ผลของไซโคลเดกซ์ทรินส์ที่ใช้ทางตาต่อการละลายและความคงตัวของสารละลายและผลิตภัณฑ์ไลโอฟิไลซ์
ของคีโตโคนาโซลที่มีความแรง 2 เปอร์เซนต์โดยน้ำหนักต่อปริมาตร

นางสาวอังคณา อิ่มเอิบสิน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชกรรม ภาควิชาเภสัชกรรม
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2545
ISBN 974-17-2763-1

EFFECTS OF CYCLODEXTRINS FOR OPHTHALMIC USE ON SOLUBILITY AND STABILITY OF 2% W/V KETOCONAZOLE SOLUTIONS AND LYOPHILIZED PRODUCTS

Miss Angkana Im-erbsin

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Sciences in Pharmacy

Department of Pharmacy
Faculty of Pharmaceutical Sciences
Chulalongkorn University

Academic Year 2002
ISBN 974-17-2763-1

Thesis Title	Effects of cyclodextrins for ophthalmic use on solubility and stability of 2% w/v ketoconazole solutions and lyophilized products
By	Miss Angkana Im-erbsin
Field of study	Pharmacy
Thesis Advisor	Associate Professor Suchada Chutimaworapan, Ph.D.

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....…….…. Dean of Faculty of Pharmaceutical Sciences

(Associate Professor Boonyong Tantisira, Ph.D.)

THESIS COMMITTEE

…….. Chairman

(Associate Professor Porntip Nimmannitya, M.Sc. in Pharm.)

…….…… Thesis Advisor

(Associate Professor Suchada Chutimaworapan, Ph.D.)

…….. Member

(Associate Professor Phensri Thongnopnua, Ph.D.)

…….. Member

(Associate Professor Waraporn Suwakul, M.Sc. in Pharm.)

…….. Member

(Assistant Professor Panida Vayumhasuwan , Ph.D.)

อังคณา อิ่มเอิบสิน : ผลของไซโคลเดกซ์ทรินส์ที่ใช้ทางตาต่อการละลายและความคงตัวของสารละลายและผลิตภัณฑ์ไลโอฟิไลซ์ของคีโตโคนาโซลที่มีความแรง 2 เปอร์เซนต์โดยน้ำหนักต่อปริมาตร. (EFFECTS OF CYCLODEXTRINS FOR OPHTHALMIC USE ON SOLUBILITY AND STABILITY OF 2% W/V KETOCONAZOLE SOLUTIONS AND LYOPHILIZED PRODUCTS)
อ. ที่ปรึกษา : รศ.ดร. สุชาดา ชุติมาวรพันธ์, 132 หน้า. ISBN 974-17-2763-1.

การพัฒนาตำรับคีโตโคนาโซลในรูปสารละลาย และผลิตภัณฑ์ไลโอฟิไลซ์สำหรับใช้ทางตาได้ศึกษาโดยการใช้ไซโคลเดกซ์ทรินส์เป็นสารเพิ่มการละลายและสารเพิ่มความคงตัว จากการศึกษาเฟสการละลายพบว่าการละลายของคีโตโคนาโซลในสารละลายบัฟเฟอร์เพิ่มขึ้นเป็นเชิงเส้นตรงกับความเข้มข้นของไฮดรอกซีโพรพิลเบตาไซโคลเดกซ์ทริน (เอชพีเบตาซีดี) และไฮดรอกซีโพลพิลแกมมาไซโคลเดกซ์ทริน (เอชพีแกมมาซีดี) ซึ่งจัดเป็นแผนภูมิเฟสชนิด A_L ค่าคงที่ความคงตัวของสารประกอบเชิงซ้อนอินคลูชันคีโตโคนาโซล:เอชพีเบตาซีดีที่พีเอช 5 มีค่าสูงกว่าของสารประกอบเชิงซ้อนอินคลูชันคีโตโคนาโซล:เอชพีแกมมาซีดีอย่างมาก ส่วนค่าคงที่ความคงตัวของสารประกอบเชิงซ้อนเอชพีเบตาซีดีที่พีเอช 5 และพีเอช 7 มีค่าเท่ากับ 1263 และ 6904 โมลาร์$^{-1}$ ตามลำดับ เนื่องจากความคงตัวทางกายภาพและทางเคมีที่สูง จึงเตรียมสารละลายและผลิตภัณฑ์ไลโอฟิไลซ์คีโตโคนาโซลที่มีความแรง 2 เปอร์เซนต์โดยน้ำหนักต่อปริมาตรโดยใช้เอชพีเบตาซีดีที่พีเอช 7 การศึกษาความคงตัวแบบสภาวะเร่งของสารละลายคีโตโคนาโซลที่ 50, 60 และ 70°ซ รวมทั้งที่ 5 และ 30°ซ ใช้ไฮเปอฟอร์มานซ์ลิควิดโครมาโทกราฟีในการวิเคราะห์ปริมาณคีโตโคนาโซลที่เหลือ การเพิ่มขึ้นของสีของสารละลายซึ่งเปลี่ยนแปลงตามการเสื่อมสลายของยาสามารถตรวจสอบได้จากการวัดการดูดกลืนแสงที่ 410 นาโนเมตร จากการเขียนกราฟตามสมการอาร์เรเนียส สามารถทำนายอายุการเก็บยาของสูตรตำรับในตู้เย็นและอุณหภูมิห้อง เท่ากับ 556 และ 182 วัน ตามลำดับ โดยค่าอายุการเก็บยาจากการทำนายมีค่าสูงกว่า อายุการเก็บยาที่ได้จากการทดสอบเท่ากับ 111 และ 107 วัน ตามลำดับ พบว่าการนึ่งอัดไอสารละลายมีผลทำให้ตัวยาเสื่อมสลายเพียง 3 เปอร์เซนต์ นอกจากนี้ผลิตภัณฑ์ไลโอฟิไลซ์ของคีโตโคนาโซลมีความคงตัวมากกว่าในรูปสารละลายเมื่อศึกษาในสภาวะเค้นที่ 45°ซ และความชื้นสัมพัทธ์ 75 เปอร์เซนต์ เป็นระยะเวลา 3 เดือน การศึกษาลักษณะทางเคมีกายภาพของสารละลายคีโตโคนาโซลที่มีความแรง 2 เปอร์เซนต์โดยน้ำหนัก ต่อปริมาตร พบว่ามีค่าพีเอชเหมาะสมเท่ากับ 7.06±0.01, ไฮเปอร์โทนิกซิตี้มีค่า 753±12 มิลลิออสโมลต่อกก. และความหนืดต่ำเท่ากับ 2.50±0.01 เซนติพอยส์ ลักษณะของเพาว์เดอร์เอกซ์เรย์ดิฟแฟรกชันและ ดิฟเฟอเรนเชียลสแกนนิงแคลอริมิทรีเทอร์โมแกรมของผลิตภัณฑ์ไลโอฟิไลซ์คีโตโคนาโซล แสดงให้เห็นถึงการเกิดสารประกอบเชิงซ้อนอินคลูชันในรูปอสัณฐานระหว่างคีโตโคนาโซลกับเอชพีเบตาซีดี นอกจากนี้สูตรตำรับสารละลายคีโตโคนาโซลที่ได้ทำให้ไร้เชื้อไม่ก่อให้เกิดการระคายเคืองและความเป็นพิษต่อตาในกระต่ายขาว

ภาควิชา .....เภสัชกรรม.....	ลายมือชื่อนิสิต .....
สาขาวิชา .....เภสัชกรรม…….....	ลายมือชื่ออาจารย์ที่ปรึกษา .....
ปีการศึกษา .....2545.....	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม .....

4476639133 : MAJOR PHARMACY

KEY WORD : CYCLODEXTRINS/ OPHTHALMIC / KETOCONAZOLE / SOLUBILITY/ STABILITY/ SOLUTIONS/ LYOPHILIZED PRODUCTS

ANGKANA IM-ERBSIN : THESIS TITLE. (EFFECTS OF CYCLODEXTRINS FOR OPHTHALMIC USE ON SOLUBILITY AND STABILITY OF 2% W/V KETOCONAZOLE SOLUTIONS AND LYOPHILIZED PRODUCTS) THESIS ADVISOR : ASSOC. PROF. SUCHADA CHUTIMAWORAPAN, 132 pp. ISBN 974-17-2763-1.

The formulation of ketoconazole as aqueous solutions and lyophilized products for ophthalmic use was investigated by using cyclodextrins as a solubilizer and stabilizer. From the phase solubility study, the solubility of ketoconazole in buffer solutions increased as a function of hydroxypropyl-β-cyclodextrin (HP-β-CD) and hydroxypropyl-γ-cyclodextrin (HP-γ-CD) concentrations as type A_L phase diagram. The stability constants of ketoconazole:HP-β-CD inclusion complexes at pH 5 was much higher than that of ketoconazole: HP-γ-CD inclusion complexes. The stability constants of HP-β-CD complexes at pH 5 and pH 7 were 1263 and 6904 M^{-1}. Due to higher physical and chemical stabilities, the 2% w/v ketoconazole solutions and lyophilized products were prepared using HP-β-CD at pH 7. Accelerated stability study at 50, 60 and 70°C, including 5 and 30°C was performed using high performance liquid chromatography to analyze the ketoconazole remaining. The color change which increased accordingly to the drug degradation could be detected by the determination of the absorbance at 410 nm. From the Arrhenius plot, the extrapolated shelf-lives of the formulation at refrigerator temperature and room temperature were 556 and 182 days, respectively. The extrapolated shelf-lives were longer than the observed shelf-lives, which were 111 and 107 days, respectively. Autoclaving of the solution was demonstrated to affect only 3% degradation. Moreover, the ketoconazole lyophilized products were more stable under the stressed condition at 45°C/ 75% relative humidity for three months than its solution form. The physiochemical characterization of 2% w/v ketoconazole solution showed the optimal pH of 7.06±0.01, hypertonicity with 753±12 miliosmole/kg and low viscosity at 2.50±0.01 cps. The powder X-ray diffraction patterns and differential scanning calorimetric thermograms of ketoconazole lyophilized products revealed the formation of amorphous inclusion complexes between ketoconazole with HP-β-CD. Furthermore, the sterile ketoconazole formulation did not produce ocular irritation and toxicity in the albino rabbits.

Department.....Pharmacy..... Student's signature.....

Field of study.....Pharmacy..... Advisor's signature.....

Academic year.....2002..... Co-advisor's signature.....

ACKNOWLEDGEMENTS

First of all, I would like to express my profound gratitude to my advisor, Associate Professor Suchada Chutimaworapan, Ph.D. for her invaluable advice, guidance, enthusiastic encouragement throughout my research study. Her understanding, kindness and patience are honesty appreciated.

I wish to express appreciation to Associate Professor Phensri Thongnopnua, Ph.D. for consultation about the analysis and to all members of the thesis committee for their suggestions and comments.

I would like to express appreciation to Veterinarian Pasakorn Briksawan, Department of Surgery, Faculty of Veterinary Science for spending his valuable time and suggestion for the ocular examination in the ocular irritation and toxicity studies in rabbits.

Special thanks are given to Choongwae Chemical Co., Ltd. for their supplying ketoconazole and the Graduate School, Chulalongkorn University for granting partial financial support to my thesis work.

I wish to thank friends, staffs of the Department of Pharmacy and other people whose names have not mentioned for their assistance and great helpful support.

Also, a special thanks are given to Dr. Pramote Sujinporm, M.D. Director of Surin Hospital and Mr. Sanach Sujinporm, Head of Pharmacy Department, Surin Hospital for their permission of my study.

Ultimately, I would like to express my family for their endless love, understanding, encouragement and cheerfulness throughout this study.

CONTENTS

LIST OF TABLES

LIST OF TABLES (cont.)

LIST OF FIGURES

LIST OF FIGURES (cont.)

LIST OF FIGURES (cont.)

LIST OF ABBREVIATIONS

BZCl	=	benzalkonium chloride
°C	=	degree Celsius
CD	=	cyclodextrin
cm	=	centimetre
cps	=	centipoise
CV	=	coefficient of variation
Cu	=	copper
DSC	=	differential scanning calorimetry
Ea	=	activation energy
FTIR	=	Fourier transform infrared
XRD	=	X-ray diffractometry
g	=	gram
HP-β-CD	=	hydroxypropy1-β-cyclodextrin
HP-γ-CD	=	hydroxypropy1-γ-cyclodextrin
HPLC	=	high performance liquid chromatography
J	=	joule
k	=	degradation rate constant
k_{obs}	=	observed degradation rate constant
$k_{extrapolated}$	=	extrapolated degradation rate constant
°K	=	degree Kelvin
K_C	=	association constant
kcal	=	kilocalorie
kg	=	kilogram
kV	=	kilovolt
M	=	molar
mA	=	milliampere
mg	=	milligram
min	=	minute
ml	=	millilitre
mm	=	millimetre
mOsm	=	milliosmole

LIST OF ABBREVIATIONS (cont.)

mT	=	millitore
nm	=	nanometre
pp	=	page
ppm	=	part per million
psi	=	pound per square inch
R^2	=	coefficient of determination
RH	=	relative humidity
SD	=	standard deviation
µg	=	microgram
µl	=	microlitre
µm	=	micrometre
UV	=	ultraviolet
w/v	=	weight by volume
v/v	=	volume by volume

CHAPTER I
INTRODUCTION

Ketoconazole is a broad spectrum imidazole antifungal drug (Lyman and Walsh, 1992). Topical ketoconazole eye drops of 1-5% w/v have been used for the treatment of fungal corneal infection (Mauger, 1994; Moroi and Lichter, 1996; Wright, 1997; McEvoy, 1999). This drug is a weak base and is water insoluble, having a solubility of 40 μg/ml at 23°C (Quanyun, 1999). Owing to the poor water solubility of ketoconazole, it is usually formulated as topical ophthalmic suspension. Topical ophthalmic suspension has a number of disadvantages compared to solution, for example, physically unstability: caking during storage, eye irritation and needs of being adequately shaken before use (Davies, Wang and Tucker, 1997; Bary, Tucker and Davies, 2000). Furthermore, ketoconazole is a photosensitive and temperature sensitive drug (McEvoy, 1999). It has low aqueous stability and the chemical reaction of ketoconazole degradation has been shown to be oxidation and hydrolysis in aqueous media, in which the major degradation pathway is specific acid catalysis (Suwanna Technowanich, 1999; Skiba et al., 2000).

From numerous studies, cyclodextrins (CDs) have been shown as useful additives in ophthalmic formulations for increasing the aqueous solubility, aqueous stability and bioavailability of ophthalmic drugs and for decreasing drug irritation (Doorne, 1993; Loftsson and Stefansson, 1997; Loftsson and Jarvinen, 1999). Consequently, to overcome these problems of ketoconazole, CDs have received attention as potential solubilizing and stabilizing agents for 2% w/v ketoconazole topical ophthalmic solution.

CDs are cyclic oligosaccharides with a hydrophilic outer surface and a lipophilic central cavity capable of forming inclusion complexes with many water insoluble drugs, which take up a whole drug molecule or some part of it into the central cavity (Loftsson and Stefansson, 1997). The most commonly applied CD in ophthalmic formulations is hydroxypropyl-β-cyclodextrin (HP-β-CD). Numerous studies in animal as well as in human have shown that HP-β-CD is nontoxic to the eye and is well tolerated to the eye, even at high concentration as 45% w/v (Javitt, Javitt and McDonnell, 1994; Loftsson and Stefansson, 1997; Loftsson and Jarvinen, 1999).

Moreover, other CDs that might be considered safe upon topical administration in ophthalmic formulations include maltosyl-β-cyclodextrin, γ-cyclodextrin and hydroxypropyl-γ-cyclodextrin (HP-γ-CD) (Loftsson and Jarvinen, 1999).

Several ophthalmic drugs are prepared as sterile powder for reconstitution, because in powder form these drugs have a much longer shelf life than that of their solution form. The sterile powder for reconstitution is usually manufactured by lyophilization (Hecht et al., 1996). Lyophilization is a drying process where the solvent, normally water, is first frozen and then removed by sublimation in a vacuum environment. Generally, a formulation is prepared as a lyophilized product if the aqueous solution formulation dose not has sufficient stability for marketing (Pikal, 1993). Therefore, it is expected that the ketoconazole formulation which is produced in lyophilized form may have a much longer shelf life than its solution form. This study is aimed to develop the formulation of ketoconazole as aqueous solution and lyophilized product for ophthalmic use by using CDs as a solubilizer and stabilizer.

The objectives of this study were as follows:

1. To study the effects of HP-β-CD and HP-γ-CD on the solubility of ketoconazole in buffer pH 5 and 7
2. To study the preparation of 2% w/v ketoconazole solutions and lyophilized products by using HP-β-CD
3. To study the chemical and physical stabilities of 2% w/v ketoconazole solutions and lyophilized products
4. To study the physicochemical properties of 2% w/v ketoconazole solutions and lyophilized products
5. To study the ocular irritation and toxicity of 2% w/v ketoconazole solution

CHAPTER II
LITERATURE REVIEW

I. Ketoconazole

1. Physicochemical Properties of Ketoconazole

1.1 Chemical Name

The chemical name of ketoconazole is *cis*-l-Acetyl-4-[4-[[2-(2,4-dichlorophenyl)-2-(l*H*-imidazol-l-yl-methyl)-1,3-dioxolan-4-yl]methoxy]phenyl] piperazine (Moffat et al., eds., 1986; BP 1999; Quanyun, 1999; USP 24, 2000). Its structure is shown in Figure 1.

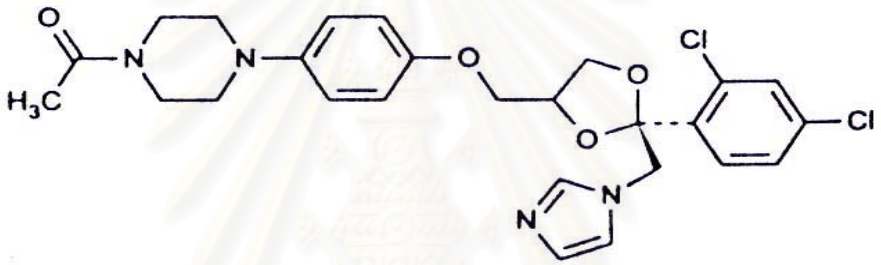


Figure 1 Chemical structure of ketoconazole (USP 24, 2000)

1.2 Chemical Formula

The chemical formula of ketoconazole is $C_{26}H_{28}Cl_2N_4O_4$ (Moffat et al., eds., 1986; Quanyun, 1999). Its chemical abstracts service (CAS) registry number is 65277-42-1 (BP 1999; USP 24, 2000).

1.3 Molecular Weight

The molecular weight of ketoconazole is 531.44 g/mole (USP 24, 2000).

1.4 Description

Ketoconazole is a white to slightly beige powder (McEvoy, ed., 1999; Quanyun, 1999).

1.5 Solubility

Ketoconazole is practically insoluble in water and has the solubility of 40 μg/ml at 23°C. It is sparingly soluble in alcohol, freely soluble in methylene chloride and is very slightly soluble in ether. It is soluble 1 part in 54 parts of ethanol, 1 part in 9 parts of methanol and 1 part in 2 parts of chloroform (Moffat et al., eds.,

1986; BP 1999; McEvoy, ed., 1999; Quanyun, 1999).

1.6 Dissociation Constant

Ketoconazole is a weak dibasic compound, which has pK_as of 2.9 and 6.5, respectively (McEvoy, ed., 1999; Quanyun, 1999).

1.7 Partition Coefficient

Ketoconazole is a lipophilic drug of which logarithm of the partition coefficient (log P) is equal to 4.3 (Jack, 1992).

1.8 Melting Point

The melting point of ketoconazole is between 148°C and 152°C (BP 1999; USP 24, 2000).

1.9 Specific Rotation

Ketoconazole 40 mg/ml in methanol at 20°C has specific rotation between -1^o and $+1^o$ (USP 24, 2000).

1.10 Ultraviolet (UV) Spectrum

In aqueous acid, ketoconazole has maximum absorption at 269 nm and in aqueous alkali, ketoconazole has maximum absorption at 287 nm. In addition, ketoconazole has two maximum absorptions at 244 nm and 296 nm, respectively in absolute methanol (Moffat et al., eds., 1986).

1.11 Infra-red (IR) Spectrum

The principle peaks of IR spectrum of ketoconazole in KBr disk are obtained at wavelengths of 1507, 1640, 1240, 1258, 1200 and 1221 cm^{-1}, respectively (Moffat et al., eds., 1986), as shown in Figure 2.

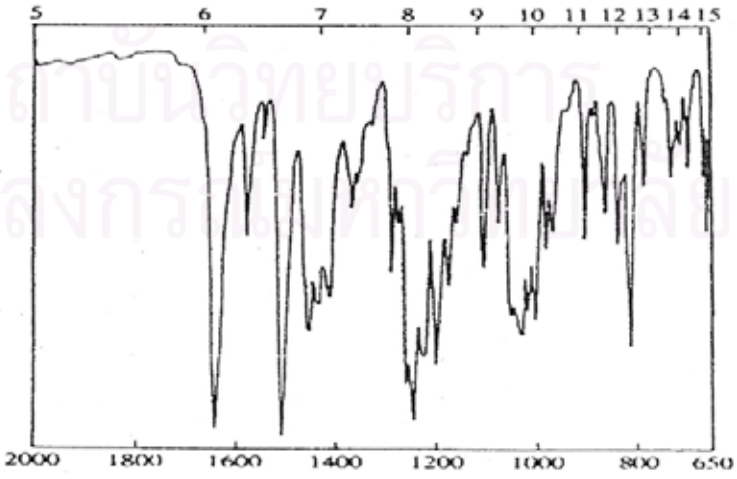


Figure 2 The principle peaks of IR spectrum of ketoconazole in KBr disk (Moffat et al., eds., 1986)

2. Synthesis of Ketoconazole (Heeres et al., 1979)

The synthesis is started from 2,4-dichloroacetaphenone 2 and outlined in Figure 3. Ketalization of 2 with glycerin was performed in a benzene, *n*-butanol medium with azeotropic removal of water in the presence of a catalytic amount of *p*-toluenesulfonic acid. Without isolation, the ketal 3 was brominated at 30°C to bromo ketal 4.

Benzoylation of 4 in pyridine afforded the ester as a cis/trans mixture, from which the cis form 5 could be isolated by crystallization from EtOH. The pure trans isomer could be obtained by liquid chromatography of the mother liquor.

Coupling of bromo ketal 5 in dry DMA with imidazole gave the imidazole derivative 6. The ester 6 was saponified at reflux with NaOH in dioxane-water medium to the alcohol 7. This alcohol was converted to methanesulfonate 8, which was coupled with the sodium salt of 9 to give ketoconazole 1.

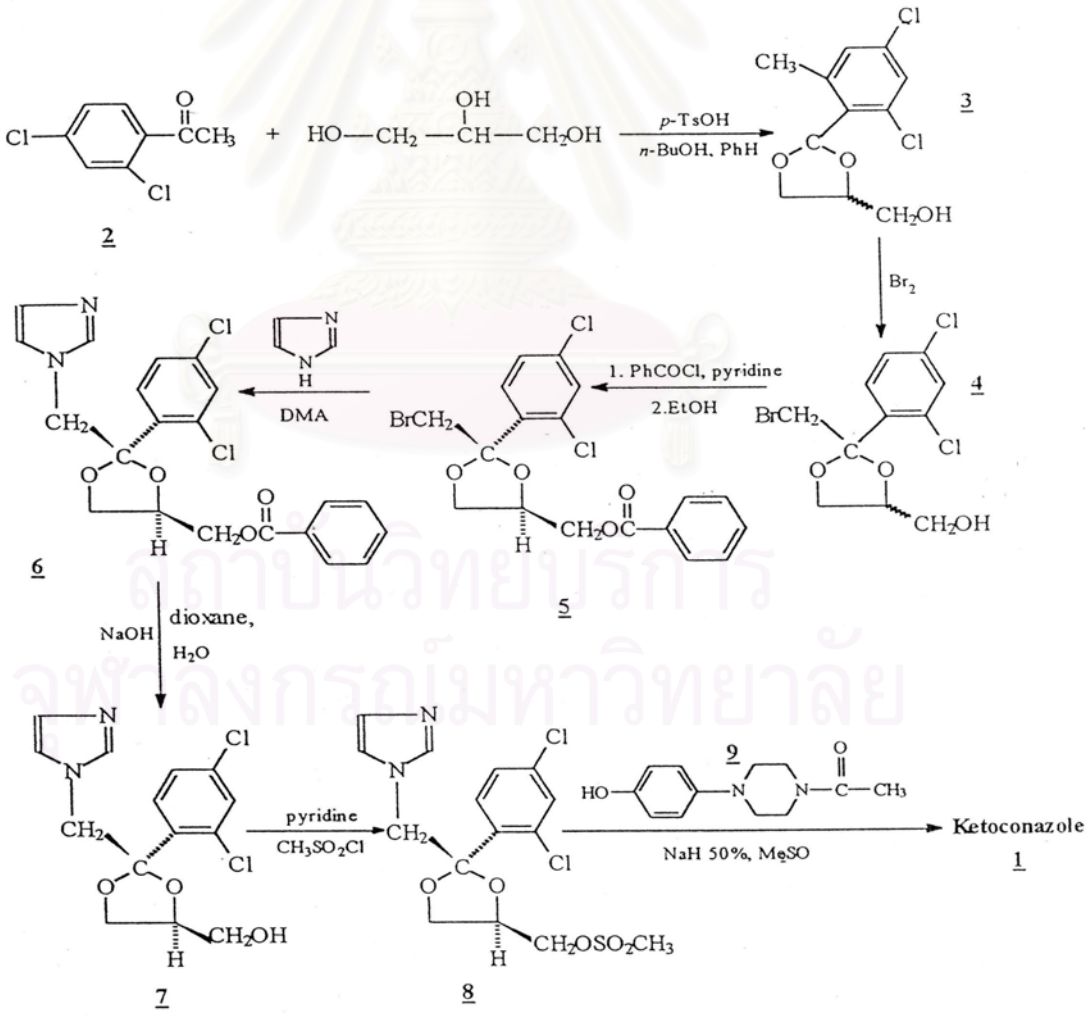


Figure 3 The synthesis scheme of ketoconazole (Heeres et al., 1979)

3. Method of Analysis of Ketoconazole

Many methods have been used to assay ketoconazole in pharmaceutical preparations, raw materials and human serum.

3.1 Potentiometric Method

Potentiometric method for the determination of ketoconazole in raw material was recommended by the USP 24 (2000). Ketoconazole 200 mg was dissolved in 40 ml of glacial acetic acid and titrated with 0.1 N perchloric acid, determining the endpoint potentiometrically. Each ml of 0.1 N perchloric acid is equivalent to 26.57 mg of $C_{26}H_{28}Cl_2N_4O_4$ (USP 24, 2000).

3.2 Chromatographic Method

3.2.1 Thin Layer Chromatographic (TLC) Method

USP 24 suggests that a solvent system for TLC method consists of a mixture of *n*-hexane, ethyl acetate, methanol, water and glacial acetic acid in a proportion of 42:40:15:2:1 to develop a spot of ketoconazole on a thin layer chromatographic plate coated with a 0.25 mm layer of silica gel mixture. The iodine vapor is used as a detection reagent.

3.2.2 High Performance Liquid Chromatographic (HPLC) Method

Different methods of extraction, column types, compositions of mobile phase, type of detectors, detection wavelengths, types of internal standard and conditions have been used for each HPLC method (Alton, 1980; Cavrini, Di Pietra, and Raggi, 1982; Swezey et al., 1982; Pascucci et al., 1983; Badcock, 1984; Di Pietra et al., 1992). For example, Di pietra et al. (1992) assayed ketoconazole in a variety of pharmaceutical formulations (tablets, creams, etc.) by using 5μm Hypersil C-18 column (250×4.6 mm i.d.), methanol - 0.05M triethylammonium (TEA) phosphate buffer (pH 7) (85:15,v/v) as the mobile phase at flow rate of 1 ml min^{-1} and the UV detection at 230 nm.

3.3 Colorimetric Method

In 1988, Sane et al. had reported the use of the colorimetric method for the determination of ketoconazole in pharmaceutical preparation. The method was based on the formation of ion-pair complexes of the drug with reagents like bromocresol green, bromocresol purple, bromophenol blue and bromophenol red in acidic medium. The ion-pair complexes formed were quantitatively extracted in

chloroform and its absorbance was measured at 420 nm. The method was statistically validated and was found to be precise and accurate.

3.4 Ultraviolet (UV) Spectrophotometric Method

Ketoconazole in phosphate buffer solutions of pH 5 and pH 6 was assayed by using UV spectrophotometric method at 225 nm (Diaz, Mendez et al., 1996; Diaz, Otero et al., 1996).

4. Pharmacology of Ketoconazole

Ketoconazole was first marketed in the United Kingdom in March 1981 by Janssen Pharmaceutical Ltd., under the trade name of Nizoral® and subsequently in other countries (Koch, 1983). The Food and Drug Administration (FDA) has approved ketoconazole for the treatment of systemic fungal infections (Hume and Kerkering, 1983). Ketoconazole received FDA's 1-A classification (Hussar, 1982).

4.1 Antimicrobial Activity

Ketoconazole has a broad spectrum of antifungal activity so it is used to treat a wide variety of superficial fungal infections, such as dermatomycoses, onychomycoses, paronychia, pityriasis versicolor, oral candidosis, vaginal candidosis, chronic mucocutaneous candidosis and systemic fungal infections, such as candidal infection, coccidioidomycosis, paracoccidioidomycosis, histoplasmosis, subcutaneous mycoses (Heel et al., 1982; Graybill and Craven, 1983; Daneshmend and Warnock, 1988; Lyman and Walsh, 1992). The spectrum of acitivity includes dermatophytes (e.g. *Microsporum spp., Trichophyton spp., epidermophyton spp.*), yeasts (e.g. *Candida spp., Cryptococcus neoformans*), dimorphic fungi (e.g. *Coccidioides immitis, Histoplasma capsulatam, Paracocidioides brasiliensis*) and various other fungi (e.g. *Asperagillus spp.*) (Heel et al., 1982; Graybill and Craven, 1983). The drug also has in vitro activity against some gram positive bacteria, including *Staphylococcus aurues, S. epidermidis, enterococci spp., Nocardia spp.,* and *Actinomadura spp.* (Heel et al., 1982; McEvoy, ed., 1999). Interestingly, it is also active in vitro against some parasites, including chloroquine-sensitive and -resistant *Plasmodium falciparum* and *Leishmania tropical* (Heel et al., 1982; McEvoy, ed., 1999). Herpes simplex virus (HSV) type I and II are also susceptible to ketoconazole (McEvoy, ed., 1999).

4.2 Mechanism of Action

Ketoconazole is usually fungistatic in action, but may be fungicidal at high concentration (McEvoy, ed., 1999). The drug alters the permeability of the

fungal cellular membranes by interfering with ergosterol synthesis or incorporation. It appears to function via a common mechanism of action, namely inhibitation of a cytochrome P450-dependent enzyme that is involved in the synthesis of ergosterol. One the molecular level, one of the nitrogen atoms of the azole ring is thought to bind to the haem moiety of the fungal cytochrome P450 enzyme lanosterol 14 α-demethylase, thereby interrupting the conversion of lanosterol to ergosterol. In addition, ketoconazole appears to influence enzymes involved in generation and detoxification of intracellular hydrogen peroxide including NADH oxidase, peroxidase, and catalase. The net effect appears to be an increase in intracellular hydrogen peroxide which damages the fungal cell (Heel et al., 1982; Graybill and Craven, 1983; Lyman and Walsh, 1992; McEvoy, ed., 1999).

5. Pharmacokinetic of Ketoconazole

5.1 Absorption

Ketoconazole appears to be well absorbed from the gastrointestinal tract. Absorption of ketoconazole is variable after oral administration, with large variability in peak serum concentrations. The bioavailability of oral ketoconazole depends on the pH of the gastric content in the stomach; an increase in the pH results in decrease absorption of the drug. Ketoconazole is not absorbed systemically after topical administration and minimally absorbed from the vagina. (Heel et al., 1982; Hume and Kerkering, 1983; Daneshmend and Warnock, 1988).

5.2 Distribution

Distribution of ketoconazole varies according to the tissue sample, the underlying diseases and the dose and duration of treatment. Ketoconazole has been detected in urine, bile, saliva, sebum, tears and synovial fluid following oral administration of a 200 mg dose of drug in adult. In human blood, only 1% of ketoconazole is presented as free drug in plasma, 83.7% is bound to plasma proteins, primarily albumin and 15.3% is bound to erythrocytes (Daneshmend and Warnock, 1988).

5.3 Elimination

Ketoconazole is partially metabolized in the liver to several inactive metabolites by oxidation and degradation of the imidazole and piperazine rings, by oxidative O-dealkylation and by aromatic hydroxylation. The major route of elimination of ketoconazole and the metabolites appears to be excretion into the feces

via the bile. Less than 1% of active drug is excreted in the urine (Lyman and Walsh, 1992; McEvoy, ed., 1999).

6. Adverse Reactions of Ketoconazole

Ketoconazole is well tolerated by most patients. Nausea and vomiting occur in 3-10% of patients receiving the drug. Abdominal pain, pruritis, headache, fever, chills, and photophobia develop in 1% or less. Abdominal complaints were reported in up to 23% of patients during a three year study period. Gynecomastia occurs in 3-8% of patients receiving ketoconazole. Transiently elevated liver enzymes occurred in about 10% of patients, and symptomatic liver dysfunction during ketoconazole administration has occurred in a few patients but resolved on discontinuing therapy (Heel et al., 1982; Hume and Kerkering, 1983; Lacy et al., eds. 1999-2000).

7. Drug Interaction of Ketoconazole

Concurrent use of drugs that decrease gastric acidity, such as the H_2-receptor blockers, antacids, and probably omeprazole, decreases absorption of ketoconazole. Ketoconazole inhibits certain hepatic oxidative enzymes especially cytochrom P450. So that, ketoconazole increases the effect and the toxicity or decreases the effect of some hepatically metabolized drugs such as, astemizole, cisapride, corticosteriod, cyclosporin, oral anticoagulants, phenytoin, terfenadine, rifampicin, isoniazid and theophylline (Hume and Kerkering, 1983; Kallet and Aaron, 1992; Lacy et al., eds. 1999-2000).

8. Dosage and Administration of Ketoconazole

The usual adult dosage of ketoconazole is 200 mg daily as single dose and may increase to 400 or 600 mg daily as single dose for severe infections or if the expected response is not achieved. The usual dosage for children who are older than 2 years of age is 3.3-6.6 mg/kg daily as single dose. The duration of ketoconazole therapy depends on the infecting organism and the site of infection. Generally they should be continued until clinical and mycologic tests show that the fungal infection has subsided (Heel et al., 1982; Hume and Kerkering, 1983; Lacy et al., eds. 1999-2000).

9. Dosage Forms of Ketoconazole

Ketoconazole is available in various dosage forms, such as 200 mg tablet, 100 mg/ml ketoconazole suspension, 2% topical cream and 2% shampoo (Reynolds, Parsons, and Sweetman, eds. 1989). In particular use, the tablets are often crushed to a

fine powder and mixed with suitable vehicle for pediatric and geriatric patients (Allen and Erickson, 1996).

Additionally, the following formulations had been developed for pharmacokinetics investigation or being evaluated in clinical trial, such as 80 mg/5ml or 20 mg/5ml oral suspensions (Daneshmend and Warnock, 1988), 20 mg/ml suspension (Allen and Erickson, 1996), 2% w/v ophthalmic suspension (Chedsada Noppawinyoowong and Yosanan Yospaiboon, 1990; Yosanan Yospaiboon, Wilailuk Limpawuthiwaranond and Chedsada Noppawinyoowong, 1990; Torres et al., 1985; Guzek et al., 1998), solutions (Daneshmend and Warnock, 1988; Kumer et al., 1991), 200 mg capsule on a citric acid carrier (Daneshmend and Warnock, 1988) and 400 mg vaginal tablet or passarie (Daneshmend and Warnock, 1988).

10. Stability Studies of Ketoconazole

Ketoconazole is available in both oral and topical preparations. It was reported that ketoconazole is photosensitive and temperature-sensitive drug, therefore its manufacturing procedure and its storage condition should be protected from light and kept at room temperature (McEvoy, ed., 1999).

Ketoconazole tablet should be protected from moisture and stored in well closed containers at 15-30°C. Ketoconazole topical cream should be stored at room temperature and should not be frozen. The cream should not be stored at high temperatures (e.g., warmer than 37°C), since cream generally separate at these temperatures. Ketoconazole shampoo should be stored at temperatures not exceeding 25°C and should be protected from light (McEvoy, ed., 1999).

Kumer et al. (1991) studied the stability of ketoconazole 50 ng/20µl and 100 ng/20 µl in ethanolic solution for the patient who could not be able to ingest a solid dosage form. It was concluded that ketoconazole exhibited good stability in ethanolic solution with and without protected form light within 29 days at ambient temperature and at 8°C. The higher stability was found in suspension dosage form. Allen and Erickson (1996) prepared three preparations of ketoconazole 20 mg/ml suspension in a 1:1 mixture of Ora-Sweet and Ora-Plus, a 1:1 mixture of Ora-Sweet SF and Ora-Plus, and cherry syrup. They found that these preparations were stable for up to 60 days at 5 and 25°C when protected from light.

A possible pathway for the photodegradation of ketoconazole in methanol was demonstrated by Paiboon Nuntankorn (1996) in Figure 4. The photolysis of

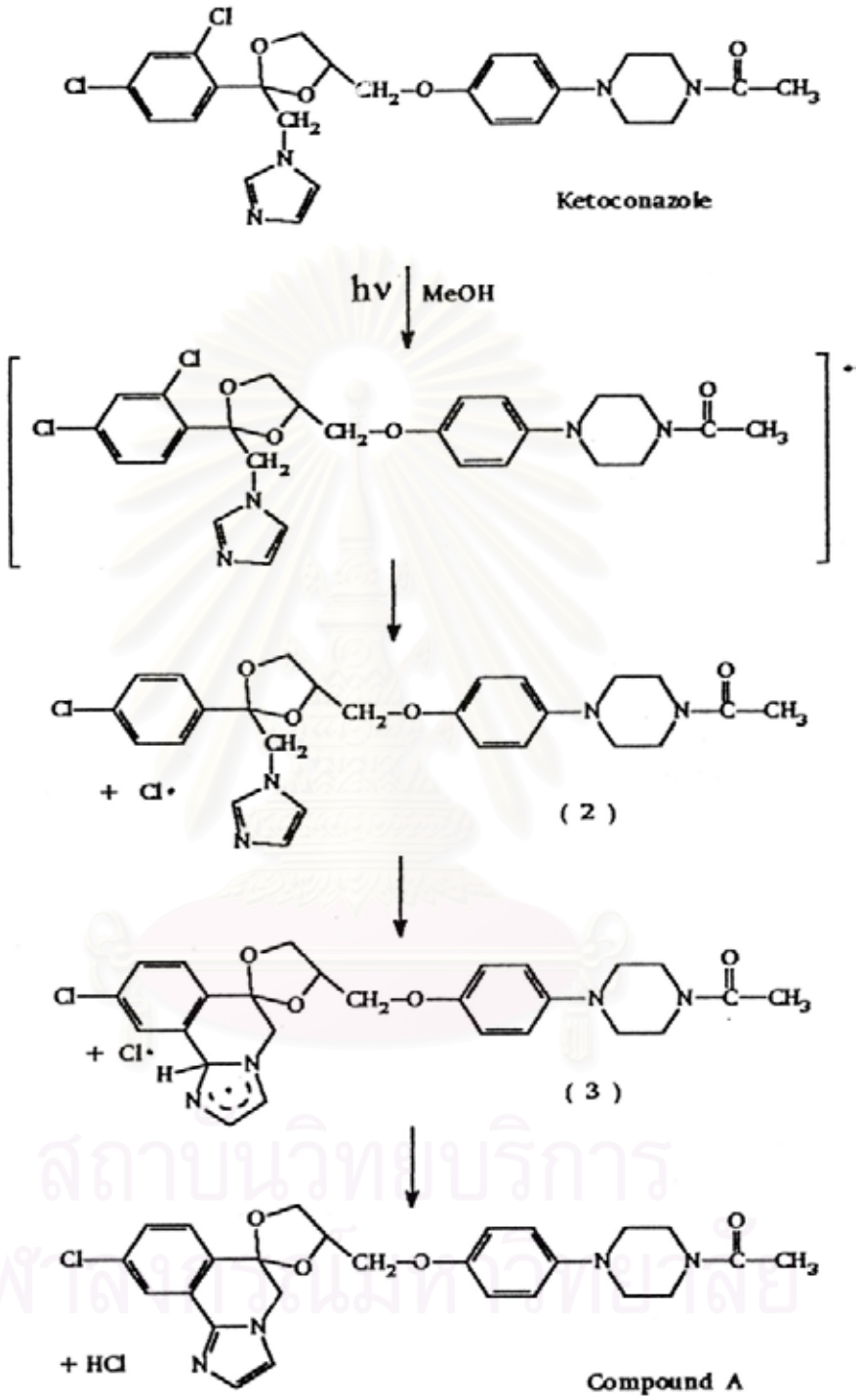


Figure 4 The photodegradation of ketoconazole in methanol (Paiboon Nuntankorn, 1996)

ketoconazole in methanol, acetone, ethylacetate and chloroform with free access to air was investigated. The process was performed by taking ketoconazole dissolved in four organic solvents and irradiated with UV lamp (wavelength 254 nm). The degradation product (compound A) was 1-acetyl-4-[-4-[[(1*H*-imidazo[2,1-a]3,4-dihydro-7-chloro-isoquinolyl)-6-spiro-2′-(1,3-dioxolan-4yl)]methoxy]phenyl] piperazine. It was found that the degradation product occurred when methanol was used as solvent gave better yield than acetone, ethylacetate and chloroform.

The degradation kinetic of 0.1 mg/ml ketoconazole in phosphate buffer solution was studied (Suwanna Techowanich, 1999). The results showed that the degradation rate followed first-order kinetic. The pH rate profile indicated that the ketoconazole degradation at pH between 2.97 and 5.21 in 0.1 M phosphate buffer solution with ionic strength of 0.35 M at 65°C was specific acid catalysis (Figure 5). In addition, from the Arrhenius study, it was found that the predicted shelf-lives at 30°C were 91.3, 113.9 and 114.6 days at pH 3, 4 and 5, respectively. Ketoconazole in aqueous formulations was least stable at pH 1 among the pH values studied (pH 1-9) and the major degradation pathway was specific acid catalysis (Skiba et al., 2000)

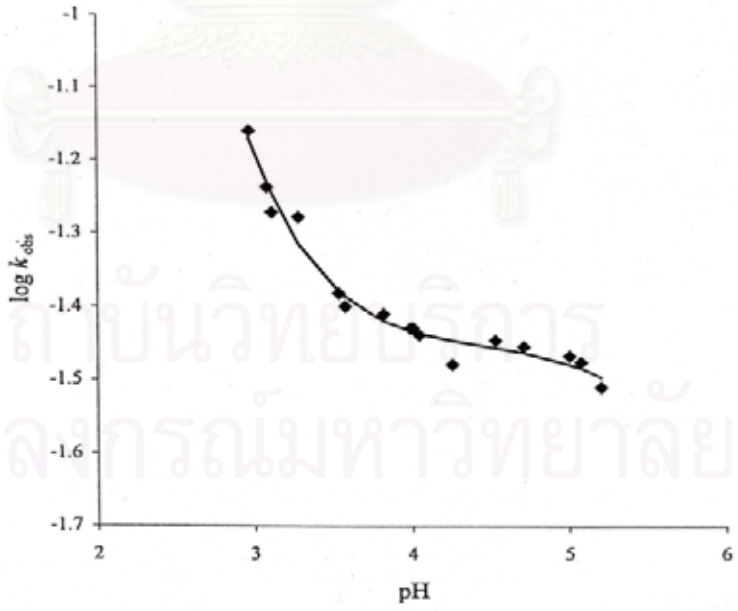


Figure 5 The pH rate profile of ketoconazole degradation in 0.1 M phosphate buffer solution and ioinc strength of 0.35 M at 65°C (Suwanna Techowanich, 1999)

Chemical instability of 2% w/v ketoconazole ophthalmic suspension prepared extemporaneously either from powders or pulverized commercial tablets was found at 50°C (Arunsri Sunthornpit et al., 2000). The results were observed from the color change with time of the supernatant. Microbial susceptibility of the drug in suspensions was within the recommended range but reduced with time especially when stored at 50°C.

II. Cyclodextrins

Cyclodextrins (CDs) are cyclic oligosaccharides consisting of a variable number of D-glucose residues attached by α-1,4 linkages (Figure 6) (Tong, 2000). CDs are enzymic conversion-products of starch. Partially prehydrolysed starch, a mixture of acyclic dextrins, is converted by cyclodextrin glucosyltransferase enzyme to a mixture of cyclic and acyclic dextrins. This enzyme can be produced by various micro-organisms, such as *Bacillus macerans*. From the mixture of acyclic and cyclic dextrins, the pure homogeneous, crystalline cyclodextrins are isolated (Szejtli, 1990). CDs are somewhat core-shaped. The outside of CDs is hydrophilic and the inside of the cavity is hydrophobic in character (Loftsson and Brewster, 1997). These characteristics enable the CDs to entrap various kinds of guest molecule.

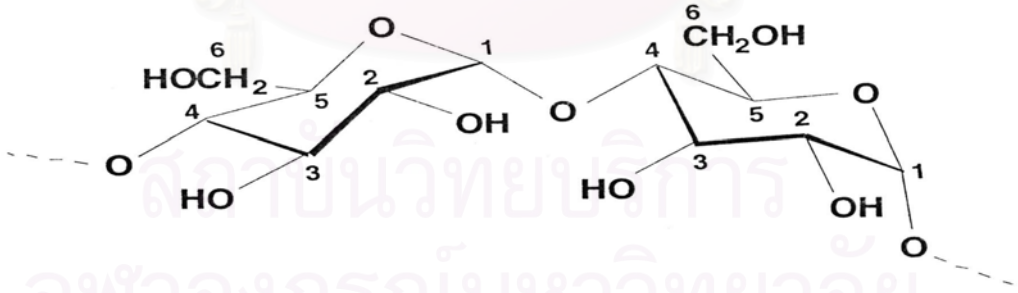


Figure 6 D-glucose residues attached by α-1,4 linkages (Tong, 2000)

1. Structure and Physicochemical Properties of Cyclodextrins

1.1 Natural Cyclodextrins

Three natural CDs are α-CD (cyclohexaamylose), β-CD (cycloheptaamylose) and γ-CD (cyclooctaamylose), consisting of 6,7 and 8 glucose units, respectively (Duchene and Wouessidjewe, 1990a; Martin, 1993). The molecular

structure of natural CDs and their spatial arrangement are shown in Figure 7. The dimensions of the CDs alter with the number of glucose units. Because of their different internal cavity diameters, each CD shows a different capability of complex formation with different sized guest molecules. Table 1 lists the dimensional size as well as some of the important physicochemical properties of natural CDs.

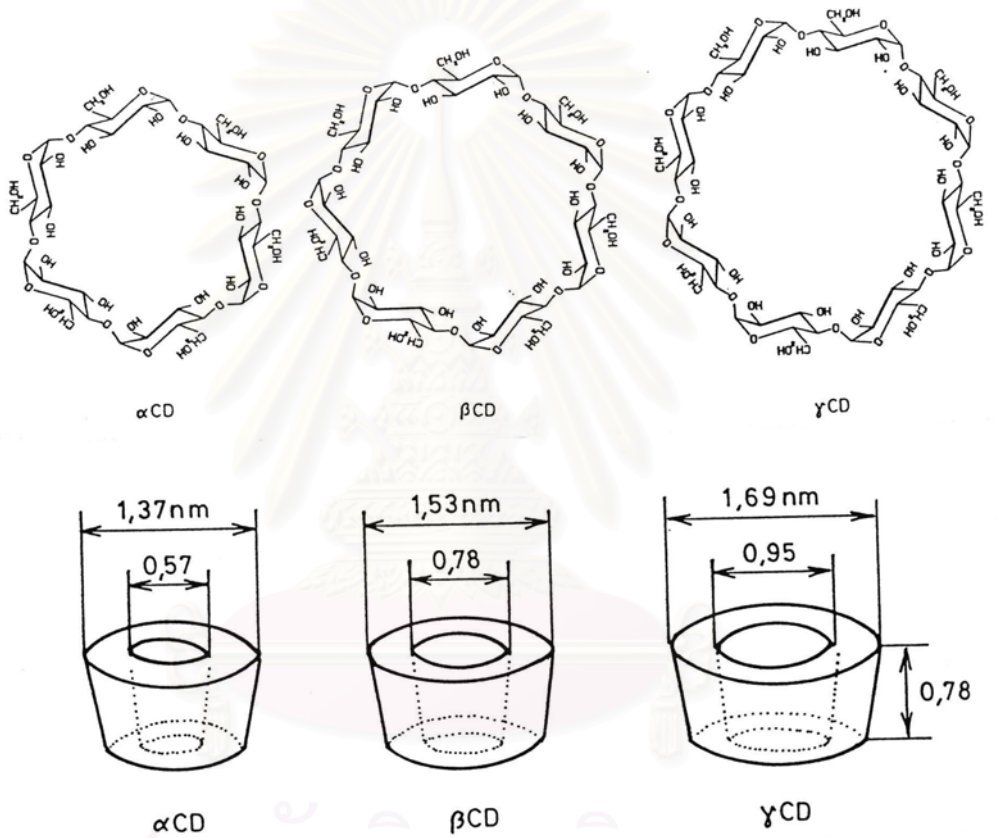


Figure 7 The molecular structure of natural CDs and their spatial arrangement (Li and Purdy, 1992)

Table 1 Physicochemical properties of natural CDs (Szejtli, 1998)

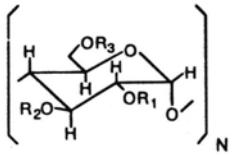
	α-CD	β-CD	γ-CD
no. of glucose units	6	7	8
molecular weight	972	1135	1297
solubility in water (g/100ml at room temp)	14.5	1.85	23.2
$[\alpha]_D^{25^\circ}$ (optical rotation)	150 ± 0.5	162.5 ±0.5	177.4 ± 0.5
cavity diameter (A°)	4.7-5.3	6.0-6.5	7.5-8.3
height of torus (A°)	7.9 ± 0.1	7.9 ± 0.1	7.9 ± 0.1
diameter of outer periphery (A°)	14.6 ± 0.4	15.4 ± 0.4	17.5 ± 0.4
approx. volume of cavity ($A^{\circ 3}$)	174	262	427
approx. cavity volume			
in 1 mol CD (ml)	104	157	256
in 1 g CD (ml)	0.10	0.14	0.20
pK_a (by potentiometry) at 25°C	12.332	12.202	12.081
crystal forms (from water)	hexagonal plates	monoclinic parallelograms	quadratic prisms

Table 1 shows that β-CD is the least soluble which results from intramolecular hydrogen bonds between the C_2-OH group of one glucose unit and C_3-OH group of the adjacent glucose unit. These intramolecular H-bonds stabilize the macrocycle of the CD molecule and turn it into a rigid structure (Bekers et al., 1991).

1.2 Modified Cyclodextrins

Natural CDs can be modified for many different purposes, for example to improve the low aqueous solubility or to decrease the toxicity in parenteral applications. This characteristic has been obtained by alkylation or hydroxyalkylation of the primary (C_6-OH) or secondary (C_2, C_3-OH) hydroxyl groups, by substitutions of primary hydroxyl groups with saccharides, or by polymerization of CDs (Duchene and Wouessidjewe, 1990b). These modified CDs can be classified into three types: hydrophilic, hydrophobic, and ionizable CDs (Irie and Uekama, 1997). The chemical structures of modified CDs and their abbreviations are listed in Table 2.

Table 2 The chemical structures of modified CDs and their abbreviations (Irie and Uekama, 1997)



Compound	Abbreviation	R_1	R_2	R_3
		Hydrophilic Derivatives		
Methylated CDs				
3-Mono-*O*-methyl-CDs		H	CH_3	H
2,6-Di-*O*-methyl-CDs	DM-CDs	CH_3	H	CH_3
2,3,6-Tri-*O*-methyl-CDs	TM-CDs	CH_3	CH_3	CH_3
Romdomly methyl-CDs	RM-CDs		$R_1, R_2, R_3 = H$ or CH_3	
Hydroxyalkylated CDs				
2-Hydroxyethyl-CDs	HE-CDs		$R_1, R_2, R_3 = H$ or CH_2CH_2OH	
2-Hydroxypropyl-CDs	HP-CDs		$R_1, R_2, R_3 = H$ or $CH_2CH(OH)CH_3$	
3-Hydroxypropyl-CDs	3-HP-CDs		$R_1, R_2, R_3 = H$ or $CH_2CH_2CH_2OH$	
2,3-Dihydroxypropyl-CDs	DHP-CDs		$R_1, R_2, R_3 = H$ or $CH_2CH(OH)CH_2OH$	
Branched CDs				
6-*O*-Glucosyl-CDs	G_1-CDs	H	H	H or glucose
6-*O*-Maltosyl-CDs	G_2-CDs	H	H	H or maltose
6-*O*-Dimaltosyl-CDs	$(G_2)_2$-CDs	H	H	H or $(maltose)_2$
		Hydrophobic Derivatives		
Alkylated CDs				
2,6-Di-*O*-ethyl-CDs	DE-CDs	C_2H_5	H	C_2H_5
2,3,6-Tri-*O*-ethyl-CDs	TE-CDs	C_2H_5	C_2H_5	C_2H_5
Acylated CDs				
2,3-Di-*O*-hexanoyl-CDs		COC_5H_{11}	H	COC_5H_{11}
2,3,6-Tri-*O*-acetyl-CDs	TA-CDs	$COCH_3$	$COCH_3$	$COCH_3$
2,3,6-Tri-*O*-propanoyl-CDs		COC_2H_5	COC_2H_5	COC_2H_5
2,3,6-Tri-*O*-butanoyl-CDs	TB-CDs	COC_3H_7	COC_3H_7	COC_3H_7
2,3,6-Tri-*O*-hexanoyl-CDs		COC_5H_{11}	COC_5H_{11}	COC_5H_{11}
		Ionizable Derivatives		
Anionic CDs				
6-*O*-Carboxymethyl-CDs	CM-CDsH	H	H	H or CH_2COONa
Sulfated CDs	S-CDs		$R_1, R_2, R_3 = H$ or SO_3Na	
Sulfobutyl-CDs	SBE-CDs		$R_1, R_2, R_3 = H$ or $(CH_2)_4SO_3Na$	

[a] N = 6: α-CDs; N = 7: β-CDs; N = 8: γ-CDs; N = 9: δ-CDs.

The hydrophilic CDs such as methylated CDs, hydroxyalkylated CDs, and branched CDs merit special study because they exhibit very high water solubilities; these derivatives may be applied to solubilize lipophilic drugs. In contrast, hydrophobic CDs have the ability to decrease the solubility of guest molecules so they may be used as sustained release drug carriers of water soluble drugs. While the ionizable CDs can modify the release rate of drugs depending on the pH of solution (Uekama and Irie, 1990).

Hydroxypropyl CDs such as hydroxypropyl-β-cyclodextrin (HP-β-CD) and hydroxypropyl-γ-cyclodextrin (HP-γ-CD) are mostly utilized in the series of hydroxyalkylated CDs because of their high water solubility. The main reason for their high water solubility is that chemical manipulation frequently transforms the crystalline structure into amorphous structure (Loftsson and Brewster, 1996).

Hydroxypropyl CDs were prepared by condensation of CDs with propylene oxide in a sodium hydroxide solution resulting in substitution of the CD-hydroxyls with hydroxypropyl groups (Szente and Szejtli, 1999; Kibbe, ed., 2000). The water solubility of HP-β-CD and HP-γ-CD are more than 50 g/100 ml at 25°C. The surface tension of HP-β-CD is equal to 52-69 mN/m (dynes/cm) at 25°C (Kibbe, ed., 2000). HP-β-CD is 50-60% w/w soluble in ethanol (95%). HP-β-CD with degree of substituation less than 7 have limited solubility in acetone, whereas one with degree of substituation 11-14 is soluble in acetone or dicholromethane but insoluble in cyclohexane (Pitha et al., 1986). Furthermore, these amorphous compounds are also less hygroscopic than the mother crystalline CDs. The low hygroscopicity of HP-β-CD may be of advantage in pharmaceutical applications since the moisture sorption often initiates hydrolytic degradation of drugs in a solid state (Yoshida et al., 1988).

2. Cyclodextrin Inclusion Complexes

CDs are able to form inclusion complexes with a variety of compounds by trapping various external molecules (guest molecules) inside the cavity of a CD (host) as shown in Figure 8 (Frank, 1975; Martin, 1993; Mosher and Thompson, 2000). The minimum requirment for this inclusion complex formation is that the guest molecule must fit, entirely or at least partially, into the CD cavity (Duchene and Wouessidjewe, 1990a). Stable complexes will not be formed with guest molecules which are too small to be enclosed by the CD molecules because they will slip out the cavity. Complex formation is also impossible with molecules which are too bulky to penetrate into the CD cavity, but possible if certain groups or side chains of the bulky molecule can penetrate into the CD cavity. Not only the steriochemistry but also the polarity of the guest molecules determines whether inclusion may occur. In general, hydrophobic molecules or residues rather than hydrophilic ones have higher affinity to the CD cavity in aqueous solution. The complex of an ionic specie is much less stable than that of a non-ionized one, the hydrophobic CD cavity favoring uncharged molecules to enter.

The interaction force for inclusion complex formation cannot be a classical non-polar binding. Thus the CD complexes formed should be stabilized by various intermolecular forces such as (Bekers et al., 1991; Li and Purdy, 1992):

-Van der Waals interaction between the guest and host, the van der Waals forces here include both permanent induced-dipole-dipole interaction and London dispersion forces

-Hydrogen bonding between the guest and host

-Release of high energy water molecules in complex formation

-Release of strain energy in the macromolecular ring of the CD (change from the high energy conformation of the CD-water complex to the lower energy conformation of the CD-guest complex)

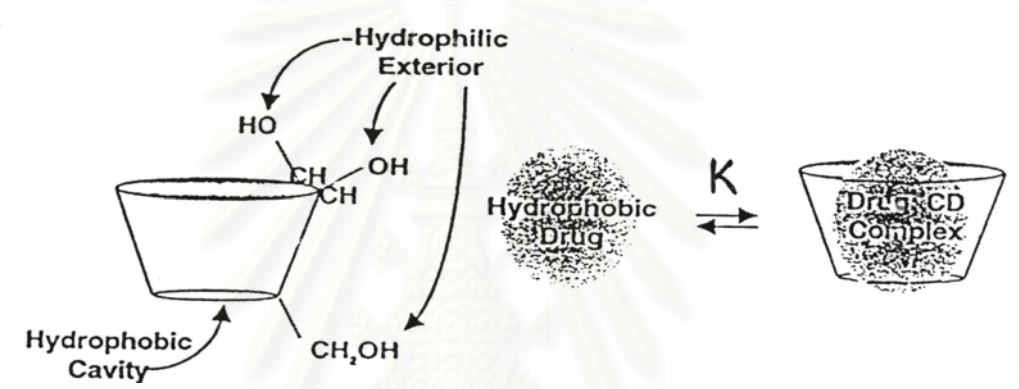


Figure 8 Inclusion complexes of drugs inside the hydrophobic cavity of a CD (Mosher and Thompson, 2000)

3. The Complex Stability Constant

Effects which can be achieved by means of cyclodextrin inclusion complex formation, such as enhancement of the solubility, pharmaceutical availability and bioavailability of the drug, the influence on drug absorption and the stabilization of a drug in solution and in the solid state, all depend on the stability and solubility of the complex. A complexation may be defined as the reversible association of 'm' molecules of a substrate (S=drug) with 'n' molecules of a ligand species (L=CD) to form a new species 'S_mL_n' as shown in Eqaution (1)

$$mS + nL \overset{K_C}{\longleftrightarrow} S_mL_n \qquad \text{Equation (1)}$$

The association constant (stability constant, equilibrium constant, K_C) for the interaction may be defined as (Higuchi and Connors, 1965; Connors, 1997; Mosher and Thompson, 2000)

$$K_C = \frac{[S_mL_n]}{[S]^m[L]^n} \qquad \text{Equation (2)}$$

As defined in Equation (2), K_C is an association or stability constant, where m and n represent the molar ratio (stoichiometric ratio) of the sequestered drug molecule to the CD.

The magnitude of this association constant can be used to compare the binding effectiveness of different CDs. Various complexes with different ratios of drug to CD molecules can be formed, depending on the type of CDs and the size and physicochemical characteristics of the drug molecules (Mosher and Thompson, 2000). Most frequently the CD:drug ratio is 1:1 (Szejtli, 1998).

4. Preparation of Inclusion Complexes

Various methods have been described for preparing the inclusion complexes. However, each applied complexation method may obtain differences in the complexation effectiveness. Types of drug and CD used and the molar ratio of drug-CD for complex formation are also important factors affecting the complexation effectiveness.

In solution the complexes are usually prepared by the addition of an excess amount of the drug to an aqueous CD solution. The suspension formed is equilibrated (for periods of up to one week at the desired temperature) and then filtered or centrifuged to form a clear drug-CD complex solution. For preparation of the solid complexes, the water is removed from the aqueous drug-CD solution by evaporation or sublimation, for example spray-drying or freeze-drying (Szejtli, 1988). Other methods can also be applied to prepare solid drug-CD complexes, and these include co-precipitation, neutralization, kneading and grinding techniques (Loftsson and Brewster, 1997).

In the kneading method, the solid complexes are formed by adding the drug to a slurry of the CDs. The mixture is thoroughly mixed, often at elevated temperatures, to yield a paste which is then dried. However, for water-insoluble compounds, these

methods are not useful unless organic solvents can be used. Organic solvents are also often utilized in the co-precipitation method. One major problem with using organic solvents is that most organic solvents will compete for inclusion in the CD cavity, and thus inhibit complex formation (Tong, 2000).

The neutralization method takes advantage of acidic or basic functional groups and is rather useful for insoluble compounds. It is important, however, to make sure that compounds are stable in the acidic or basic conditions. Finally, solid drug-CD complexes can be formed by the grinding of a physical mixture of the drug and CD and then heating the mixture in a sealed container to 60^{o}-90^{o}C (Loftsson and Brewster, 1997).

5. Detection of Inclusion Complexes

One of the most interesting properties of cyclodextrin is their ability to form inclusion complexes with a wide variety of guest molecules. Molecular encapsulation is occurred both in solution and in solid state. In solution there is an equilibrium between complexes and non-complexes guest molecules. In solid state guest molecules can be enclosed within the cavity or may be aggregated to the outside of the cyclodextrin molecules. Upon inclusion within the cyclodextrin cavity, a guest molecule experiences changes in its physicochemical properties. These changes make it possible to detect whether guest molecules are really included in the cyclodextrin cavity (Beker et al., 1991).

5.1 Inclusion Complexes in Solution (Szejtli, 1988; Becker et al., 1991; Althal, Udupa and Sreenivasan, 1995; Hedges, 1998; Tong, 2000)

Inclusion complexation of cyclodextrin in solution can be studied by a number of physicochemical methods. For examples; in the solubility method, 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 CD concentration, complex formation in solution is indicated.

Spectroscopic methods are ultraviolet/visible (UV/VIS) spectroscopy, circular dichroism spectroscopy, fluorescence spectroscopy and nuclear magnetic resonance (NMR) spectroscopy. Other methods are pH-potentiometric titration, microcalorimetry and surface tension technique.

5.2 Inclusion Complexes in Solid State (Szejtli, 1988; Becker et al., 1991; Althal, Udupa and Sreenivasan, 1995; Hedges, 1998; Tong, 2000)

Several methods have been used to characterize complexes in solid state. Among the most oftenly used methods are differential scanning calorimetry (DSC), powder X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and X-ray crystallography.

5.2.1 Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry has been utilized by many studies to investigate inclusion complexes in solid state. The melting endotherm of the substrate typically is changed as a result of complexation. The complex formed may have a different melting point or no melting endotherm because of its amorphous nature. The physical mixture in most cases will still exhibit the melting endotherms of the substrate and the CD (if it is crystalline).

5.2.2 Powder X-Ray Diffraction (XRD)

X-ray diffractometry is also a useful tool to study complexes in solid state. The complex should give a different powder X-ray diffraction pattern compared to the physical mixture of the host and guest molecules. If the complex formed is crystalline, it is possible to get the single crystal X-ray pattern to elucidate the structure of the complex.

5.2.3 Fourier Transform Infrared Spectroscopy (FTIR)

IR spectroscopy is used to assess the interaction between CD and guest molecules in solid state. The technique is not generally suitable to detect inclusion complexes and is less clarifying than other methods. CD bands often change only slightly upon complex formation and if the fraction of 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 CD. The application of IR spectroscopy is limited to guests having some characteristic bands, such as carbonyl or sulfonyl groups.

5.2.4 X-Ray Crystallography

X-ray crystallography should be the ultimate tool for understanding crystalline complex structures. Many crystalline structures of complexes formed by natural CDs have been reported (Connors, 1997). This technique is less useful for studying complexes formed with CD derivative since almost all the complexes formed with water soluble CD derivatives are amorphous.

6. Phase Solubility Analysis

Phase solubility analysis was described by Higuchi and Connors (1965). Phase solubility analysis is used to determine the relationship between the total concentrations of dissolved drug and the concentration of added CD. The solubility method is based on monitoring changes in solubility of drug by the addition of CD. Excess amount of the drug was added to a solution of CD in various concentrations, shaken at a constant temperature. After equilibrium is attained, the solution phase is analyzed for total concentration of the drug in the solution. The heart of the solubility method is the phase solubility diagram which is constructed by plotting the molar concentration of dissolved drug found on the vertical axis against the molar concentration of CD added on the horizontal axis. Two types of phase solubility profiles can be generated; Type A where soluble complexes are formed, and Type B where complexes of limited solubility are formed, as shown in Figure 9 (Higuchi and Connors, 1965; Mosher and Thompson, 2000; Tong, 2000).

In Type A diagrams, an increase in solubility of the drug occurs as the amount of CD increases. Soluble complexes are formed between the drug and the CD, thereby increasing the total amount of drug in solution. Depending on the nature of the complexes formed, the diagram can be linear, A_L, or curved in a positive, A_P, or negative, A_N, fashion (Figure 9). Linear diagrams are formed when each complex contains only one molecule of CD. When more than one molecule of CD is found in the complex, an A_p-type diagram is formed. The A_N diagrams are uncommon but may result in the presence of self-associations or if high concentrations of CD cause alterations in the nature of the solvent.

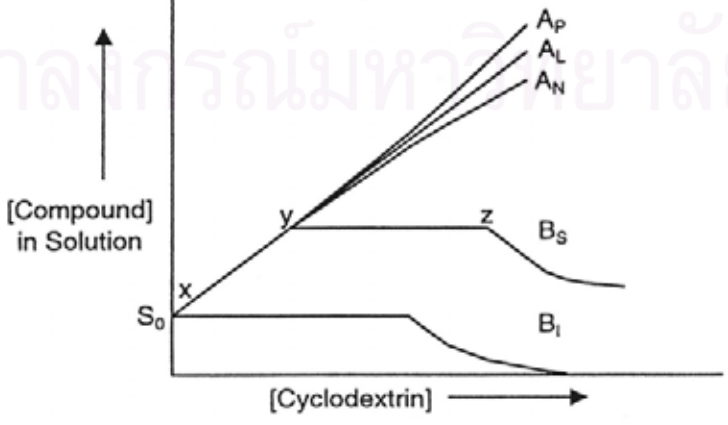


Figure 9 Types of phase solubility diagram (Mosher and Thompson, 2000)

Type B diagrams are observed when complexes of limited solubility are formed. In Figure 9, the segment xy in curve B_S shows the formation of a complex which increases the total solubility of the drug. This is similar to a Type A diagram. At point y, however, the solubility of the complex is reached and as additional drug goes into solution, some solid complex precipitates. At point z, all of the excess solid drug added to the vials has been consumed. Further addition of CD beyond point z results in depletion of the drug from solution by complex formation. Curve B_1 is interpreted in a similar manner except that the complex formed is so insoluble that no increase in solubility is observed. Normally, a stoichiometric ratio can be calculated in type B diagrams (Tong, 2000).

The stoichiometric ratio of the complexes can often be determined from the ascending and descending portions of these diagrams if certain assumptions can be made (Higuchi and Connors, 1965). If a 1:1 complex is formed, the association constants K_C can be determined from the slope of the initial linear portion of the phase solubility curve, and the intrinsic solubility, S_o, of the drug, using Equation (3) (Higuchi and Connors, 1965; Mosher and Thompson, 2000; Tong, 2000).

$$K_C = \frac{\text{slope}}{S_o\,(1\text{-slope})} \qquad \text{Equation (3)}$$

7. Toxicity of Cyclodextrins

The toxicological profiles of CDs have recently been reviewed (Irie and Uekama, 1997). In general, the natural CDs and hydrophilic CDs are only able to permeate lipophilic biological membranes such as the eye cornea, with considerable difficulty (Doorne, 1993; Loftsson and Stefansson, 1997; Loftsson and Jarvinen, 1999; Loftsson and Masson, 2001). After topical administration of a CD containing eye drop solutions, the CD is washed rather rapidly from the surface of the eye through the nose into the gastrointestinal tract. Studies in various animal species and human have shown that the CDs are essentially nontoxic when given orally, due to lack of absorption from the gastrointestinal tract (Doorne, 1993; Loftsson and Stefansson, 1997; Loftsson and Jarvinen, 1999). Thus, no systemic toxicity would be expected after topical administration of CDs in eye drop solution (Doorne, 1993; Loftsson and Stefansson, 1997; Loftsson and Jarvinen, 1999).

Natural α-CD, β-CD and dimethyl-β-cyclodextrins (DM-β-CD) are not suitable for parenteral administration because of renal toxicity, but γ-CD, hydroxypropyl-β-cyclodextrin (HP-β-CD), sulfobutylether-β-cyclodextrin (maltosyl-β-CD) appear to be safe even when administered parenterally (Brewster, Estes and Bodor, 1990; Carpenter, Gerloczy and Pitha, 1995; Stella and Rajewski, 1997; Hirayama et al., 1999). No dermal, ophthalmic, or muscle irritation has been shown from aqueous HP-β-CD concentrations that are as high as 20, 40, or 50% w/v, respectively (Strattan, 1992).

The most commonly applied CD in aqueous eye drop formulations is HP-β-CD (Loftsson and Jarvinen, 1999). Numerous studies in animals as well as in human have shown that HP-β-CD is well tolerated in eye drop solutions. The effects of single and multiple applications of 12.5% HP-β-CD and 5% and 12.5% DM-β-CD eye drop solutions on the corneal epithelium of rabbits with slit lamp biomicroscopy and scanning electron microscopy were studied. It was concluded that DM-β-CD at both 5% and 12.5% caused conjuctival hyperemia and desquamation of corneal epithelial cells with the effects becoming severe at the high dose, while HP-β-CD at 12.5% did not cause any alterations in the corneal tissues (Rajewski and Stella, 1996; Irie and Uekama, 1997; Loftsson and Stefansson, 1997; Stella and Rajewski, 1997). For this reason, DM-β-CD should not be considered for ophthalmic formulations.

Moreover, application of one drop of acetazolamide aqueous eye drop solution containing 18% HP-β-CD to humans, three times a day for 28 days, was well tolerated in the eye (Loftsson and Jarvinen, 1999). Javitt, Javitt and McDonnell (1994) studies the effect of aqueous 45%w/v HP-β-CD solutions containing acetazolamide or methazolamide in the rabbit eyes and showed that these preparations are also non irritating and non-toxic. Furthermore, Savolainen et al. (1998) reported the cytotoxic effects of various CDs on human corneal epithelial cell line. The results revealed the following cytotoxic ranking: α-CD > DM-β-CD > SBE7-β-CD ≈ HP-β-CD > γ-CD.

Other CDs which might be considered safe upon topical administration in aqueous eye drop solutions include maltosyl-β-CD, HP-γ-CD, and at low concentration α-CD. There is some concern that α-CD at concentration > 4% caused superficial epithelial toxicity, such as loss of microvill, and microerosion in the cornea of rabbits (Irie and Uekama, 1997; Loftsson and Jarvinen, 1999). The lack of

available toxicological data will, more than anything else, limit the selection of CDs available for ophthalmic drug formulation.

III. Pharmaceutical Application of Cyclodextrins in Ophthalmic Formulations

Physicochemical and pharmaceutical properties of guest molecules may be altered , resulted from complexation with CDs. These alterations may lead to suitable formulations for potential drugs. A drug may dissolve better and faster, have a better bioavailability, fewer side effects and also be more stable (Duchene, 1987, 1988; Szejtli, 1988, 1991; Duchene and Wouessidjewe, 1990c; Hedges, 1998). Several papers have shown that CDs are useful additives in ophthalmic formulations for increasing the aqueous solubility, aqueous stability and bioavailability of opthalmic drugs, and to decrease drug irritation in the eye (Doorne, 1993; Loftsson and Stefansson, 1997; Loftsson and Jarvinen, 1999; Loftsson and Masson, 2001).

1. Enhancement of Drug Solubility

Generally, poorly water soluble drugs are ophthalmically administered as suspensions or in ointment dosage forms. Both delivery systems are less than ideal, the former leading to irritation if the particles are too large and the latter leading to blurred vision (Rajewski and Stella, 1996; Loftsson and Jarvinen, 1999). Thus, the CDs are mainly added to the aqueous eye drop solutions of poorly water-soluble drugs as drug solubilizers (Loftsson and Stefansson, 1997).

CDs have been used successfully to solubilize opthalmic drugs, including dexamethasone, dexamethasone acetate, diclofenac sodium, acetazolamide, ethoxyzolamide, hydrocortisone, methazolamide, dipivefrin, pilocarpine prodrug [0, 0'- dipropionyl-(1, 4-xylylene) bispilocarpate], anandamide and tropicamide (Usayapant, Karara and Narurkar, 1991; Loftsson et al., 1994a, 1994b; Reer, Bock and Muller, 1994; Jarho, Jarvinen et al., 1996, 1997; Jarho, Urtti et al., 1996; Davies, Wang and Tucker, 1997; Fridriksdottir, Loftsson and Stefansson, 1997; Cappello et al., 2001).

The solubility abilities of CDs depends largely on their abilities to form complexes. The efficacy of the complexation is frequently very low, in which case large amount of CDs is needed to solubilize relatively small amounts of poorly water soluble drugs. In addition to this difficulty, vehicle additives commonly used in

aqueous eye drop formulations, such as sodium chloride, buffer salts, and lipophilic preservatives can displace the drug molecule form the CD cavity, thus reducing the solublizing effect of the CD (Lehner, Muller and Seydel, 1993; Matsuda et al., 1993). In contrast, hydrophilic preservatives, like thimerosal, bronopol, benzalkonium chloride and chlorohexidine gluconate, are not as effectively bound to CDs, so hydrophilic preservatives should be used in aqueous CD containing ophthalmic formulations (Loftsson et al., 1992; Lehner, Muller and Seydel, 1994).

2. Enhancement of Drug Stability

The most common dosage form for topical ocular administration is the aqueous eye drop solutions, which is easy to use and does not blurr vision. However, in aqueous solutions, most drugs are subject to chemical degradation. The consequence of drug degradation is a decrease of drug potency and the formation of degradation products, which may be harmful. Drug stability in eye drop solutions has traditionally been increased by appropriate adjustment of pH and storage conditions (e.g. regarding temperature and light). One of the most common pharmaceutical applications of CDs is to enhance drug stability in aqueous solutions by formation of inclusion complexes (Loftsson and Brewster, 1996).

Inclusion complex formation can be regarded as an “encapsulation” of the drug molecule, or at least the labile part of the molecule. This encapsulation protects the drug molecule against attack by various reactive molecules and in this way reduces the rate of, for example, hydrolysis, oxidation, steric rearrangement, racemization, polymerization and even enzymatic decomposition. In addition, CDs can decrease the photodegradation of various light sensitive drug. However, if the labile part of the drug molecules is located outside the CD, stability may not be affected. (Bekers et al., 1991; Loftsson and Brewster, 1996; Mosher and Thompson, 2000). Several studies have shown that CDs significantly improve the aqueous stability of ophthalmic drugs such as dexamethasone acetate, anandamide, hydrocortisone and dipivefrin, which are very unstable in aqueous solution and are able to produce a useful eye drop solution (Usayapant, Karara and Narurkar, 1991; Jarho, Urtti et al., 1996; Davies, Wang and Tucker, 1997; Jarho, Jarvinen et al., 1997).

3. Enhancement of Drug Bioavailability

In general, the ocular bioavailability of topically applied drugs is very low, frequently less than 5% (Loftsson and Jarvinen, 1999). The main ocular barrier to

drug permeability, into the eye consist of lipophilic membranes (i.e. cornea, conjunctiva and sclera) but the membrane exterior consist of aqueous tear fluid and a hydrophilic mucin layer at the membrane surface and, thus the drug molecule must be somewhat hydrophilic and hydrophobic (Loftsson and Stefansson, 1997). In the case of aqueous eye drop solutions, CDs act as true carriers by keeping the hydrophobic drug molecules in solution without changing their molecular structure and deliver them through the aqueous mucin layer to the surface of the ocular barrier where they partition into the barrier (Loftsson and Stefansson, 1997; Loftsson and Jarvinen, 1999). For this reason, CDs can improve the membrane permeability and ophthalmic bioavailability of poorly water soluble drugs such as dexamethasone, dexamethasone acetate and hydrocortisone (Usayapant, Karara and Narurkar, 1991; Bary, Tucker and Davies, 2000).

It is generally accepted that only the free drug, and not the drug/CD complexes, can penetrate lipophilic membranes and, therefore, the drug must be released from the inclusion complexes before absorption (Frijlink et al., 1990). Thus, in ophthalmic applications, complexation of water-soluble drugs or excess complexation of poorly water soluble drugs may decrease permeability and bioavailability of the applied drug. Several in vitro (Loftsson et al., 1994a, 1994b; Jarho, Urtti et al., 1996) and in vivo (Jarho, Jarvinen et al., 1996; Davies, Wang and Tucker, 1997) studies have shown that excess complexation of poorly water soluble drugs decreases their membrane permeability and ophthalmic bioavailability. Thus, it is important to use only the minimum amount of CDs necessary to solubilize the poorly water soluble drugs because a large excess of CD is present, the fraction of free drug will be decreased and at the same time the fraction of complexed drug will be increased.

4. Reduction of Drug Irritability

Eye drops are commonly dosed at high drug concentrations, directly on the corneal surface and thus, it is not surprising that ophthalmic irritation is a common drawback in ophthalmic drug development and in their clinical use. Ophthalmic irritation was thought to be due to rapid absorption of lipophilic drug into lipophilic corneal epithelium or precipitation of drug molecules in the pre-corneal area (Suhonen et al., 1995). Ophthalmic irritation may decrease patient compliance or in the case of a strong irritation, may even be a reason for patients to stop their medication. In principal, CDs decreased the irritation of ophthalmic drugs such as

diclofenac sodium, pilocarpine prodrug [O, O′-dipropionyl-(1,4-xylylene) bispilocarpate] and cetirizine by formation of inclusion complexes, thereby masking the irritating drugs to decrease the rate of drug absorption (Reer et al., 1994; Jarho, Jarvinen et al., 1996; Loftsson and Jarvinen, 1999).

In addition to ophthalmic formulations, CDs have been applied in order to decrease drug irritation in oral and injectable dosage forms (Duchene and Wouessidjewe, 1990c; Rajewski and Stella, 1996; Stella and Rajewski, 1996).

IV. Ophthalmic Solutions

For diseases and conditions affecting the eye, topical or local administration is preferred over systemic administration for obvious reasons, for example, the systemic toxicity of many ophthalmic drugs, the rapid onset of action, and the smaller dose required compared to the systemic route. Various types of ophthalmic dosage forms are commercially available, such as aqueous solutions, aqueous suspensions, ointments, powder for reconstitution and solid inserts, but the most commonly used are aqueous solutions (Desai and Blanchard, 1995). Other topically applied drug formulations, such as suspensions, oily drops, gels, ointments and solid inserts, have also been used but most of these formulations give rise to unwanted side effects (e.g., eye irritation and blurred vision).

Ophthalmic solutions are sterile solutions, essentially free from foreign particles, suitably compounded and packaged for instillation into eyes. Several ophthalmic drugs are prepared as sterile powder for reconstitution, because of in powder form these drugs have a much longer shelf life than that of their solution forms. The sterile powder is usually manufactured by lyophilization (Hecht et al., 1996).

1. Compositions

In addition to the active drugs, ophthalmic preparations contain a number of excipients, including vehicles, buffers, preservatives, tonicity adjusting agents, antioxidants and viscosity enhancers . The most important factor in the formulation process is the use of ingredients that are nonirritating and compatible with the eyes.

2. Pharmaceutic Requirements

Preparation of an ophthalmic solution requires careful considerations of such factors as clarity, sterility, preservation, pH, buffering, tonicity and viscosity. Many of

these factors are interrelated and are often a compromise among optimal properties and components.

2.1 Clarity

The official definition of ophthalmic solutions requires that they must be free of particulate matter. Solution clarity is usually achieved by filtration, either with a clarifying filter or as part of a sterile filtration procedure. It is essential that these procedures be performed in a "clean-room" environment, for example, in a laminar-flow hood, with personnel properly attired in nonshedding clothing (Desai and Blanchard, 1995).

2.2 Sterility

Sterility is defined as the absence of viable microbial contamination. Sterility is an absolute requirement of all ophthalmic formulations. Contaminated ophthalmic formulations may result in eye infections that could ultimately cause blindness, especially if the *Pseudomonas aeruginosa* microbe is involved.

Common methods of sterilization include moist heat under pressure (autoclave) at 121°C, 15 psi for 15 minutes, dry heat, membrance filtration, gas and ionizing radiation sterilization (Desai and Blanchard, 1995). The method chosen is often dictated by the resistance of the active ingredient and the resultant product to heat and to the type of packaging used.

2.3 Preservation

Ophthalmic solutions are generally packaged in multiple dose containers. Since there is the possibility of inadvertent bacterial contamination of the formulation with repeated patient use, a preservative should be added. Preservatives used should not cause patient sensitivity or are not incompatible with other ingredients in the formulation. Common ophthalmic preservatives for ophthalmic products are shown in Table 3.

2.4 pH and Buffer

The pH of an ophthalmic preparation may be adjusted and buffered for one or more of the following purposes: 1) for greater comfort to the eye; 2) to render the formulation more stable; 3) to enhance the aqueous solubility of the drug; 4) to enhance the drug bioavailability (i.e., by favoring unionized molecular species); and 5) to maximize preservative efficacy (Ansel, Allen and Popovich, 1999).

Table 3 Ophthalmic Preservatives (Desai and Blanchard, 1995)

Type	Concentration Range (%)	Incompatibilities	Remarks
Quaternary ammonium compounds	0.004-0.02 0.01 most common	Soaps, anionic materials, salicylates	Benzalkonium chloride is the single most frequently used ophthalmic preservative; EDTA increases effectiveness
Organic mercurials	0.001-0.01	Certain halides with phenylmercuric acetate	Typically used as substitute for benzalkonium where latter is incompatiable
Parahydroxybenzoates	0.1 maximun	Adsorpion by macromolecules	Infrequently used; activty limited to bacteriostasis
Chlorobutanol	0.5	Stability is pH-dependent; active concentration is near solubility maximum	Diffuses through low-density polyethylene containers
Aromatic alcohols	0.5-0.9	Low solubility in water	As above; occasionally used in combination with other preservatives

The physiologic pH of tears is approximately 7.4 (Ansel, Allen and Popovich, 1999). Thus, from a comfort and safety standpoint, this would be the optimal pH of ophthalmic solutions. This may not be possible, however, from a perspective of solubility, chemical stability or therapeutic activity. Thus, some compromise must be made and product stability must be considered paramount.

When a formulation is administered to the eyes, it stimulates the flow of tears. Tear fluid is capable of quickly diluting and buffering small volumes of instilled solution, thus the eye can tolerate a fairly wide pH range (Hecht et al., 1996). In addition, the buffers are included to resist any change in pH during the storage life of the drug; this can result from absorbed CO_2 from the air or from hydroxyl ions from a glass container. Change in pH can affect the solubility and the stability of drugs, consequently, it is important to minimize fluctuations in pH. The buffer system should be designed sufficient to maintain the pH throughout the expected shelf-life of the product but with a low buffer capacity so as soon as the ophthalmic solutions are dropped into the eye, the buffer system of the tears will rapidly bring the pH of the solution back to that of the tears. This is accomplished by using as low a concentration of the buffer salts as possible but still be effective. Generally a buffer capacity less than 0.05 is desired.

2.5 Tonicity

Tonicity refers to the osmotic pressure exerted by a solution due to the solutes present. Tears have an osmotic pressure of 302 to 318 mOsm/kg, which is approximately equivalent to that of a 0.9% w/v sodium chloride solution (normal saline) (Desai and blanchard, 1995). Tears and normal saline are said to be isotonic, that is, to have equal osmotic pressure. Solutions with osmotic pressure lower than that of normal saline are said to be hypotonic, where as those with higher osmotic pressure are termed hypertonic.

Ideally the ophthalmic solution should have the same osmotic pressure, as tears, but in actual practice, it has been observed that the eye can tolerate a range of osmotic pressure equivalent to 0.6 to 2.0% sodium chloride without marked discomfort (Hecht et al., 1996; Ansel, Allen and Popovich, 1999). Some ophthalmic solutions will be hypertonic by nature of the high concentration required of the drug substance. This is the case for sodium sulfacetamide, for which the isotonic concentration is about 3.5%, but the drug is used in 10-30% concentrations (Hecht et al., 1996). Fortunately, the eye seems to tolerate hypertonic solutions better than hypotonic solutions. Where the amount of such solutions used is small, dilution with tears takes place rapidly so that discomfort from the hypertonicity is only temporary. However, any adjustment toward isotonicity by dilution with tears is negligible where large volumes of hypertonic solutions are used as collyria to wash the eye; it is therefore important that solutions used for this purpose be approximately isotonic

(USP 24, 2000). Tonicity-adjusting ingredients usually used include NaCl, KCl, boric acid, dextrose, glycerin, and propylene glycol.

2.6 Viscosity

The viscosity of ophthalmic solutions is often increased in order to prolong the corneal contact time, decrease the drainage rate, and thus increase the bioavailability of the active ingredient. The most common viscosity desired in the ophthalmic solutions is between 25 and 50 cps, which range significantly improves contact time in the eye (Desai and Blanchard, 1995; Hecht et al., 1996). Numerous viscosity enhancers are used, among which methylcellulose is the most common, generally in a concentration of about 0.25% if the 4000 cps grade or about 1% if the 25cps grade is used. Hydroxypropyl methylcellulose in the range of 0.5 to 1% is a good viscosity enhancer, while polyvinyl alcohol 0.5 to 1.5% w/v is an alternative.

3. Packaging and Storage

Ophthalmic solutions should be packaged in such a way that they are easy to administer and maintain in a sterile condition. Other considerations include the protection of light-sensitive drugs from exposure to light and the use of an "inert" container which does not allow materials to leach out. Such materials may affect product stability (e.g., leaching of alkaline materials from certain types of glass) or patient acceptance by generating particulate matter or other irritants. Originally, ophthalmic solutions were packaged in glass containers with an accompanying glass, dropper. Since the introduction of the low-density polyethylene "Droptainer" in the 1950s, glass containers have largely been replaced. The advantages of the Droptainer include a lower contamination potential, increased convenience of use, lower weight, and lower cost (Desai and Blanchard, 1995; Hecht et al., 1996). Generally, ophthalmic preparations should be stored at either room or refrigerated temperatures and should not be frozen.

V. Chemical Kinetics (Carstensen, 1990, 2000)

The consideration of the basic chemical kinetics is necessary to understand the pharmaceutical problems. The kinetic principles are always of great importance in stability investigations.

Chemical kinetics grant the models for the intermediate steps through which reactions are converted to other compounds, so that it is a useful tool to elucidate the

mechanism of the reactions. In application to pharmaceutics, such information permits a rational approach to the stabilization of pharmaceutical products, prediction of shelf-life and optimum storage conditions.

1. The Order of a Reaction

1.1 Zero-Order Reaction

In zero-order reactions, the rate is constant and independent of the concentrations of any of the reactants. The equation for zero-order reactions is

$$-\frac{d[A]}{dt} = k_0 \qquad \text{Equation (4)}$$

where k_0 is the zero-order rate constant. The integrated form of Equation (4) between the initial concentration at time equal to zero, A_0 and the concentration at time equal to t, A_t is;

$$A_t - A_0 = -k_0 t \qquad \text{Equation (5)}$$

hence, for a zero-order reaction, a plot of concentration against time is linear with a slope of $-k_0$. The unit of k_0 is concentration per time.

The terms that are often utilized are the half-life ($t_{1/2}$) and shelf-life (t_{90}). The $t_{1/2}$ and t_{90} are the time required for one-half and 10%, respectively, of the reactant to disappear. For zero-order reaction, these two terms are calculated by:

$$t_{1/2} = \frac{0.5A_0}{k_0} \qquad \text{Equation (6)}$$

$$t_{90} = \frac{0.1A_0}{k_0} \qquad \text{Equation (7)}$$

It is noted that $t_{1/2}$ and t_{90} of zero-order reaction are dependent on the initial concentration, but the rate is otherwise.

1.2 First-Order Reaction

The rate of reaction that is proportional to the first power of the concentration of the reactant is called first-order reaction. The rate equation for any first-order reaction is:

$$-\frac{d[A]}{dt} = k_1 [A] \qquad \text{Equation (8)}$$

where k_1 is the first-order rate constant. The Equation (8) can be integrated to:

$$\ln A_t = -k_1 t + \ln A_0 \qquad \text{Equation (9)}$$

hence, for a first-order reaction, a plot of natural logarithm function of concentration against time is linear with a slope of $-k_1$. The unit of k_1 is 1/time.

The $t_{1/2}$ and t_{90} of first-order reaction are, respectively,

$$t_{1/2} = \frac{0.693}{k_1} \qquad \text{Equation (10)}$$

$$t_{90} = \frac{0.105}{k_1} \qquad \text{Equation (11)}$$

It is obvious that, for first-order reaction, the $t_{1/2}$ and t_{90} of the reactant are constant irrespective of the concentration of the reactant and independent to the initial concentration. Theoretically, the time required for a reactant to decompose completely takes an infinite period of time.

2. Arrhenius Equation

Temperature is important in pharmaceutical stability for both practical and theoretical reasons. Experimentally, the reaction rate constant showed an exponential dependence on temperature:

$$k = Ae^{-Ea/RT} \qquad \text{Equation (12)}$$

The relationship is called the Arrhenius equation. The equation (12) is usually in its natural logarithmic from as:

$$\ln k = \frac{-Ea}{RT} + \ln A \qquad \text{Equation (13)}$$

where k = the rate constant

Ea = the activation energy (kcal/mol)

R = the gas constant (1.987 cal/mol/degree)

T = the absolute temperature (°C+273.15 degree Kelvin)

A = the frequency factor

A graph of ln k against the reciprocal absolute temperature will give a straight line with a slope of -Ea/R and an intercept of ln A. Since the Arrhenius plot is linear and the Ea is known, it is possible to predict the specific rate constant of the drug at room temperature or at any lower temperature by extrapolation of rate kinetic data obtained at higher temperature. Once the k value is obtained, it can be used to estimate the shelf-life. This procedure is referred to as accelerated stability testing. It is most useful when the reaction in room temperature is too slow to monitor conveniently and when Ea is relatively high.

CHAPTER III
MATERIALS AND METHODS

Materials

Ketoconazole (supplied by Choongwae Chemical, Korea, Lot number KTC 1008)

Hydroxypropyl-β-cyclodextrin (HP-β-CD) with degree of substitution ~ 4.5, molecular weight ~ 1396.3 (Nihon shokuhin kako, Japan, Lot number 121F9)

Hydroxypropyl-γ-cyclodextrin (HP-γ-CD) with degree of substitution ~ 0.6, molecular weight ~ 1580 (Fluka, Switzerland, Lot number 417981/1 52901)

Caffeine anhydrous (Sigma, USA, Lot number 110K0264)

Glacial acetic acid (Merck, Germany, Lot number K29822263 141)

Sodium acetate anhydrous (Merck, Germany, Lot number TA948368 134)

Sodium dihydrogen phosphate monohydrate (Merck, Germany, Lot number A243146 125)

Sodium hydrogen phosphate anhydrous (Merck, Germany, Lot number F1021786 125)

Benzalkonium chloride (distributed by Srichand United Dispensary, Thailand)

Methanol, HPLC grade (distributed by Labscan Asia, Thailand)

Triethylamine (Merck, Germany, Lot number G230418)

Orthophosphoric acid, 85% (Carlo Erba, Italy, Lot number 1G369191L)

Purified water (GPO, Thailand)

Syringe filter cellulose acetate membrane 13 mm, 0.45 μm (distributed by Amani, Thailand, Lot number 007110014052)

Syringe filter cellulose acetate membrane 25 mm, 0.2 μm (Sartorius, Germany, Lot number 16534 020221)

Equipments

Analytical balance (Sartorius model 1615, Germany)

Differential scanning calorimeter (NETZCH DSC 200, Germany)

Freeze dryer (Dura-Dry model FD-6-85DMPO, Japan)

High performance liquid chromatography

- Liquid chromatograph pump (LC-10AD, Shimadzu, Japan)
- UV-VIS detector (SPD-10A, Shimadzu, Japan)
- Auto Injector (SIL-10A, Shimadzu, Japan)
- Column (μBondapak C18, 10 μm, 300 × 3.9 mm, Waters, USA)

Hot air oven (Memmert, Germany)

Laminar air flow (Bassaire model A6HB, England)

Low pressure sodium lamp, SOX-EXWC 121K, Philips, UK)

Osmometer (Osmomat model 030-DM, Germany)

Ostwald viscometer (Arthur H. Thomas, USA)

Pcynometer (Brand Western, Germany)

pH meter (Orion model 420A, USA)

Powder X-ray diffractometer (Bruker model D8 advance, Germany)

Shell-freezer (Just-A-Tilt model SF-4, USA)

Stopwatch (Heuer-Leonidas SA, Switzerland)

Top to bottom rotator (EWPC 902T, Eliwell)

UV-visible spectrophotometer (UV-1601, Shimadzu, Japan)

Methods

Since ketoconazole is photosensitive (McEvoy, ed., 1999), all experiments in this investigation were performed with protection from light. This was achieved by carrying out the experiment in dark room and wrapping all containers with aluminium foil.

I. Phase Solubility Analysis

1. Analysis of Ketoconazole by UV Spectrophotometric Method

In phase solubility analysis, UV spectrophotometric method (UV-1601, Shimadzu, Japan) was used for quantitation of ketoconazole because the method was convenient and rapid.

1.1 Standard Solutions of Ketoconazole

An accurately weighed amount 125 mg of ketoconazole was transferred to a 50 ml volumetric flask. Methanol was used to dissolve and adjust to volume. Four millilitres of this solution were pipetted into 100 ml volumetric flasks and adjusted to 100 ml with 0.1 M acetate buffer pH 5 and 0.1 M phosphate buffer pH 7. These solutions were stock solution of ketoconazole.

Standard solutions of ketoconazole were prepared by pipetting 0.5, 1, 1.5, 2, 3, 4, 5 and 6 ml of ketoconazole stock solutions into 25 ml volumetric flasks. These solutions were diluted with 0.1 M acetate buffer pH 5 and 0.1 M phosphate buffer pH 7 to 25 ml so that the concentrations of standard solution were 2, 4, 6, 8, 12, 16, 20 and 24 μg/ml, respectively.

All of standard solutions were analyzed spectrophotometrically at 225 nm. Acetate buffer 0.1 M, pH 5 and phosphate buffer 0.1 M, pH 7 were used as blank. Each concentration was determined in triplicate. The standard curve was plotted between absorbance against the concentration of standard solutions and the equation of linear regression was calculated.

1.2 Validation of UV Spectrophotometric Method

The analytical parameters used for the assay validation were specificity, linearity, accuracy and precision (USP 24, 2000).

1.2.1 Specificity

Under the UV absorption spectrophotometric method used, the absorbance of ketoconazole must not be interfered by the absorbance of other components in the sample.

Sample solutions of ketoconazole alone, ketoconazole:HP-β-CD inclusion complexes and ketoconazole:HP-γ-CD inclusion complexes in buffer solutions pH 5 and 7 were prepared. The drug complexes solutions were prepared by mixing 2% w/v of ketoconazole with 40% w/v of HP-β-CD and 0.5% w/v of ketoconazole (maximal amount that can be dissolved) with 40% w/v of HP-γ-CD, respectively. These solutions were diluted with 0.1 M acetate buffer pH 5 and 0.1 M phosphate buffer pH 7 so that these solutions containing 24 μg/ml of ketoconazole, 0.48 mg/ml of HP-β-CD and 1.92 mg/ml of HP-γ-CD, respectively. The UV absorption spectra at a wavelength in the range of 200-400 nm of the drug complexes solutions were studied and evaluated by comparing with those of 24 μg/ml standard solution of ketoconazole without HP-β-CD and HP-γ-CD.

1.2.2 Linearity

Eight standard solutions of ketoconazole ranging from 2 to 24 μg/ml were prepared as described in 1.1 and analyzed. Linear regression analysis of the absorbances versus their concentrations was performed. The linearity was determined from the coefficient of determination (R^2).

1.2.3 Accuracy

Three sets of the six standard solutions of ketoconazole were prepared and analyzed spectrophotometrically at 225 nm. The percentage of analytical recovery of each standard solution was calculated.

1.2.4 Precision

a) Within Run Precision

The within run precision was evaluated by analyzing three sets of the six standard solutions of ketoconazole in three intervals of time in the same day. The mean, standard deviation (SD) and the percent coefficient of variation (%CV) of each standard solution were determined.

b) Between Run Precision

The between run precision was evaluated by comparing each concentration of three sets of standard solutions prepared and analyzed in different days. The mean, standard deviation (SD) and the percent coefficient of variation (%CV) of each standard solution were determined.

2. Phase Solubility Study of Ketoconazole with Cyclodextrins at pH 5 and 7

2.1 Phase Solubility Study of Ketoconazole with HP-β-CD and HP-γ-CD at pH 5

The complexation of ketoconazole with HP-β-CD and HP-γ-CD was determined by using the phase-solubility method of Higuchi and Connors (1965). Excessed amount of ketoconazole (150 mg) was added into 2 ml of 0.1 M acetate buffer pH 5 containing 0-30% w/v of either HP-β-CD or HP-γ-CD in the screw-cap tubes. The tubes were continuously rotated (50 rpm) using the top to bottom rotator (EWPC 902T, Eliwell) at room temperature (30±3°C) for a further 72 hours to achieve equilibration (Appendix A).

At equilibrium, the suspensions were filtered through 0.45 μm cellulose acetate membrane filters and then diluted with buffer solution pH 5 to make up appropriate concentrations. The ketoconazole concentration was determined by UV spectrophotometry at 225 nm using buffer solution pH 5 as blank. There was no interference from HP-β-CD and HP-γ-CD at this wavelength. The experiment was carried out in triplicate and protected from light.

The concentrations of dissolved ketoconazole were determined from the standard curve and the molarity of dissolved ketoconazole in each solution was calculated in moles per litre. The phase solubility diagram was constructed by plotting the molar concentration of dissolved ketoconazole on the vertical axis versus the molar concentration of CD added on the horizontal axis. The association constant (K_C) was calculated from Equation (3).

2.2 Phase Solubility Study of Ketoconazole with HP-β-CD at pH 5 and 7

For the pH 7 solutions, ketoconazole (150 mg) was added into 2 ml of 0.1 M phosphate buffer pH 7 containing 0-35% w/v of HP-β-CD. The complexation of ketoconazole with HP-β-CD at pH 5 and 7 was studied by the same method as described in 2.1.

II. Preparation of 2% w/v Ketoconazole Solutions and Lyophilized Products

1. 2% w/v Ketoconazole Solutions at pH 5 and pH 7

Following the phase solubility diagrams in the previous study (I, 2.1). HP-β-CD was chosen to prepare the 2% w/v ketoconazole solutions and lyophilized products. HP-γ-CD was excluded because it could not increase ketoconazole solubility to the extent of 2% w/v. Therefore, two formulations of 2% w/v ketoconazole solutions (pH 5 and pH 7) as shown in Table 4 (Formula I and II) were prepared.

Table 4 Formulation of ketoconazole solutions and lyophilized products

Composition	Formula I pH 5	Formula II pH 7	Formula III pH 7
Ketoconazole (g)	2	2	2
HP-β-CD (g)	25	30	30
Benzalkonium chloride (g)	-	-	0.01
Buffer solution to	100 ml	100 ml	100 ml

The formulation of 2% w/v ketoconazole solution pH 5 (Formula I) was prepared by dissolving ketoconazole and HP-β-CD in 0.1 M acetate buffer pH 5. The solution was mixed by stirring for 48 hours at room temperature, sterilized by filtration through 0.2 μm cellulose acetate membrane filter in larminar air flow (Bassaire model A6HB, England). All steps were protected from light.

The formulation of 2% w/v ketoconazole solution at pH 7 (Formula II) was prepared by dissolving ketoconazole and HP-β-CD in 0.1 M phosphate buffer pH 7. The solution was mixed by stirring for 48 hours at room temperature and filtered through a 0.2 μm cellulose acetate membrane filter in larminar air flow (Bassaire model A6HB, England). All steps have to be protected from light.

2. 2% w/v Ketoconazole Solutions at pH 7 with and without benzalkonium chloride

Following the stability study, ketoconazole solutions at pH 7 were chosen for further studies. The solutions with and without benzalkonium chloride (Formula II and III) were prepared.

3. 2% w/v Ketoconazole lyophilized products with and without benzalkonium chloride at pH 7

In the preparation of 2% w/v ketoconazole lyophilized products with and without benzalkonium chloride, the solutions (Formula II and III) were firstly prepared in the same manner as mentioned above. Afterthat the solutions were then freeze dried by freeze-dryer. (Dura-Dry model FD-6-85DMPO, Japan) for 48 hours. All steps were protected from light.

Freeze-Drying Condition:

The solutions were dipped in a shell freezer (Just-A-Tilt model SF-4, USA) containing ethanol at -45^0C until frozen and then were dried by using a Dura-Dry freeze-dryer (Dura-Dry model FD-6-85DMPO, Japan). When the drying process began, the temperature and pressure were about -74°C to -70°C and 200-300 mT, respectively. The time required to dry these solutions was 48 hours.

III. Stability Studies of 2% w/v Ketoconazole Solutions and Lyophilized Products

1. Analysis of Ketoconazole by HPLC Method

In stability studies, HPLC method was used for quantitation of ketoconazole because of specificity and high sensitivity. The condition of HPLC analysis for remaining ketoconazole in solutions and lyophilized products was modified from Di Pietra et al. (1992).

1.1 Chromatographic Condition

The HPLC conditions for the analysis of ketoconazole remaining were as follows:

Column	: μ Bondapak (C18, 10 μm, 300×3.9 mm)
Mobile phase	: methanol : 0.05M triethylammonium phosphate buffer pH 7.0 (75:25 v/v)

Injection volume : 20 μl
Flow rate : 1.0 ml/min
Detector : UV detector 230 nm
Temperature : ambient
Run time : 10 min
Internal standard : caffeine 50 μg/ml

The 0.05 M triethylammonium phosphate buffer pH 7.0 was prepared by adding phosphoric acid to 0.05 M triethylamine solution to adjust the pH to 7.0.

The mobile phase was prepared by using methanol : 0.05M triethylammonium phosphate buffer pH 7.0 with the ratio of 75 : 25 v/v. The mixture solution was thoroughly mixed, filtered through 0.45 μm membrane filter and then degassed by sonication for 30 min prior to use.

1.2 Standard Solutions of HPLC Method

From the preliminary study, caffeine was chosen to be the internal standard. A stock solution of internal standard was prepared by accurately weighing of 50 mg of caffeine in a 50 ml volumetric flask. Methanol was added to dissolve the internal standard and adjust the final volume to give the final concentration of 1.0 mg/ml.

A stock solution of ketoconazole was prepared by accurately weighing of 25 mg of ketoconazole in a 50 ml volumetric flask. Mobile phase was used to dissolve the drug and adjust the final volume. This stock solution had a final concentration of 0.5 mg/ml.

Standard solutions of ketoconazole were prepared by pipetting 0.2, 0.5, 1, 1.5, 2 and 2.5 ml of the ketoconazole stock solution into 10 ml volumetric flasks, respectively. Then 500 μL of the caffeine stock solution was added into each of these volumetric flasks. The solutions were adjusted to volume with mobile phase so that the concentrations of ketoconazole the standard solutions were 10, 25, 50, 75, 100, and 125 μg/ml, respectively, and that of caffeine was 50 μg/ml. Three sets of standard solutions were prepared for each HPLC run.

As a result, the standard curve of ketoconazole between concentration and peak area ratio was plotted.

1.3 Sample Solutions of HPLC Method

Sample solutions of 2% w/v ketoconazole solutions were prepared by pipetting 50 μl of 2% w/v ketoconazole solutions and 500 μl of the caffeine stock solution into 10 ml volumetric flasks. The solutions were adjusted to volume with mobile phase to give the sample solution of 100 μg/ml ketoconazole and 50 μg/ml of caffeine, respectively, and then injected into the HPLC column. The peak area ratio of ketoconazole and caffeine was calculated and the concentration of remaining ketoconazole was determined from the daily standard curve.

In the case of 2% w/v ketoconazole lyophilized products, they were reconstituted with purified water. These sample solutions were then prepared further as the same manner as described above.

1.4 Validation of HPLC Method

The analytical parameters used for validation of the HPLC method were specificity, linearity, accuracy and precision (USP 24, 2000).

1.4.1 Specificity

Under the chromatographic conditions used, the peak of ketoconazole must be completely separated from and not be interfered by the peaks of other components in the sample.

a) In the Presence of Buffer, HP-β-CD and Benzalkonium Chloride

Sample solutions of blank buffer solutions, including buffer solution pH 5 or buffer solution pH 7, 25% w/v HP-β-CD in buffer solution pH 5, 30% w/v HP-β-CD in buffer solution pH 7 and 0.01% w/v benzalkonium chloride in buffer solution pH 7 were prepared. The pipetting 50 μl of these solution into 10 ml volumemetric flasks were pipetted and adjusted to volume with mobile phase. Chromatograms of these solutions were evaluated by comparing with that of the 100 μg/ml standard solution of ketoconazole.

b) In the Presence of Degradation Products of Ketoconazole

The formulations of 2% w/v ketoconazole solutions pH 5 and pH 7 containing HP-β-CD concentration of 25% w/v in buffer solution pH 5 and 30% w/v in buffer solution pH 7, respectively were kept storing them in hot air oven at 70°C for 2 months to accelerate the degradation of ketoconazole. The sample solutions were then prepared by pipetting 50 μl of 2% w/v ketoconazole solutions and 500 μl of

the caffeine stock solution into 10 ml volumetric flasks. Then solutions were adjusted to volume with mobile phase. Their chromatograms were compared with a that of sample solutions of 2% w/v ketoconazole solutions pH 5 and pH 7, which were not forced to decompose.

1.4.2 Linearity

Six ketoconazole standard solutions were prepared and analyzed. Linear regression analysis of the peak area ratios versus their concentrations was performed. The linearity was determined from the coefficient of determination (R^2).

1.4.3 Accuracy

Three sets of the six standard solutions of ketoconazole were prepared and injected. The percentage of analysis recovery of each standard solution was calculated.

1.4.4 Precision

a) Within Run Precision

The within run precision was determined by analyzing three sets of the six standard solutions of ketoconazole in three intervals of time in the same day. Peak area ratios of ketoconazole to caffeine were compared and the percent coefficient of variation (%CV) of each concentration was determined.

b) Between Run Precision

The between run precision was determined by comparing each concentration of three sets of ketoconazole standard solutions prepared and injected in different days. The percent coefficient of variation (%CV) of ketoconazole of each concentration was determined.

2. Comparison of the Stability of 2% w/v Ketoconazole Solutions pH 5 and pH 7

The formulations (Formula I and II) were kept in amber-glass vials, tightly closed with rubber closures and aluminium caps. They were stored in a hot air oven at 70$\pm$1°C. Three vials were sampling for analysis of remaining ketoconazole at appropriate time intervals. The observed degradation rate constants (K_{obs}) of ketoconazole solutions pH 5 and pH 7 were calculated and compared by Mann-Whitney test at significant level (α) = 0.05 using SPSS for windows version 10.0.

The physical appearances including color and clarity of the preparations were determined before and during the stability studies. Clarity was determined visually by

observation for the absence of precipitation of ketoconazole. Although color change occurred as the drug degraded, it cannot be detected appropriately by visual observation. Therefore, spectral absorbances in the visible region were determined for color change of the solutions. Sample solutions were prepared by pipetting 2 ml of 2% w/v ketoconazole solutions and 2 ml of purified water into 10 ml screwed cap tubes. All of the sample solutions were determined spectrophotometrically at 410 nm (Spectrophotometer UV-1601, Shimadzu, Japan). Purified water was used as blank. However, the physical appearances of the solutions were also visually observed.

3. Stability of 2% w/v Ketoconazole Solution pH 7

3.1 Accelerated Stability Studies

The formulation of 2% w/v ketoconazole solution pH 7 (Formula II) was kept in amber-glass vials at controlled temperatures of 50, 60 and 70°C with $\pm$ 1° C deviation, at refrigerator temperature (5$\pm$3°C) and at room temperature (30$\pm$3° C). Three vials were sampled for analysis of remaining ketoconazole at appropriate time intervals.

The observed degradation rate constants (k_{obs}) of ketoconazole were determined. The ln k_{obs} and reciprocal absolute temperature (1/T) were plotted according to the Arrhenius equation.

From the Arrhenius equation, extrapolated degradation rate constant ($K_{extrapolated}$) and extrapolated shelf-lives ($t_{90,\ extrapolated}$) at 5 and 30°C, respectively, were determined. The observed shelf-lives values ($t_{90,obsered}$) at refrigerator temperature (5$\pm$3°C) and at room temperature (30$\pm$3°C) were calculated by using the observed degradation rate constants (K_{obs}) at refrigerator temperature (5$\pm$3°C) and at room temperature (30$\pm$3°C).

The physical properties including color and clarity were determined by the same method as described in 2.

3.2 Stability Studies after Autoclaving

The formulation of 2% w/v ketoconazole solution pH 7 (Formula II) was kept in clear-glass vials, tightly closed with rubber closures and aluminium caps. The vials were wrapped with aluminium foil to protect the solution from light. These vials were autoclaved at 120°C, 15 psi for 15 minutes (Autoclave Hirayama model HA-3D, Japan). Three vials were analyzed for ketoconazole concentration before and after autoclaving. The statistical difference of percent remaining of ketoconazole

before and after autoclaving was tested by Mann-Whitney test at significant level (α) = 0.05 using SPSS for windows version 10.0.

The physical appearances including color and clarity were determined before and after autoclave by the same method as described in 2.

4. Stability of 2% w/v Ketoconazole Lyophilized Products

The formulations of 2% w/v ketoconazole lyophilized products with and without benzalkonium chloride were kept in clear-glass vials. These vials were wrapped with aluminium foil to protect from light and kept at room temperature (30$\pm$3°C) and at 75% relative humidity (RH) and 45°C (**จุไรรัตน์ รักวาทิน**, 2543) for three months. The latter condition was achieved by keeping the samples under the atmosphere exerting by saturated sodium chloride solution in a desiccator keeping in a incubator at 45°C. Three vials were sampled for analysis of remaining ketoconazole in each formula by using the HPLC method at appropriate time intervals.

The physical appearances including reconstitution time, color and clarity were determined before and during the stability studies by:

a) Reconstitution time was recorded by stopwatch (Heuer-Leonidas SA, Switzerland) for the time needed to reconstitute the lyophilized product into a clear solution by hand shaking with uniform shaking rate and force.

b) Color and clarity were determined by the same method as in 2.

The statistical difference of percent remaining of ketoconazole of the products before and during the storage was tested by multiple-factors analysis of variance (Multiple-Factors ANOVA) at significant level (α) = 0.05, SPSS for windows version 10.0.

.

IV. Physicochemical Properties of 2% w/v Ketoconazole Solutions and Lyophilied Products

1. Physicochemical Properties of 2% w/v Ketoconazole Solutions pH 7 with and without Benzalkonium Chloride

The formulations of 2% w/v ketoconazole solutions pH 7 with and without benzalkonium chloride were determined for their physicochemical properties such as pH, viscosity, and tonicity at room temperature.

The measurements were as follows:

1.1 pH was measured by pH meter (Orion model 420A, USA).

1.2 Viscosity was determined by Ostwald viscometer (Arthur H. Thomas, USA). The time required for measured solutions and the purified water flow between two marks on the vertical capillary tube at room temperature (30°C) were determined. The viscosity of 2% w/v ketoconazole solutions pH 7 with and without benzalkonium chloride were calculated by substituting the experimental values in the Equation (14) (Martin, 1993).

$$\frac{\eta_1}{\eta_2} = \frac{\rho_1 t_1}{\rho_2 t_2} \qquad \text{Equation (14)}$$

η_1 = Viscosity of 2% w/v ketoconazole solutions pH 7 with and without benzalkonium chloride

η_2 = Viscosity of purified water, 0.7977 cps at room temperature (30° C) (Lide and Frederikse, 1995-1996)

ρ_1 = Density of 2% w/v ketoconazole solutions pH 7 with and without benzalkonium chloride, 1.0955 and 1.0956 g/ml at room temperature (30°C) (determined by using a pycnometer in Appendix B)

ρ_2 = Density of purified water, 0.9787 g/ml at room temperature (30° C) (determined by using a pycnometer in Appendix B)

t_1 = Flow time of 2% w/v ketoconazole solutions pH 7 with and without benzalkonium chloride at room temperature (30°C) (Appendix C)

t_2 = Flow time of purified water, 1.54 minutes at room temperature (30°C) (Appendix C)

1.3 Tonicity was measured by the Osmometer (Osmomat model 030-DM, Germany).

2. Physicochemical Properties of 2% w/v Ketoconazole Lyophilized Products

Physicochemical properties of 2% w/v ketoconazole lyophilized products with and without benzalkonium chloride were investigated by using powder X-ray diffractometry and differential scanning calorimetry (DSC) and compared with those

ketoconazole, HP-β-CD and physical mixture of ketoconazole with HP-β-CD which was triturated with a mortar and pestle for 5 minutes.

2.1 Powder X-ray Diffractometry

Powder X-ray diffractograms were carried out by using powder X-ray diffractometer (Bruker model D8 advance, Germany) with Nickel filtered and Cu-K_α radiation as the source of X-ray. The measurement conditions were as follows: voltage of 40 kV, current of 40 mA, scanning speed of 3°/min in the 2-θ angle range of 4-40°.

2.2 Differential Scanning Calorimetry (DSC)

DSC thermograms were determined by using differential scanning calorimeter **(**NETZSCH DSC 200, Germany). An accurately weighed amount of 3-7 mg of powder samples were placed in an aluminium pan and pierced lid. The run was performed at a heating rate of 10°C/min, in the temperature range from 25-250°C under nitrogen gas atmosphere.

V. Ocular Irritation and Toxicity Test of 2% w/v Ketoconazole Solution pH 7 in Rabbits

The Draize test (Auletta, 2000) was used to study the ocular irritation and toxicity of sterile 2% w/v ketoconazole solution pH 7, which had benzalkonium chloride as preservative in rabbits. Six young adult male albino (New Zealand White) rabbits, weighing approximately 2-3 kg were used in the study.

An approximate volume of 0.1 ml (2 drops) of 2% w/v ketoconazole solution pH 7 was instilled into conjunction sac of the right eye of a rabbit three times a day (10.00 a.m., 12.00 a.m. and 14.00 p.m.) for four weeks. The left eye of a rabbit was dropped with 0.9% sodium chloride as control. Ocular irritation and toxicity were evaluated by a veterinarian according to the criteria proposed by Auletta (2000) as specified in Table 5. The ocular irritation and toxicity scores range from 0 to 110 by examining before testing and at 1 hour, 1, 2, 3, 7, 10, 14, 21, and 28 days after dose administration.

Then ocular irritation and toxicity scores of 2% w/v ketoconazole solution pH 7 were compared with 0.9% sodium chloride. Before the study, the experimental procedure was submitted for allowance to the ethic committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Table 5 Grade for ocular irritation and toxicity lesions (Auletta, 2000)

Lesions	Grade
I. Cornea	
A. Opacity	
No Opacity	0
Scattered or diffuse areas of opacity, details of iris clearly visible	1
Easily discernible translucent areas; details of iris slightly obscured	2
Nacreous area, no details of iris visible, size of pupil barely discernible	3
Opaque cornea, iris not discernible through opacity	4
B. Ulceration	
No Ulceration	0
One quarter (or less) but not zero	1
Greater than one quarter, but less than half	2
Greater than half, but less than three quarters	3
Greater than three quarters, up to whole area	4
Scores equals A × B × 5 = total maximum = 80	
II. Iris	
A.Values	
Normal	0
Markedly deepened folds (above normal), congestion, swelling, moderate circumcorneal hyperemia or injection (any or all of these or combination of there of), iris still reacting to light (sluggish reaction is positive)	1
No reaction to light, hemorrhage, gross destruction (any or all of these)	2
Scores equals A × 5 = total maximum = 10	
III.Conjunctiva	
A. Redness	
Vessels normal	0

Table 5 Grade for ocular irritation and toxicity lesions (Auletta, 2000) (cont.)

Lesions	Grade
Some vessels definitely hyperemic (injected above normal)	1
Diffuse, crimson red, individual vessels not easily discernible	2
Diffuse beefy red	3
B. Chemosis (lids and/or nictitating membranes)	
No swelling	0
Any swelling above normal (includes nictitating membrane)	1
Obvious swelling with partial eversion of lids	2
Swelling with lids about half closed	3
Swelling with lids more than half closed	4
C. Discharge	
No discharge	0
Any amount different from normal (dose not include small amounts observed in inner canthus or normal animals)	1
Discharge with moistening of the lids and hairs just adjacent to lids	2
Discharge with moistening of the lids and hairs and considerable area around eye	3
Scores equals (A + B + C) × 2 = total maximum = 20	
*** Total scores equals = 80 + 10 + 20 = 110**	

CHAPTER IV
RESULTS AND DISCUSSION

I. Phase Solubility Analysis

1. Analysis of Ketoconazole by UV Spectrophotometric Method

1.1 Validation of UV Spectrophotometric Method

The validation of an analytical method is the process by which performance characteristics of the method are established to meet the requirements for the intended analytical applications. The performance characteristics are expressed in terms of analytical parameters. The analytical parameters used for the assay validation were specificity, linearity, accuracy and precision.

1.1.1 Specificity

The specificity of an analytical method is its ability to measure the analyte accurately and specificity in the presence of other components in the sample. The UV absorption spectra (Figures 10-12) indicated that the wavelength 225 nm was the optimal wavelength giving the highest sensitivity without interference of HP-β-CD and HP-γ-CD. Both HP-β-CD (Figures 10 and 11) and HP-γ-CD (Figure 12) showed no absorbance at the wavelength 200-400 nm.

Furthermore, The UV absorption spectra of ketoconazole:HP-β-CD inclusion complexes and ketoconazole:HP-γ-CD inclusion complexes in 0.1 M acetate buffer pH 5 and 0.1 M phosphate buffer pH 7 did not change and were similar to the the UV absorption spectra that obtained from ketoconazole in 0.1 M acetate buffer pH 5 and 0.1 M phosphate buffer pH 7. These results suggested that HP-β-CD and HP-γ-CD did not affect the UV absorption spectra of ketoconazole in the inclusion complexes. Consequently, this method had high specificity for analysis of ketoconazole both in its free form and inclusion complexes.

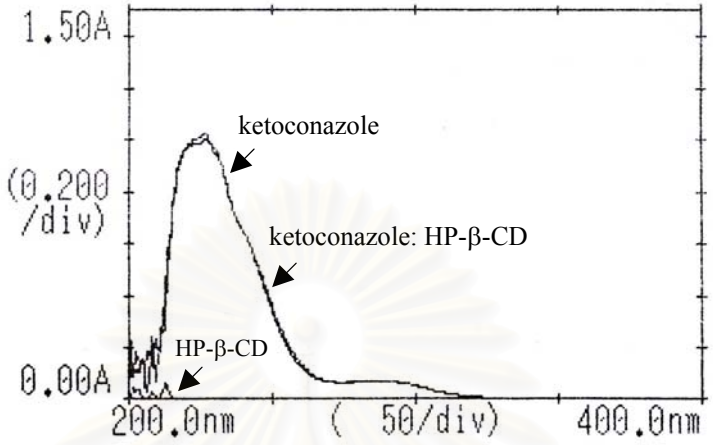


Figure 10 UV absorption spectra of ketoconazole, HP-β-CD and ketoconazole : HP-β-CD inclusion complexes in 0.1 M acetate buffer pH 5

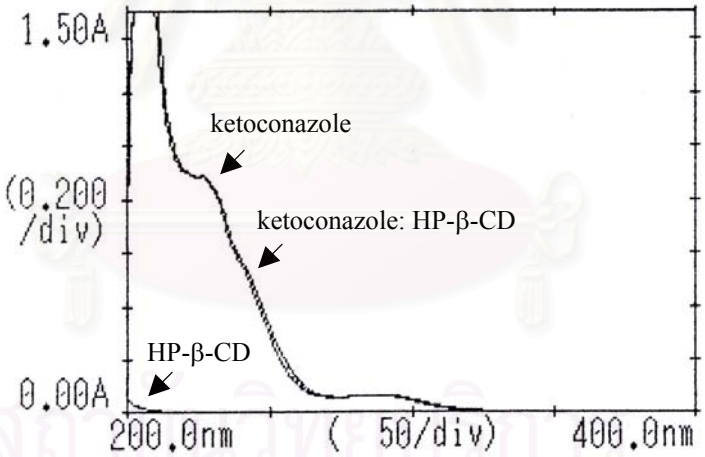


Figure 11 UV absorption spectra of ketoconazole, HP-β-CD and ketoconazole : HP-β-CD inclusion complexes in 0.1 M phosphate buffer pH 7

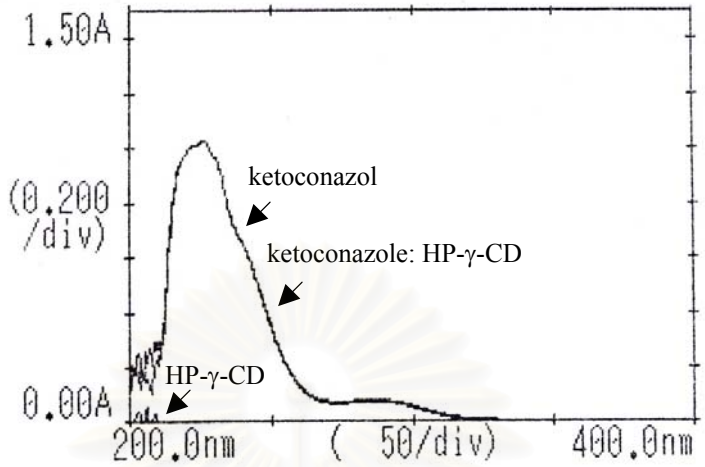


Figure 12 UV absorption spectra of ketoconazole, HP-γ-CD and ketoconazole : HP-γ-CD inclusion complexes in 0.1 M acetate buffer pH 5

1.1.2 Linearity

The linearity of an analytical method is its ability to elicit test results that are directly or by a well-defined mathematical transformation, proportional to the concentration of the analyte in samples within a given range. The linearity is usually expressed in terms of the variance around the slope of the regression line calculated according to an established mathematical relationship from test results obtained by the analysis of samples with varying concentrations of analyte.

The standard curves of ketoconazole in 0.1 M acetate buffer pH 5 and 0.1 M phosphate buffer pH 7 are shown in Figures 13 and 14, respectively.

The standard curves were found to be linear with excellent coefficient of determination (R^2). The coefficient of determination (R^2) were 0.9999 and 0.9996, respectively. These results indicated that UV spectrophotometric method was acceptable for quantitative analysis of ketoconazole in the range studied. The equations of standard curves according to Beer's Law plot were used for calculating the concentration of ketoconazole in HP-β-CD solution and HP-γ-CD solution.

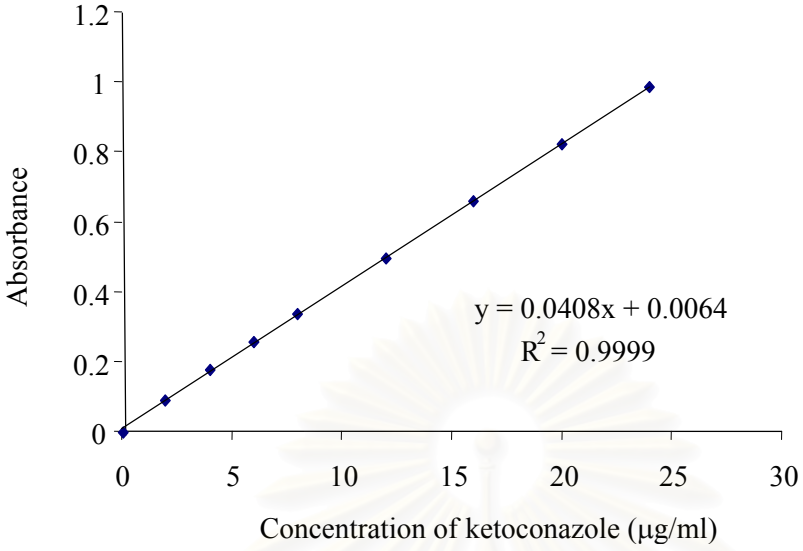


Figure 13 Standard curve of ketoconazole in 0.1 M acetate buffer pH 5 (UV spectrophotometric method)

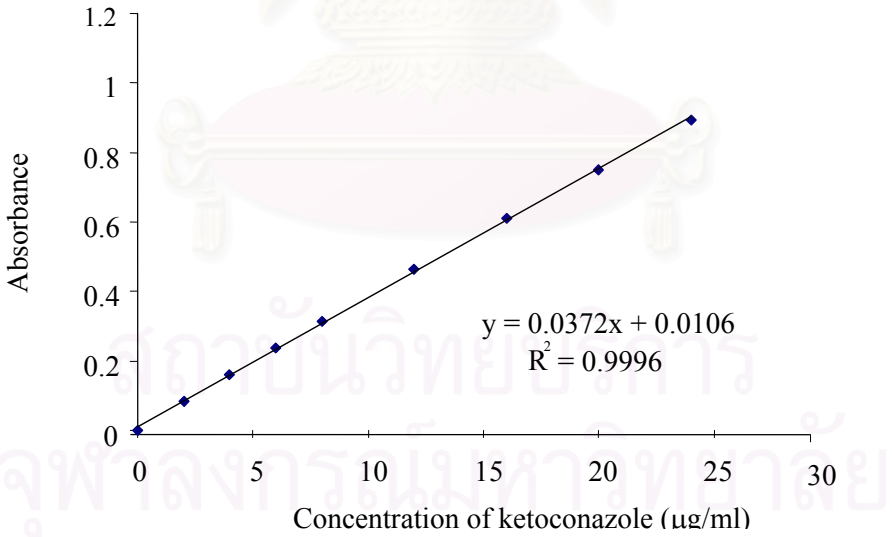


Figure 14 Standard curve of ketoconazole in 0.1 M phosphate buffer pH 7 (UV spectrophotometric method)

1.1.3 Accuracy

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. Accuracy may often be expressed as percent recovery by the assay of known, added amount of analyte. The percentages of analytical recovery of each ketoconazole concentration in 0.1 M acetate buffer pH 5 and 0.1 M phosphate buffer pH 7 are shown in Tables 6 and 7. All the percentage analytical recovery of all drug concentrations in 0.1 M acetate buffer pH 5 with a mean of 101.01% and a %CV of 0.57, and in 0.1 M phosphate buffer pH 7 with a mean of 100.96% and a %CV of 1.10, indicated the high accuracy of this method. Thus, it could be used for analysis of ketoconazole in all concentrations studied.

Table 6 The percentages of analytical recovery of ketoconazole in 0.1 M acetate buffer pH 5 by UV spectrophotometric method

Actual concentration of ketoconazole (μg/ml)	Calculated concentration of ketoconazole (μg/ml)	% Analytical recovery
2.0	2.03±0.04	101.63±1.87
6.0	6.06±0.04	101.01±0.62
12.0	12.20±0.05	101.70±0.43
16.0	16.13±0.01	100.79±0.09
20.0	20.15±0.12	100.73±0.60
24.0	24.05±0.04	100.21±0.16
	mean	101.01
	SD	0.57
	%CV	0.57

Table 7 The percentages of analytical recovery of ketoconazole in 0.1 M phosphate buffer pH 7 by UV spectrophotometric method

Actual concentration of ketoconazole (μg/ml)	Calculated concentration of ketoconazole (μg/ml)	% Analytical recovery
2.0	2.04±0.10	102.24±5.09
6.0	6.15±0.09	102.48±1.57
12.0	12.19±0.07	101.57±0.59
16.0	16.07±0.06	100.43±0.35
20.0	20.00±0.02	100.01±0.08
24.0	23.77±0.14	99.06±0.58
	mean	100.96
	SD	1.35
	%CV	1.34

1.1.4 Precision

The precision of an analytical method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample. The precision of an analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation).

The precision of the analysis of ketoconazole in 0.1 M acetate buffer pH 5 and 0.1 M phosphate buffer pH 7 by UV spectrophotometric method was determined both within run precision and between run precision as illustrated in Tables 8-11. All percentage coefficient of variation values were very low. The coefficient of variation of an analytical method should generally be less than 2% (USP 24, 2000). Therefore, the UV spectrophotometric method used were precise for quantitative analysis of ketoconazole in the range studied.

In conclusion, the analysis of ketoconazole in 0.1 M acetate buffer pH 5 and 0.1 M phosphate buffer pH 7 by UV spectrophotometric method developed in this study showed good specificity, linearity, accuracy and precision. Thus this

method was used for the determination of the content of ketoconazole in the phase solubility study.

Table 8 The within run precision of ketoconazole in 0.1 M acetate buffer pH 5 by UV spectrophotometric method

Ketoconazole concentration (μg/ml)	Absorbance of ketoconazole					
	Set 1	Set 2	Set 3	Mean	SD	%CV
2	0.0907	0.0893	0.0880	0.0893	0.0013	1.49
6	0.2563	0.2537	0.2560	0.2553	0.0015	0.57
12	0.5100	0.5043	0.5103	0.5082	0.0034	0.66
16	0.6670	0.6643	0.6660	0.6658	0.0013	0.20
20	0.8307	0.8283	0.8300	0.8297	0.0012	0.14
24	0.9870	0.9877	0.9853	0.9867	0.0012	0.12

Table 9 The between run precision of ketoconazole in 0.1 M acetate buffer pH 5 by UV spectrophotometric method

Ketoconazole concentration (μg/ml)	Absorbance of ketoconazole					
	Day 1	Day 2	Day 3	Mean	SD	%CV
2	0.0877	0.0880	0.0893	0.0883	0.0009	1.00
6	0.2497	0.2550	0.2537	0.2528	0.0028	1.10
12	0.4907	0.4963	0.5043	0.4971	0.0069	1.38
16	0.6570	0.6573	0.6643	0.6596	0.0041	0.63
20	0.8183	0.8227	0.8283	0.8231	0.0050	0.61
24	0.9840	0.9860	0.9877	0.9859	0.0018	0.19

Table 10 The within run precision of ketoconazole in 0.1 M phosphate buffer pH 7 by UV spectrophotometric method

Ketoconazole concentration (μg/ml)	Absorbance of ketoconazole					
	Set 1	Set 2	Set 3	Mean	SD	%CV
2	0.0823	0.0807	0.0813	0.0814	0.0008	1.03
6	0.2320	0.2287	0.2270	0.2292	0.0025	1.11
12	0.4610	0.4577	0.4607	0.4598	0.0018	0.40
16	0.6067	0.6063	0.6083	0.6071	0.0011	0.18
20	0.7590	0.7553	0.7513	0.7552	0.0038	0.51
24	0.8977	0.8977	0.8970	0.8974	0.0004	0.04

Table 11 The between run precision of ketoconazole in 0.1 M phosphate buffer pH 7 by UV spectrophotometric method

Ketoconazole concentration (μg/ml)	Absorbance of ketoconazole					
	Day 1	Day 2	Day 3	Mean	SD	%CV
2	0.0867	0.0837	0.0847	0.0850	0.0015	1.80
6	0.2393	0.2357	0.2373	0.2374	0.0018	0.77
12	0.4640	0.4677	0.4630	0.4649	0.0025	0.53
16	0.6083	0.6023	0.6120	0.6076	0.0049	0.80
20	0.7547	0.7540	0.7513	0.7533	0.0018	0.23
24	0.8950	0.8847	0.8943	0.8913	0.0058	0.65

2. Phase Solubility Study of Ketoconazole with Cyclodextrins at pH 5 and pH 7

Since the equilibration time to obtain phase solubility diagram varied depending on types of drug, complexing agents, temperatures, etc, it was necessary to determine prior to the phase solubility study. The equilibrium of ketoconazole and cyclodextrins was demonstrated to nearly approach since 1 day. However, to ensure the equilibrium, the equilibration time was 3 days (Appendix A). Phase Solubility data of

ketoconazole with HP-β-CD and HP-γ-CD in 0.1 M acetate buffer pH 5 and 0.1 M phosphate buffer pH 7 at room temperature (30±3°C) are shown in Appendix D. Phase solubility diagram was constructed by plotting the molarity of ketoconazole found in solution against the molarity of HP-β-CD and HP-γ-CD added.

2.1 Phase Solubility Study of Ketoconazole with HP-β-CD and HP-γ-CD at pH 5

It was found that the solubility of ketoconazole increased linearly as a function of HP-β-CD and HP-γ-CD concentrations and showed the features of an A_L type following Higuchi and Connors (1965), as shown in Figure 15. It was obvious that HP-β-CD increased the solubility of ketoconazole in the much greater extent than HP-γ-CD. The solubility enhancement can be attributed to the formation of an inclusion complex in solution. This result was in agreement with the study reported by Diaz, Mendez et al. (1996).

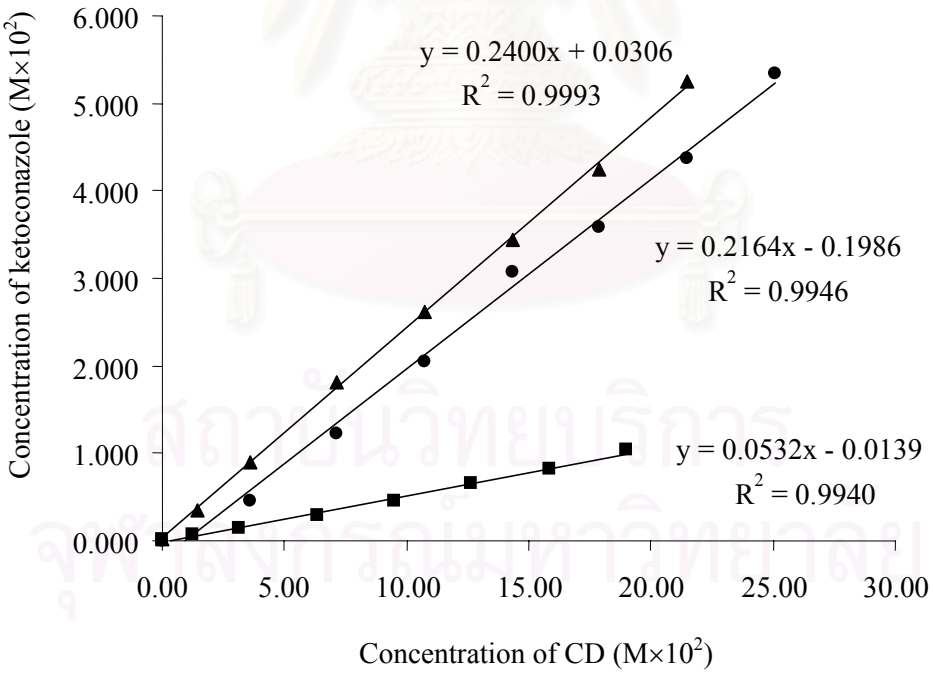


Figure 15 Phase solubility diagram of ketoconazole with cyclodextrins in buffer solutions at room temperature (30±3°C): (▲) HP-β-CD in 0.1 M acetate buffer pH 5; (●) 2HP-β-CD in 0.1 M phosphate buffer pH 7 and (■) HP-γ-CD in 0.1 M acetate buffer pH 5

An attempt to determine the association constants or stability constants (K_C) was calculated from the A_L phase solubility diagrams. Since generally, linear diagrams are formed when each complex contains only one molecule of CD. Phase solubility diagrams were made by assuming that only 1:1 (M:M) complexes were formed (Higuchi and Connors, 1965). So the association constants were calculated based on the formation of 1:1 complexes according to the Equation (3) (Appendix D). Consequently, the association constants (K_C) of ketoconazole:HP-β-CD inclusion complexes and ketoconazole:HP-γ-CD inclusion complexes in 0.1 M acetate buffer pH 5 were determined to be 1263 and 244 M^{-1}, respectively.

The K_C for ketoconazole:HP-β-CD inclusion complexes in 0.1 M acetate buffer pH 5 was much higher than ketoconazole:HP-γ-CD inclusion complexes in 0.1 M acetate buffer pH 5, which meant a better interaction of ketoconazole with HP-β-CD or formed more stable inclusion complexes with HP-β-CD. This results was consistent with a previous study by Jarho, Urtti et al. (1996) that HP-β-CD formed more stable inclusion complexes with arachidonylethanolamide than HP-γ-CD. This result may reflect that the size of the HP-β-CD cavity is more suitable to form stable inclusion complexes with ketoconazole than HP-γ-CD. Because CDs are capable of forming inclusion complexes with drugs of a size compatible with the dimensions of the CDs cavity (Mosher and Thompson, 2000). The most important requirement for the formation of the stable inclusion complexes is the tight fitting of the drug molecule within the cavity of CD (Szejtli, 1991).

2.2 Phase Solubility Study of Ketoconazole with HP-β-CD at pH 5 and 7

From the result of ketoconazole:HP-γ-CD inclusion complexes, it was clear that 2% w/v ketoconazole (3.76×10^{-2} M) could not be prepared by the enhancement of HP-γ-CD. In the further study, the effect of pH 5 and 7, consequently, was investigated from the ketoconazole:HP-β-CD inclusion complexes (Figure 15). The K_C of the complexation at pH 5 and pH 7 were calculated to be 1263 and 6904 M^{-1}, respectively. These results demonstrated that the value of K_C for ketoconazole:HP-β-CD inclusion complexes in 0.1 M phosphate buffer pH 7 is higher than in 0.1 M acetate buffer pH 5 due to the lower ionization of the ketoconazole in 0.1 M phosphate buffer pH 7. Because drug-cyclodextrin complexation has been found to be better with unionized drug or usually the complex of an ionized species is much less stable than that of unionized species, the hydrophobic CD cavity favour

uncharged molecules to enter (Bekers et al.,1991; Diaz, Mendez et al., 1996; Diaz, Otero et al., 1996).

From the results obtained, it could be concluded that HP-β-CD at pH 5 and pH 7 increased ketoconazole solubility by formation of inclusion complexes. The K_C of the complex formation of ketoconazole with HP-β-CD at pH 7 was 5.5 fold higher than that of at pH 5. However, from the phase solubility diagram of both systems, 2% w/v ketoconazole solution could be prepared. Inspite of lower K_C at pH 5, the greater solubility enhancement of ketoconazole at lower pH was demonstrated. Thus the optimal amount of HP-β-CD at pH 5 needed for the preparation of 2% w/v ketoconazole solution was 25% w/v, whereas to be 30% w/v at pH 7 (Table 4).

II. Stability Studies of 2% w/v Ketoconazole Solutions and Lyophilized Products

1. Analysis of Ketoconazole by HPLC Method

1.1 Validation of HPLC Method

The validation of an analytical method is the process by which performance characteristics of the method are established to meet the requirements for the intended analytical applications. The performance characteristics are expressed in terms of analytical parameters. For HPLC assay validation, these include specificity, linearity, accuracy and precision.

1.1.1 Specificity

The specificity of an analytical method is its ability to measure the analyte accurately and specificity in the presence of other components in the sample.

a) In the Presence of Buffer, HP-β-CD and Benzalkonium Chloride

Figure 16 shows typical chromatogram of 100 μg/ml ketoconazole standard solution. Among several compounds tested, caffeine gave a sharp peak with a high resolution, it was thus selected as the internal standard. The retention times of ketoconazole and caffeine were about 7.2 minutes and 3.4 minutes, respectively. As shown in Figures 17-19, since blank buffer solutions, HP-β-CD and benzalkonium chloride showed no absorbance at the wavelength of 230 nm, peaks of they did not interfere with peaks of ketoconazole and caffeine. Therefore, these two peaks were completely separated from each other.

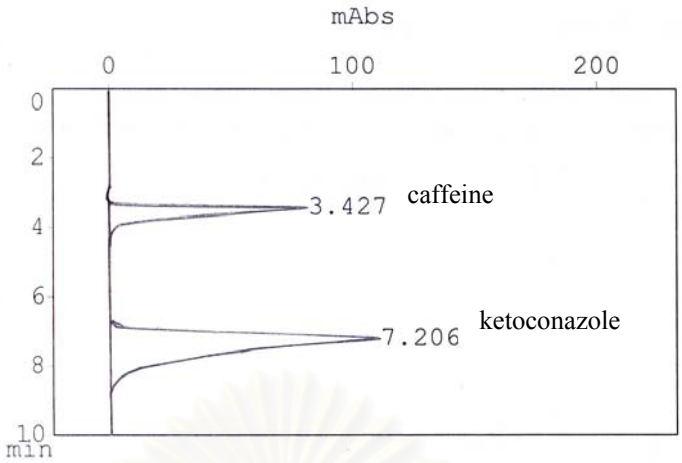
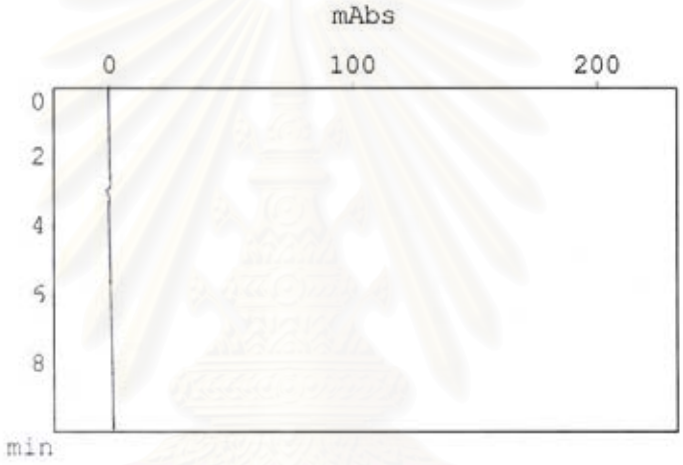
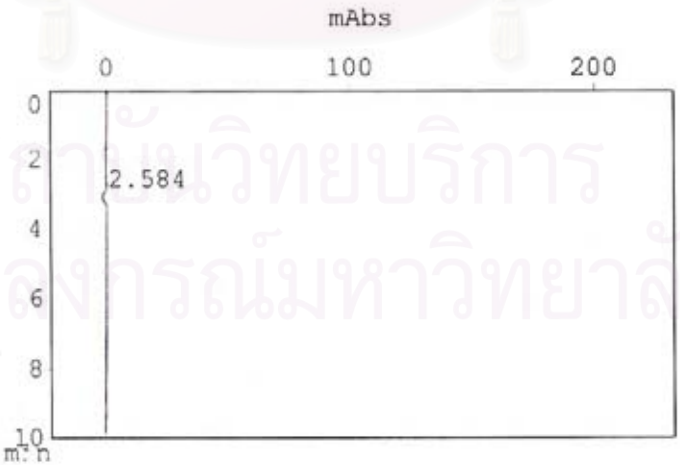


Figure 16 HPLC chromatogram of 100 μg/ml ketoconazole standard solution

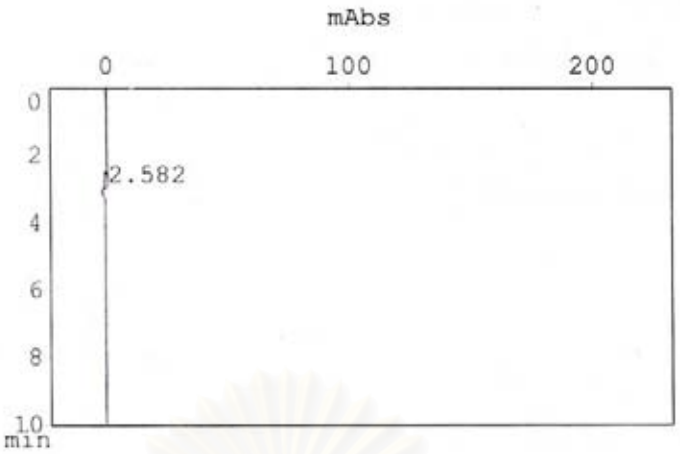


A

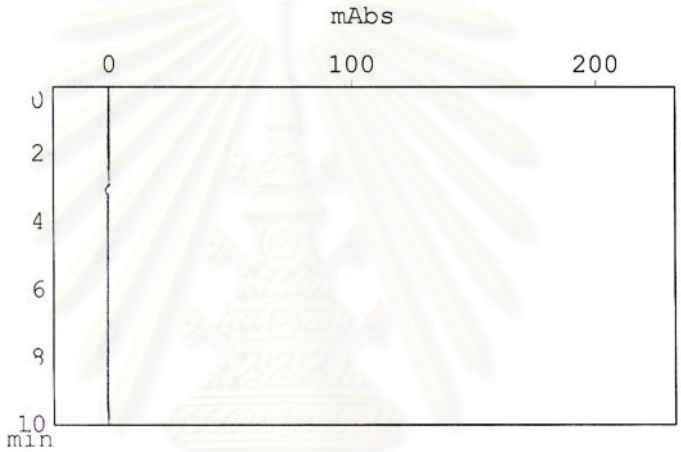


B

Figure 17 HPLC chromatograms of sample solutions of blank buffer solutions (A; 0.1 M acetate buffer pH 5, B; 0.1 M phosphate buffer pH 7)



A



B

Figure 18 HPLC chromatograms of sample solutions of HP-β-CD (A; 25% w/v HP-β-CD in 0.1 M acetate buffer pH 5, B; 30% w/v HP-β-CD in 0.1 M phosphate buffer pH 7)

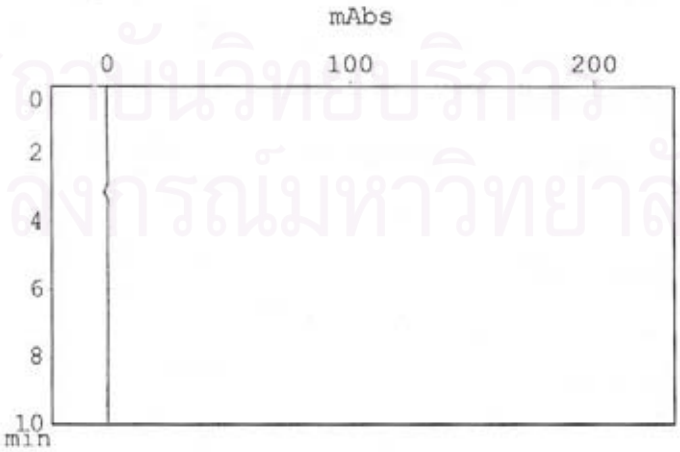


Figure 19 HPLC chromatograms of sample solution of 0.01% w/v benzalkonium chloride in 0.1 M phosphate buffer pH 7

Furthermore, the chromatograms of 100 μg/ml sample solutions of 2% w/v ketoconazole solutions containing HP-β-CD at pH 5 and pH 7 (Figures 20 and 21) were similar to the chromatograms of 100 μg/ml ketoconazole standard solution (containing without HP-β-CD). This results suggested that HP-β-CD did not affect the peak of ketoconazole.

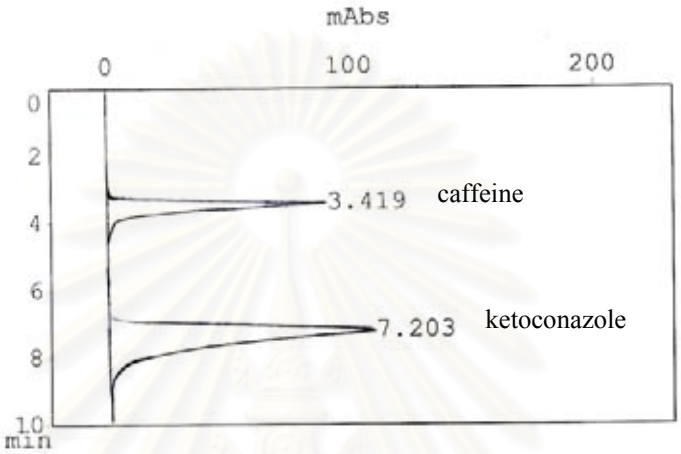


Figure 20 HPLC chromatograms of 100 μg/ml sample solution of 2% w/v ketoconazole solution with 25% w/v HP-β-CD at pH 5

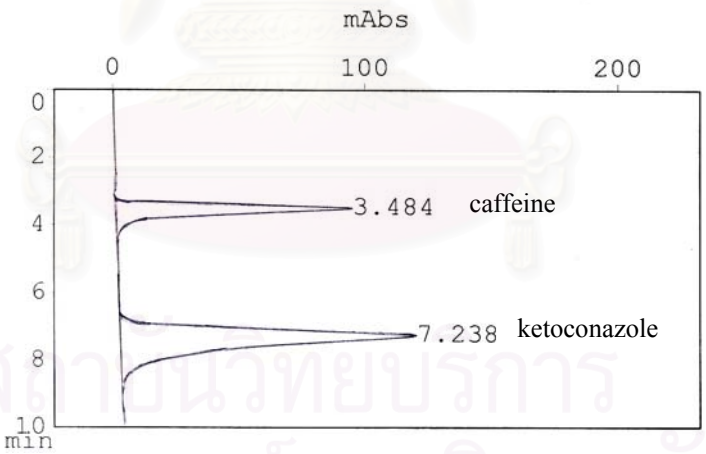


Figure 21 HPLC chromatograms of 100 μg/ml sample solution of 2% w/v ketoconazole solution with 30% w/v HP-β-CD at pH 7

b) In the Presence of Degradation Products of Ketoconazole

The HPLC method for analysis of ketoconazole in the stability studies should be capable of separating the parent drug from its degradation products. The chromatograms are presented in Figures 22 and 23. Peaks of degradation products were eluted before that of ketoconazole and were about 5.2 minutes and

5.6 minutes. Furthermore, resolution values of both degradation products-ketoconazole peaks and degradation products-caffeine peaks were more than 1.0 (USP 24, 2000). Therefore, the degradation products did not interfere with peak of ketoconazole and caffeine.

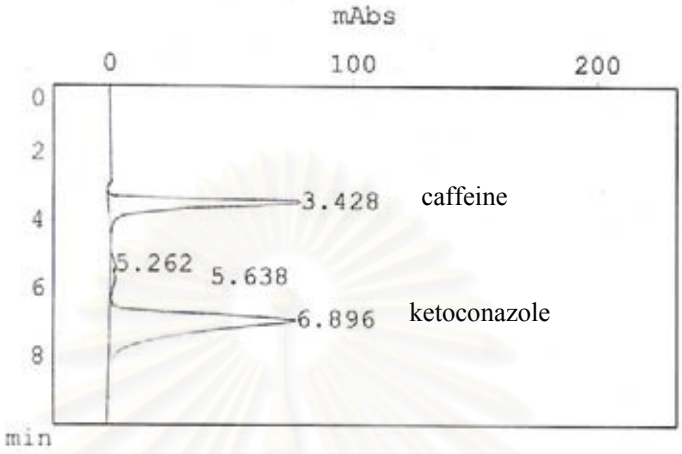


Figure 22 HPLC chromatograms of sample solution of decomposed 2% w/v ketoconazole solution pH 5

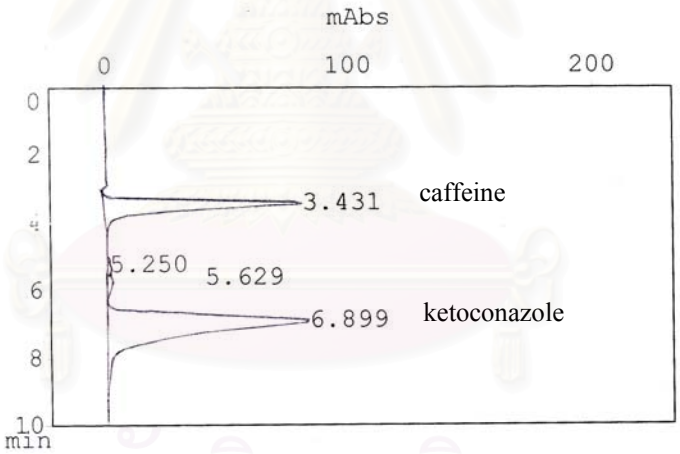


Figure 23 HPLC chromatograms of sample solution of decomposed 2% w/v ketoconazole solution pH 7

Thus, this method had high specificity for analysis of ketoconazole, both in its free form and inclusion complexes and in the presence of its degradation products.

1.1.2 Linearity

The linearity of an analytical method is its ability to elicit test results that are directly or by a well-defined mathematical transformation, proportional to the concentration of the analyte in samples within a given range. The linearity is usually

expressed in terms of the variance around the slope of the regression line calculated according to an established mathematical relationship from test results obtained by the analysis of samples with varying concentrations of analyte. The standard curve data is shown in Table 12. The plot of ketoconazole concentrations versus the peak area ratios of ketoconazole and its internal standard (Figure 24) illustrated the linear correlation in the concentration range studied, 10-125 μg/ml. The coefficient of determination (R^2) of this line was 0.9999. These results indicated that HPLC method was acceptable for quantitative analysis of ketoconazole in the range studied.

Table 12 Data for standard curve of ketoconazole by HPLC method

Concentration of ketoconazole (μg/ml)	Peak area ratio			Mean	SD	%CV
	Set 1	Set 2	Set 3			
10	0.2769	0.2723	0.2746	0.2746	0.0023	0.83
25	0.6656	0.6711	0.6694	0.6687	0.0029	0.43
50	1.3276	1.3492	1.3022	1.3263	0.0235	1.78
75	1.9789	1.9768	1.9552	1.9703	0.0131	0.67
100	2.6138	2.6530	2.6246	2.6305	0.0202	0.77
125	3.3552	3.2424	3.3276	3.3084	0.0588	1.78

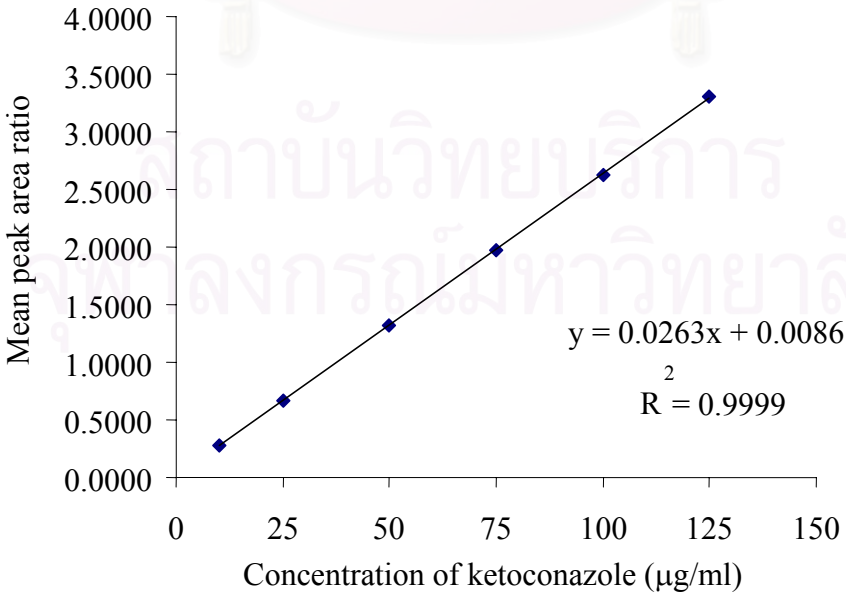


Figure 24 Standard curve of ketoconazole by HPLC method

1.1.3 Accuracy

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. Accuracy may often be expressed as percent recovery by the assay of known, added amount of analyte. The determination of accuracy of the analysis of ketoconazole by HPLC method was performed by analyzing percentages analytical recovery of three sets of six standard solutions. The percentages of analytical recovery of each ketoconazole concentration are shown in Table 13. All the percentages analytical recovery of all drug concentrations with a mean of 100.65% and a %CV of 1.54 indicated that this method could be used for analysis of ketoconazole in all concentrations studied with high accuracy.

Table 13 The percentages of analytical recovery of ketoconazole by HPLC method

Actual concentration of ketoconazole (μg/ml)	Calculated concentration of ketoconazole (μg/ml)	% Analytical recovery
10.0	9.88±0.02	98.77±0.16
25.0	24.88±0.19	99.52±0.77
50.0	51.31±0.72	102.61±1.44
75.0	76.51±1.07	102.01±1.42
100.0	101.37±2.92	101.37±2.92
125.0	124.55±3.28	99.64±2.63
	mean	100.65
	SD	1.55
	%CV	1.54

1.1.4 Precision

The precision of an analytical method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample. The precision of an analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation).

Tables 14 and 15 illustrated the data of within run precision and between run precision, respectively. All coefficient of variation values were small, as 0.87-1.85% and 1.09-1.94%, respectively. The coefficient of variation of an analytical method should generally be less than 2% (USP 24, 2000). Therefore, the HPLC method used were precise for quantitative analysis of ketoconazole in the range studied.

Table 14 Data of within run precision by HPLC method

Ketoconazole concentration (μg/ml)	Peak area ratio					
	Set 1	Set 2	Set 3	Mean	SD	%CV
10	0.2672	0.2735	0.2735	0.2714	0.0036	1.34
25	0.6375	0.6233	0.6396	0.6335	0.0089	1.40
50	1.2820	1.2603	1.2753	1.2725	0.0111	0.87
75	1.9236	1.8884	1.9183	1.9101	0.0190	1.00
100	2.6258	2.5495	2.5799	2.5851	0.0384	1.49
125	3.2359	3.1293	3.1402	3.1685	0.0586	1.85

Table 15 Data of between run precision by HPLC method

Ketoconazole concentration (μg/ml)	Peak area ratio					
	Day 1	Day 2	Day 3	Mean	SD	%CV
10	0.2684	0.2642	0.2746	0.2691	0.0052	1.94
25	0.6630	0.6532	0.6687	0.6616	0.0078	1.18
50	1.3579	1.3303	1.3263	1.3382	0.0172	1.29
75	2.0208	1.9535	1.9703	1.9815	0.0350	1.77
100	2.6746	2.6208	2.6305	2.6420	0.0287	1.09
125	3.2844	3.2101	3.3084	3.2676	0.0512	1.57

In conclusion, the analysis of ketoconazole by HPLC method developed in this study showed good specificity, linearity, accuracy and precision. Thus this method was used for the determination of the content of ketoconazole in the study.

2. Comparison of the Stability of 2% w/v Ketoconazole Solutions pH 5 and pH 7

In this study, the stability study of aqueous ketoconazole solution without HP-β-CD added was not possible since the extremely poor water solubility of ketoconazole disabled the preparation of the ketoconazole solution. Comparison of the stability of 2% w/v ketoconazole solutions pH 5 and pH 7 (Formula I and II) was performed at 70±1°C. Percent remaining of ketoconazole in both formulations were shown in Table 16.

Table 16 Percent remaining of ketoconazole in 2% w/v ketoconazole solutions pH 5 and pH 7 at 70±1°C

Time (day)	Percent remaining of ketoconazole[a]	
	2% w/v ketoconazole solution pH 5	2% w/v ketoconazole solution pH 7
0	100.00±1.66	100.00±1.15
5	99.06±0.49	101.30±1.55
14	96.21±3.02	99.14±0.94
37	83.29±1.68	89.10±0.35
45	83.07±0.49	88.21±1.37
52	82.45±2.83	87.91±0.85
60	81.34±4.69	86.67±0.87

a – mean±SD, n = 3

The result showed that the degradation of both formulations is not large. For data corresponding to a zero-, first-, or second-order degradation pattern, it is impossible to distinguish one order from another, when the total degraded drug is not large. The degradation reaction order can be determined, when at least 50% decomposition must occur. As the loss with pharmaceuticals generally is less, zero-order kinetics should be assumed (Vadas, 2000). Therefore, the degradation reaction of both formulations was assumed to the zero-order kinetics.

The observed degradation rate constant (k_{obs}) is determined by its slope of the plot of percent remaining of ketoconazole versus time (Figure 25). The observed degradation rate constants of both formulations at 70±1°C are shown in Table 17. It was found that the observed degradation rate constant (k_{obs}) of 2% w/v ketoconazole solution pH 5 was significantly higher than that of 2% w/v ketoconazole pH 7 ($p \leq 0.05$) (Appendix F, Table 1F). The similar result was also reported by Skiba et al. (2000) in that degradation rate of ketoconazole in acidic region was faster than in an alkaline environment since the degradation pathway was specific acid catalysis.

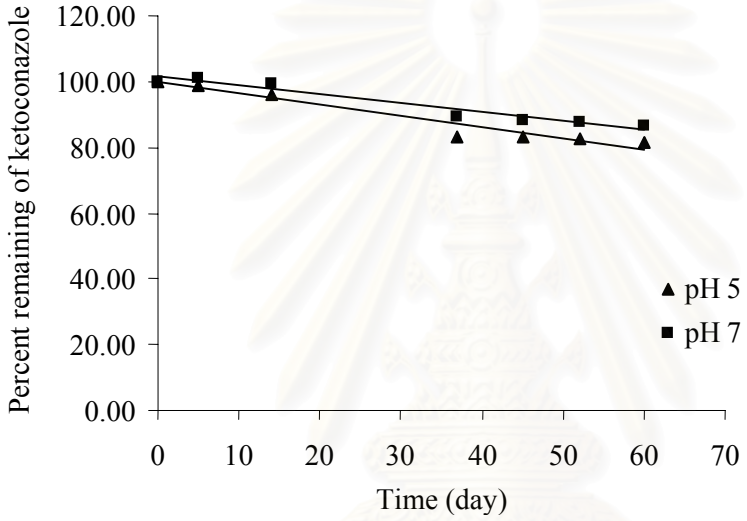


Figure 25 Percent remaining of ketoconazole in 2% w/v ketoconazole solutions pH 5 and pH 7 at 70±1°C

Table 17 The observed degradation rate constants (k_{obs}) of 2% w/v ketoconazole solutions pH 5 and pH 7 at 70±1°C

Formulation	Observed degradation rate constant (k_{obs}) ($\%.day^{-1}$)
2% w/v ketoconazole solution pH 5	$34.92\times10^{-2}\pm3.00\times10^{-2}$
2% w/v ketoconazole solution pH 7	$26.69\times10^{-2}\pm4.00\times10^{-2}$

a – mean±SD, n = 3

For the physical stability, clarity of the solutions was obserbed visually. No precipitation was obseved in all solutions. It was found that the color of 2% w/v

ketoconazole solution pH 5 changed from colorless to “brown” after about 5 days, but the color of 2% w/v ketoconazole solution pH 7 changed into “yellow” after about 2 weeks. From the spectral absorbances in visible wavelength (400-800 nm) of the ketoconazole solution at initial time and during the stability study (Appendix G), the color change of the solution could be detected more sensitively and quantitatively from the spectral change at 410 nm.

Table 18 and Figure 26 demonstrate the visible spectral absorbance of both formulations. It was found that the spectral absorbance at 410 nm of 2% w/v ketoconazole solution pH 5 was higher than that of 2% w/v ketoconazole solution pH 7. Hence, HP-β-CD could not stop the degradation of ketoconazole at high temperature. Because of the association constant or stability constant decreased rapidly with increasing temperature due to dissociation of the inclusion complexes (Mosher and Thompson, 2000). Therefore, it may said that at high temperature as in this study, there was dissociation of the ketoconazole:HP-β-CD inclusion complexes so that free ketoconazole was exposed to the degradation reaction.

Table 18 Visible spectral absorbance at 410 nm of 2% w/v ketoconazole solution pH 5 and pH 7 at 70±1°C

Time (day)	Absorbance at 410 nm[a]	
	2% w/v ketoconazole solution pH 5	2% w/v ketoconazole solution pH 7
0	0.005±0.000	0.007±0.000
5	0.674±0.000	0.017±0.000
14	1.547±0.001	0.043±0.000
21	1.792±0.002	0.070±0.001
30	1.932±0.001	0.109±0.002
37	2.088±0.004	0.147±0.004
45	2.103±0.002	0.202±0.001
52	2.198±0.001	0.231±0.001
60	2.232±0.009	0.271±0.001

a – mean±SD, n = 3

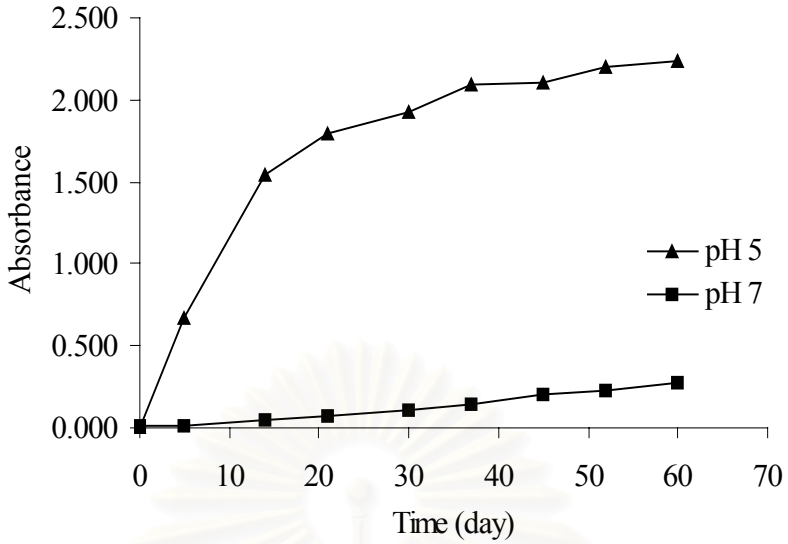


Figure 26 Visible spectral absorbance at 410 nm of 2% w/v ketoconazole solutions pH 5 and pH 7 at 70±1°C

From all results obtained, it could be concluded that the physical and chemical stabilities of 2% w/v ketoconazole solution pH 7 were more stable than 2% w/v ketoconazole solution pH 5. Thus, in the further study, only the ketoconazole solution pH 7 was focused.

3. Stability of 2% w/v Ketoconazole Solution pH 7

3.1 Accelerated Stability Studies

The accelerated stability studies were performed at 50, 60, 70±1°C, refrigerator temperature (5±3°C) and room temperature (30±3°C). The degradation data of 2% w/v ketoconazole solution pH 7 at various temperatures are presented in Table 19. It was found that the degradation of this formulation is not large. For data corresponding to a zero-, first-, or second-order degradation pattern, it is impossible to distinguish one order from another, when the total degraded drug is not large. The degradation reaction order can be determined, when at least 50% decomposition must occur. As the loss with pharmaceuticals generally is less, zero-order kinetics should be assumed (Vadas, 2000). Therefore, the degradation reaction of both formulations was assumed to the zero-order kinetics. The kinetic plots of percent remaining of ketoconazole against sampling time are shown in Figure 27. The obserbed degradation rate constants (k_{obs}) were calculated from the slope of each linear regression line at various temperatures, are shown in Table 20.

Table 19 Percent remaining of ketoconazole in 2% w/v ketoconazole solution pH 7 at various temperatures

Time (day)	Percent remaining of ketoconazole[a]				
	refrigerator temperature (5±3°C)	room temperature (30±3°C)	50°C	60°C	70°C
0	100.00±1.15	100.00±1.15	100.00±1.15	100.00±1.15	100.00±1.15
5	99.08±3.24	99.63±1.45	100.77±0.64	102.07±0.67	101.30±1.55
14	100.67±1.34	98.30±1.82	102.09±0.77	101.68±0.67	99.14±0.94
37	-	-	88.38±1.79	91.38±3.43	89.10±0.35
45	94.33±0.85	92.59±1.12	91.69±0.36	90.82±3.99	88.21±1.37
52	-	-	92.36±0.39	90.23±1.70	87.91±0.85
60	93.56±0.77	93.72±2.03	92.80±0.59	89.64±2.81	86.67±0.87
75	94.23±2.56	93.35±1.63	-	-	-
90	92.45±1.36	91.76±1.62	92.53±1.28	88.97±3.02	81.50±1.74

a – mean±SD, n = 3

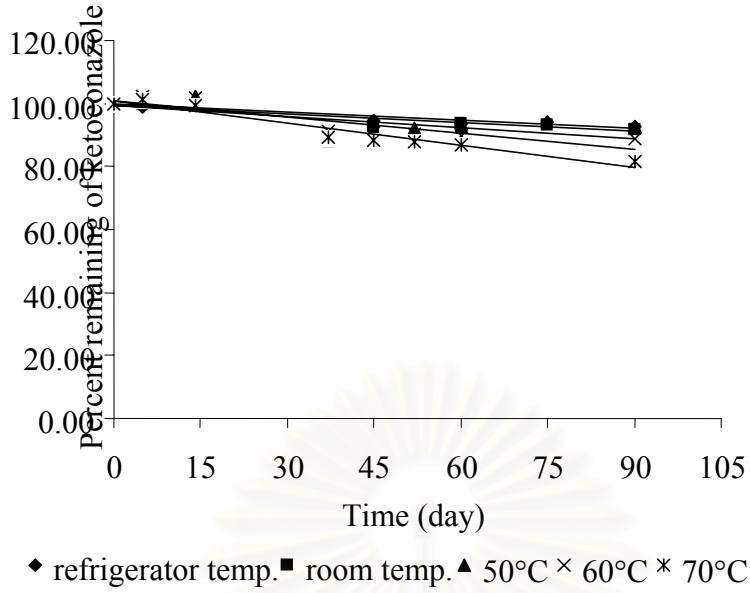


Figure 27 Percent remaining of ketoconazole in 2% w/v ketoconazole solution pH 7 at various temperatures

Table 20 Obserbed degradation rate constants (k_{obs}) of 2% w/v ketoconazole solution pH 7 at various temperatures

Temperature (°C)	Observed degradation rate constant (k_{obs}) ($\%.day^{-1}$)
refrigerator temperature (5±3°C)	$9.02\times10^{-2}\pm0.74\times10^{-2}$
room temperature (30±3°C)	$9.31\times10^{-2}\pm1.29\times10^{-2}$
50	$11.93\times10^{-2}\pm1.91\times10^{-2}$
60	$16.93\times10^{-2}\pm3.15\times10^{-2}$
70	$23.34\times10^{-2}\pm1.30\times10^{-2}$

a – mean±SD, n = 3

Arrhenius plot of this formulation was plotted between ln k versus the reciprocal of temperature (1/T) and shown in Figure 28. Arrhenius equation and kinetic parameters of the degradation of ketoconazole were calculated by linear regression analysis and summarized in Table 21. From the slope of Arrhenius equation, the activation energy (Ea) was calculated, which was 7.47 kcal/mol.

Theorectically, the lower Ea reveals the faster rate of reaction. The apparent activation energy or heat of activation obtained in the study was rather lower than the usual range of Ea about 10 to 30 kcal/mol.

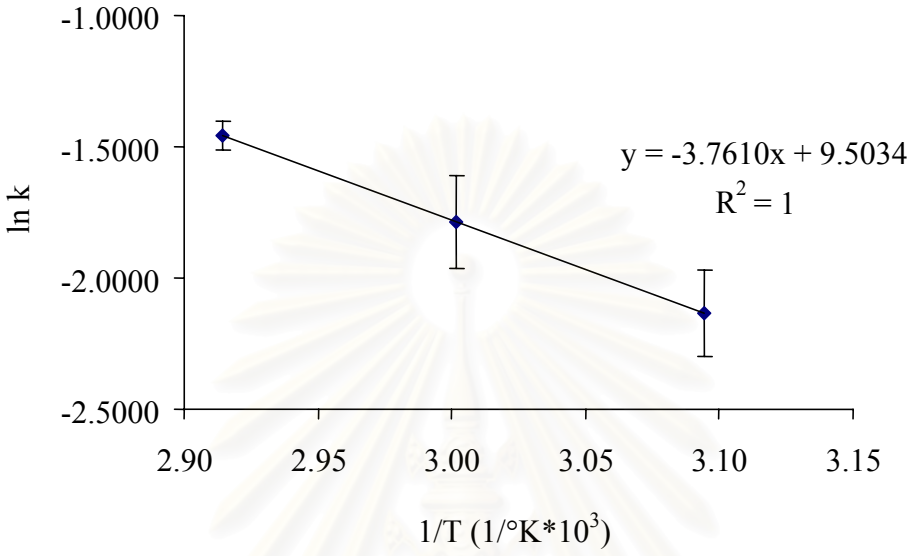


Figure 28 Arrhenius plot of 2% w/v ketoconazole solution pH 7

Table 21 Arrhenius equation and kinetics parameters of the degradation of 2% w/v ketoconazole solution pH 7

	2% w/v ketoconazole solution pH 7
Arrhenius equation	ln k = 9.5034 – 3.7610 (1/T)
Correlation of determination (R^2)	1
Activation energy, Ea (kcal/mol)	7.47
$k_{extrapolated}$ at 5°C (%.day^{-1})	1.80×10^{-2}
$k_{extrapolated}$ at 30°C (%.day^{-1})	5.49×10^{-2}

In addition, the extrapolated degradation rate constants ($k_{extrapolated}$) at 5 and 30°C of this formulation were calculated from Arrhenius equation. The k_{obs} at refrigerator temperature (5±3°C) and room temperature (30±3°C), $k_{extrapolated}$ at 5 and 30°C and corresponding shelf-lives which were calculated from Eqaution (7) of this formulation were compared in Table 22.

Table 22 Comparison the extrapolated degradation rate constants ($k_{extrapolated}$) and the extrapolated shelf-lives at 5 and 30°C with those experimentally observed at refrigerator temperature (5±3°C) and room temperature (30±3°C)

Temperature	k_{obs} (%.day^{-1})	$t_{90,\ obserbed}$ (days)	$k_{extrapolated}$ (%.day^{-1})	$t_{90,\ extrapolated}$ (days)
Refrigerator (5°C)	9.02×10^{-2}	111	1.80×10^{-2}	556
Room (30°C)	9.31×10^{-2}	107	5.49×10^{-2}	182

It was found that k_{obs} and $k_{extrapolated}$ at refrigerator temperature (5°C) were lower than those at room temperature (30°C). However, there was no statistical difference of k_{obs} at refrigerator temperature (5°C) and room temperature (30°C) ($p > 0.05$) (Appendix F, Table 2F). The obserbed shelf-lives at refrigerator temperature (5±3°C) and room temperature (30±3°C), were 111 and 107 days, respectively. The obserbed shelf-lives were much lower than the extrapolated values from Arrhenius equation. The explanation of the difference between the extrapolated shelf-lives and the observed shelf-lives might be explained in terms of the effect of temperature on the inclusion complex association constant. As the temperature increased in the stability testing, the association constant decreased, which lead to the increase of free drug (Mosher and Thompson, 2000). As the free drug concentration was not constant, the degradation rate constant at refrigerator temperature and room temperature could not be predicted accurately by the Arrhenius equation. This was likely similar to that observed in the suspension dosage form that the drug solubility increased as the temperature increased.

For the physical stability, the color of 2% w/v ketoconazole solution pH 7 kept in refrigerator and room temperature as observed visually, did not change throughout the study. However, the color of this formulation kept at 50, 60 and 70°C changed into “yellow”. In addition, Table 23 and Figure 29 presented the visible spectral absorbance of this formulation at various temperatures. It was found that the visible spectral absorbance of the solutions at refrigerator and room temperature did not change and absorbance revealing of color change at 70°C was higher than at 60 and 50°C, respectively.

Table 23 Visible spectral absorbance at 410 nm of 2% w/v ketoconazole solution pH 7 at various temperatures

Time (day)	Absorbance at 410 nm[a]				
	refrigerator temperature (5±3°C)	room temperature (30±3°C)	50°C	60°C	70°C
0	0.007±0.000	0.007±0.000	0.007±0.000	0.007±0.000	0.007±0.000
5	0.003±0.000	0.003±0.000	0.007±0.000	0.008±0.000	0.017±0.000
14	0.007±0.000	0.007±0.000	0.012±0.000	0.021±0.001	0.043±0.000
21	0.003±0.000	0.003±0.000	0.013±0.000	0.026±0.001	0.070±0.001
30	0.004±0.000	0.007±0.000	0.017±0.001	0.032±0.000	0.109±0.002
37	-		0.029±0.001	0.050±0.001	0.147±0.004
45	0.006±0.000	0.006±0.001	0.035±0.002	0.050±0.000	0.202±0.001
52	-	-	0.035±0.002	0.068±0.002	0.231±0.001
60	0.003±0.001	0.010±0.000	0.036±0.002	0.070±0.001	0.271±0.001
75	0.004±0.000	0.010±0.000	-	-	-
90	0.008±0.001	0.020±0.001	0.063±0.001	0.144±0.004	0.500±0.0009

a – mean±SD, n = 3

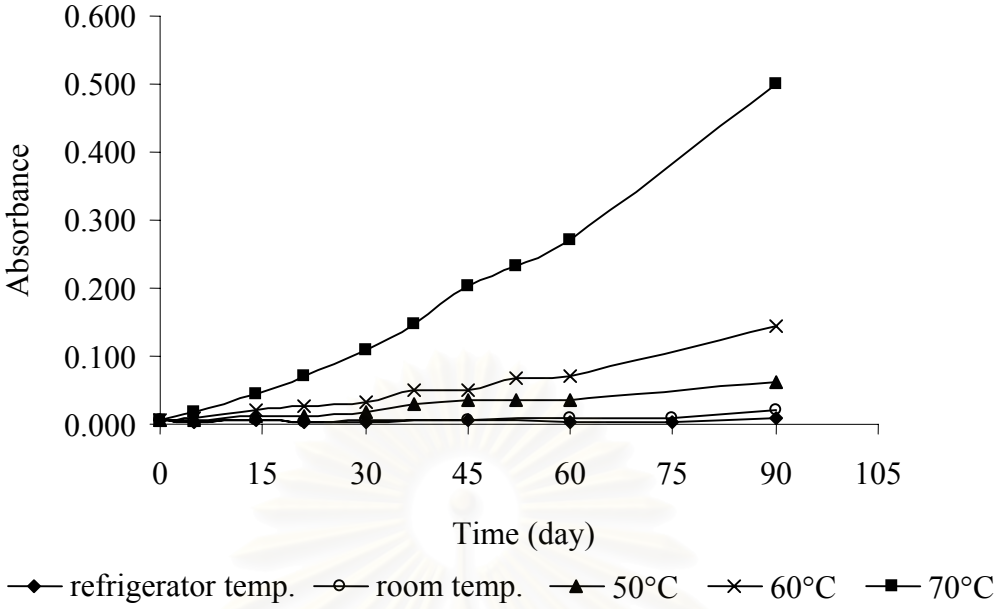


Figure 29 Visible spectral absorbance at 410 nm of 2% w/v ketoconazole solution pH 7 at various temperatures

From the data obtained, it was concluded that 2% w/v ketoconazole solution pH 7 should be stored at either refrigerator or room temperature, due to the high temperature affected on the physical and chemical stability of 2% w/v ketoconazole solution pH 7.

3.2 Stability Studies after Autoclaving

Table 24 shows percent drug remaining of 2% w/v ketoconazole pH 7 before and after autoclaving. The statistical difference in percent drug remaining of formulation before and after autoclaving were determined by using Man-Whitney test at significant level (α) = 0.05. It was found that the percent remaining of ketoconazole significantly decreased after autoclaving ($p \leq 0.05$) (Appendix F, Table 3F). However, it was interesting that the degradation during the autoclaving process was limited up to 3% degradation.

For the physical stability, the color of this formulation did not change after autoclaving by visual observation. However, the visible spectral absorbance at 410 nm of 2% w/v ketoconazole solution pH 7 slightly increased after autoclaving (Table 25).

Table 24 Percent drug remaining of 2% w/v ketoconazole solution pH 7 before and after autoclaving

	Percent remaining of ketoconazole			Mean	SD
	n_1	n_2	n_3		
Before autoclaving	99.91	99.13	100.95	100.00	0.91
After autoclaving	96.26	97.52	98.65	97.48	1.20

Table 25 Visible spectral absorbance at 410 nm of 2% w/v ketoconazole solution pH 7 before and after autoclaving

	Absorbance at 410 nm			Mean	SD
	n_1	n_2	n_3		
Before autoclaving	0.003	0.003	0.003	0.003	0.000
After autoclaving	0.028	0.028	0.028	0.028	0.000

From these results obtained, it was shown that 2% w/v ketoconazole solution pH 7 after autoclaving showed only slight decomposition. Therefore, the autoclaving may be chosen as an alternative sterililization method other than nonthermal method, for example, sterile membrane filtration.

4. Stability of 2% w/v Ketoconazole Lyophilized Products

The 2% w/v ketoconazole lyophilized products were prepared from the solutions at pH 7 with and without benzalkonium chloride (Formula II and III). Since benzalkonium chloride was accepted to be the “preservative of choice” for ophthalmic products, it was included in the formula III (Desai and Blanchard, 1995).

Stability of 2% w/v ketoconazole lyophilized products with and without benzalkonium chloride was studied when storaged at room temperature (30±3°C) and 45°C/ 75% RH for 3 months. Table 26 and Figure 30 showed stability data of both lyophilized products. It was summarized that the percent remaining of ketoconazole of two preparations at room temperature (30±3°C) and 45°C/ 75% RH were more than 90%. It could be concluded that two preparations appeared to be similarly stable at room temperature (30±3°C) and 45°C/ 75% RH.

Table 26 Percent remaining of ketoconazole of 2% w/v ketoconazole lyophilized products at room temperature (30±3°C) and 45°C/ 75% RH

Time (day)	2% w/v ketoconazole lyophilized products without BZCl		2% w/v ketoconazole lyophilized products with BZCl	
	room temperature (30±3°C)	45°C/ 75% RH	room temperature (30±3°C)	45°C/ 75% RH
0	100.00±1.97	100.00±1.97	100.00±1.53	100.00±1.53
15	98.49±5.33	97.19±4.05	98.89±0.92	98.60±0.85
30	100.89±3.52	99.23±2.61	97.30±1.17	95.88±0.63
60	104.70±4.36	102.04±3.40	100.66±1.57	97.59±3.01
90	98.56±5.62	103.39±3.00	95.97±0.99	94.32±1.82

a – mean±SD, n = 3

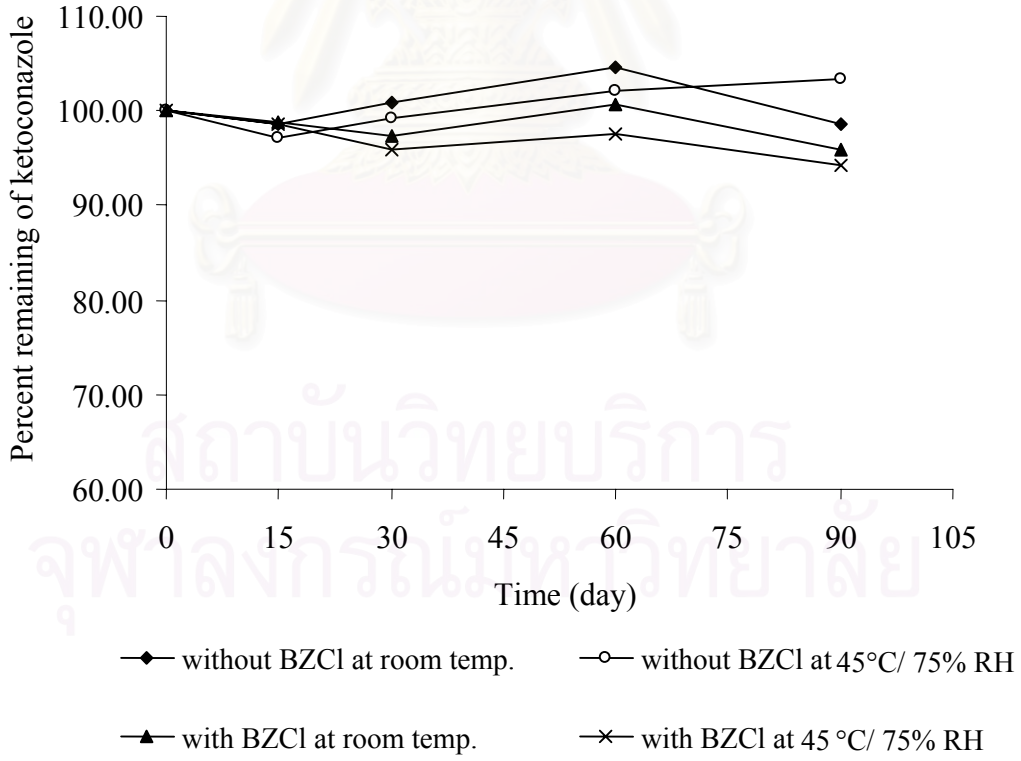


Figure 30 Percent remaining of ketoconazole of 2% w/v ketoconazole lyophilized products at room temperature (30±3°C) and 45°C/ 75% RH

The statistical differences of percent remaining of ketoconazole of the 2% w/v ketoconazole lyophilized products with and without benzalkonium chloride before and during the storage at room temperature (30±3°C) and 45°C/ 75% RH were tested by multiple-factors analysis of variance (Multiple-Factors ANOVA) at significant level (α) = 0.05, SPSS for windows version 10.0. It was found that percent remaining of ketoconazole of the 2% w/v ketoconazole lyophilized products with and without benzalkonium chloride was significantly different ($p \leq 0.05$), whereas percent remaining of ketoconazole at room temperature (30±3°C) and 45°C/ 75% RH was not significantly different ($p > 0.05$) and percent remaining of ketoconazole at different time was significantly different ($p \leq 0.05$) (Appendix F, Table 4F). In addition, there was no interaction among formulation, temperature and time effects on the stability of the products ($p > 0.05$) (Appendix F, Table 4F).

Therefore, it was concluded that benzalkonium chloride and time affected on the stability of 2% w/v ketoconazole lyophilized products, whereas temperature did not affect on the stability of the products. The degradation of ketoconazole in the formulations with and without benzalkonium chloride was different. This might be explained to the competition of benzalkonium chloride with ketoconazole in the inclusion complex formation. However, as shown in Table 26, all values of percent ketoconazole remaining were greater than 90% labeled amount. This meant that ketoconazole lyophilized products were stable in both at room temperature and in stressed condition. Furthermore, it was found that when storaged at room temperature for 90 days the percent ketoconazole remaining of 2% w/v ketoconazole lyophilized products at pH 7 without BZCl was significantly higher than that of 2% w/v ketoconazole solution pH 7 ($p \leq 0.05$) (Appendix F, Table 5F).

For the physical stability studies at room temperature (30±3°C) and 45° C/ 75% RH, it was found that the reconstitution time and color of lyophilized products by visual observation and by visible spectral absorbance, did not change throughout the study (Tables 27 and 28, Figures 31and 32).

Thus, the stability of 2% w/v ketoconazole solution could be improved successfully by the preparation as powder reconstitution by lyophilization technique.

Table 27 Reconstitution time of 2% w/v ketoconazole lyophilized products at room temperature (30±3°C) and 45°C/ 75% RH

Time (day)	2% w/v ketoconazole lyophilized products without BZCl (minutes)		2% w/v ketoconazole lyophilized products with BZCl (minutes)	
	room temperature (30±3°C)	45°C/ 75% RH	room temperature (30±3°C)	45°C/ 75% RH
0	2.44±0.08	2.44±0.08	2.42±0.09	2.42±0.09
15	2.66±0.41	2.99±0.63	2.61±0.80	3.34±0.24
30	1.83±0.45	2.61±0.63	2.51±0.07	2.90±0.33
60	2.05±0.45	2.27±0.24	1.89±0.33	2.74±0.31
90	1.94±0.34	2.17±0.16	2.30±0.14	2.78±0.42

a – mean±SD, n = 3

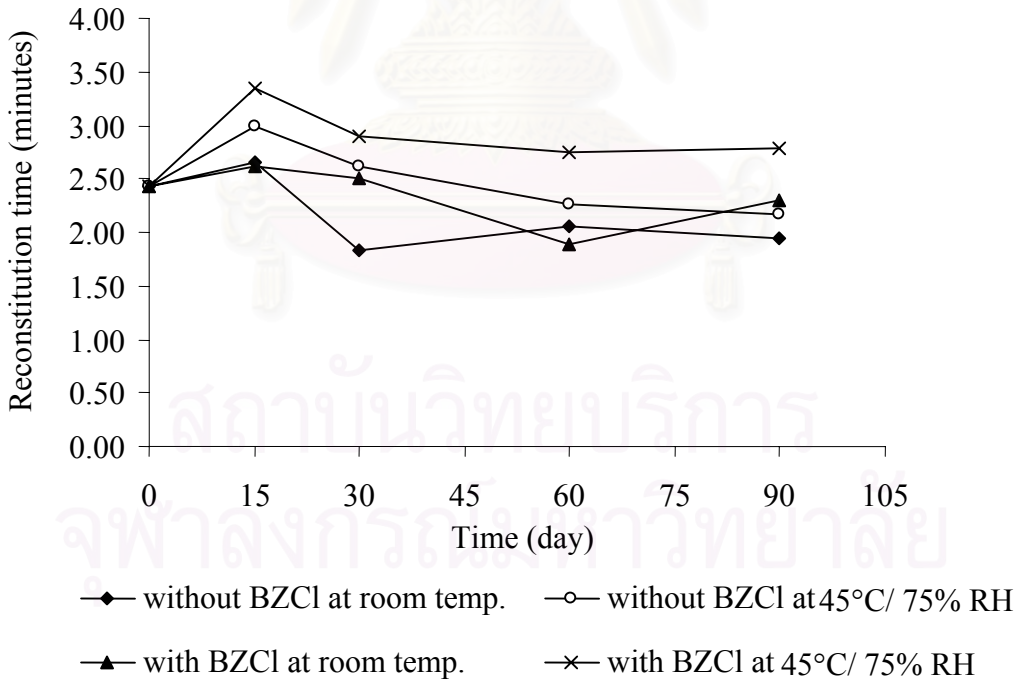


Figure 31 Reconstitution time of 2% w/v ketoconazole lyophilized products at room temperature (30±3°C) and 45°C/ 75% RH

Table 28 Visible spectral absorbance at 410 nm of 2% w/v ketoconazole lyophilized products at room temperature (30±3°C) and 45°C/ 75% RH

Time (day)	2% w/v ketoconazole lyophilized products without BZCl (minutes)		2% w/v ketoconazole lyophilized products with BZCl (minutes)	
	room temperature (30±3°C)	45°C/ 75% RH	room temperature (30±3°C)	45°C/ 75% RH
0	0.006±0.000	0.006±0.000	0.008±0.000	0.008±0.000
15	0.008±0.000	0.009±0.000	0.009±0.000	0.008±0.000
30	0.005±0.001	0.004±0.000	0.005±0.000	0.005±0.000
60	0.008±0.001	0.010±0.001	0.009±0.000	0.010±0.000
90	0.005±0.000	0.007±0.000	0.006±0.000	0.007±0.000

a – mean±SD, n = 3

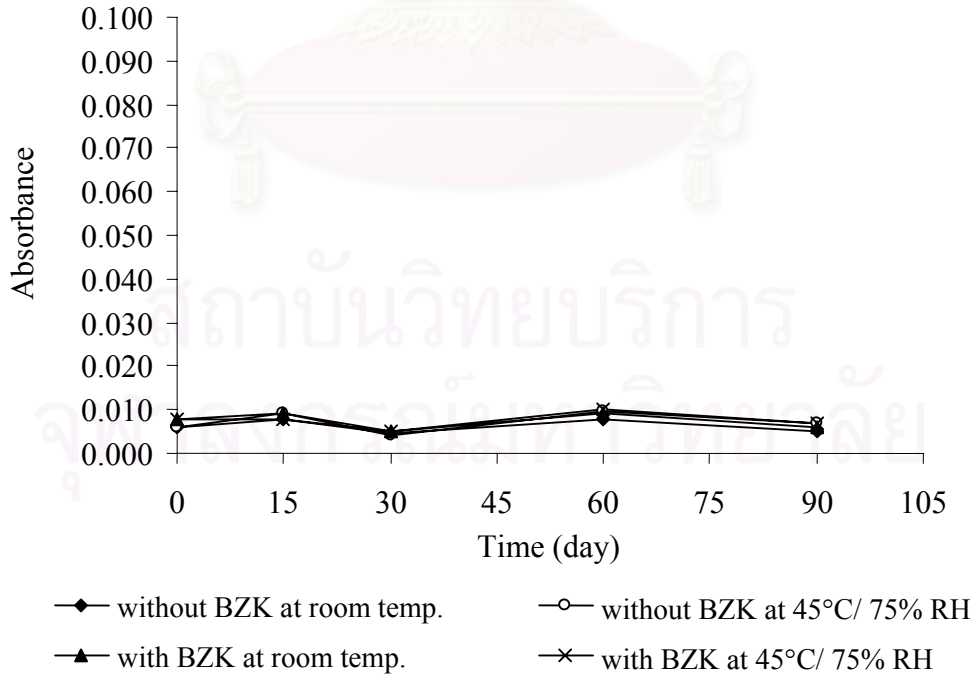


Figure 32 Visible spectral absorbance at 410 nm of 2% w/v ketoconazole lyophilized products at room temperature (30±3°C) and 45°C/ 75% RH

III. Physicochemical Properties of 2% w/v Ketoconazole Solutions and Lyophilized Products

1. Physicochemical Properties of 2% w/v Ketoconazole Solutions pH 7 with and without Benzalkonium Chloride

1.1 pH

Table 29 showed pH values of 2% w/v ketoconazole solutions pH 7 with and without benzalkonium chloride. The pH values were 7.04±0.01 and 7.06± 0.01, respectively. It was shown that addition of benzalkonium chloride did not affect the pH of the solution due to its low concentration used. Therefore, ocular discomfort and irritation did not occur when 2% w/v ketoconazole solutions pH 7 was instilled into the eye, because the formulations have pH values closed to pH of tears which is approximately 7.4 (Hecht et al.,1996; Ansel, Allen and Popovich, 1999).

Table 29 pH values of 2% w/v ketoconazole solutions pH 7 with and without benzalkonium chloride

	pH values of 2% w/v ketoconazole solutions (pH 7)	
	With benzalkonium chloride	Without benzalkonium chloride
n_1	7.05	7.05
n_2	7.06	7.06
n_3	7.06	7.07
n_4	7.05	7.05
n_5	7.04	7.07
n_6	7.03	7.06
n_7	7.04	7.07
n_8	7.03	7.07
n_9	7.03	7.06
mean	7.04	7.06
SD	0.01	0.01

Additionally, ketoconazole was a weak dibasic compound, which had pK_a of 2.9 and 6.5 (McEvoy, ed., 1999; Quanyum, 1999) and pH of these formulations were 7.04±0.01 and 7.06±0.01, respectively. Thus, most molecules of ketoconazole in the solutions was in unionized form which was the species that can more easily and rapidly penetrate the corneal epithelium (Hecht et al., 1996; Ansel, Allen and Popovich, 1999; Loftsson and Jarvinen, 1999). Moreover, the pH values of these formulations was the stable pH for ketoconazole solutions, since the degradation pathway was specific acid catalysis (Suwanna Techowanich, 1999; Skiba et al., 2000)

1.2 Viscosity

Viscosity at room temperature (30°C) of 2% w/v ketoconazole solutions pH 7 with and without benzalkonium chloride was determined by Ostwald viscometer (Arthur H. Thomas, USA) and viscosity values were shown in Table 30. The viscosity values were between 2.49-2.52 cps and 2.48-2.52 cps, respectively, these values were very low viscosity.

Table 30 Viscosity values of 2% w/v ketoconazole solutions pH 7 with and without benzalkonium chloride

	Viscosity values (cps) of 2% w/v ketoconazole solutions (pH 7)	
	With benzalkonium chloride	Without benzalkonium chloride
n_1	2.52	2.49
n_2	2.52	2.52
n_3	2.51	2.51
n_4	2.50	2.48
n_5	2.50	2.50
n_6	2.49	2.50
n_7	2.49	2.49
n_8	2.50	2.49
n_9	2.50	2.50
mean	2.50	2.50
SD	0.01	0.01

The viscosity of ophthalmic solution is often increased in order to prolong the corneal contact time, decreased the drainage rate and increased the bioavailability of the active ingredient. In general, viscosity increased up to the 25 to 50 cps range significantly improves contact time in the eye (Desai and Blanchard, 1995; Hecht et al., 1996). Therefore, for the formulations of 2% w/v ketoconazole solutions pH 7, the suitable viscosity enhancers should be added to increase the viscosity, thereby aid in maintaining the drug in contact with the tissues to enhance bioavailability of the drug.

Furthermore, the addition of small portions of viscosity enhancers has been reported to enhance the effect of CDs (Loftsson et al., 1994a, 1994c; Cappello et al., 2001), thus allowing reduction of the CDs amount required for drug solubilization. The common viscosity enhancers used in ophthalmic solution, such as methylcellulose, hydroxypropyl methylcellulose, polyvinyl alcohol and polyvinylpyrrolidone (Desantis and Patil, 1994). Generally, the criteria of selection of viscosity enhancers should be considered much as their compatibility with other components of the formulations.

However, in the case of powder for reconstitution, addition of any viscosity enhancers into the formulation might lead to the difficulty to redisperse and increase the reconstitution time. Consequently, it was reasonable to not include a viscosity enhancer into ketoconazole lyophilized products.

1.3 Tonicity

Tonicity of 2% w/v ketoconazole solutions pH 7 with and without benzalkonium chloride is presented in Table 31. The tonicity values were between 733-769 mOsm/kg and 736-767 mOsm/kg, respectively. These values have the higher tonicity than tonicity of which eye can tolerlate without eye discomfort and irritation which equivalent to 2% sodium chloride or approximately 685 mOsm/kg (Hecht et al., 1996; Ansel, Allen and Popovich, 1999).

Although, the amount of solution administered is small, dilution with tears take place rapidly so that discomfort and irritation from the hypertonicity is only temporary (USP 24, 2000), on this basis, both formulations may irritate and toxic to the eye. To support the safety of the eyes after administration of the formulations, ocular irritation and toxicity test was designed and included in this study. Additionally, the survey of the tonicity or osmolarity of an eyedrop product available in any drugstores was carried out. Sulfacetamide eyedrops as 10-30% w/v strength

have been accepted practically for eye bacterial infections. The tonicity value of 10% w/v sulfacetamide eyedrops was measured in this study to be 1026 mOsm/kg.

Table 31 Tonicity values of 2% w/v ketoconazole solutions pH 7 with and without benzalkonium chloride

	Tonicity values (mOsm/kg) of 2% w/v ketoconazole solutions (pH 7)	
	With benzalkonium chloride	Without benzalkonium chloride
n_1	733	743
n_2	752	763
n_3	769	736
n_4	762	767
n_5	756	744
n_6	754	745
n_7	743	765
n_8	752	753
n_9	736	765
mean	751	753
SD	12	12

2. Physicochemical Properties of 2% w/v Ketoconazole Lyophilized Products

2.1 Powder X-ray Diffractometry

Powder X-ray diffractometry may be used to detect inclusion complexation in the solid state (Tong, 2000). Figure 33 and 34 showed the X-ray diffractograms of ketoconazole, HP-β-CD, physical mixture and 2% w/v ketoconazole lyophilized products with and without benzalkonium chloride. Ketoconazole gave a sharp and intense diffraction peaks due to its crystalline character at 7, 10.5, 17, 19.5 and 27.5°. This was contrast to the case of HP-β-CD that gave a diffuse diffraction pattern due to its amorphous character. In the case of physical mixture, the X-ray diffractogram is a mere superimposition of the diffraction pattern of ketoconazole and HP-β-CD with the peaks having lower intensity at 7, 10.5, 17, 19.5 and 27.5°.

On the other hand, the diffraction patterns of lyophilized products showed a broad, diffuse diffraction pattern indicating amorphous form of drug or inclusion complexation occurred or both (Diaz, Mendez et al., 1996; Diaz, Otero et al., 1996). Because the powder X-ray diffraction pattern of drug is considerably affected by complexation with CD owing to change in their crystalline nature. Normally, formation of amorphous inclusion complexes leads to the disappearance of certain peaks or peaks become less sharp than that of the pure compound or physical mixture. Occasionally, an appearance of a few new peaks and shifting of certain peaks are associated with crystalline inclusion complexes formation (Athal, Udapa and Sreenivasan, 1995). However, supporting evidence for the amorphous inclusion complexes formation was obtained from the DSC study.

The comparison of the powder X-ray diffraction patterns of lyophilized products with and without benzalkonium chloride (Figures 33 and 34) showed no difference. This was due to the amount of the preservative included was too small to the detected.

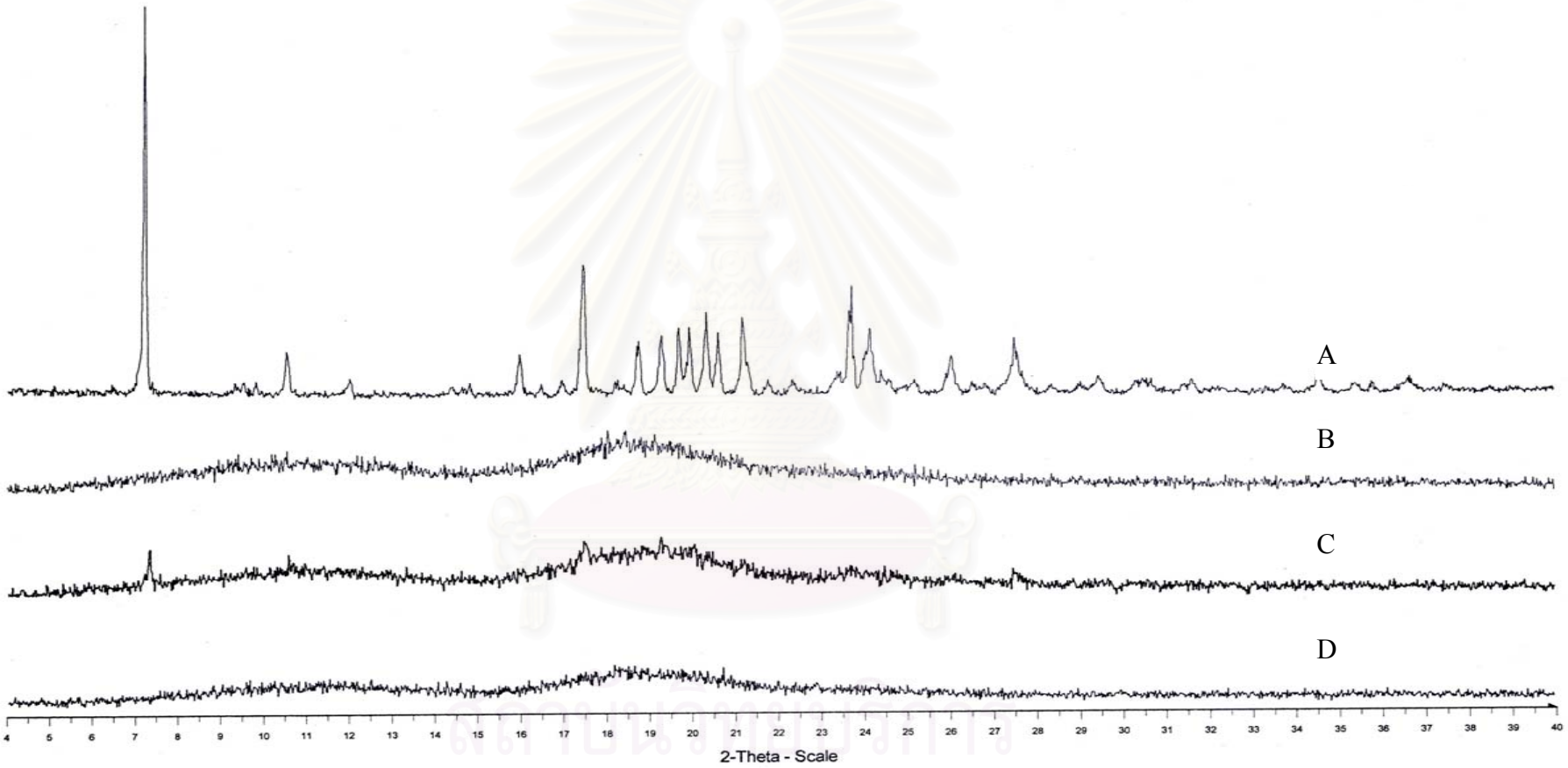


Figure 33 Powder X-ray diffractograms of: (A) ketoconazole; (B) HP-β-CD; (C) physical mixture; (D) 2% w/v ketoconazole lyophilized products with benzalkonium chloride

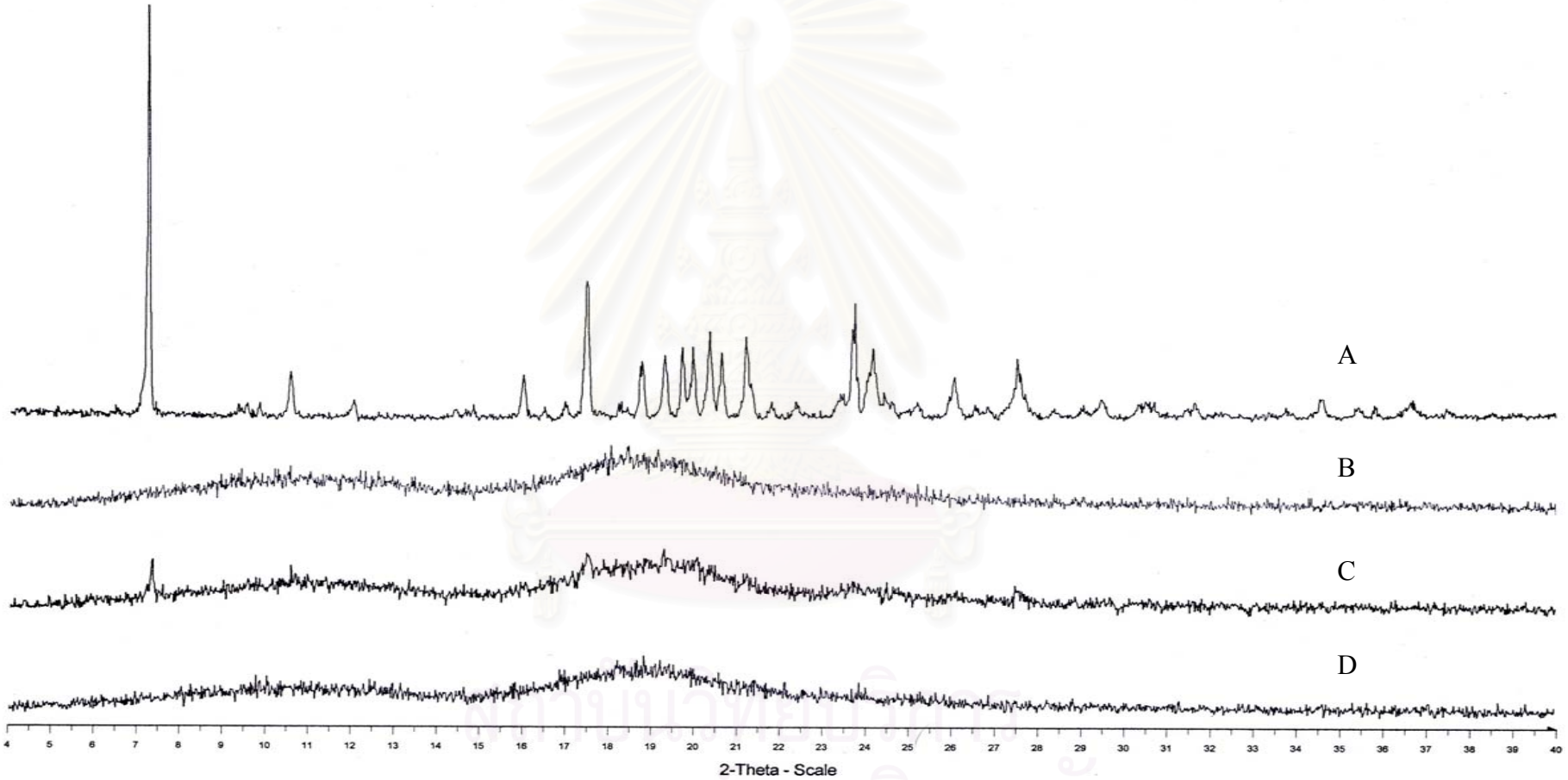


Figure 34 Powder X-ray diffractograms of: (A) ketoconazole; (B) HP-β-CD; (C) physical mixture; (D) 2% w/v ketoconazole lyophilized products without benzalkonium chloride

91

2.2 Differential Scanning Calorimetry (DSC)

The DSC thermograms of ketoconazole, HP-β-CD, physical mixture and 2% w/v ketoconazole lyophilized products with and without benzalkonium chloride were compared in Figures 35 and 36. The DSC thermogram of ketoconazole showed an endothermic melting peak at 151.6°C with heat of fusion (ΔH_f) of 97.81 J/g. While the DSC thermogram of HP-β-CD showed broad endotherm ranging from 30-130°C, corresponding to water evaporation from HP-β-CD (Ruiz and Paronen, 1997). In the case of physical mixture, the DSC thermogram showed the shift of endothermic peak of ketoconazole form 151.6°C to 150.3°C. This revealed that ketoconazole in the physical mixture still existed in a crystalline form with a lesser heat of fusion. This was consistant to the powder X-ray diffraction pattern that some diffraction peaks of ketoconazole were still observed (Figures 33, C and 34, C)

On the contrary, the DSC thermograms of 2% w/v ketoconazole lyophilized products with and without benzalkonium chloride showed broad endotherms of HP-β-CD, whereas the endothermic melting peak of ketoconazole at 151.6°C disappeared. The absence of the melting peak of ketoconazole in the DSC thermograms of lyophilized products may be attributed to the transformation of ketoconazole in the amorphous state or the inclusion complexes formation or both (Diaz, Mendez et al., 1996; Diaz, Otero et al., 1996). The effects of CD on the DSC thermogram of the guest molecule could be observed as the broadening, shifting and appearance of new peaks or disappearance of certain peaks (Athal, Udapa and Sreenivasan, 1995). In the case of the absence of melting peak, no energy absorption is observed at the melting temperature of the guest when the guest is inclusion complexes, since the guest is surrounded by the CD and not interacting with other guest molecules, there is no crystalline guest structure to absorb energy (Hedges, 1998). It was interesting that the presence of dehydration endotherm of HP-β-CD was observed in the lyophilized products. This revealed that water molecules in the HP-β-CD could not be normally removed by the lyophilization process or oppositely lyophilized products might absorb moisture from atmosphere during storage.

Consequently, from the powder X-ray diffractometry and DSC studies it was likely that the formation of amorphous inclusion complexes between ketoconazole with HP-β-CD occurred. HP-β-CD generally gives a highly water soluble amorphous inclusion complexes when forming complexes with poorly water

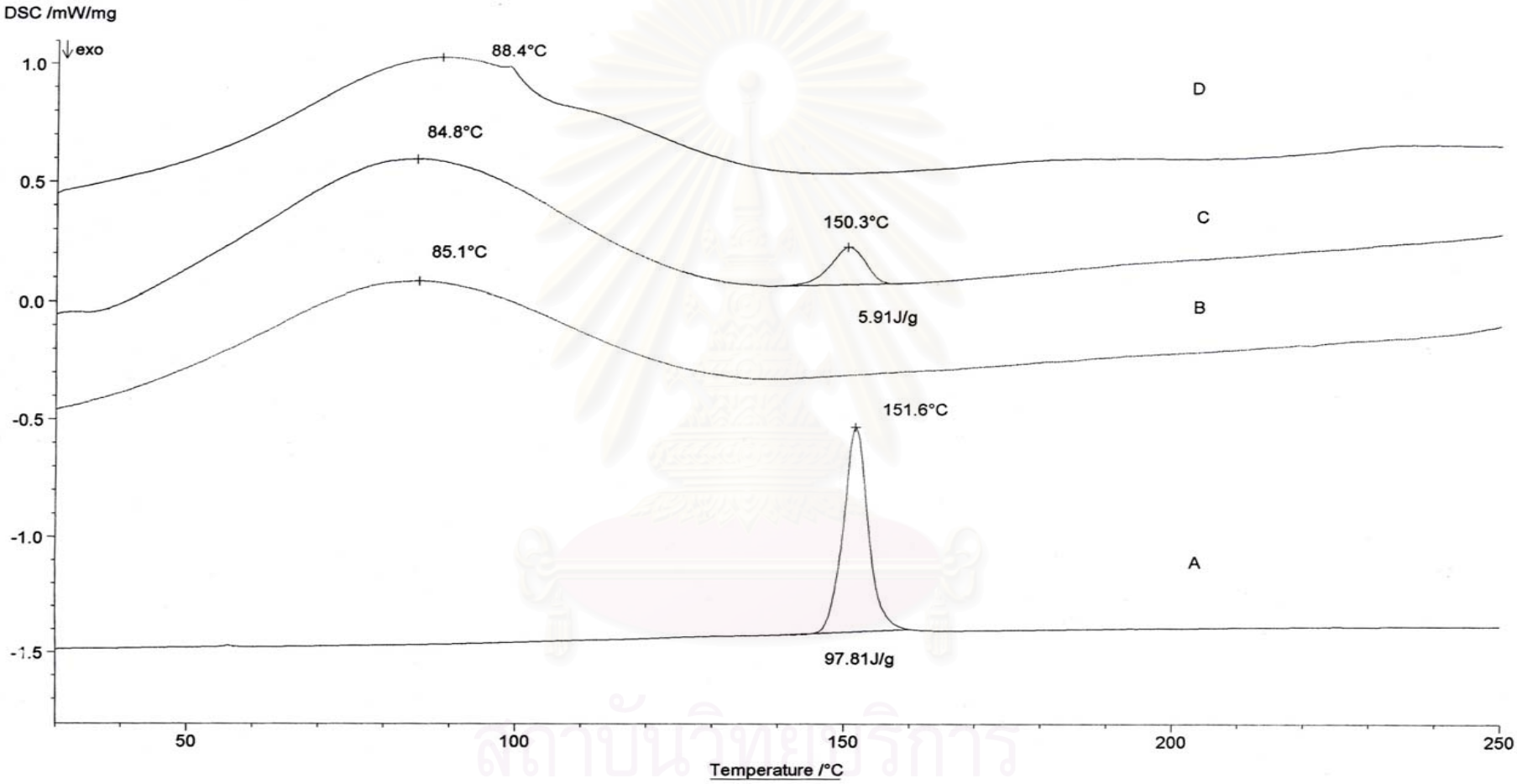


Figure 35 DSC thermograms of: (A) ketoconazole; (B) HP-β-CD; (C) physical mixture; (D) 2% w/v ketoconazole lyophilized products with benzalkonium chloride

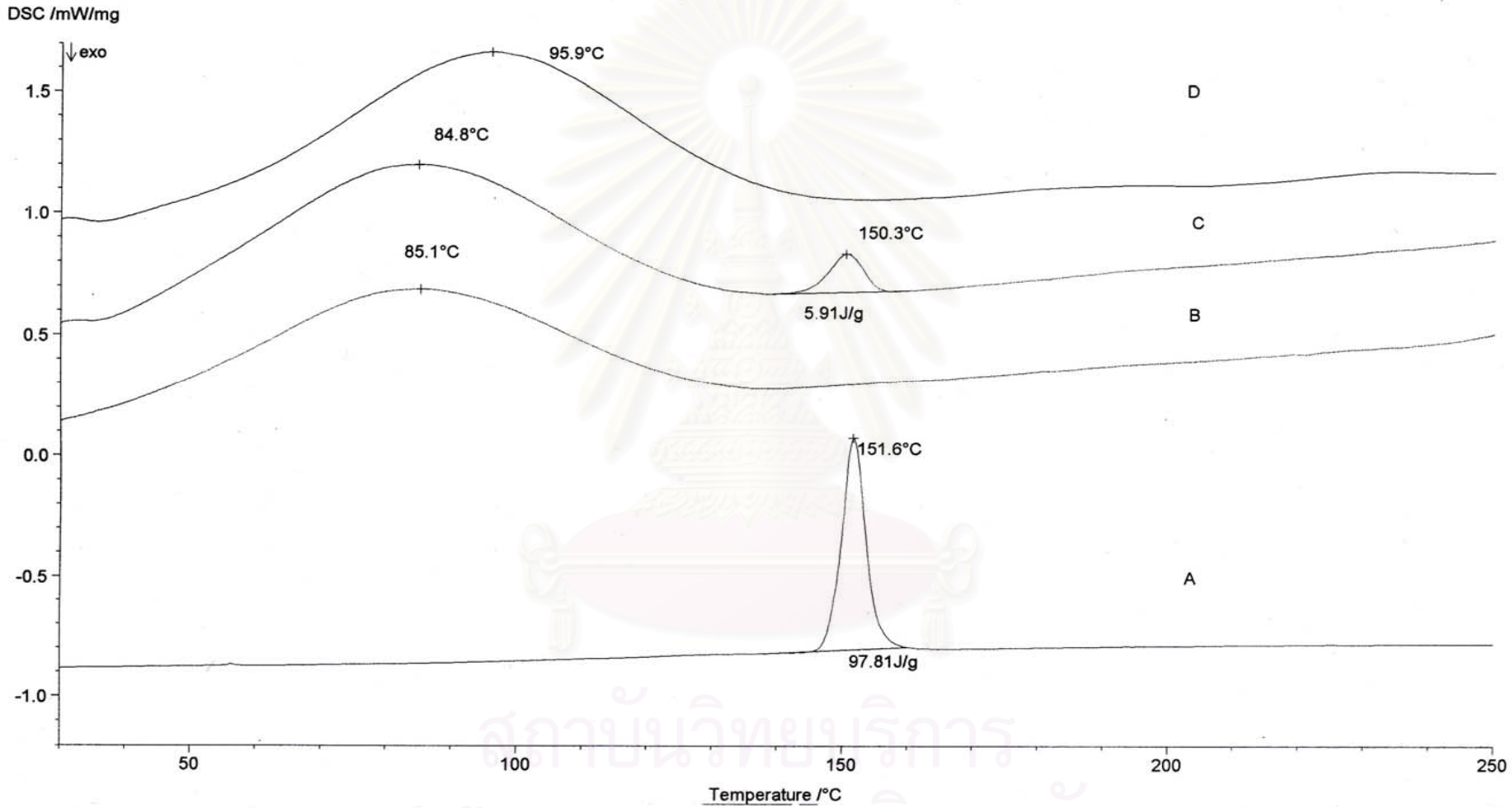


Figure 36 DSC thermograms of: (A) ketoconazole; (B) HP-β-CD; (C) physical mixture; (D) 2% w/v ketoconazole lyophilized products without benzalkonium chloride

soluble drugs (Szente and Szejtli, 1999; Duchene and Wouessidjewe, 1990a, 1990b; Uekama and Irie, 1990).

IV. Ocular Irritation and Toxicity Test of 2% w/v Ketoconazole Solution pH 7 in Rabbits

The traditional animal model for ocular irritation and toxicity evaluations is the albino (New Zealand White) rabbit. The ocular irritation and toxicity test in the rabbit is now commonly referred to as " the Draize test ". The sensitivity of the albino rabbit to ocularly administered materials, the availability and ease of handling and husbandry, the relatively large size of the eye, and the lack of pigmentation make this model useful in performing studies predicting potential ocular irritation and toxicity (Hecht et al., 1996; Auletta, 2000). Moreover, because of its extreme sensitivity to ocular irritants, the rational is that this model will over predict any hazards and that any material that is nonirritating to the rabbit eye is very unlikely to be irritating to the human eye (Auletta, 2000).

Therefore, the Draize test was used to study the ocular irritation and toxicity of sterile 2% w/v ketoconazole solution pH 7 containing HP-β-CD and benzalkonium chloride by using albino rabbits as test animal and compared to the effects of 0.9% sodium chloride solution as control. The results of this study were presented in Table 32. It was found that the effects of both solutions did not cause opacity, ulceration of cornea or swelling, congestion, hemorrhage or change reaction to light of the iris and did not produce conjunctivitis. Thus, it was concluded that 2% w/v ketoconazole solution pH 7 containing 30% w/v HP-β-CD and 0.01% w/v benzalkonium chloride did not produce eye irritation and toxicity in the albino rabbits.

Although, the administration of 2% w/v ketoconazole solution pH 7 showed no eye irritation and toxicity in the albino rabbits throughout the period of study (1 month), the results of this study in the rabbits was not used to predict accurately the actual eye irritation and toxicity in human. Because the anatomy and physiology between the eye of rabbit and human were slightly different. The primary differences between rabbit and human relate to decreased tearing in rabbit, decreased blinking rate, presence of a nictating membrane, and a slower reepithelialization of the rabbit cornea (Hecht et al., 1996). Therefore, a more realistic prediction of ocular irritation and toxicity in human may be obtained using a nonhuman primate model, generally

Table 32 Ocular irritation and toxicity test of 2% w/v ketoconazole solution pH 7 in rabbits (n = 6)

Day	Ocular irritation and toxicity scores	
	0.9% sodium chloride solution	2% w/v ketoconazole solution pH 7
0	0	0
1 (hour)	0	0
1	0	0
2	0	0
3	0	0
7	0	0
10	0	0
14	0	0
21	0	0
28	0	0

the rhesus or cynomolgus monkey, because nonhuman primate model has ocular structure, function and pigmentation close to those in the human (Auletta, 2000).

In conclusion, the outcomes of this investigation for the solving problems of ketoconazole water insolubility and instability were demonstrated. The recommendation from the study was the solubilization of ketoconazole by inclusion complex formation into aqueous solution, and then lyophilization. Under the stressed condition for 90 days, the lyophilized products was shown to be stable at room temperature. After reconstitution, the solution could be storaged with accepted stability at either room temperature or refrigerator temperature and protected from light for approximately 100 days. Finally, the further study of this formulation for clinical use in the treatment of fungal corneal infection should be performed.

CHAPTER V
CONCLUSIONS

The effects of cyclodextrins for ophthalmic use on solubility and stability of 2% w/v ketoconazole solutions and lyophilized products were investigated. The conclusions of this study were as follows:

1. The solubility of ketoconazole in buffer solutions increased as a function of HP-β-CD and HP-γ-CD concentration. The phase solubility diagram can be classified as type A_L.

2. The association constants of ketoconazole with HP-β-CD and HP-γ-CD in buffer pH 5 were 1263 and 244 M^{-1}, respectively. Therefore, HP-β-CD was more suitable as a solubility enhancer for ketoconazole than HP-γ-CD. The association constants of ketoconazole:HP-β-CD inclusion complexes in buffer pH 5 and buffer pH 7 were 1263 M^{-1} and 6904 M^{-1}, respectively. Thus, HP-β-CD was found to form much more stable inclusion complexes with unionized drug than with ionized drug.

3. From the phase solubility diagrams, HP-β-CD was applied successfully to prepare the 2% w/v ketoconazole solutions and lyophilized products, whereas HP-γ-CD was not able to increase solubility of ketoconazole to 2% w/v.

4. The physical and chemical stability of 2% w/v ketoconazole solution pH 7 was much better than 2% w/v ketoconazole solution pH 5.

5. From accelerated stability study, the extrapolated shelf lifes of 2% w/v ketoconazole solution pH 7 at refrigerator temperature and room temperature were 556 and 182 days that were longer than the observed shelf lifes, 111 and 107 days.

6. The sterilization method for 2% w/v ketoconazole solution by autoclaving might be used since it caused only 3% degradation of the drug.

7. The ketoconazole powder for reconstitution produced by lyophilization technique was more stable than its solution form. In this standpoint, 2% w/v ketoconazole ophthalmic solution should be prepared commercially in lyophilized products in amber glass containers. After reconstitution, the storage of the reconstituted solution might be in either refrigerator temperature or in room temperature and the solution should be stable for about 100 days.

8. The pH values of 2% w/v ketoconazole solutions pH 7 were around 7 that closed to pH of tears, the tonicity of formulation was hypertonic and the viscosity was slightly higher than that of purified water.

9. From the powder X-ray diffractometry and DSC studies of 2% w/v ketoconazole lyophilized products gave some clues indicating that the formation of amorphous inclusion complexes between ketoconazole with HP-β-CD might occurr.

10. The test of ocular irritation and toxicity in the albino rabbits, the preparation of 2% w/v ketoconzole solution pH 7 containing 30% w/v HP-β-CD with 0.01% w/v benzalkonium chloride did not produce ocular irritation and toxicity in the albino rabbits.

REFERENCES

Thai

จุไรรัตน์ รักวาทิน. 2543. แนวทางการเสนอรายงานความคงสภาพของตำรับยา. กรุงเทพมหานคร: กองยา กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข.

English

Allen, L. V., and Erickson, M. A. 1996. Stability of ketoconazole, metolazone, metronidazole, procainamide hydrochloride, and spironolactone in extemporaneously compounded oral liquids. Am. J. Health-Syst. Pharm. 53: 2073-2078.

Althal, K. S., Udupa, N., and Sreenivasan, K. K. 1995. Physicochemical properties of drug-cyclodextrin complexes. Indian Drugs 32(7): 293-305.

Alton, K. B. 1980. Determination of the antifungal, ketoconazole in human plasma by high performance liquid chromatography. J. Chromatogr. 221: 337-344.

Ansel, H. C., Allen, L. V., and Popovich, N. G. 1999. Pharmaceutical Dosage Forms and Drug Delivery. 7 th ed. Philadelphia: Lippincott Williams & Wilkins, pp. 469-486.

Auletta, C. S. 2000. Routes of Exposure: Other. In M. L. Weiner; and L. A. Kotkoskie (eds.), Excipient Toxicity and Safety, pp. 231-265.New york: Marcel Dekker.

Badcock, N. R. 1984. Micro-determination of ketoconazole in plasma or serum by high-pressure liquid chromatography. J. Chromatogr. 306: 436-440.

Bary, A. R., Tucker, I. G., and Davies, N. M. 2000. Considerations in the use of hydroxypropyl-β-cyclodextrin in the formulation of aqueous ophthalmic solutions of hydrocortisone. Eur. J. Pharm. Biopharm. 50: 237-244.

Bekers, O., Uijtendaal, E. V., Beijnen, J. H., Bult, A., and Underberg, W. J. M. 1991. Cyclodextrins in the pharmaceutical field. Drug Dev. Ind. Pharm. 17(11): 1503-1549.

Brewster, M. E., Estes, K. S., and Bodor, N. 1990. An intravenous toxicity study of 2-hydroxypropyl-β-cyclodextrin, a useful drug solubilizer, in rats and monkeys. Int. J. Pharm. 59: 231-243.

Cappello, B., Carmignani, C., Iervolino, M., La Rontonda, M. I., and Saettone, M. F. 2001. Solubilization of tropicamide by hydroxypropyl-β-cyclodextrin and water-soluble polymers: in vitro/in vivo studies. Int. J. Pharm. 213: 75-81.

Carpenter, T. O., Gerloczy, A., and Pitha, J. 1995. Safety of parenteral hydroxypropyl-β-cyclodextrin. J. Pharm. Sci. 84(2): 222-225.

Carstensen, J. T. 1990. Chemical Kinetics and Drug Stability. In J. Swarbrick; and J. C. Boylan (eds.), Encyclopedia of pharmaceutical technology 2, pp. 355-395.New york: Marcel Dekker.

Carstensen, J. T. 2000. Solution Kinetics. In J. T. Carstensen; and C. T. Rhodes (eds.), Drug Stability Principles and Practices, pp. 19-55.New york: Marcel Dekker.

Cavrini, V., Di Pietra, A. M., and Raggi, M. A. 1982. High-pressure liquid chromatographic (HPLC) analysis of imidazole antifungals in commercial dosage forms. Int. J. Pharm. 10: 119-124.

Connors, K. A. 1997. The stability of cyclodextrin complexes in solution. Chem. Rev. 97: 1325-1357.

Daneshmend, T. K., and Warnock, D. W. 1988. Clinical pharmacokinetics of ketoconazole. Clin. Pharmacokinet. 14(1): 13-34.

Daveis, N., Wang, G., and Tucker, I.G. 1997. Evaluation of a hydrocortisone/hydroxypropyl-β-cyclodextrin solution for ocular drug delivery. Int. J. Pharm. 156: 201-209.

Desai, S. D., and Blanchard, J. 1995. Ocular Drug Formulation and Delivery. In J. Swarbrick; and J. C. Boylan (eds.), Encyclopedia of pharmaceutical technology 11, pp. 43-75. New york: Marcel Dekker.

Desantis, L. M., and Patil, P. N. 1994. Pharmacokinetics. In T. F. Mauger; and E. L. Craig (eds.), Havener's Ocular Pharmacology. 6 th ed, pp. 22-52. St. Louis: Mosby.

Diaz, M. T. E., Mendez, M. G., Marcos, M. B. P., Jato, J. L. V., and Labandeira, J. J. T. 1996. Characterization and in vitro dissolution behaviour of ketoconazole / β- and 2-hydroxypropyl-β-cyclodextrin inclusion compounds. Int. J. Pharm. 143: 203-210.

Diaz, M. T. E., Otero, M. G., Marcos, M. B. P., Jato, J. L. V., and Labandeira, J. J. T. 1996. Preparation and evaluation of ketoconazole-β-cyclodextrin muticomponent complexes. Int. J. Pharm. 142: 183-187.

Di Pietra, A. M., Cavrini, V.,Andrisano, V., and Gatti, R. 1992. HPLC analysis of imidazole antimycotic drugs in pharmaceutical formulations. J. Pharm. Biomed. Anal. 10(10-12): 873-879.

Doorne, H. V. 1993. Interactions between cyclodextrins and ophthalmic drugs. Eur. J. Pharm. Biopharm. 39(4): 133-139.

Duchene, D. 1987. Pharmaceutical applications of cyclodextrins. In D. Duchene (ed.), Cyclodextrins and their industrial uses, pp. 213-254. Paris: Editions de Sante.

Duchene, D. 1988. New trends in pharmaceutical applications of cyclodextrin inclusion compounds. In O. Huber; and J. Szejtli (eds.), Proceedings of the fourth international symposium on cyclodextrins, pp. 265-275. Dordrecht: Kluwer Academic.

Duchene, D., and Wouessidjewe, D. 1990a. Pharmaceutical uses of cyclodextrins and derivatives. Drug Dev. Ind. Pharm. 16(17): 2487-2499.

Duchene, D., and Wouessidjewe, D. 1990b. Physicochemical characteristics and pharmaceutical uses of cyclodextrin derivatives, part I. Pharm. Technol. 14 (6): 26-34.

Duchene, D., and Wouessidjewe, D. 1990c. Physicochemical characteristics and pharmaceutical uses of cyclodextrin derivatives, part II. Pharm. Technol. 14 (8): 22-28.

Frank, S. G. 1975. Inclusion compounds. J. Pharm. Sci. 64(10): 1585-1604.

Frijlink, H. W., Eissens, A. C., Schonen, A. J. M., and Lerk, C. F. 1990. The effects of cyclodextrins on drug absorption II. In vivo observations. Int. J. Pharm. 64: 195-205.

Fridriksdottir, H., Loftson, T., and Stefansson, E. 1997. Formulation and testing of methazolamide cyclodextrin eye drop solutions. J. Contr. Rel. 44: 95-99.

Graybill, J. R., and Craven, P. C. 1983. Antifungal agents used in systemic mycoses activity and therapeutic use. Drugs 25: 41-62.

Guzek,J. P., Roosenberg, J. M., Gano, D. L., and Wessels, I. F. 1998. The effect of vehicle on corneal penetration of triturated ketoconazole and itraconazole. Ophthalmic Surgery and Lasers 29(11): 926-929.

Hecht, G., Roehrs, R. E., Lang, L. C., Rodeheaver, D. P., and Chowhan, M. A. 1996. Desigh and Evaluation of Ophthalmic Pharmaceutical Products. In G. S.

Banker; and C. T. Rhodes (eds.), Modern Pharmaceutics. 3 rd ed, pp. 489-546. New york: Marcel Dekker.

Hedges, A. R. 1998. Industrial applications of cyclodextrins. Chem. Rev. 98: 2035-2044.

Heel, R. C., et al. 1982. Ketoconazole: a review of its therapeutic efficacy in superficial and systemic fungal infections. Drugs 23: 1-36.

Heeres, J., Backx, L. J. J., Mostmans, J. H., and Cutsem, J. V. 1979. Antimycotic imidazoles. Part 4. Synthesis and antifungal activity of ketoconazole, a new potent orally active broad-spectrum antifungal agent. J. Med. Chem. 22(8): 1003-1005.

Higuchi, T., and Connors, K. A. 1965. Phase solubility techniques. Adv. Anal. Chem. Instrum. 4: 117-212.

Hirayama, F., Mieda, S., Miyamoto, Y., Arima, H., and Uekama, K. 1999. Heptakis (2,6-di-0-methyl-3-0-acetyl-β-cyclodextrin: A water-soluble cyclodextrin derivative with low hemolytic activity. J. Pharm. Sci. 88(10): 970-975.

Hume, A. L., and Kerkering, T. M. 1983. New drug evaluations: ketoconazole. Drug Intell. Clin. Pharm. 17: 169-174.

Hussar, D. A. 1982. New drugs of year. Am. Pharm. 22(3): 29-47.

Irie, T., and Uekama, K. 1997. Pharmaceutical applications of cyclodextrins. III. Toxicological issues and safety evaluation. J. Pharm. Sci. 86(2): 147-162.

Jack, D. B. 1992. Handbook of clinical pharmacokinetic data. Basingstoke, Hants: Macmillan, pp. 28.

Jarho, P., Jarvinen, K., Urtti, A., Stella, V. J., and Jarvinen, T. 1996. Modified β-cyclodextrin (SBE7-β-CyD) with viscous vehicle improves the ocular delivery and tolerability of pilocarpine prodrug in rabbits. J. Pharm. Pharmacol. 48: 263-269.

Jarho, P., Jarvinen, K., Urtti, A., Stella, V.J., and Jarvinen, T. 1997. The use of cyclodextrins in ophthalmic formulations of dipivefrin. Int. J. Pharm. 153: 225-233.

Jarho, P., Urtti, A., Pate, D.W., Suhonen, P., and Jarvinen, T. 1996. Increase in aqueous solubility, stability and in vitro corneal permeability of anandamide by hydroxypropyl-β-cyclodextrin. Int. J. Pharm. 137: 209-216.

Javitt, J. C., Javitt, N. B., and McDonnell, P. 1994. Topical compositions for the eye comprising a beta-cyclodextrin derivative and a carbonic anhydrase inhibitor. International Patent Application No. WO 94/15582.

Kallet, A., and Aaron, H. 1992. Drugs for treatment of fungal infection. Med. Lett. Drugs Ther. 34(21): 14-16.

Kibbe, A. H., ed. 2000. Handbook of pharmaceutical excipients. 3 rd ed. London: Pharmaceutical Press, pp. 165-168.

Koch, H. 1983. Ketoconazole: broad-spectrum antifungal agent. Pharm. Int. 4(7): 151-152.

Kumer, K. P., Okonomah, A. D., Bradshaw, W. G., and Soliman, K. F. A. 1991. Stability of ketoconazole in ethalnolic solutions. Drug Dev. Ind. Pharm. 17 (4): 577-580.

Lacy, C. F., Armstrong, L. L., Goldman, M. P., and Lance, L. L., eds. 1999-2000. Drug information handbook. 7 th ed. Hudson: Lexi-comp, pp. 649-650.

Lehner, S. J., Muller, B. W., and Seydel, J. K. 1993. Interactions between *p*-hydroxybenzoic acid esters and hydroxypropyl-β-cyclodextrin and their antimicrobial effect against *Candida albicans*. Int. J. Pharm. 93: 201-208.

Lehner, S. J., Muller, B. W., and Seydel, J. K. 1994. Effect of hydroxypropyl-β-cyclodextrin on the antimicrobial action of preservatives. J. Pharm. Pharmacol. 46: 186-191.

Li, S., and Purdy, W. C. 1992. Cyclodextrins and their applications in analytical chemistry. Chem. Rev. 92: 1457-1470.

Lide, D. R., and Frederikse, H. P. R., eds. 1995-1996. CRC Handbook of chemistry and physics. 76 th ed. London: CRC Press, pp. 6-10.

Loftson, T., et al.1994b. 2-Hydroxypropyl-β-cyclodextrin in topical carbonic anhydrase inhibitor formulations. Eur. J. Pharm. Sci. 1: 175-180.

Loftson, T., and Brewster, M. 1996. Pharmaceutical applications of cyclodextrins. 1. Drug solubilization and stabilization. J. Pharm. Sci. 85(10): 1017-1025.

Loftsson, T., and Brewster, M. E. 1997. Cyclodextrins as pharmaceutical excipients. Pharm. Tech. Eur. May: 26-34.

Loftson, T., Frioriksdottir, H., Siguroardottir, A. M., and Ueda, H. 1994c. The effect of water-soluble polymers on drug-cyclodextrin complexation. Int. J. Pharm. 110: 169-177.

Loftson, T., Frioriksdottir, H., Thorisdottir, S., and Stefansson, E. 1994a. The effect of hydroxypropyl methylcellulose on the release of dexamethasone from aqueous 2-hydroxypropyl-β-cyclodextrin formulations. Int. J. Pharm. 104: 181-184.

Loftsson, T., and Jarvinen, T. 1999. Cyclodextrins in ophthalmic drug delivery. Adv. Drug Deliv. Rev. 36: 59-79.

Loftsson, T., and Masson, M. 2001. Cyclodextrins in topical drug formulations: Theory and practice. Int. J. Pharm. 225: 15-30.

Loftsson, T., and Stefansson, E. 1997. Effect of cyclodextrins on topical drug delivery to the eye. Drug Dev. Ind. Pharm. 23(5): 473-481.

Loftson, T., Stefansson, E., Frioriksdottir, H., and Guomundsson, O. 1992. Interactions between preservatives and 2-hydroxypropyl-β-cyclodextrin. Drug Dev. Ind. Pharm. 18(13): 1477-1484.

Lyman, C. A., and Walsh, T. J. 1992. Systemically administered antifungal agents: a review of their clinical pharmacology and therapeutic applications. Drugs 44 (1): 9-35.

Martin, A. 1993. Physical Pharmacy. 4 th ed. London: Lea & Febiger, pp. 251-283, 453-476.

Matsuda, H., et al. 1993. Inclusion complexation of *p*-hydroxybenzoic acid esters with 2-hydroxypropyl-β-cyclodextrins. On changes in solubility and antimicrobial activity. Chem. Pharm. Bull. 41(8): 1448-1452.

Mauger, T. F. 1994. Antimicrobials. In T. F. Mauger; and E. L. Craig (eds.), Havener's Ocular Pharmacology. 6 th ed, pp. 234-349. St. Louis: Mosby.

McEvoy, G. K., ed. 1999. AHFS drug information. Bethesda, MD: American society of health-system pharmacists, pp. 2403-2405.

Moffat, A. C., Jackson, J. V., Moss, M. S., and Widdop, B., eds. 1986. Clarke's isolation and identification of drugs. 2 nd ed. London: The Pharmaceutical Press, pp. 696-697.

Moroi, S. E., and Lichter, P. R. 1996. Ocular Pharmacology. In J. G. Hardman et al. (eds.), Goodman & Gilman' s The Pharmacological Basis of Therapeutics. 9 th ed, pp. 1619-1645. New york: Marcel Dekker.

Mosher, G., and Thompson, D. 2000. Complexation and cyclodextrins. In J. Swarbrick; and J. C. Boylan (eds.), Encyclopedia of pharmaceutical technology 19, pp. 49-88. New york: Marcel Dekker.

Noppawinyoowong, C., and Yospaiboon, Y. 1990. Preparation of 2% ketoconazole eyedrops in batch size. Thai J. Ophthalmol. 4(2): 125-127.

Nuntanakorn, P. 1996. Degradation products of ketoconazole. Master's Thesis, Department of Pharmaceutical Chemistry, Graduate School, Chulalongkorn University.

Pascucci, V. L., Bennett, J., Narang, P. K., and Chatterji, D. C. 1983. Quantitation of ketoconazole in biological fluids using high-pressure liquid chromatography. J. Pharm. Sci. 72(12): 1467-1469.

Pikal, M. J. 1993. Freeze Drying. In J. Swarbrick; and J. C. Boylan (eds.), Encyclopedia of pharmaceutical technology 6, pp. 275-303. New york: Marcel Dekker.

Pitha, J., Milecki, J., Fales, H., Pannell, L., and Uekama, K. 1986. Hydroxypropyl-β-cyclodextrin: preparation and characterization; effects on solubility of drugs. Int. J. Pharm. 29: 73-82.

Quanyun, A. Xu. 1999. Stability-indicating HPLC methods for drug analysis. London: American Pharmaceutical Association, pp. 215-216.

Rajewski, R. A., and Stella, V. J. 1996. Pharmaceutical applications of cyclodextrins. 2. In vivo Drug Delivery. J. Pharm. Sci. 85(11): 1142-1169.

Reer, O. Bock, T.K., and Muller, B.W. 1994. In vitro corneal permeability of diclofenac sodium in formulations containing cyclodextrins compared to the commercial product Voltaren Ophtha. J. Pharm. Sci. 83(9): 1345-1349.

Reynolds, J. E. F., Parsons, A. V., and Sweetman, S. C., eds. 1989. Martindale The Extra Pharmacopoeia. 29 th ed. London: The Pharmaceutical Press, pp. 426-429.

Ruiz, A. M., and Paronen, P. 1997. Particle and powder properties of cyclodextrins. Int. J. Pharm. 148: 33-39.

Sane, R. T., Tendolkar, R. V., Gangal, D. P., Ladage, K. D., and Kolthorkar, R. M. 1988. Extraction colorimetric method for the determination of ketoconazole from pharmaceutical preparation. Indian J. Pharm. Sci. 50: 347-348.

Savolainen, P. S., Jarvinen, T., Sasaki, K. A., Watanabe, H., and Urtti, A. 1998. Evaluation of cytotoxicity of various ophthalmic drugs, eye drop excipients and cyclodextrins in an immortalized human corneal epithelial cell line. Pharm. Res. 15(8): 1275-1280.

Skiba, M., Lahiani, M. S., Marchais, H., Duclos, R., and Arnaud, P. 2000. Stability assessment of ketoconazole in aqueous formulations. Int. J. Pharm. 198: 1-6.

Stella, V. J., and Rajewski, R. A. 1997. Cyclodextrins: Their future in drug formulation and delivery. Pharm. Res. 14(5): 556-567.

Strattan, C. H. 1992. 2-Hydroxypropyl-β-cyclodextrin, part II: Safety and manufacturing issues. Pharm. Technol. 16(1-3): 52-58.

Suhonen, P., Jarvinen, T., Lehmussaari, K., Reunamaki, T., and Urtti, A. 1995. Ocular absorption and irritation of pilocarpine prodrug is modified with buffer, polymer, and cyclodextrin in the eyedrop. Pharm. Res. 12(4): 529-533.

Sunthornpit, A., Kaewnopparat, N., Songkarak, S., and Faroongsarng, D. 2000. Evaluation of methylcellulose and carbopol solutions as viscous vehicles for ketoconazole ophthalmic suspensions. Songklanakarin J. Sci. Technol. 22 (4):435-445.

Swezey, S. F., et al. 1982. Measurement of ketoconazole, a new antifungal agent, by high-pressure liquid chromatography. J. Chromatogr. 227: 510-515.

Szejtli, J. 1988. Cyclodextrin technology. Dordrecht: Kluwer Academic, pp. 79-170.

Szejtli, J. 1990. Cyclodextrins properties and applications. Drug Invest. 2(Suppl. 4): 11-21.

Szejtli, J. 1991. Cyclodextrins in drug formulation: part I. Pharm. Technol. Int. 2: 15-22.

Szejtli, J. 1998. Introduction and general overview of cyclodextrin chemistry. Chem. Rev. 98: 1743-1753.

Szente, L., and Szejtli, J. 1999. Highly soluble cyclodextrin derivatives: chemistry, properties, and trends in development. Adv. Drug Del. Rev. 36: 17-28.

Techowanich, S. 1999. Chemical stability of ketoconazole in aqueous solution. Master's Thesis, Faculty of Graduate Studies, Mahidol University.

The British Pharmacopoeia. 1999. London: The stationery office, pp. 840-841.

The United States Pharmacopeia 24 and the National Formulary 19. 2000. Philadelphia: The United States Pharmacopeia Convention, pp. 864-865, 1920-1924, 2113-2114, 2149-2152.

Tong, W. 2000. Applications of complexation in the formulation of insoluble compounds. In L. Rong (ed.), Water – insoluble drug formulation, pp. 111-140. Colorado: Interpharm Press.

Torres, M. A., Mohamed, J., Adema, H. C., and Martinez, L. A. 1985. Topical ketoconazole for fungal keratitis. Am. J. Ophthalmol. 100(2): 293-298.

Uekama, K., and Irie, T. 1990. New perspectives in cyclodextrins pharmaceutical applications cyclodextrin derivatives as new drug carriers. Drug Invest. 2 (Suppl. 4): 22-28.

Usayapant, A., Karara, A. H., and Narurkar, M. M. 1991. Effect of 2-hydroxypropyl-β-cyclodextrin on the ocular absorption of dexamethasone and dexamethasone acetate. Pharm. Res. 8(12): 1495-1499.

Vadas, E. B. 2000. Stability of pharmaceutical products. In A. R. Gennaro et al. (eds.), Remington : The Science and Practice of Pharmacy, pp. 986-994. Baltimore: Lippincott Williams & Wilkins.

Wright, K. W. 1997. Textbook of Ophthalmology. 1 st ed. Baltimore: Williams & Wilkins, pp. 729-732.

Yoshida, A., Arima, H., Uekama, K., and Pitha, J. 1988. Pharmaceutical evaluation of hydroxyalkyl ethers of β-cyclodextrin. Int. J. Pharm. 46:217-222.

Yospaiboon, Y., Limpawuthiwaranond, W., and Noppawinyoowong, C. 1990. Topical ketoconazole for treatment of keratomysis. Thai J. Ophthalmol. 4(2): 129-133.

APPENDICES

APPENDIX A

Equilibrium time data of phase solubility analysis

Table 1A Equilibrium time data of ketoconazole with 40% w/v HP-β-CD in 0.1 M phosphate buffer pH 7 at room temperature (30±3°C)

Time	Concentration of ketoconazole ($M\times10^2$)			Mean	SD
(day)	n_1	n_2	n_3		
0	0.00	0.00	0.00	0.00	0.00
1	7.77	8.05	7.75	7.85	0.17
2	8.23	7.85	8.02	8.03	0.19
3	8.27	8.12	8.48	8.29	0.18
4	8.20	8.50	8.18	8.29	0.18
5	8.66	8.15	8.17	8.33	0.29
6	8.51	8.39	8.33	8.41	0.09
7	8.42	8.49	8.38	8.43	0.06

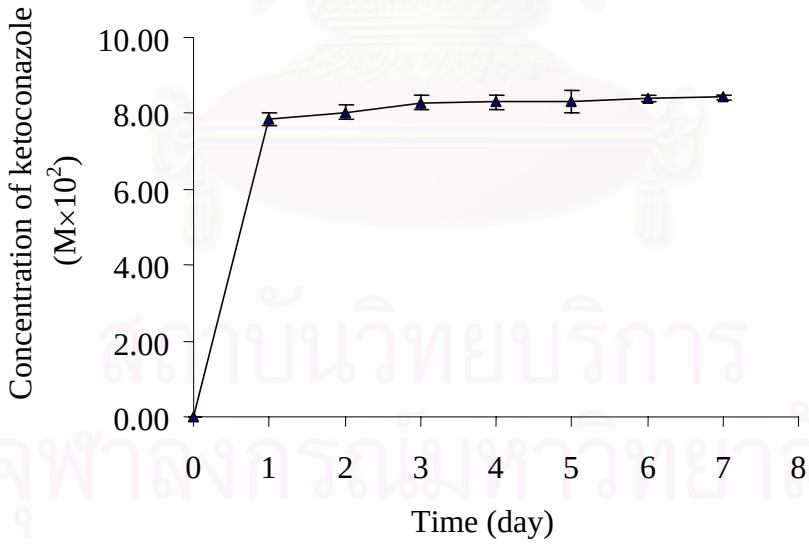


Figure 1A Equilibrium time curve of ketoconazole with 40% w/v HP-β-CD in 0.1 M phosphate buffer pH 7 at room temperature (30±3°C)

APPENDIX B

Density data of 2% w/v ketoconazole solutions pH 7
with and without benzalkonium chloride and purified water
at room temperature (30°C)

Table 1B Density data of 2% w/v ketoconazole solutions pH 7 with benzalkonium chloride at room temperature (30°C)

Weight (g)	n_1	n_2	n_3	mean
1. pycnometer	16.3222	16.3223	16.3222	16.3222
2. pycnometer+10 ml of 2% w/v ketoconazole solution pH 7 with benzalkonium chloride	27.2774	27.2771	27.2769	27.2771
3. 10 ml of 2% w/v ketoconazole solution pH 7 with benzalkonium chloride (2-1)				10.9549
4. density of 2% w/v ketoconazole solution pH 7 with benzalkonium chloride				1.0955

Table 2B Density data of 2% w/v ketoconazole solutions pH 7 without benzalkonium chloride at room temperature (30°C)

Weight (g)	n_1	n_2	n_3	mean
1. pycnometer	16.3139	16.3138	16.3137	16.3138
2. pycnometer+10 ml of 2% w/v ketoconazole solution pH 7 without benzalkonium chloride	27.2703	27.2701	27.2697	27.2700
3. 10 ml of 2% w/v ketoconazole solution pH 7 without benzalkonium chloride (2-1)				10.9562
4. density of 2% w/v ketoconazole solution pH 7 without benzalkonium chloride				1.0956

Table 3B Density data of purified water at room temperature (30°C)

Weight (g)	n_1	n_2	n_3	mean
1. pycnometer	16.3094	16.3095	16.3096	16.3095
2. pycnometer+10 ml of purified water	26.0965	26.0967	26.0964	26.0965
3. 10 ml of purified water (2-1)				9.7870
4. density of purified water				0.9787

APPENDIX C

Flow time data of 2% w/v ketoconazole solutions pH 7 with and without benzalkonium chloride and purified water at room temperature (30°C) a determined with Oswald capillary viscometer

Table 1C Flow time data of 2% w/v ketoconazole solutions pH 7 with and without benzalkonium chloride and purified water at room temperature (30°C)

	Flow time (minutes)		
	2% w/v ketoconazole solutions pH 7 with benzalkonium chloride	2% w/v ketoconazole solutions pH 7 without benzalkonium chloride	purified water
n_1	4.35	4.30	1.40
n_2	4.34	4.34	1.50
n_3	4.33	4.33	1.50
n_4	4.32	4.28	1.70
n_5	4.31	4.32	1.60
n_6	4.30	4.32	1.60
n_7	4.30	4.29	1.48
n_8	4.31	4.30	1.52
n_9	4.31	4.32	1.54
mean	4.32	4.31	1.54
SD	0.02	0.02	0.09

APPENDIX D

Phase solubility data and association constants (K_C)
of ketoconazole with CDs in buffer solutions
at room temperature (30±3°C)

Table 1D Phase solubility data of ketoconazole with HP-β-CD in 0.1 M acetate buffer pH 5 at room temperature (30±3°C)

Concentration of HP-β-CD		Dilution factor	Absorbance			Mean	Concentration of ketoconazole	
(%W/V)	($M\times10^2$)		1	2	3		(mg/ml)	($M\times10^2$)
0	0.00	10.00	0.550	0.532	0.545	0.5423	0.131	0.025
2	1.43	714.29	0.116	0.114	0.113	0.1143	1.890	0.356
5	3.58	714.29	0.272	0.277	0.285	0.2780	4.755	0.895
10	7.16	714.29	0.553	0.560	0.556	0.5563	9.628	1.812
15	10.74	714.29	0.793	0.805	0.801	0.7997	13.888	2.613
20	14.32	1666.67	0.456	0.447	0.460	0.4543	18.298	3.443
25	17.90	1666.67	0.571	0.556	0.547	0.5580	22.533	4.240
30	21.49	1666.67	0.684	0.688	0.699	0.6903	27.938	5.257

Table 2D Phase solubility data of ketoconazole with HP-β-CD in 0.1 M phosphate buffer pH 7 at room temperature (30±3°C)

Concentration of HP-β-CD		Dilution factor	Absorbance			Mean	Concentration of ketoconazole	
(%W/V)	($M\times10^2$)		1	2	3		(mg/ml)	($M\times10^2$)
0	0.00	1.00	0.801	0.821	0.806	0.8093	0.021	0.004
5	3.58	714.29	0.129	0.142	0.143	0.1380	2.446	0.460
10	7.16	714.29	0.351	0.355	0.341	0.3490	6.498	1.223
15	10.74	714.29	0.579	0.578	0.571	0.5760	10.856	2.043
20	14.32	1666.67	0.371	0.374	0.380	0.3750	16.326	3.072
25	17.90	1666.67	0.430	0.435	0.442	0.4357	19.044	3.584
30	21.49	1666.67	0.530	0.526	0.532	0.5293	23.241	4.373
35	25.07	1666.67	0.640	0.645	0.650	0.645	28.423	5.348

Table 3D Phase solubility data of ketoconazole with HP-γ-CD in 0.1 M acetate buffer pH 5 at room temperature (30±3°C)

Concentration of HP-γ-CD		Dilution factor	Absorbance			Mean	Concentration of ketoconazole	
(%W/V)	($M\times10^2$)		1	2	3		(mg/ml)	($M\times10^2$)
0	0.00	10.00	0.499	0.513	0.516	0.5093	0.123	0.023
2	1.27	125.00	0.125	0.129	0.135	0.1297	0.378	0.071
5	3.16	125.00	0.252	0.232	0.277	0.2537	0.758	0.143
10	6.33	125.00	0.505	0.517	0.506	0.5093	1.541	0.290
15	9.49	125.00	0.786	0.792	0.810	0.7960	2.419	0.455
20	12.66	250.00	0.556	0.566	0.587	0.5697	3.451	0.649
25	15.82	250.00	0.725	0.726	0.713	0.7213	4.381	0.824
30	18.99	250.00	0.888	0.909	0.916	0.9043	5.502	1.035

Table 4D Association constants (K_C) of ketoconazole with CDs in buffer solutions at room temperature (30±3°C)

pH	CDs	S_0 (M)	Slope	(1-Slope)	K_c (M^{-1})
5	HP-γ-CD	0.00023	0.0532	0.9468	244
	HP-β-CD	0.00025	0.2400	0.7600	1263
7	HP-β-CD	0.00004	0.2164	0.7836	6904

APPENDIX E

Degradation data of 2% w/v ketoconazole solutions and lyophilized products

Table 1E Degradation data of 2% w/v ketoconazole solution pH 5 at 70±1°C

Time	Percent remaining of ketoconazole			Mean	SD
(day)	n_1	n_2	n_3		
0	98.41	99.86	101.73	100.00	1.66
5	98.65	99.60	98.93	99.06	0.49
14	92.81	98.59	97.23	96.21	3.02
37	82.44	82.21	85.23	83.29	1.68
45	82.65	83.62	82.95	83.07	0.49
52	84.05	84.11	79.18	82.45	2.83
60	80.03	77.45	86.55	81.34	4.69
k_{obs} ($\%.day^{-1}$)	32.06×10^{-2}	38.62×10^{-2}	34.09×10^{-2}	34.92×10^{-2}	3.00×10^{-2}

Table 2E Degradation data of 2% w/v ketoconazole solution pH 7 at 70±1°C

Time	Percent remaining of ketoconazole			Mean	SD
(day)	n_1	n_2	n_3		
0	98.67	100.65	100.67	100.00	1.15
5	99.53	102.37	102.01	101.30	1.55
14	98.64	100.22	98.55	99.14	0.94
37	89.47	89.07	88.77	89.10	0.35
45	89.28	86.66	88.67	88.21	1.37
52	88.59	86.95	88.17	87.91	0.85
60	87.30	85.67	87.03	86.67	0.87
k_{obs} ($\%.day^{-1}$)	22.47×10^{-2}	30.69×10^{-2}	26.92×10^{-2}	26.69×10^{-2}	4.00×10^{-2}

Table 3E Degradation data of 2% w/v ketoconazole solution pH 7 at refrigerator temperature (5±3°C)

Time	Percent remaining of ketoconazole			Mean	SD
(day)	n_1	n_2	n_3		
0	98.67	100.65	100.67	100.00	1.15
5	96.80	97.65	102.79	99.08	3.24
14	99.14	101.65	101.22	100.67	1.34
45	95.20	94.27	93.51	94.33	0.85
60	92.88	93.40	94.39	93.56	0.77
75	91.35	95.10	96.25	94.23	2.56
90	90.88	93.14	93.32	92.45	1.36
k_{obs} ($\%.day^{-1}$)	9.07×10^{-2}	8.26×10^{-2}	9.73×10^{-2}	9.02×10^{-2}	0.74×10^{-2}

Table 4E Degradation data of 2% w/v ketoconazole solution pH 7 at room temperature (30±3°C)

Time	Percent remaining of ketoconazole			Mean	SD
(day)	n_1	n_2	n_3		
0	98.67	100.65	100.67	100.00	1.15
5	100.91	98.05	99.93	99.63	1.45
14	98.08	96.60	100.22	98.30	1.82
45	91.30	93.26	93.20	92.59	1.12
60	91.51	94.17	95.50	93.72	2.03
75	94.13	91.48	94.43	93.35	1.63
90	92.32	93.02	89.93	91.76	1.62
k_{obs} ($\%.day^{-1}$)	9.02×10^{-2}	8.18×10^{-2}	10.72×10^{-2}	9.31×10^{-2}	1.29×10^{-2}

Table 5E Degradation data of 2% w/v ketoconazole solution pH 7 at 50±1°C

Time	Percent remaining of ketoconazole			Mean	SD
(day)	n_1	n_2	n_3		
0	98.67	100.65	100.67	100.00	1.15
5	100.12	100.78	101.40	100.77	0.64
14	101.34	102.88	102.05	102.09	0.77
37	88.30	86.64	90.21	88.38	1.79
45	91.83	91.96	91.28	91.69	0.36
52	92.31	92.78	92.00	92.36	0.39
60	93.44	92.29	92.66	92.80	0.59
90	92.99	91.09	93.51	92.53	1.28
k_{obs} ($\%.day^{-1}$)	9.97×10^{-2}	13.79×10^{-2}	12.04×10^{-2}	11.93×10^{-2}	1.91×10^{-2}

Table 6E Degradation data of 2% w/v ketoconazole solution pH 7 at 60±1°C

Time	Percent remaining of ketoconazole			Mean	SD
(day)	n_1	n_2	n_3		
0	98.67	100.65	100.67	100.00	1.15
5	102.70	102.14	101.37	102.07	0.67
14	101.83	102.26	100.94	101.68	0.67
37	88.60	90.32	95.21	91.38	3.43
45	86.36	94.05	92.05	90.82	3.99
52	88.77	92.10	89.82	90.23	1.70
60	91.69	90.79	86.43	89.64	2.81
90	90.43	90.98	85.50	88.97	3.02
k_{obs} ($\%.day^{-1}$)	15.45×10^{-2}	14.80×10^{-2}	20.56×10^{-2}	16.93×10^{-2}	3.15×10^{-2}

Table 7E Degradation data of 2% w/v ketoconazole solution pH 7 at 70±1°C

Time	Percent remaining of ketoconazole			Mean	SD
(day)	n_1	n_2	n_3		
0	98.67	100.65	100.67	100.00	1.15
5	99.53	102.37	102.01	101.30	1.55
14	98.64	100.22	98.55	99.14	0.94
37	89.47	89.07	88.77	89.10	0.35
45	89.28	86.66	88.67	88.21	1.37
52	88.59	86.95	88.17	87.91	0.85
60	87.30	85.67	87.03	86.67	0.87
90	80.42	83.50	80.57	81.50	1.74
k_{obs} ($\%.day^{-1}$)	21.88×10^{-2}	23.76×10^{-2}	24.38×10^{-2}	23.34×10^{-2}	1.30×10^{-2}

Table 8E Degradation data of 2% w/v ketoconazole lyophilized products without benzalkonium chloride at room temperature (30±3°C)

Time	Percent remaining of ketoconazole			Mean	SD
(day)	n_1	n_2	n_3		
0	101.46	100.78	97.76	100.00	1.97
15	96.10	94.78	104.60	98.49	5.33
30	101.39	104.14	97.16	100.89	3.52
60	99.68	106.89	107.54	104.70	4.36
90	93.46	97.63	104.60	98.56	5.62

Table 9E Degradation data of 2% w/v ketoconazole lyophilized products without benzalkonium chloride at 45°C/ 75% RH

Time	Percent remaining of ketoconazole			Mean	SD
(day)	n_1	n_2	n_3		
0	101.46	100.78	97.76	100.00	1.97
15	92.53	99.79	99.26	97.19	4.05
30	100.41	101.04	96.24	99.23	2.61
60	98.25	103.04	104.82	102.04	3.40
90	100.73	106.65	102.80	103.39	3.00

Table 10E Degradation data of 2% w/v ketoconazole lyophilized products with benzalkonium chloride at room temperature (30±3°C)

Time	Percent remaining of ketoconazole			Mean	SD
(day)	n_1	n_2	n_3		
0	101.76	99.11	99.12	100.00	1.53
15	99.53	97.84	99.30	98.89	0.92
30	96.16	98.50	97.25	97.30	1.17
60	99.23	100.42	102.34	100.66	1.57
90	95.03	97.00	95.87	95.97	0.99

Table 11E Degradation data of 2% w/v ketoconazole lyophilized products with benzalkonium chloride at 45°C/ 75% RH

Time	Percent remaining of ketoconazole			Mean	SD
(day)	n_1	n_2	n_3		
0	101.76	99.11	99.12	100.00	1.53
15	99.32	97.66	98.82	98.60	0.85
30	95.46	95.58	96.61	95.88	0.63
60	100.55	97.69	94.53	97.59	3.01
90	92.57	96.19	94.20	94.32	1.82

APPENDIX F

Statistics test

Table 1F Statistical comparison of the degradation rate constant between 2% w/v ketoconazole solution pH 5 and pH 7 at 70±1°C

Mann-Whitney Test

Ranks

	pH	N	Mean Rank	Sum of Ranks
degradation rate constant	5	3	5.00	15.00
	7	3	2.00	6.00
	Total	6		

Test Statistics[b]

	degradation rate constant
Mann-Whitney U	.000
Wilcoxon W	6.000
Z	-1.964
Asymp. Sig. (2-tailed)	.050
Exact Sig. [2*(1-tailed Sig.)]	.100[a]

a. Not corrected for ties.

b. Grouping Variable: pH

Table 2F Statistical comparison of the degradation rate constant of 2% w/v ketoconazole solution pH 7 at refrigerator temperature (5±3°C) and at room temperature (30±3°C)

Mann-Whitney Test

Ranks

	temperature	N	Mean Rank	Sum of Ranks
degradation rate constant	refrigerator	3	3.67	11.00
	room	3	3.33	10.00
	Total	6		

Test Statistics[b]

	degradation rate constant
Mann-Whitney U	4.000
Wilcoxon W	10.000
Z	-.218
Asymp. Sig. (2-tailed)	.827
Exact Sig. [2*(1-tailed Sig.)]	1.000[a]

a. Not corrected for ties.

b. Grouping Variable: temperature

Table 3F Statistical comparison of the percent remaining of ketoconazole before and after autoclaving

Mann-Whitney Test

Ranks

	autoclave	N	Mean Rank	Sum of Ranks
percent remaining of ketoconazole	before	3	5.00	15.00
	after	3	2.00	6.00
	Total	6		

Test Statistics[b]

	percent remaining of ketoconazole
Mann-Whitney U	.000
Wilcoxon W	6.000
Z	-1.964
Asymp. Sig. (2-tailed)	.050
Exact Sig. [2*(1-tailed Sig.)]	.100[a]

a. Not corrected for ties.

b. Grouping Variable: autoclave

Table 4F Statistical comparison of the percent remaining of ketoconazole of 2% w/v ketoconazole lyophilized products with and without benzalkonium chloride at room temperature (30±3°C) and 45°C/ 75% RH

Multiple-Factors Analysis of Variance

Tests of Between-Subjects Effects

Dependent Variable: percent remaining of ketoconazole

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	360.990[a]	19	18.999	2.278	.014
Intercept	590271.756	1	590271.756	70782.68	.000
FORMULA	96.014	1	96.014	11.513	.002
TEMP	7.848	1	7.848	.941	.338
TIME	92.521	4	23.130	2.774	.040
FORMULA * TEMP * TIME	164.607	13	12.662	1.518	.153
Error	333.568	40	8.339		
Total	590966.314	60			
Corrected Total	694.558	59			

a. R Squared = .520 (Adjusted R Squared = .292)

Post Hoc Tests

TIME

Multiple Comparisons

Dependent Variable: percent remaining of ketoconazole

LSD

(I) TIME	(J) TIME	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	15	1.7042	1.1789	.156	-.6785	4.0869
	30	1.6700	1.1789	.164	-.7127	4.0527
	60	-1.2500	1.1789	.295	-3.6327	1.1327
	90	1.9375	1.1789	.108	-.4452	4.3202
15	0	-1.7042	1.1789	.156	-4.0869	.6785
	30	-3.4167E-02	1.1789	.977	-2.4169	2.3485
	60	-2.9542*	1.1789	.016	-5.3369	-.5715
	90	.2333	1.1789	.844	-2.1494	2.6160
30	0	-1.6700	1.1789	.164	-4.0527	.7127
	15	3.417E-02	1.1789	.977	-2.3485	2.4169
	60	-2.9200*	1.1789	.018	-5.3027	-.5373
	90	.2675	1.1789	.822	-2.1152	2.6502
60	0	1.2500	1.1789	.295	-1.1327	3.6327
	15	2.9542*	1.1789	.016	.5715	5.3369
	30	2.9200*	1.1789	.018	.5373	5.3027
	90	3.1875*	1.1789	.010	.8048	5.5702
90	0	-1.9375	1.1789	.108	-4.3202	.4452
	15	-.2333	1.1789	.844	-2.6160	2.1494
	30	-.2675	1.1789	.822	-2.6502	2.1152
	60	-3.1875*	1.1789	.010	-5.5702	-.8048

Based on observed means.

*. The mean difference is significant at the .05 level.

Table 5F Statistical comparison of the percent ketoconazole remaining of 2% w/v ketoconazole lyophilized products at pH 7 without benzalkonium chloride with 2% w/v ketoconazole solution pH 7 without benzalkonium chloride when storaged at room temperature (30±3°C) for 90 days

Mann-Whitney Test

Ranks

	TYPE	N	Mean Rank	Sum of Ranks
Percent ketoconazole remaining	Lyophilized product	3	5.00	15.00
	Solution	3	2.00	6.00
	Total	6		

Test Statistics[b]

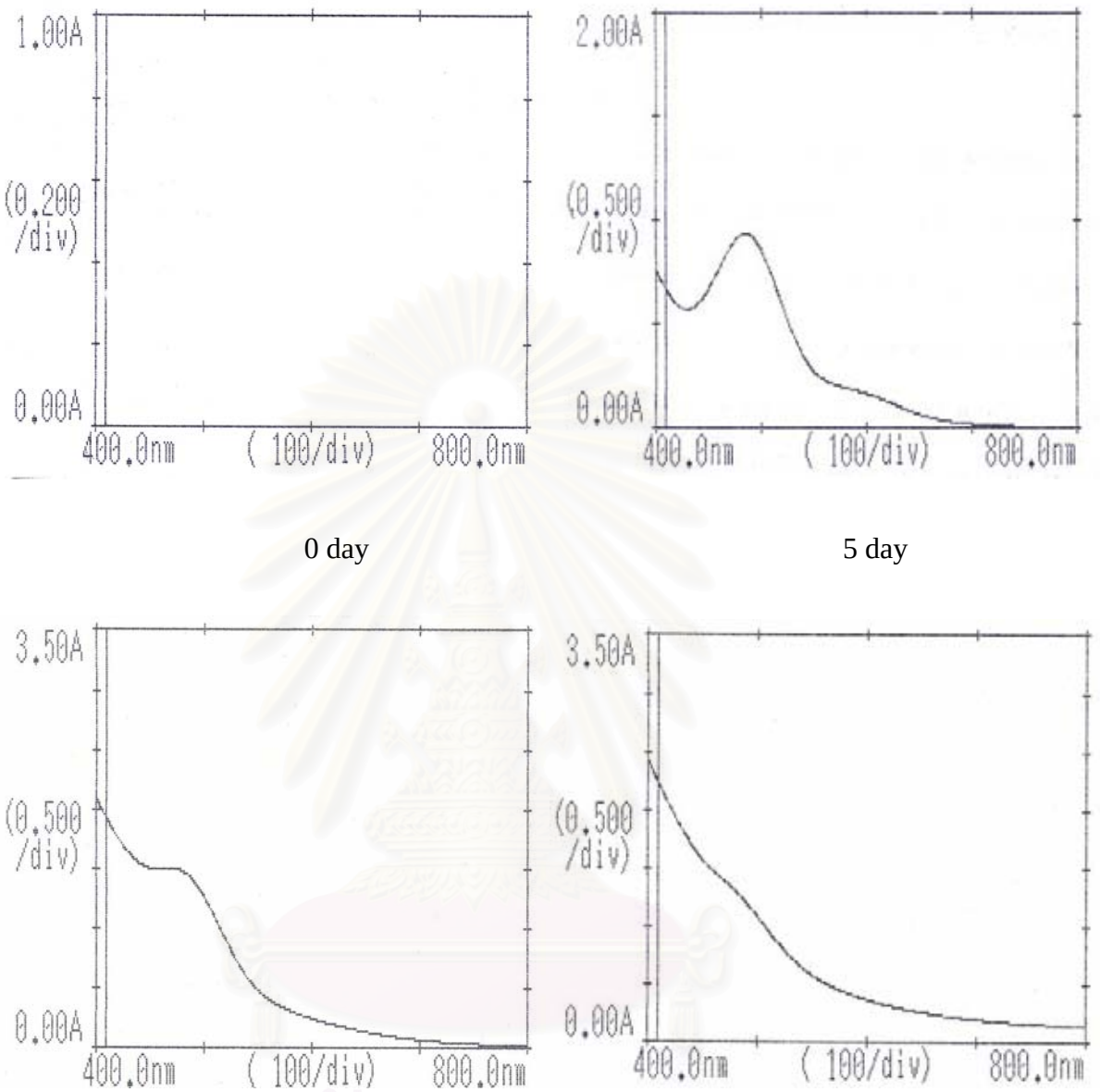
	Percent ketoconazole remaining
Mann-Whitney U	.000
Wilcoxon W	6.000
Z	-1.964
Asymp. Sig. (2-tailed)	.050
Exact Sig. [2*(1-tailed Sig.)]	.100[a]

a. Not corrected for ties.

b. Grouping Variable: TYPE

APPENDIX G

Spectral absorbance in visible wavelengths (400-800 nm) of ketoconazole solution pH 5 and pH 7 at initial time and during the stability study (70±1°C)



0 day 5 day

30 day 60 day

Figure 1G Spectral absorbance in visible wavelengths (400-800 nm) of ketoconazole solution pH 5 at initial time and during the stability study

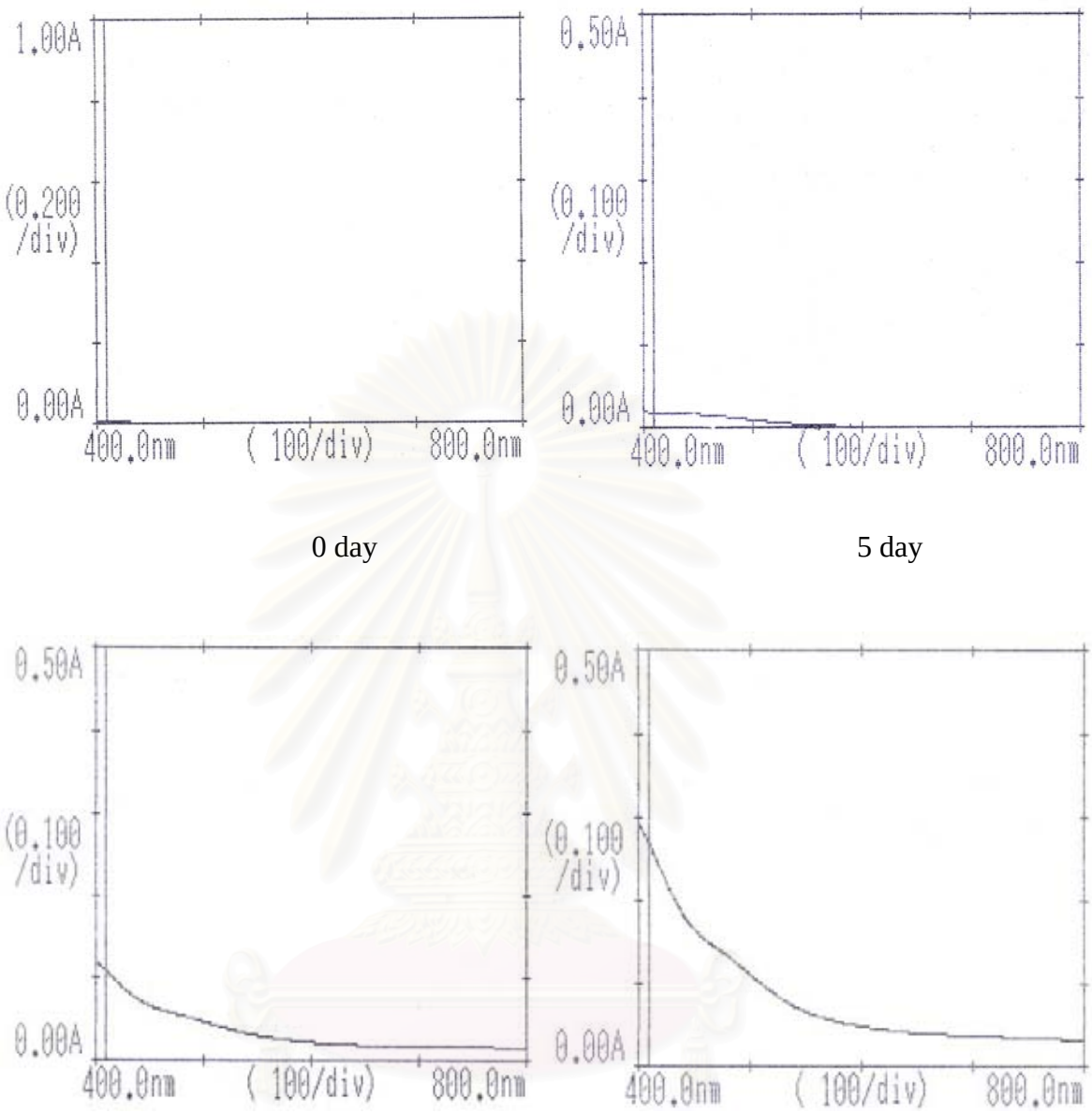


Figure 2G Spectral absorbance in visible wavelengths (400-800 nm) of ketoconazole solution pH 7 at initial time and during the stability study

VITA

Miss Angkana Im-erbsin was born on September 21, 1976 in Surin, Thailand. She received the Bachelor degree of Pharmacy from the Faculty of Pharmaceutical Science, Ubonrachathanee University, Ubonrachathanee, Thailand in 1998. After graduation, she has worked at Surin Hospital for two years before she entered the Master's Degree program in Pharmacy at Chulalongkorn University in 2001.