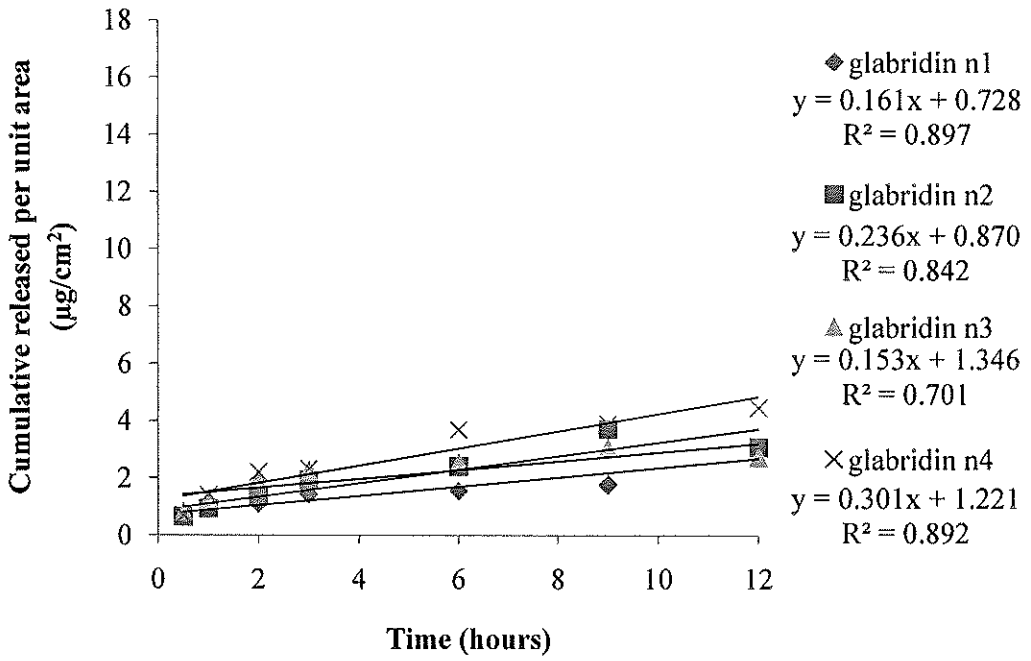

Figure 4.8 Zero order release model of glabridin from lotion containing pure glabridin (n1, n2, n3, n4).

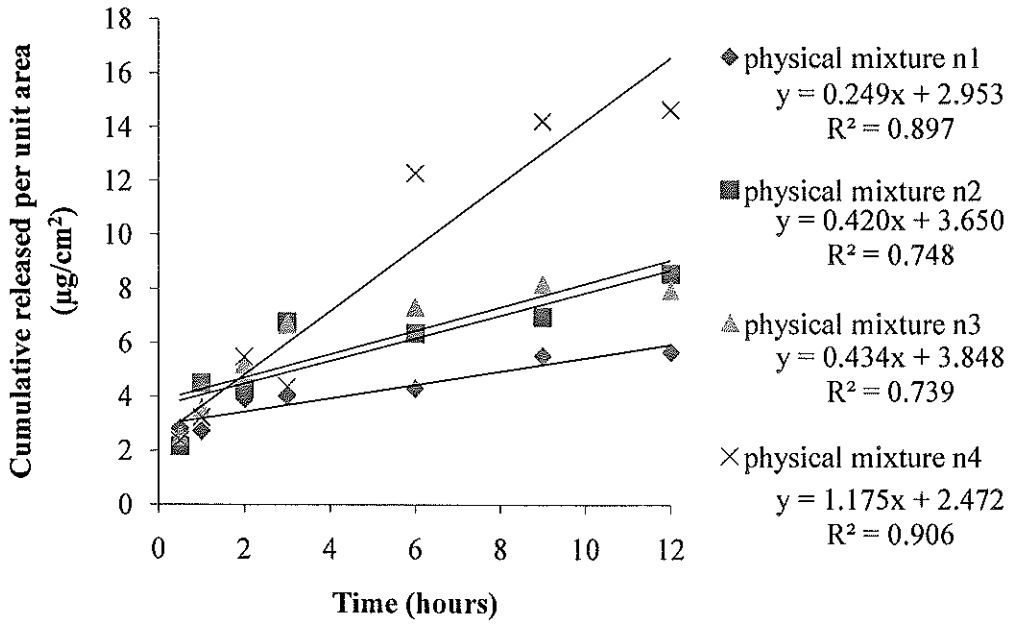

Figure 4.9 Zero order release model of glabridin from lotion containing glabridin-HP-β-CD physical mixture (n1, n2, n3, n4).

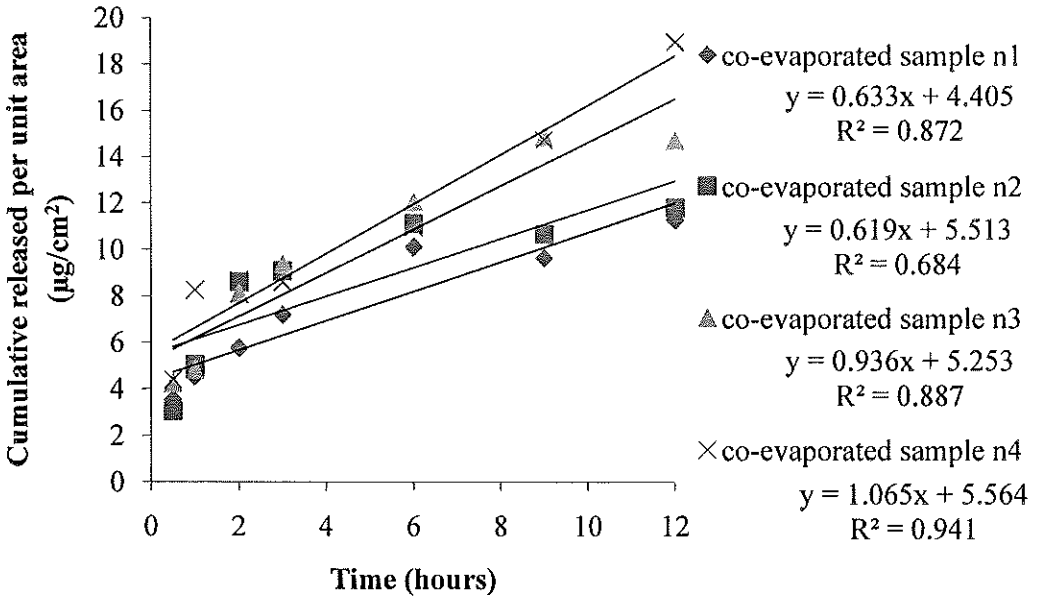

Figure 4.10 Zero order release model of glabridin from lotion containing glabridin-HP-β-CD co-evaporated sample (n1, n2, n3, n4).

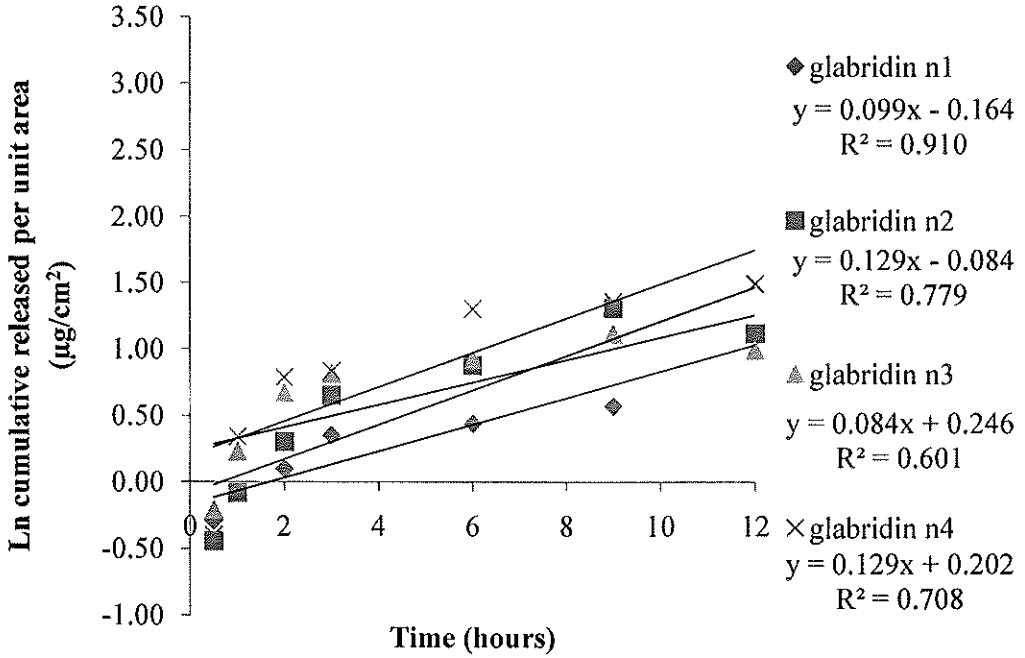

Figure 4.8 First order release model of glabridin from lotion containing pure glabridin (n1, n2, n3, n4).

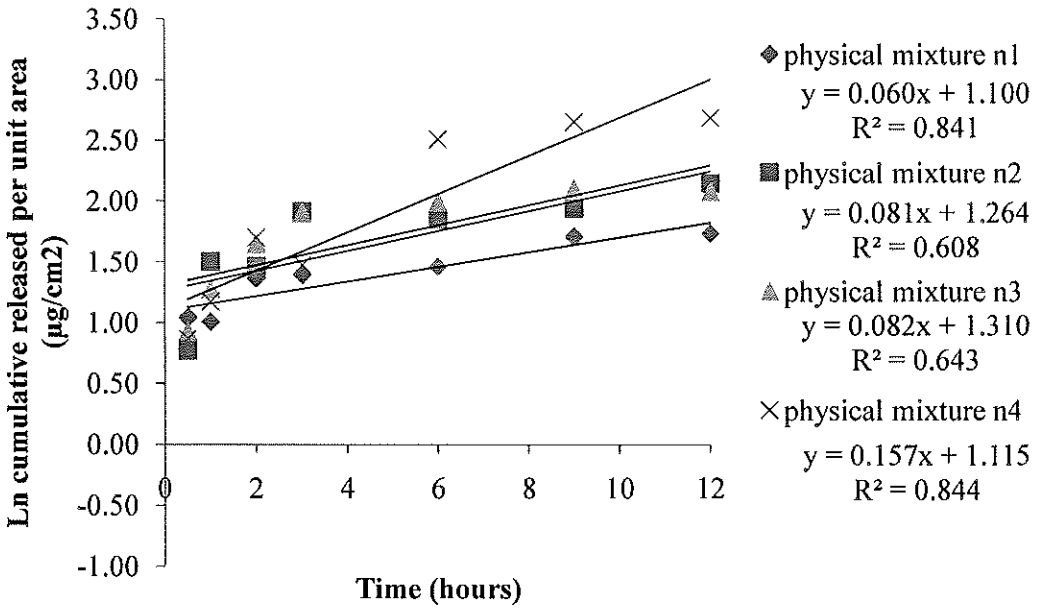

Figure 4.12 First order release model of glabridin from lotion containing glabridin-HP-β-CD physical mixture (n1, n2, n3, n4).

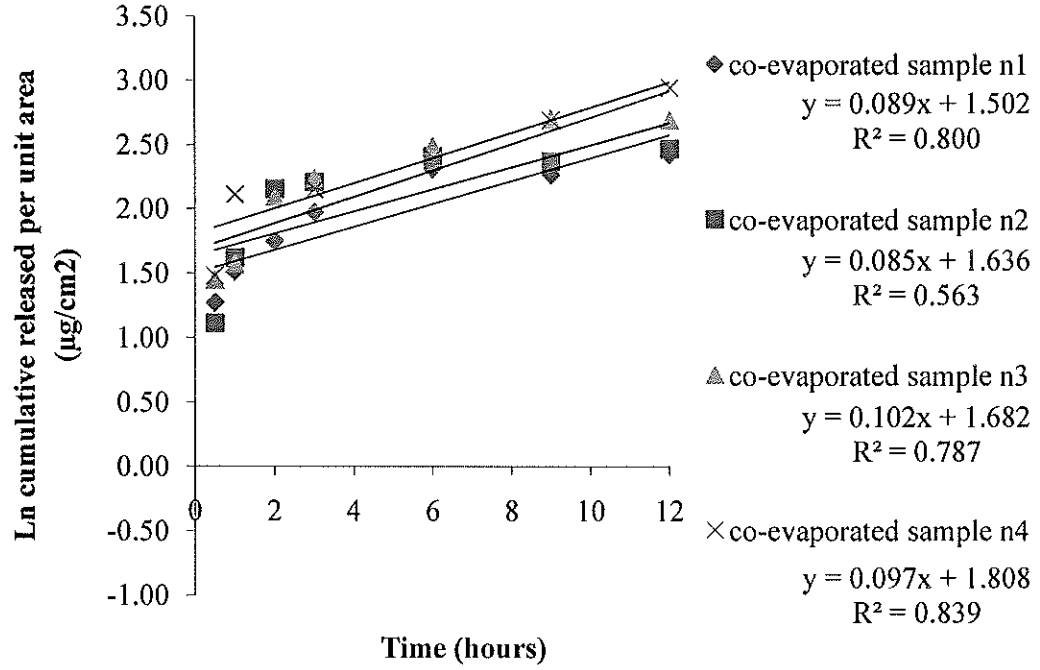

Figure 4.13 First order release model of glabridin from lotion containing glabridin-HP-β-CD co-evaporated sample (n1, n2, n3, n4).

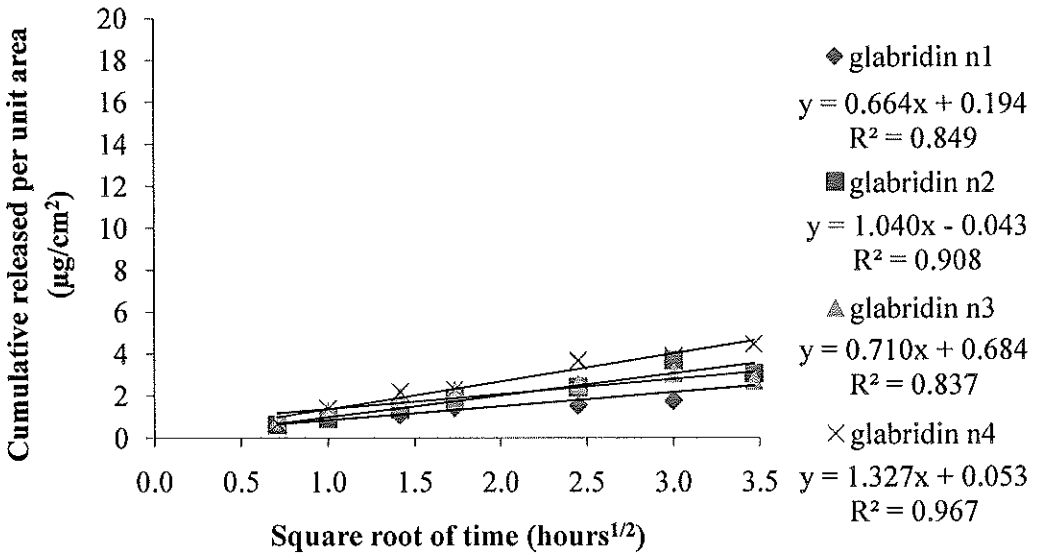

Figure 4.14 Higuchi release model of glabridin from lotion containing pure glabridin (n1, n2, n3, n4).

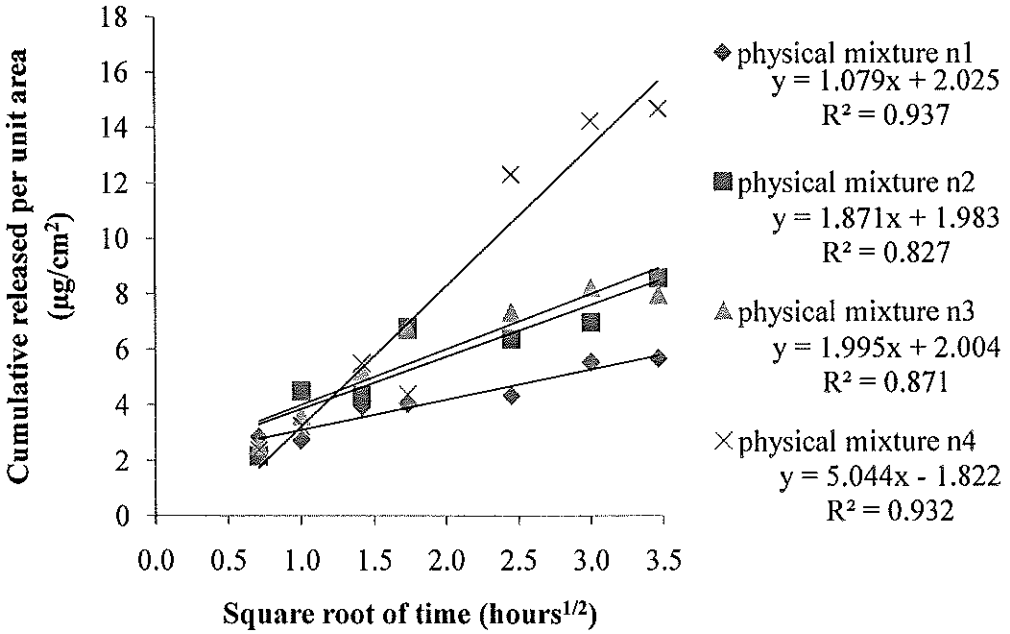

Figure 4.15 Higuchi release model of glabridin from lotion containing glabridin-HP-β-CD physical mixture (n1, n2, n3, n4).

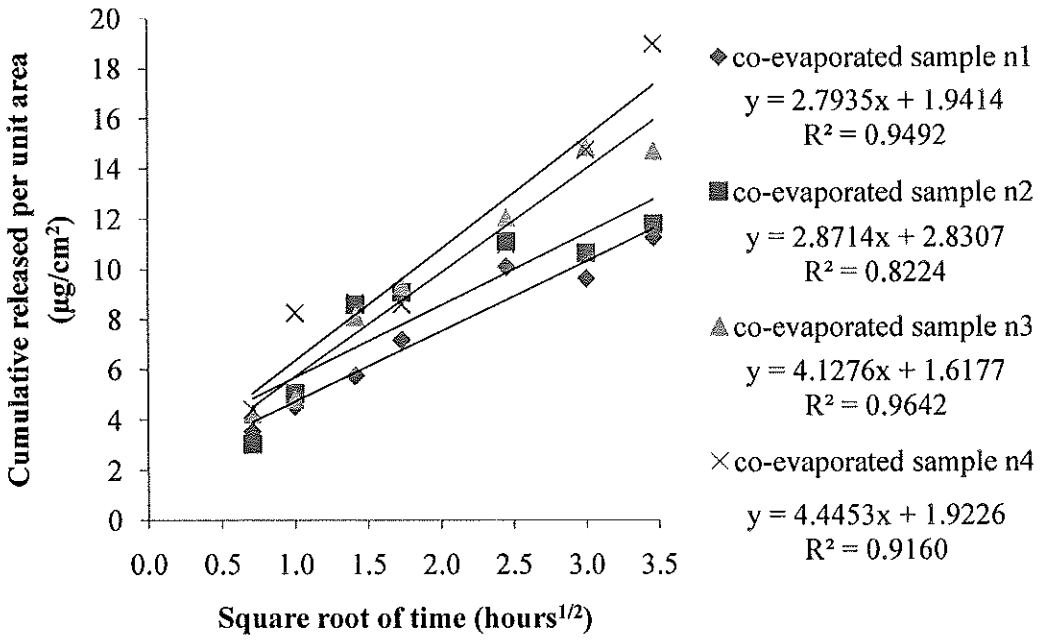

Figure 4.16 Higuchi release model of glabridin from lotion containing glabridin-HP-β-CD co-evaporated sample (n1, n2, n3, n4).

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List of Publication and Proceedings

Piphob Pitaksakseree and Nattha Kaewnopparat (2008). Increased glabridin solubility by complexation with beta-cyclodextrin and gamma-cyclodextrin. Proceedings of the 14th International Cyclodextrin Symposium. May 8-11, 2008, 226-229, Kyoto, Japan (Full text proceedings).

Piphob Pitaksakseree and Nattha Kaewnopparat (2008). Solubility enhancement and tyrosinase inhibition of a glabridin and hydroxypropyl beta-cyclodextrin complex. Oral presented at 34th Congress on Science and Technology of Thailand, Queen Sirikit National Convention Center, Bangkok, Thailand, 31 October – 2 November 2008 (Oral presentation and Full text proceedings).