ผลของ 2-ไฮดรอกซีโพรพิลเบต้าไซโคลเด็กซ์ทรินต่อการละลายและความคงตัวของเมโทรนิดาโซลในน้ำ

นางสาว วารี ลิมป์วิกรานต์

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต

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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

EFFECTS OF 2-HYDROXYPROPYL-β-CYCLODEXTRIN ON
THE SOLUBILITY AND STABILITY OF METRONIDAZOLE IN AQUEOUS SOLUTION

MISS WAREE LIMWIKRANT

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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จุฬาลงกรณ์มหาวิทยาลัย

วารี ลิมป์วิกรานต์ : ผลของ 2-ไฮดรอกซีโพรพิลเบต้าไซโคลเด็กซ์ทรินต่อการละลายและความคงตัวของเมโทรนิดาโซลในน้ำ. (EFFECTS OF 2-HYDROXYPROPYL-β-CYCLODEXTRIN ON THE SOLUBILITY AND STABILITY OF METRONIDAZOLE IN AQUEOUS SOLUTION) อ. ที่ปรึกษา: รศ.ดร. พจน์ กุลวานิช, อ.ที่ปรึกษาร่วม : ผศ. ศิริศักดิ์ ดำรงพิศุทธิกุล, 92 หน้า. ISBN 974-03-0326-9.

วัตถุประสงค์ของงานวิจัยนี้คือ การศึกษาผลของ 2-ไฮดรอกซีโพรพิลเบต้าไซโคลเด็กซ์ทรินต่อการละลายและความคงตัวของเมโทรนิดาโซลในน้ำ จากการศึกษาเฟสการละลาย การละลายในน้ำของเมโทรนิดาโซลเพิ่มขึ้นเป็นเชิงเส้นกับฟังก์ชันความเข้มข้นของ 2-ไฮดรอกซีโพรพิลเบต้าไซโคลเด็กซ์ทริน แผนภูมิเฟสการละลายของเมโทรนิดาโซลจัดเป็นชนิด A_L ค่าคงที่ความคงตัวประมาณ 11.24 โมลาร์$^{-1}$ ได้เตรียมสารประกอบเชิงซ้อนของแข็งของเมโทรนิดาโซล : 2-ไฮดรอกซีโพรพิลเบต้าไซโคลเด็กซ์ทริน (1:2 อัตราส่วนโมลาร์) ด้วยวิธีการทำแห้งโดยใช้ความเย็นแล้วนำสารประกอบเชิงซ้อนที่ได้ในลักษณะเฟสของแข็งมาทำการตรวจวิเคราะห์โดยวิธีฟูเรียร์ทรานฟอร์มอินฟราเรดสเปกโทรสโกปี ดิฟเฟอเรนเชียลสแกนนิงแคลอริมิทรี และเอกซ์เรย์ดิฟแฟรกโทมิทรี และในเฟสของเหลวโดยวิธีโปรตอนนิวเคลียร์แมกเนติกเรโซแนนซ์สเปกโทรสโกปี ผลที่ได้จากโปรตอนนิวเคลียร์แมกเนติกเรโซแนนซ์สเปกโทรสโกปียืนยันว่าเมโทรนิดาโซลเกิดสารประกอบเชิงซ้อนอินครูชันกับ 2-ไฮดรอกซีโพรพิลเบต้าไซโคลเด็กซ์ทริน การศึกษาผลของอุณหภูมิ พีเอช และ แสงที่มีต่อความคงตัวของสารประกอบเชิงซ้อนระหว่างเมโทรนิดาโซลและ 2-ไฮดรอกซีโพรพิลเบต้าไซโคลเด็กซ์ทรินพบว่า อันดับปฏิกิริยาการเสื่อมของสารประกอบเชิงซ้อนระหว่างเมโทรนิดาโซลกับ 2-ไฮดรอกซีโพรพิลเบต้าไซโคลเด็กซ์ทรินเป็นปฏิกิริยาอันดับหนึ่ง ยกเว้นปฏิกิริยาการเสื่อมจากแสงเป็นปฏิกิริยาอันดับศูนย์ 2-ไฮดรอกซีโพรพิลเบต้าไซโคลเด็กซ์ทรินสามารถลดการเสื่อมของเมโทรนิดาโซลได้โดยการเกิดสารประกอบเชิงซ้อนยกเว้นที่พีเอช 3.1 แต่อย่างไรก็ตาม 2-ไฮดรอกซีโพรพิลเบต้าไซโคลเด็กซ์ทรินมีแนวโน้มเร่งการเสื่อมจากแสงของเมโทรนิดาโซล

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ภาควิชา……เภสัชอุตสาหกรรม…… ลายมือชื่อนิสิต…………………………………………..

สาขาวิชา…..เภสัชอุตสาหกรรม…… ลายมือชื่ออาจารย์ที่ปรึกษา……………………………..

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The purpose of this present study was to explore the effects of 2-hydroxypropyl-β-cyclodextrin (HPBCD) on the solubility and stability of metronidazole in aqueous solution. From the phase solubility study, the aqueous solubility of metronidazole increased linearly as function of HPBCD concentration. The phase solubility diagram of metronidazole can be classified as type A_L and the stability constant was estimated as 11.24 M^{-1}. Freeze-drying method was used to prepare solid complex of metronidazole:HPBCD (1:2 molar ratio). The complexes obtained were characterized in the solid phase by Fourier transform infrared spectroscopy, differential scanning calorimetry and X-ray diffractometry, and in the liquid phase by proton nuclear magnetic resonance spectroscopy. The results obtained from proton nuclear magnetic resonance spectroscopy indicated that metronidazole was shown to form inclusion complex with HPBCD. The studies of effects of temperature, pH and light on the stability of complex showed that the kinetic of metronidazole:HPBCD degradation followed first-order except for the photochemical degradation which followed zero-order. HPBCD appears to retard the degradation of metronidazole by forming inclusion complex except at pH 3.1. On the other hand, HPBCD has a tendency to accelerate the photodecomposition of metronidazole.

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LIST OF ABBREVIATIONS

°C	degree Celsius
CD	cyclodextrin
cm	centrimetre
CV	coefficient of variation
DSC	differential scanning calorimetry
FTIR	fourier transform infrared
g	gram
HPBCD	2-hydroxypropyl-β-cyclodextrin
HPLC	high performance liquid chromatography
k	degradation rate constant
K	degree Kelvin
K_c	stability constant
Kcal	kilocalories
kV	kilovolt
M	molar
mA	milliampere
mg	milligram
min	minute
ml	millilitre
mm	millimetre
nm	nanometre
NMR	nuclear magnetic resonance
ppm	part per million
r	correlation coefficient
R^2	coefficient of determination
SD	standard deviation
μ	ionic strength
μg	microgram
μl	microlitre
μm	micrometre

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LIST OF ABBREVIATIONS (cont.)

UV-VIS	ultraviolet-visible
w/v	weight by volume
λ	wavelength

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CHAPTER I
INTRODUCTION

Metronidazole is a widely used drug for the prevention and treatment of infections caused by anaerobic bacteria such as intraabdominal infections, gynecologic infections and lower respiratory tract infections (Brodgen et al., 1978; Jensen, Bundgaard and Falch, 1990). Available dosage forms are tablet, capsule, suspension, gel, vaginal suppository and parenteral dosage form. Although the drug is usually administered orally, intravenous infusion providing rapid onset of action is often required (Jensen et al., 1990).

In the systemic treatment of anaerobic infections, a parenteral mode of administration is preferable for patients who are seriously ill and for whom oral administration is not feasible or for drugs that are known to be subjected to extensive hepatic first-pass elimination or to produce a gastrointestinal irritation. However, a practical way of administering a parenteral dose (500 mg/dose) of metronidazole in a single injectable dosage form (with a volume of 10 ml or less) is not available, since the aqueous solubility of metronidazole is relatively low (~1% w/v at 25°C) (Cho et al., 1982; Chien, 1984; Jensen et al., 1990). The intravenous administration of this drug is presently performed in the form of infusion, using 0.5% w/v aqueous solution. To meet the required doses it is usually necessary to give 100-200 ml of such solution every 8 hours.

Metronidazole has been shown to be subject to hydrolysis in aqueous media, a reaction which is often accelerated by the presence of hydroxyl radicals (Barnes and Sugden, 1986; Kendall, Stark and Sugden, 1989). Stability study of metronidazole in aqueous solution from previous work indicated a pseudo-first order degradation kinetic and in the pH range of 3.9-6.6, metronidazole was more stable than in other pH range (Wang and Yeh, 1993). Metronidazole has been reported to be sensitive to light and the photodegradation products absorbed at very short wavelengths (Habib and Asker, 1989; Karim, Ibrahim and Adam, 1991). Kendall et al. (1989) found that light irradiation had more effect on the degradation of metronidazole in solution than irradiation with sonic energy and the addition of 5% D-glucose retarded the rate of the

photolysed reaction of metronidazole by acting as a hydroxyl radical scavenger. Habib and Asker (1989) also reported that the complex formation between metronidazole and sodium urate accounted for the photostabilization of metronidazole.

Cyclodextrins (CDs) are fascinating molecules and have become the subject of growing interest for several decades. CDs have a doughnut-shaped hydrophobic cavity in which various types of drugs (guest molecules) may be encased. Non-covalently bonded inclusion complexes either in the solid phase or in aqueous solutions are formed (Szejtli, 1982). This phenomenon has received extensive attention in the pharmaceutical field because of its ability to improve the aqueous solubility, dissolution and release rates, bioavailability, chemical stability (e.g. photodegradation, hydrolysis, oxidation, racemization and isomerization), physical stability and modification of the pharmacokinetics of various drug molecules. In addition, the complexation may also suppress the volatility and unpleasant odors or tastes associated with the drug and reduce the local irritance of drug (Saenger, 1980; Szejtli, 1982; Jones et al., 1984a, 1984b; Duchene, Vaution and Glomot, 1986; Duchene and Wouessidjewe, 1990; Bekers et al., 1991; Mosher and Thompson, 2000).

To overcome the aforementioned solubility and stability problems of metronidazole, the formation of inclusion complex with CDs could be a feasible approach. However, there is no study that investigates about inclusion complex of metronidazole with CDs. Consequently, the objective of this study was to examine effects of CDs on the solubility and stability of metronidazole.

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In preliminary study, phase solubility diagrams of metronidazole-cyclodextrins systems (β-cyclodextrin or 2-hydroxypropyl-β-cyclodextrin) were carried out according to the method of Higuchi and Connors (1965). It was found that only 2-hydroxypropyl-β-cyclodextrin enhanced the solubility of metronidazole. From this reason, 2-hydroxypropyl-β-cyclodextrin was chosen for further study the possibility of increasing solubility and stability of metronidazole.

Objectives

The aims of this study are as followings:

1. To investigate the effects of 2-hydroxypropyl-β-cyclodextrin on the solubility of metronidazole.
2. To study the stability of metronidazole: 2-hydroxypropyl-β-cyclodextrin inclusion complex under storage at different temperature, pH and light conditions.
3. To determine the degradation kinetics of metronidazole and its inclusion complex with 2-hydroxypropyl-β-cyclodextrin in the investigated conditions.

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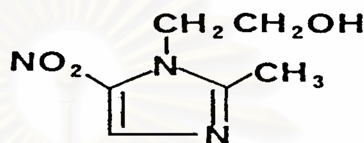
CHAPTER II
LITERATURE REVIEW

Metronidazole

Physical Properties of Metronidazole

(Wearley and Anthony, 1976; Moffat, 1986)



Structure formula	:
Empirical formula	: $C_6H_9N_3O_3$
Chemical name	: 2-methyl-5-nitroimidazole-1-ethanol
Molecular weight	: 171.16
Description	: A white to pale yellow, odorless crystalline powder or crystals
Melting range	: 159° C-163° C
Solubility	: 1 in 100 of water 1 in 200 of ethanol 1 in 250 of chloroform Slightly soluble in ether
Dissociation constant, pKa	: 2.5

Stability of Metronidazole

The degradation kinetics of metronidazole in aqueous solutions of pH 3.1 to 9.9 at 90±0.2°C were studied by Wang and Yeh (1993). The stability of metronidazole in solutions containing propylene glycol or polyethylene glycol 400 was also investigated. The reaction order for metronidazole in these aqueous and solvent systems followed pseudo-first-order degradation kinetics. The degradation rate of metronidazole was invariant under various total buffer concentrations at each specific pH within the investigated pH range. These results indicated that no general acid/base catalysis imposed by acetate, phosphate and borate buffer species was

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responsible for the degradation of metronidazole. The pH rate profile (Figure 1) shows a pH-independent region of pH 3.9-6.6. Maximum stability of metronidazole was at pH 5.6 under zero total buffer species conditions.

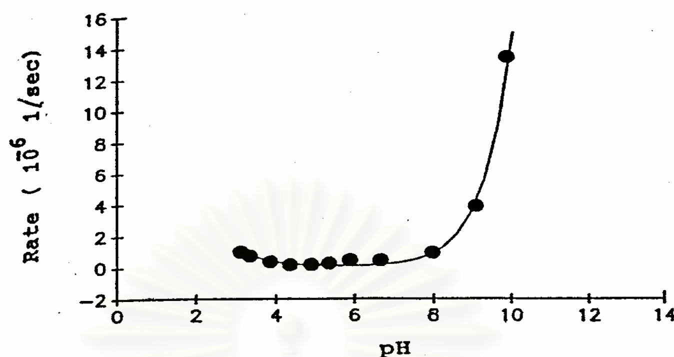

Figure 1 The pH-rate profile of metronidazole in aqueous solution under zero buffer concentration and constant μ (0.5) at 90±0.2°C

source : *J. Pharm. Sci.* 1993, 82(1): 95-98

Kendall et al. (1989) investigated the degradation of metronidazole in buffer solution (pH 9.2) on exposure to light and sonic energy. It was found that metronidazole in buffer solution (pH 9.2) underwent degradation very slowly at 40 and 70°C on irradiation with sonic energy. Irradiation with light (360 nm) at 20,48 and 63°C produced more rapid degradation. The photodecomposition kinetics of metronidazole followed zero order kinetic at 20°C but followed first order kinetic at both 48 and 63°C. The addition of hydrogen peroxide increased the reaction rate constant. The addition of glucose (5% w/v) as well as hydrogen peroxide retarded the reaction at 20 and 48°C but accelerated it to above the rate of the photolysed reaction without additives at 63°C.

Metronidazole has been reported to be sensitive to light (Wilkins and Moore, 1988). Previous work (Karim et al., 1991) has indicated that the photodecomposition reaction of metronidazole appeared to follow pseudo-first-order kinetic. The rate of decomposition was found to increase with temperature, pH and intensity of radiation and to decrease with increasing in drug concentration. The presence or absence of oxygen was found to exert very little effect on the photodecomposition rate of

metronidazole. The degradation rate of metronidazole in different solvents decreased in the order: chloroform > isopropanol > methanol > water.

Aqueous solutions of metronidazole 0.5% in citrate:phosphate buffer at pH 5 became bright yellow after exposure to daylight for 18 months but faded to colorless after further exposure to daylight for 21 months. These products have been characterized by UV, IR, proton NMR, mass spectroscopy and melting point determination. It is proposed that the initial yellow degradation product is an "excimer ion-pair" formed by the stabilization of metronidazole in its first electronic excited state by the citrate molecule (Godfrey and Edwards, 1991).

Suwakul (2000) has pointed out that photodegradation of metronidazole gel followed first-order reaction. After exposure to accelerated light throughout 24 weeks, the color of metronidazole gels changed to yellow whereas that of metronidazole gels wrapped in aluminum foil showed no change and no degradation occurred. The formulations composing of poloxamer 407 as gelling agent were less stable than the formulations composing of hydroxyethyl cellulose. Acetate buffer, D-glucose and sodium urate showed no effect on the chemical stability of metronidazole in the formulations containing hydroxyethyl cellulose except for the formulations containing poloxamer 407 which acetate buffer retarded degradation rate constants. Between D-glucose and sodium urate, it was found that sodium urate was the best photostabilizer in this study.

Cyclodextrins

Structure and Physicochemical Properties

Cyclodextrins (CDs) are cyclic (α-1,4)-linked oligosaccharides of α-D-glucopyranose containing a relatively hydrophobic central cavity and hydrophillic outer surface. Owing to lack of free rotation about the bonds connecting the glucopyranose units, the CDs are not perfectly cylindrical molecules but are toroidal or cone shaped. Based on this architecture, the primary hydroxyl groups are located on the narrow side of the torus while the secondary hydroxyl groups are located on

the wide range edge (Figure 2). The most common CDs are α-cyclodextrin, β-cyclodextrin, and γ-cyclodextrin, which consist of six, seven, and eight glucopyranose units, respectively. Although it is thought that CDs with fewer than six glucopyranose units do not exist because of steric factor, CDs containing more than eight units have been identified. However, their complexing abilities are generally lower than those of β-cyclodextrin and, thus, they are of less pharmaceutical interest (Bekers et al., 1991; Loftsson and Brewster, 1996; 1997).

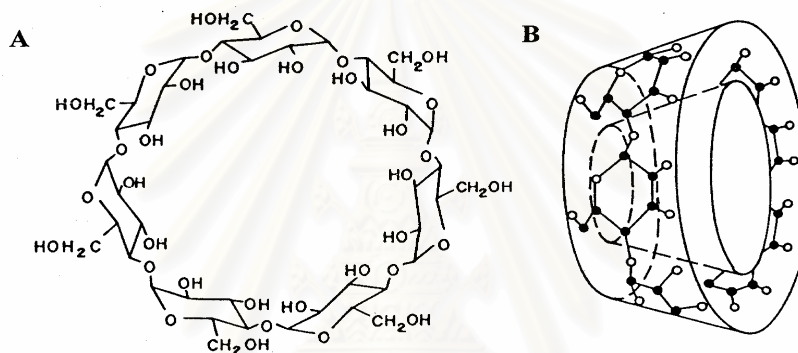

Figure 2 A) The chemical structure and B) the toroidal shape of the β–cyclodextrin molecule

source : *J. Pharm. Sci.* 1996, 85(10): 1017-1025

The cavity of the torus consists of a ring of C3-H groups, C5-H groups and a ring of glucosidic oxygen. For this reason, the cavity of the torus is non-polar (compare to water). This makes CDs exterior decidedly hydrophilic whereas the interior of the cavity is rather hydrophobic. Free rotation of primary hydroxyls reduces the effective diameter of the cavity on the side they occur, while the secondary hydroxyl groups on the relatively rigid chains cannot rotate (Szejtli, 1982; Bekers et al., 1991).

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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 differently sized guest molecules. Table 1 lists some of the important physicochemical properties of CDs and some of their derivatives.

Most striking from Table 1 is the low solubility of β-CD in water. Many intramolecular hydrogen bonds exist between the secondary hydroxyl groups. The C2-OH group of one glucopyranose unit can form a hydrogen bond with the C3-OH group of the adjacent glucopyranose unit. These intramolecular H-bonds stabilize the macrocycle of the CD molecule and turn the CD molecule into a rigid structure. These hydrogen bonds also prevent hydration of the CD molecule which is probably an explanation for the low solubility of β-CD.

Table 1 Physical properties of the CDs and some derivatives

	α	β	γ	DM-β[1)]	HP-β[2)]
Number of glucose residues	6	7	8	7	7
Cavity dimensions (A°)					
cavity diameter	5	6	8	6	6
height of torus	7.9	7.9	7.9	10.0	
diameter of periphery	14.6	15.4	17.5		
Molecular weight	973	1135	1297	1331	±1300
Aqueous solubility [3)]	14.5	1.85	23.2	57	>50
Melting point (°C)	275	280	275	295-300	
pK_a [4)]	12.3	12.2	12.1		
Half-life of ring opening [5)](hr)	6.2	5.4	3.0	8.5	
Enzymatic hydrolysis [6)]	negligible	slow	rapid		

1) - Heptakis-2,6,-di-O-methyl-β-CD

2) - 2-hydroxypropyl-β-CD

3) - in grams per 100 ml water at ambient temperature

4) - pK_a: by potentiometry at 25°C

5) - Half-life of ring opening: in 1 N HCl at 60°C

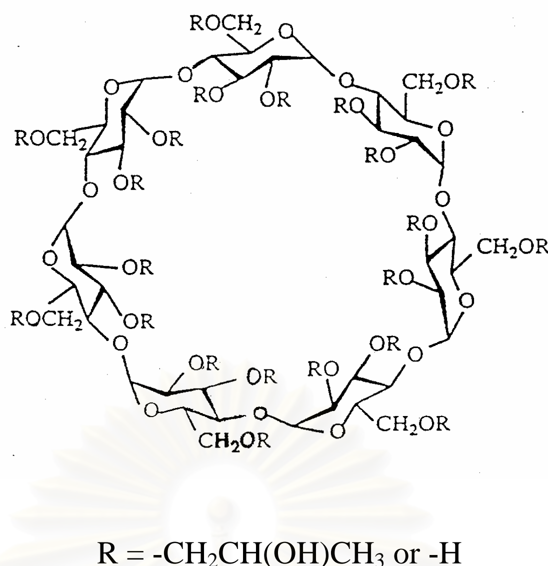
6) – by Aspergillus oryzae α-amylase

source : *Drug Dev. Ind. Pharm.* 1991, 17(11): 1503-1549

The parent CDs, in particular β-cyclodextrin, have limited aqueous solubility, and their complex formation with lipophilic drugs, and other compounds with limited aqueous solubility, frequently gives rise to B-type phase-solubility diagrams as defined by Higuchi and Connors (1965). Thus, substitution of any of the hydrogen bond forming hydroxyl groups, even by hydrophobic moieties such as methoxy and ethoxy functions, will result in a dramatic increase in water solubility. For example, the aqueous solubility of β-cyclodextrin is only 1.85% (w/v) at room temperature but increases with increasing degree of methylation e.g. heptakis(2,6-O-dimethyl)-β-cyclodextrin.

Other common cyclodextrin derivatives are formed by other types of alkylation or hydroxyalkylation of the hydroxyl groups. The main reason for the solubility enhancement in these derivatives is that chemical manipulation frequently transforms the crystalline CDs into amorphous mixtures of isomeric derivatives. For example, 2-hydroxypropyl-β-cyclodextrin is obtained by treating a base-solubilized solution of β-cyclodextrin with propylene oxide, resulting in an isomeric system that has an aqueous solubility well in excess of 60%(w/v).

2-hydroxypropyl-β-cyclodextrin (HPBCD) (Figure 3) is mostly utilized in the series of hydroxyalkylated-β-cyclodextrin derivatives because of its high water solubility (more than 50 g/100ml at 25°C). Furthermore, these amorphous compounds are also less hygroscopic than the mother crystalline β-cyclodextrin. The low hygroscopicity of HPBCD 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). HPBCD can be safely administered by parenteral route whereas parenterally administered natural CDs may cause severe nephrotoxicity, particularly β-cyclodextrin, and also cause shape changes and hemolysis of human erythrocytes (Duchene and Wouessidjewe, 1990; Carpenter et al., 1995; Rajewski and Stella, 1996).



R = $-CH_2CH(OH)CH_3$ or -H

Figure 3 Structure of 2-hydroxypropyl-β-cyclodextrin

Cyclodextrin Inclusion Complexes

CDs are able to form inclusion complexes with a variety of compounds, i.e., the trapping of various external molecules (guest molecules) inside the cavity of a CD (host). The minimum requirement for this inclusion complex formation is that the guest molecule must fit, entirely, or at least partially, into the CD cavity. 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 if certain groups or side chains of the bulky molecule can penetrate into the CD cavity, complex formation remains possible. 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

- 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).

Various methods have been applied to the preparation of drug-CD complexes. In the solution phase, the procedure is generally as follows: an excess amount of the drug is added to an aqueous CD solution, and the suspension is agitated for up to 1 week at the desired temperature. The suspension is then filtered or centrifuged to form a clear drug-CD complex solution. For preparation of solid formulation of the drug-CD complex, the water is removed from the aqueous drug-CD complex solution by evaporation or sublimation. It is sometimes possible to shorten this process by formation of supersaturated solutions through sonication followed by precipitation at the desired temperature (Loftsson and Brewster, 1996). Other methods to prepare solid inclusion complexes are freeze-drying, spray-drying, kneading, coprecipitation, neutralization, grinding and heating in a sealed container (Duchene, 1988).

Upon inclusion within the CD cavity, a guest molecule changes in its physicochemical properties. These changes provide methods to detect whether guest molecules are really included in the CD cavity. Several methods are applicable for detection of the inclusion complex formation such as phase-solubility, nuclear magnetic resonance spectroscopy, x-ray diffractometry, differential scanning calorimetry, thermogravimetry, differential thermal analysis, infrared spectroscopy, etc.

Pharmaceutical Applications of Cyclodextrins

CDs and their derivatives have aroused considerable interest in the pharmaceutical field due to their potential to form complexes with many varieties of drug molecules. The resulting complexes generally lead to an improvement in some of the characteristics of the guest molecules, e.g. stability, solubility and bioavailability. This review will focus on their partial applications in pharmaceutical fields about its use as solubilizing agent and stabilizer.

1. Drug Solubilization

The most common pharmaceutical application of CDs is to enhance drug solubility in aqueous solutions. HPBCD has been used successfully to solubilize various drugs such as famotidine, norfloxacin, ketoprofen, danazol, etc (Islam and Narurkar, 1991; Guyot et al., 1995; Nagarsenkar and Shenai, 1996; Badawy et al., 1996). Although prediction of compound solubilization by CDs continues to be highly empirical, various historical observations permit several general statements(Loftsson and Brewster, 1996). First, the lower the aqueous solubility of the pure drug, the greater the relative solubility enhancement obtained through CD complexation. Second, CD derivatives of lower molar substitution are better solubilizers than the same type of derivatives of higher molar substitution. Third, charged CDs can be powerful solubilizers, but their solubilizing effect appears to depend on the relative proximity of the charge to the CD cavity. The farther away the charge is located, the better the complexing abilities. Compared to neutral CDs, enhanced complexation is frequently observed when the drug and CD molecules have opposite charge but decreased complexation is observed if they carry same type of charge. Another finding is that while many ionizable drugs are able to form CD complexes, the stability constant of the complex is much larger for the un-ionized than for the ionized form.

2. Drug Stability

The effect of CDs on the chemical stability of drugs is another useful property of these excipients and has been extensively examined in the literature. CDs

interaction with labile compounds can result in several outcomes: CDs can retard degradation, can have no reactivity, or can accelerate drug degradation.

Drug-CD complexation can be regarded as molecular encapsulation, i.e., encapsulation of drug at the molecular level. The CD molecule shields, at least partly, the drug molecule from attack by various reactive molecules. That is, the CD can insulate a labile compound from a potentially corrosive environment and, in this way, reduce or even prevent drug hydrolysis, oxidation, steric rearrangement, racemization, and other forms of isomerization, polymerization, and even enzymatic decomposition of drugs.

Loftsson et al. (1989) reported that the aqueous stability of chlorambucil and melphalan can be increased by forming an inclusion complex with HPBCD since both drugs degrade at much slower rate within the HPBCD cavity than outside in the aqueous solution. About 19-fold increase in the aqueous stability of chlorambucil and about 5-fold increase in that of melphalan were obtained when 5%(w/v) of HPBCD was added to the reaction medium.

Famotidine was found to form an inclusion complex with HPBCD, with the generation of an A_L type phase-solubility diagram. At 0.143 M HPBCD, the solubility enhancement for famotidine was approximately ten-fold. Complexed famotidine degraded at a slower rate than uncomplexed famotidine at pH 2.02 and 37±0.5°C (Islam and Narurkar, 1991).

Antoniadou-Vyza et al. (1997) found that the rate of hydrolysis of methocarbamol in pH 7.4 buffered solution at 37 and 60°C was reduced by almost 50% when formed complex with HPBCD. This is due to the fact that the susceptible moiety of methocarbamol was included in the hydrophobic cavity of the HPBCD and was protected from the attack of the aqueous buffered media.

Although CD complexation of drug molecules usually results in increased drug stability, there are examples of accelerated degradation. For example, the pH-rate profile for the degradation of cephalothin is characterized by a large pH-

independent region from pH about 2-8. In this region, the HPBCD had a significant stabilizing effect, but at pH 9.7, where the specific-base catalysis dominates, the same CDs had a destabilizing effect. For aztreonam, specific-base-catalyzed degradation was dominant at pH values greater than 6 and in this region of the pH-rate profile, CDs accelerated the degradation (Loftsson and Brewster, 1996).

Phase Solubility Analysis

Phase solubility analysis was described by Higuchi and Connors (1965). It can be utilized to study the change in the solubility characteristic of drugs due to complexation. A phase diagram is constructed by plotting, on the vertical axis, total molar concentration of substrate (S) found in the solution phase against the molar concentration of ligand or complexing agent (L) added to the system. The phase diagrams are observed to fall into two main classes: type A and type B diagrams.

The type A diagram, shown in Figure 4, is obtained when the complex formed is soluble and does not form a precipitate regardless of the amount of ligand added. This can be subdivided according to the nature of the obtained phase diagram. The A_L type, exhibiting a linear relationship between $[S]_t$ and $[L]_t$, is obtained when the complexes are a first order dependence on $[L]_t$. The A_P type, showing a positive deviation from linearity, is obtained when the formed complexes contain more than one molecule of ligand. As the ligand concentration increases, the contribution of the higher order complexes increases. The remaining diagram, A_N type, exhibits a negative deviation which represents a decreasing dependence on ligand added at higher ligand concentrations. This may be explained on the basis of self-association of the ligand at high concentrations.

The B type diagram, shown in Figure 5, results when the system develops the third phase consisting of the complex. If the complex exhibits some solubility, the diagram shows an initial rise in $[S]_t$ and the diagram is said to be a B_S diagram. If the complex is significantly soluble relative to the inherent solubility of the substrate, the system gives rise to the B_I diagram.

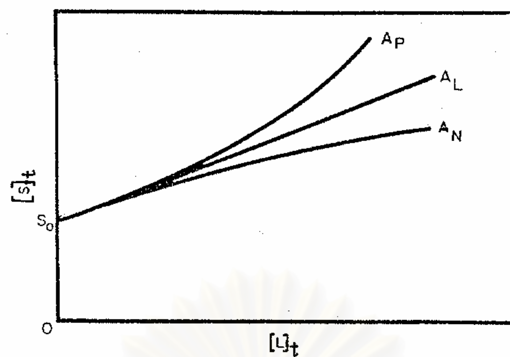


Figure 4 Phase solubility diagram of type A system

($[S]_t$ = the total molar concentration of dissolved substrate and

$[L]_t$ = the total molar concentration of ligand)

source : *Adv. Anal. Chem. Instr.* 1965, 4: 117-212

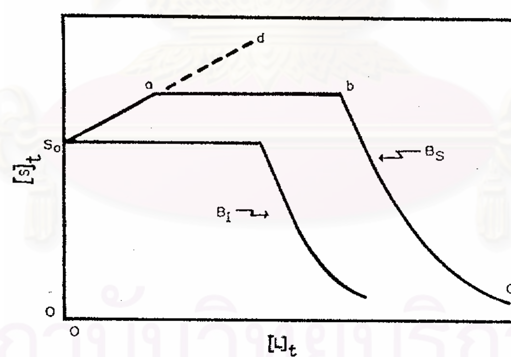


Figure 5 Phase solubility diagram of type B system

($[S]_t$ = the total molar concentration of dissolved substrate and

$[L]_t$ = the total molar concentration of ligand)

source : *Adv. Anal. Chem. Instr.* 1965, 4: 117-212

CHAPTER III

MATERIALS AND METHODS

Materials

All materials were used as received.

1. Metronidazole (supplied by Acdhon Co., Ltd., Thailand, batch no. W-212835)
2. 2-Hydroxypropyl-β-cyclodextrin with degree of substitution 0.6, molecular weight 1380 (Fluka, Switzerland, lot 399016/1)
3. Tinidazole (a gift from Siam Bheasach Co., Ltd., Thailand, batch no. 76302)
4. Methanol HPLC grade (Labscan Asia Co., Ltd., Thailand)
5. Glacial acetic acid (J.T. Baker Inc. , USA. , lot F16828)
6. Sodium acetate trihydrate (Carlo Erba, Italy, lot 2691F100)
7. Potassium dihydrogen phosphate, anhydrous (Merck, Germany, lot A894271 605)
8. Dipotassium hydrogen phosphate, anhydrous (APS Finechem, Australia, batch no.F0B063)
9. Boric acid (supplied by Srichand United Dispensary Co., Ltd., Thailand, lot ACD11/147)
10. Sodium hydroxide pellets (Mallinckrodt Baker, Mexico, lot 7708MVKK)
11. Sodium chloride (Merck, Germany, lot K26811304 952)
12. Disodium hydrogen phosphate, anhydrous (Carlo Erba, Italy, lot 9I706290B)
13. Citric acid (supplied by Srichand United Dispensary Co., Ltd., Thailand, lot ACF17)

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Equipments

1. Analytical Balance (Sartorius model A200S, Germany)
2. Top to bottom rotator
3. UV-visible spectrophotometer (UV-160A, Shimadzu, Japan)
4. High performance liquid chromatography equipped with
   - Liqiud chromatograh pump (LC-10AD, Shimadzu, Japan)
   - UV-VIS detector (SPD-10A, Shimadzu, Japan)
   - Recorder (C-R6A Chromatopac, Shimadzu, Japan)
   - C18 column 300x3.9 mm, 10 μm (Phenomenex, USA.)
   - Microsyringe 100 μl (ITO corporation, USA.)
5. pH meter (Beckman, USA.)
6. Freeze dryer (Modulyo 4K freeze-dryer, Edwards, England)
7. Hot air oven (Memmert, Germany)
8. FTIR spectrophotometer (FT-IR 1760X, Perkin Elmer, USA.)
9. Differential scanning calorimeter (NETZCH DSC 200, Germany)
10. X-ray diffractometer (JDX-8030, JEOL, Japan)
11. NMR (JNM-A500, JEOL, Japan)
12. Light cabinet equipped with TL/05 Philip lamp 15 watt, 45 cm long
13. Digital light meter (model TES1332, TES Electrical Electronics Corp., Taiwan)

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Methods

1. Analysis of Metronidazole

There are several methods available for determination of metronidazole including non-aqueous titration, spectrophotometry, colorimetry, high performance liquid chromatography (HPLC), gas-liquid chromatography and thin layer chromatography (Wearley and Anthony, 1976). In phase solubility studies, spectrophotometry was used for quantitation of metronidazole because it was convenient and rapid. But in drug stability studies, HPLC was performed because of its specificity and high sensitivity.

1.1 Spectrophotometric Method

Standard Curve of Metronidazole

100 mg of metronidazole was accurately weighed and transferred to a 100-ml volumetric flask. Deionized water was used to dissolve and adjust to volume. Ten millilitres of this solution was pipetted into a 100-ml volumetric flask and adjusted to volume with deionized water. This solution was stock solution of metronidazole.

Standard solutions of metronidazole were prepared by pipetting 2, 3, 4, 5, 6, 7 and 8 ml of metronidazole stock solution into 50-ml volumetric flasks. These solutions were diluted with deionized water to volume so that the concentrations of standard solutions were 4, 6, 8, 10, 12, 14 and 16 μg/ml, respectively.

All of standard solutions were analyzed spectrophotometrically at 320 nm. Deionized water was used as blank.

1.2 HPLC Method

1.2.1 Chromatographic Condition

The chromatographic condition used was adapted from USP24 and Jensen and Gugler (1983). Its condition was presented as follows:

Column : Phenomenex C18 column (300x3.9 mm), 10 μm

Detector wavelength : 254 nm

Flow rate : 2 ml/min

Attenuation : 8

Injection volume : 20 µl

Internal standard : Tinidazole 20 µg/ml

Mobile phase : 0.68 g of monobasic potassium phosphate in water: methanol (85:15), adjusted with 1 M phosphoric acid to pH of 4.0 ± 0.5

1.2.2 Standard Solutions

A stock solution of internal standard was prepared by transferring about 100 mg of tinidazole, accurately weighed, to a 100-ml volumetric flask and dissolving in 15 ml of methanol. Deionized water was used to adjust to volume. Then 10 ml of this solution was pipetted and transferred into a 100-ml volumetric flask. Deionized water was used to adjust to volume.

A stock solution of metronidazole was prepared by transferring about 50 mg of metronidazole, accurately weighed, to a 100-ml volumetric flask and dissolving in 10 ml of methanol. Deionized water was used to adjust to volume. Then 10 ml of this solution was pipetted and transferred into a 100-ml volumetric flask. Deionized water was used to adjust to volume.

Standard solutions of metronidazole (1, 5, 10, 15 and 20 µg/ml) containing 20 µg/ml of tinidazole were prepared from stock solution of metronidazole and tinidazole by diluting and adjusting to volume with mobile phase.

1.2.3 Validation of HPLC Method

The analytical parameters used for the assay validation were specificity, accuracy, precision and linearity (USP24).

1.2.3.1 Specificity

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

a) In the Presence of HPBCD and Buffer

Blank buffer solutions, including acetate buffer, phosphate buffer and borate buffer, and solutions of metronidazole:HPBCD inclusion complex having metronidazole concentration of 1-20 μg/ml were injected. Chromatograms were evaluated by comparing with those of the standard solutions of metronidazole.

b) In the Presence of Degradation Products of Metronidazole

The sample containing degradation products was prepared by method (Gupta,1984) below:

10 ml of 5 mg/ml metronidazole solution was mixed with 4 ml of 1 M NaOH in a 150-ml beaker and then heated until boiling on a hot plate for 25 min (water was replaced as necessary). The mixture was cooled, the pH adjusted to 6 using 0.5 M HCl, and brought to volume (250 ml) with water.

The decomposed solution was injected. Chromatogram was compared with the standard solution of metronidazole.

1.2.3.2 Accuracy

Three sets of the standard solutions of metronidazole:HPBCD inclusion complex having metronidazole concentrations of 1-20 μg/ml were prepared and injected. The percentage of analytical recovery of each standard solution was calculated.

1.2.3.3 Precision

a) Within Run Precision

The within run precision was determined by analyzing three sets of the five standard solutions of metronidazole in the same day. Peak area ratios of metronidazole to tinidazole were compared and the percentage coefficient of variation (%CV) for each concentration was determined.

b) Between Run Precision

The between run precision was determined by comparing each concentration of metronidazole standard solutions prepared and injected on different days. The percentage coefficient of variation (%CV) of metronidazole to its internal standard peak area ratios from the three sets of standard solutions having the same concentration was determined.

1.2.3.4 Linearity

Metronidazole standard solutions ranging from 1 to 20 μg/ml were prepared and analyzed. Linear regression analysis of the peak area ratios versus their concentrations was performed.

2. Phase Solubility Study

The phase solubility study was carried out according to the method reported by Higuchi and Connors (1965). Excess amounts of metronidazole were added to screwed-cap tubes containing aqueous solutions of various concentrations of HPBCD (0.01-0.25 M) and shaken in the top to bottom rotator at 37$\pm$1 °C. After equilibrium was attained (approximately 24 hours), an aliquot was filtered through a 0.45 μm membrane filter. A portion of the filtrate was adequately diluted and analyzed spectrophotometrically at 320 nm. There was no interference from HPBCD at this wavelength. The experiment was carried out in triplicate.

3. Preparation of Metronidazole: HPBCD Inclusion Complex

Solid complex of metronidazole with HPBCD was prepared using condition derived from the phase solubility study. The solid complex of metronidazole with HPBCD in 1:2 molar ratio was prepared by freeze-drying.

The appropriate amounts of metronidazole and HPBCD in molar ratio 1:2 were dissolved in distilled water and mixed by magnetic stirring for 24 hours at room temperature. The solution was filtered through a 0.45 μm membrane filter and freeze-dried in a Modulyo 4K freeze-dryer (Edwards, England).

Freeze-drying condition: The solution was kept in a freezer at $-25^{\circ}C$ until frozen and then placed into the Modulyo 4K freeze-dryer which the condenser was pre-cooled to reach a temperature of $-45^{\circ}C$ or lower. When the drying process began, the temperature and pressure started to drop to $-66^{\circ}C$ and 4.2×10^{-2} torr, respectively. The time required to dry this solution was approximately 8 hours.

4. Characterization of Metronidazole: HPBCD Inclusion Complex

Inclusion complex formulation of metronidazole:HPBCD was determined by methods described below:

4.1 Fourier Transform Infrared Spectroscopy (FTIR Spectroscopy)

The infrared spectra of the pure components, the inclusion complex and the physical mixture of metronidazole and HPBCD, prepared by simply mixing the powder together, were taken by a FTIR spectrophotometer (model 1760X, Perkin Elmer, USA.) using potassium bromide disk technique.

4.2 Differential Scanning Calorimetry (DSC)

The DSC patterns were determined by using NETZSCH DSC 200 (Germany) with a heating rate of 10 °C/min, in the temperature range from 22 to 400°C.

4.3 X-ray Diffractometry

X-ray diffractograms were obtained by using x-ray diffractometer model JDX-8030 (JEOL, Japan) with Ni-filtered CuK_{α} radiation, a voltage of 45 kV, and a current of 35 mA. The scanning speed was 1°/min over a 2θ range 5-40°.

4.4 Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR)

Proton NMR experiments were performed by using JNM-A500 (JEOL, Japan). Samples were solubilized in D_2O and water signal (4.70 ppm) was used as a reference.

5. Effect of Temperature on the Stability of Inclusion Complex of Metronidazole:HPBCD in Aqueous Solution

Formulation of metronidazole 5 mg/ml was prepared as specified for Flagyl I.V.RTU ®, SCS Pharmaceuticals in Handbook on injectable drugs (Trissel, 1998):

Metronidazole	500	mg
Disodium hydrogen phosphate , anhydrous	47.6	mg
Citric acid, anhydrous	22.9	mg
Sodium Chloride	790	mg
Water for injection to	100	ml

In case of formulation of metronidazole:HPBCD, its composition was the same as above except for adding HPBCD into the solution to make 1:2 molar ratio of metronidazole:HPBCD.

After filtered through a 0.45 μm membrane filter, both solutions were transferred to type I glass vials and packaged. These vials were wrapped with aluminum foil to protect solution from light and kept at 60, 70, 80, and 90$\pm$1°C. At appropriate time intervals, samples were tested. The remaining concentration of metronidazole in each sample was obtained by HPLC according to the method described in section 1.2. Shelf life of each formulation was calculated.

The physical properties including color, clarity and pH of the preparations were measured before and during the stability study. Solutions were left to cool down to room temperature before measuring pH.

6. Effect of pH on the Stability of Inclusion Complex of Metronidazole:HPBCD in Aqueous Solution

The hydrolysis of metronidazole and metronidazole:HPBCD were studied in aqueous buffer solution at constant temperature. The buffer used were acetate buffer (pH 3.1), phosphate buffer (pH 5.6) and borate buffer (pH 9.2). The total buffer concentration was generally 0.1 M and a constant ionic strength (μ) of 0.5 was

maintained for each buffer by adding a calculated amount of sodium chloride. Metronidazole with/without HPBCD formulations were prepared in the buffer solutions to possess the final metronidazole concentration of 5 mg/ml. The solution was filtered through a 0.45 μm membrane filter and then transferred to type I glass vials. The vials were closed with rubber closures, then covered with aluminum caps using a hand clipper and wrapped the vials with aluminum foil to protect solution from light. These vials were stored in a hot air oven at 80°C. Four vials were sampling for analysis of metronidazole remaining at appropriate time intervals. Rate constants of the reactions were calculated by using regression analysis.

The physical properties including color, clarity and pH of the solutions were measured.

7. Effect of Light on the Stability of Inclusion Complex of Metronidazole:HPBCD in Aqueous Solution

Metronidazole with/without HPBCD formulations were prepared in phosphate buffer (pH 5.6) having constant ionic strength of 0.5 to possess the final metronidazole concentration of 5 mg/ml. The solution was filtered through a 0.45 μm membrane filter and then transferred to type I glass vials. The vials were closed with rubber closures and covered with aluminum caps using a hand clipper. Half of these vials were covered with aluminum foil to protect solution from light and were used as the control group. Both wrapped and unwrapped vials were place under an UV light which wavelengths (λ) ranged from 300 to 460 nm (Philip TL/05 lamp, 15 watt, 45cm, λ max 365 nm; see Appendix I). Each point placed the vial on had the illuminance 500 lux $\pm$ 5% in the light cabinet. This stability study was conducted at 60$\pm$1°C. Four vials from each group were collected at appropriate time intervals for determining the remaining metronidazole concentration. The degradation rate constant was evaluated by using regression analysis.

The physical properties including color, clarity and pH of the solutions were studied.

CHAPTER IV
RESULTS AND DISCUSSION

1. Analysis of Metronidazole

1.1 Spectrophotometric Method

In the preliminary study, it was found that there was no interference from HPBCD at 320 nm (see Appendix II). The standard curve of metronidazole is shown in Figure 6. The coefficient of determination of regression line is highly significant (R^2 = 1). This curve was used for calculating the solubility of metronidazole in HPBCD solution.

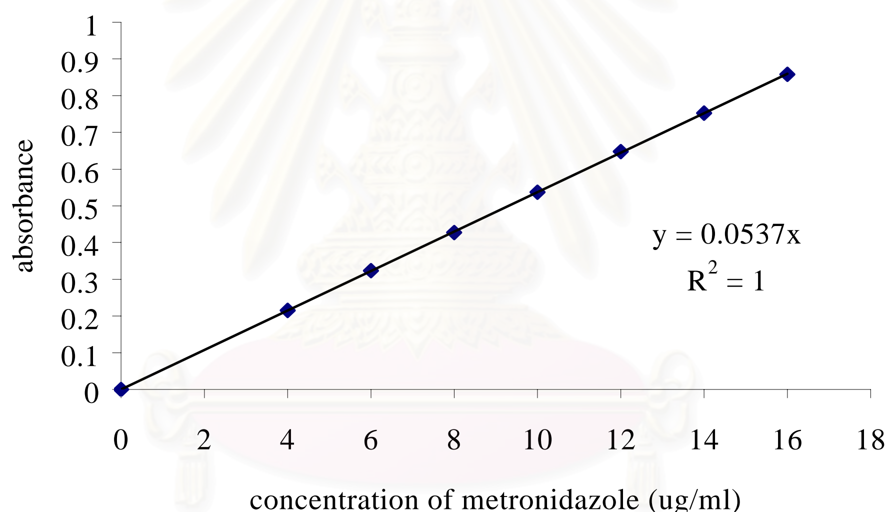

Figure 6 Standard curve of metronidazole (Spectrophotometric method)

1.2 HPLC Method

Validation of HPLC Method

1.2.1 Specificity

a) In the presence of HPBCD and buffer

As shown in Figure7, peaks of all blank buffer solution did not interfere with peaks of metronidazole and tinidazole. Figure 8 shows typical chromatograms of metronidazole standard solutions. Metronidazole and tinidazole were eluted at 4.00-

5.00 min and 6.00-7.00 min, respectively. The resolution values, which were calculated from a mean of five replicated injections, of metronidazole and its internal standard, tinidazole, are presented in Table 2. All resolution values between metronidazole and tinidazole peaks were more than 1.0. USP24 stated that two peaks are completely resoluted if their resolution values are more than 1.0. Therefore, these two peaks were separated from each other. Furthermore, the retention times of metronidazole:HPBCD inclusion complexes and tinidazole were similar to that of metronidazole standard solution (Figure 9). This result suggested that HPBCD did not affect the peak of metronidazole in the inclusion complex.

b) In the presence of degradation products of metronidazole

A stability-indicating assay is an important methodology to ensure that the method used in the stability studies is capable of separating the parent drug from its degradation products. The chromatogram of decomposed drug solution is given in Figure 10. Peaks of degradation products were eluted before that of metronidazole which almost disappeared. It indicated that the degradation products did not interfere with peak of metronidazole.

Thus, this method having high specificity could be used for analysis of metronidazole, both in its free form and in metronidazole:HPBCD inclusion complex, in the presence of its degradation products and it was not interfered by the buffer components.

1.2.2 Accuracy

The accuracy of an analytical method is the closeness of the test results obtained by that method to the true value. It is usually calculated as the percentage of recovery by the assay of the known added amount of analyte in the sample. The percentages of analytical recovery of each drug concentration are shown in Table 3. The mean of percentage of analytical recoveries closely to 100% with a low % CV indicated the high accuracy of this method. Thus, it could be used for analysis of metronidazole in all concentrations studied.

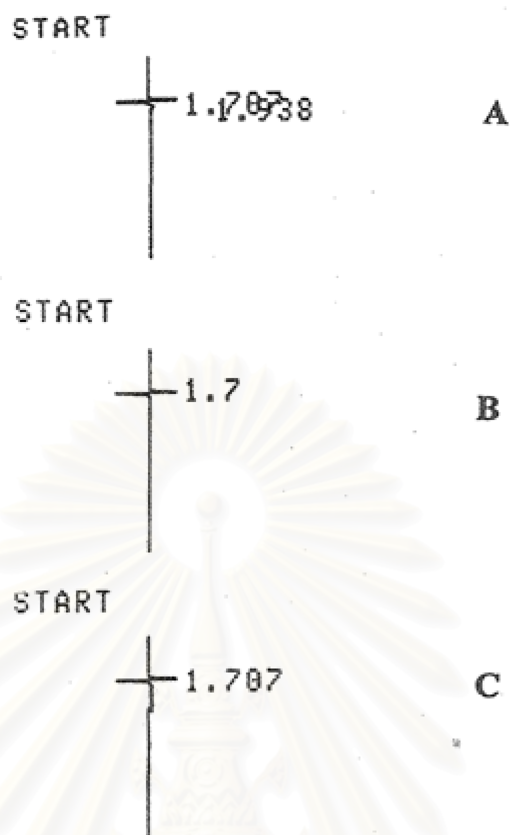

Figure 7 HPLC chromatograms of blank buffer solutions
(acetate buffer pH 3.1, (A); phosphate buffer pH 5.6, (B); and borate buffer pH 9.2 (C))

Table 2 Resolution values of metronidazole and tinidazole peaks

metronidazole concentrations (μg/ml)	resolution value
1	5.19
5	5.25
10	5.11
15	4.96
20	5.00

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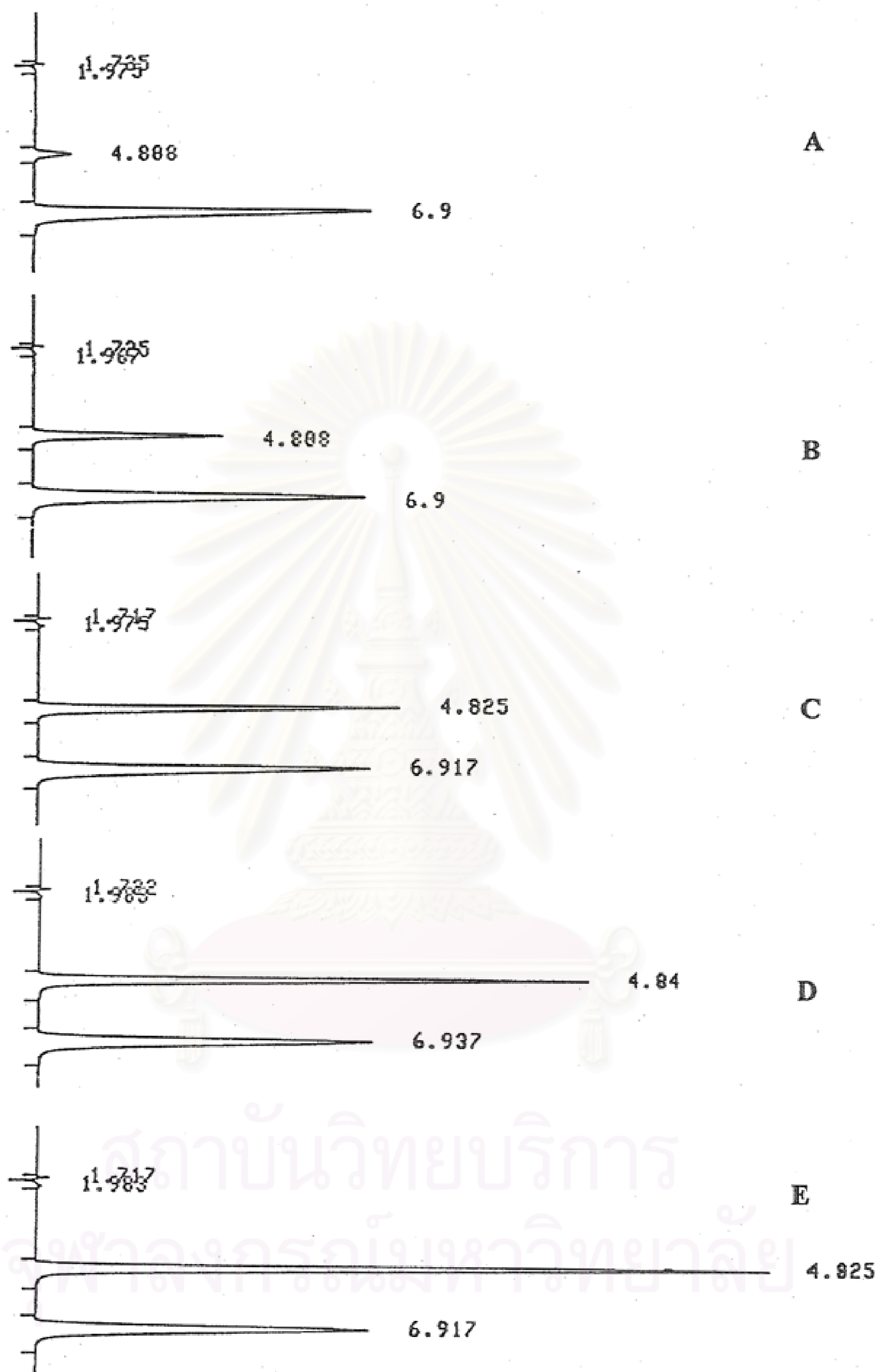

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Figure 8 HPLC chromatograms of standard solutions of metronidazole (1 μg/ml, (A); 5 μg/ml, (B); 10 μg/ml, (C); 15 μg/ml, (D); and 20 μg/ml, (E); retention time of metronidazole and tinidazole are at 4.00-5.00 min and 6.00-7.00 min, respectively)

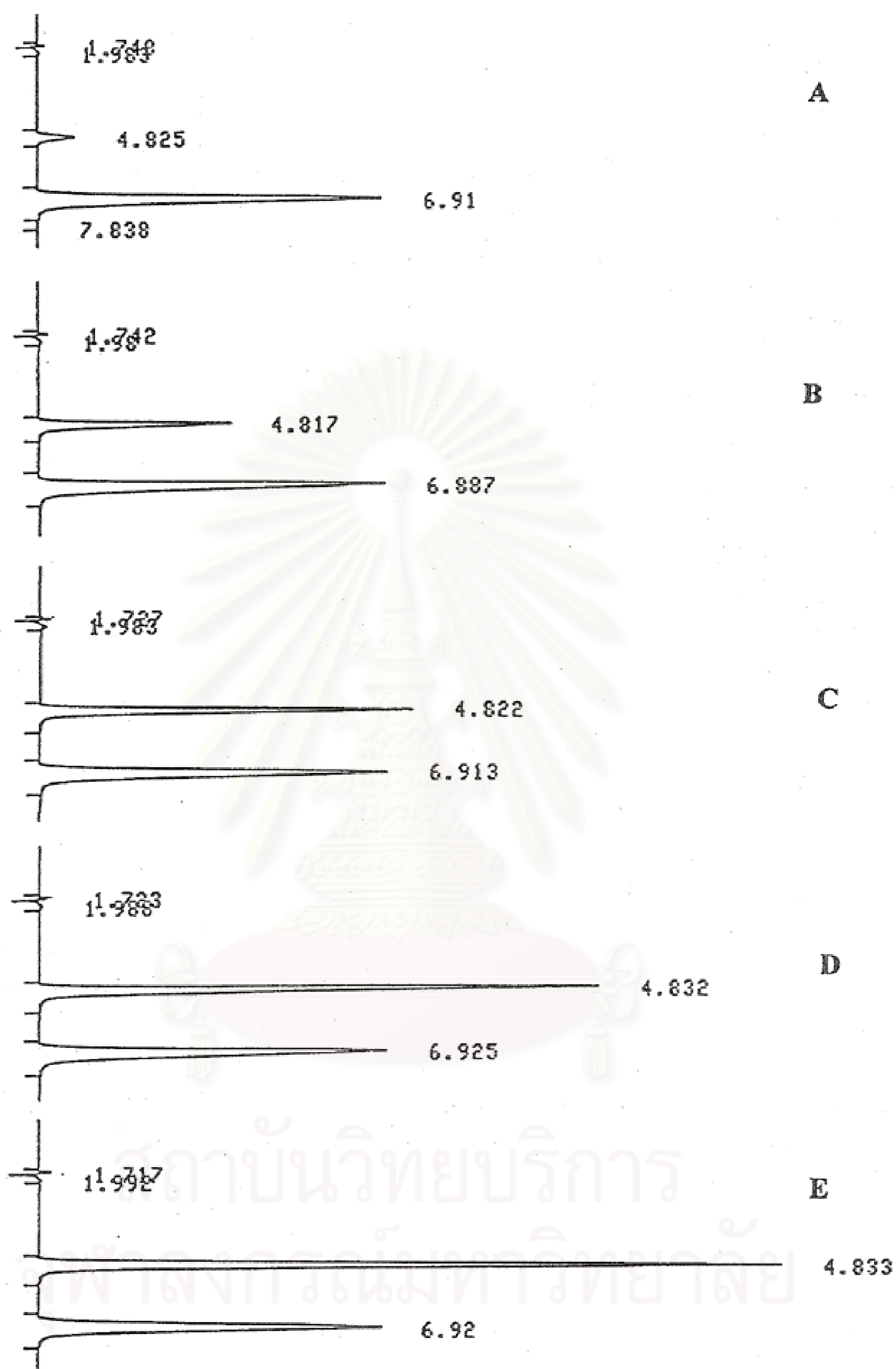

Figure 9 HPLC chromatograms of solutions of metronidazole:HPBCD inclusion complex (1 μg/ml, (A); 5 μg/ml, (B); 10 μg/ml, (C); 15 μg/ml, (D); and 20 μg/ml, (E); retention time of metronidazole and tinidazole are at 4.00-5.00 min and 6.00-7.00 min, respectively)

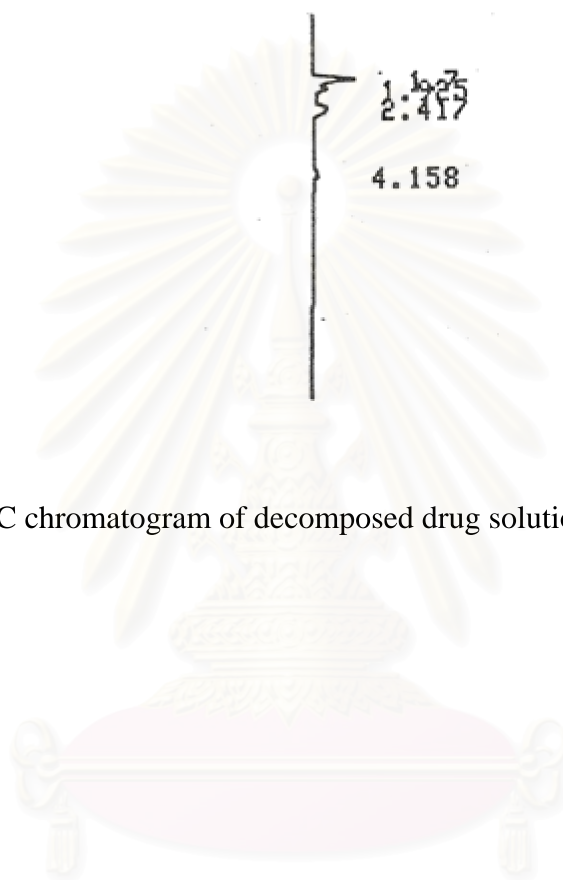

Figure 10 HPLC chromatogram of decomposed drug solution

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Table 3 Percentage of analytical recovery of metronidazole

actual concentration of metronidazole (μg/ml)	calculated concentration of metronidazole (μg/ml)	% analytical recovery
1	0.9703	97.03
	0.9846	98.46
	0.9683	96.83
5	5.0931	101.86
	5.0803	101.61
	5.1182	102.36
10	9.8307	98.31
	9.9188	99.19
	9.8235	98.23
15	14.7159	98.11
	14.9255	99.50
	14.8243	98.83
20	19.6154	98.08
	19.9211	99.61
	19.7544	98.77
mean		99.12
SD		1.66
% CV		1.67

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1.2.3 Precision

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogeneous sample. The precision of analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation). Table 4 and 5 illustrate the data of within and between run precision , respectively. All coefficients of variation values were small so it indicated that the HPLC method used was precise for quantitation of metronidazole concentration in the range studied.

Table 4 Data of within run precision

metronidazole concentrations (μg/ml)	peak area ratio					
	set no.1	set no. 2	set no. 3	mean	SD	% CV
1	0.0742	0.0740	0.0737	0.0740	0.0002	0.32
5	0.3798	0.3771	0.3740	0.3770	0.0029	0.77
10	0.7329	0.7340	0.7158	0.7276	0.0102	1.40
15	1.0989	1.1112	1.0900	1.1000	0.0106	0.97
20	1.4610	1.4782	1.4785	1.4726	0.0100	0.68

Table 5 Data of between run precision

metronidazole concentrations (μg/ml)	peak area ratio					
	day1	day2	day3	mean	SD	% CV
1	0.0734	0.0740	0.0744	0.0739	0.0005	0.64
5	0.3796	0.3770	0.3846	0.3804	0.0039	1.02
10	0.7240	0.7276	0.7413	0.7309	0.0091	1.25
15	1.0900	1.1000	1.1113	1.1004	0.0106	0.97
20	1.4472	1.4726	1.4900	1.4699	0.0216	1.47

1.2.4 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 analyte in samples within a given range. Figure 11 shows that the relationship between peak area ratios and metronidazole concentrations is linear with a coefficient of determination (R^2) value of 0.9999. This result indicated that HPLC method was acceptable for quantitative analysis of metronidazole in the range studied.

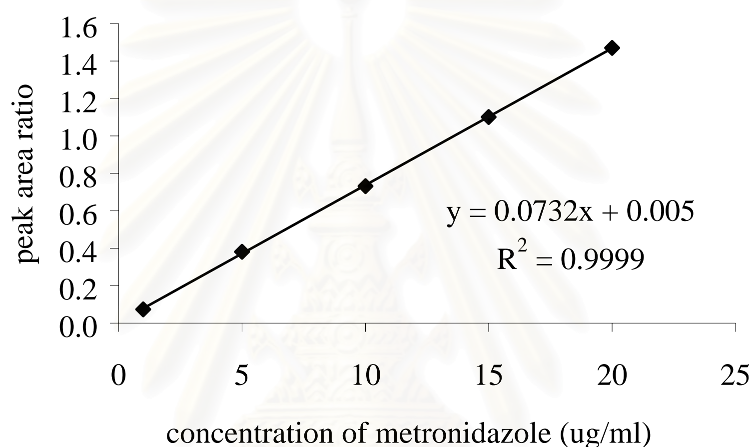

Figure 11 Standard curve of metronidazole (HPLC method)

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2. Phase Solubility Study

The phase solubility diagram for metronidazole-HPBCD system at 37±1°C is presented in Figure 12. In the absence of HPBCD, the aqueous solution of metronidazole was 7.09 X 10^{-2} M. This plot showed that the aqueous solubility of the drug increased linearly as function of HPBCD concentration. It is clearly observed that the phase solubility diagram of metronidazole-HPBCD system can be generally classified as type A_L according to Higuchi and Connors. The solubility enhancement can be attributed to the formation of an inclusion complex in solution.

Generally, if the slope of phase solubility diagram was less than 1, it was assumed that the formation of complex might occur in a 1:1 stoichiometric ratio. However, it should be kept in mind that a slope of less than unity with a type A_L diagram does not necessarily mean that only a 1:1 complex is formed (Higuchi and Connors, 1965). As Marques, Hadgraft and Kellaway (1990) have stated that the slope of phase solubility diagram was quoted as molar ratio of guest:cyclodextrins. In support of previous study, this study found that the slope which was 0.4434 (~0.5) defined as 1:2 molar ratio of metronidazole:HPBCD. Thus, it indicated the formation of the complex might occur in stoichiometric ratio of both 1:1 and 1:2 type. Due to insufficient data, it was difficult to determine the concentration of each complex. So the apparent stability constant or formation constant (K_c) was calculated based on the formation of 1:1 complex according to equation 1

$$K_c = \frac{\text{slope}}{\text{intercept (1- slope)}} \quad (1)$$

and was to be 11.24 M^{-1}, indicating a low-stability complex. Similarly low K_c values between 5-80 M^{-1} have been reported for α- and β-cyclodextrin complex with non-steroidal antiinflammatory drugs (Hamada, Nambu and Nagai, 1975), thiazides(Corrigan and Stanley, 1982), benzaldehyde (Uekama et al., 1983), furosemide (Szeman et al., 1987) and famotidine (Hassan, Suleiman, and Najib, 1990) as presented in Table 6. In spite of the low of K_c values, these complexes still exhibited favorable biopharmaceutical properties e.g. dissolution rate, stability.

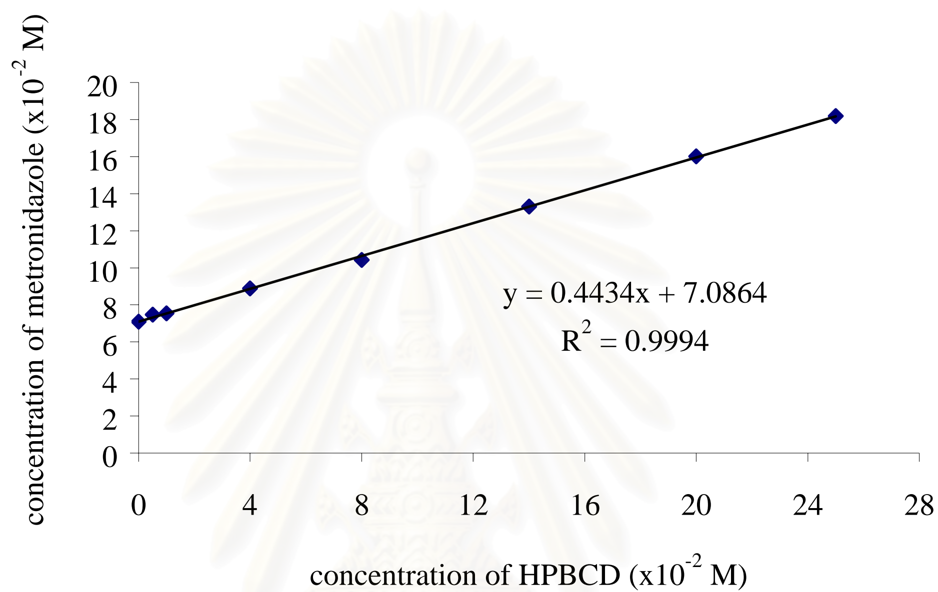

Figure 12 Phase solubility diagram of metronidazole-HPBCD system

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Table 6 Stability constants for drug-CDs systems

Drug-CDs systems	Stability constant, K_c (M^{-1})
Azapropazone - α CD	36.0
Azapropazone - β CD	36.0
Bendrofluazide - β CD	80.5
Chlorothiazide - β CD	13.8
Hydrochlorothiazide - β CD	58.5
Hydroflumethiazide - β CD	32.4
Benzaldehyde - α CD	7.0
Furosemide - β CD	74.96
Famotidine - β CD	62.0

Although the result obtained by the phase solubility study confirmed the formation of complex in solution, it is also important to clarify the formation of complex in solid state. According to Higuchi and Connors (1965), type A_L diagram indicated the formation of soluble complex between metronidazole and HPBCD,so freeze-drying was used to obtain solid complexes. The freeze-dried products were prepared and characterized in order to confirm complex formation in solid state.

In the preliminary study, solutions of metronidazole:HPBCD which concentration of metronidazole was 30 mg/ml above its aqueous solubility were prepared in different molar ratios including 1:1, 1:1.5 and 1:2. It was found that time used to dissolve all metronidazole in solution decreased in the order: 1:1 > 1:1.5 > 1:2 molar ratio. When these solutions were kept in a refrigerator, metronidazole in 1:1 and 1:1.5 molar ratio solutions precipitated out and did not redissolve after left at room temperature. Whereas solution of metronidazole:HPBCD in 1:2 molar ratio remained unchanged after refrigeration. Thus the inclusion complex of metronidazole:HPBCD was prepared by freeze drying method at the molar ratio of 1:2 for further study.

3. Characterization of Metronidazole:HPBCD Inclusion Complex

To verify the existence of the interaction between metronidazole and HPBCD in the solid state, the freeze-dried samples were analyzed by Fourier transform infrared spectroscopy, differential scanning calorimetry and X-ray diffractometry and then compared with the corresponding physical mixture in the same molar ratio. While proton nuclear magnetic resonance spectroscopy was used to obtain information about the existence of the complexes in solution.

3.1 Fourier Transform Infrared Spectroscopy (FTIR Spectroscopy)

Upon complexation of the guest, shifts or changes in the spectra occur.

The FTIR spectra of metronidazole, HPBCD, physical mixture and metronidazole:HPBCD product are shown in Figure 13 and Appendix III.

The FTIR spectrum of metronidazole showed major peaks at 3219, 3102, 1537 & 1372, 1077 and 828 cm^{-1}, indicated OH stretching vibration, C-H stretching of C=H, N-O stretching of NO_2, C-O stretching of C-OH and C-N stretching of C-NO_2, respectively.

The FTIR spectrum of HPBCD is characterized by intense bands at 3500-3300 cm^{-1} associated with the absorption of hydrogen bonded –OH groups of the CD. The vibrations of the –CH and –CH_2 groups appear 2950-2800 cm^{-1} region. Several sharp and intense bands in 1157-1035 cm^{-1} may be assigned to the stretching vibrations of the primary or secondary C-OH groups. It should be noted that HPBCD contained some moisture, which is characterized by the band at 1637 cm^{-1}. These findings are consistent with a previous study by Arias, Moyano, and Gines (1997).

Metronidazole:HPBCD product and physical mixture show spectra corresponding to a superimposition of their parent products and no significant shift of the major peaks of metronidazole.

From the data obtained, it indicated that no strong chemical interaction between metronidazole and HPBCD occurred. Nevertheless, it could not be concluded exactly that the product was a simple physical mixture. Several studies have suggested that the characteristic bands of CD representing the overwhelming part of the complex are hardly influenced by complex formation. Generally, bands due to the included part of the guest molecule will shift or their intensities will alter. But if the mass of the guest molecule does not exceed 25% of the mass of the complex, these alterations will be obscured by the spectrum of the host (Szejtli, 1982; Bekers et al., 1991). Moreover, some of the changes upon complex formation are very subtle so it requires careful interpretation of the spectrum (Hedges, 1998).

3.2 Differential Scanning Calorimetry (DSC)

For analysis by this technique, the guest must have a melting or boiling temperature below about 300°C, the temperature at which the CDs decompose. The effects of CDs on the thermogram of the guest molecule were observed for the broadening, shifting and appearance of new peaks or disappearance of certain peaks (Atha, Udapa and Sreenivasan, 1995). In case of the absence of melting peak, no energy absorption is observed at the melting temperature of the guest when the guest is complexed. 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).

Figure 14 represents DSC thermograms of metronidazole, HPBCD, physical mixture and metronidazole:HPBCD product. The thermogram of metronidazole displayed its endothermic melting peak at 162°C. The thermogram of HPBCD showed endothermic peak at 89.6°C corresponding to water evaporation from HPBCD cavities (Munoz-Ruiz and Paronen, 1997; Cwiertnia, Hladon and Stobiecki, 1999).

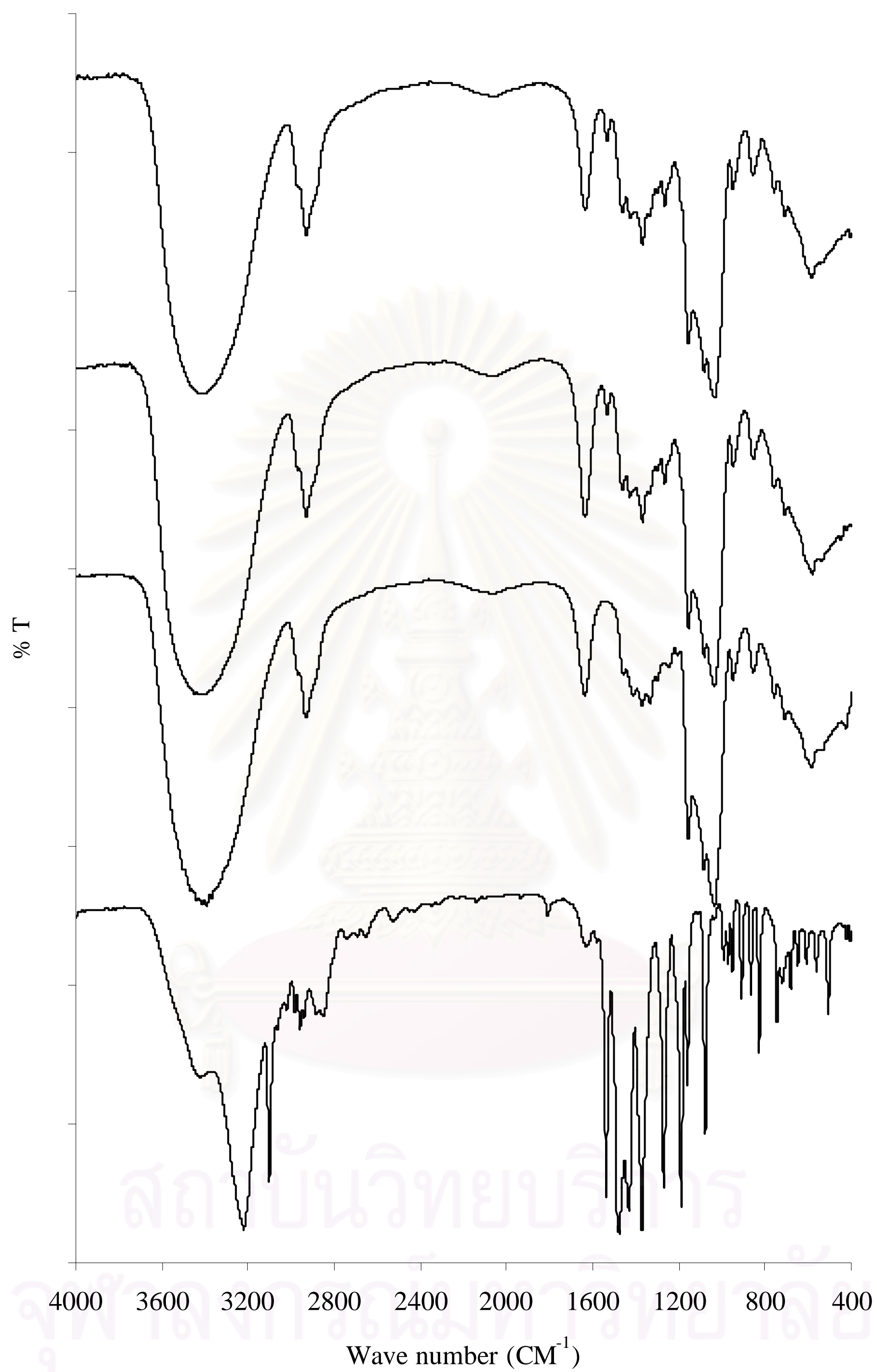

Figure 13 Infrared spectra of metronidazole, A; HPBCD, B; physical mixture of metronidazole and HPBCD, C; and metronidazole:HPBCD complex, D

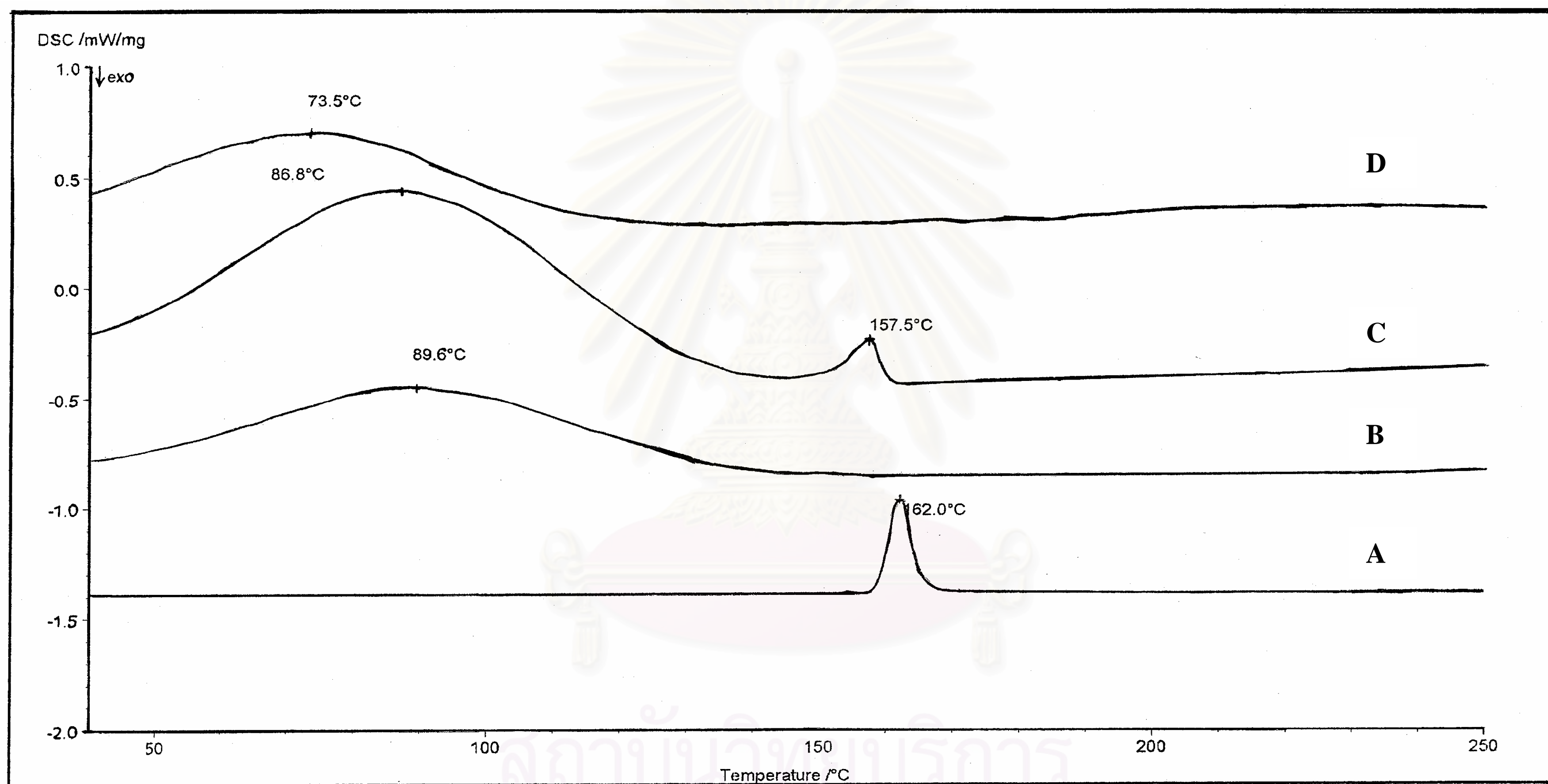

Figure 14 DSC thermograms of metronidazole, A; HPBCD, B; physical mixture of metronidazole and HPBCD, C; and metronidazole: HPBCD complex, D

The DSC thermogram of physical mixture was a combination of the thermograms of metronidazole and HPBCD. On the contrary, the thermogram of metronidazole:HPBCD product showed an endothermic peak at 73.5°C whereas the endothermic peak characteristic of metronidazole disappeared. From this data, it can be deduced that metronidazole:HPBCD product was not simple physical mixture. The absence of the melting peak of metronidazole in the DSC thermogram of the freeze-dried product may be attributed to the amorphous state or inclusion complex formation or both. Because HPBCD has an amorphous character and generally gives a highly water-soluble amorphous complex when forming complex with poorly water-soluble drugs (Duchene and Wouessidjewe, 1990).

3.3 X-ray Diffractometry

Figure 15 shows the X-ray diffraction patterns of metronidazole, HPBCD, physical mixture and freeze-dried product of metronidazole with HPBCD, respectively. It is obvious that metronidazole and physical mixture exhibited crystalline characteristics. X-ray diffraction pattern of the physical mixture of metronidazole and HPBCD was simply a superimposition of each component with the peaks having lower intensity. The freeze-dried product showed a broad, diffuse pattern indicating amorphization of drug and/or complexation occurs. Because the X-ray diffraction pattern of drugs is considerably affected by complexation with CDs owing to change in their crystalline nature. Normally sharpening of the existing peaks, appearance of a few new peaks and shifting of certain peaks are associated with crystalline complex formation. On the other hand, formation of amorphous complexes lead to the disappearance of certain peaks or the peaks become less sharp than that of the pure compound or physical mixture (Athal et al., 1995).

From the characterization of the inclusion complex of metronidazole:HPBCD in the solid state, DSC and X-ray diffractometry gave some hints on an inclusion complex formation between metronidazole and HPBCD. However, Ventura et al. (1998) have suggested that solid state characterization gives information about the interaction between drug and CDs that could interest only the external surface of the CDs. Hence, the guest molecule could be accommodated not in the cavity of CDs but

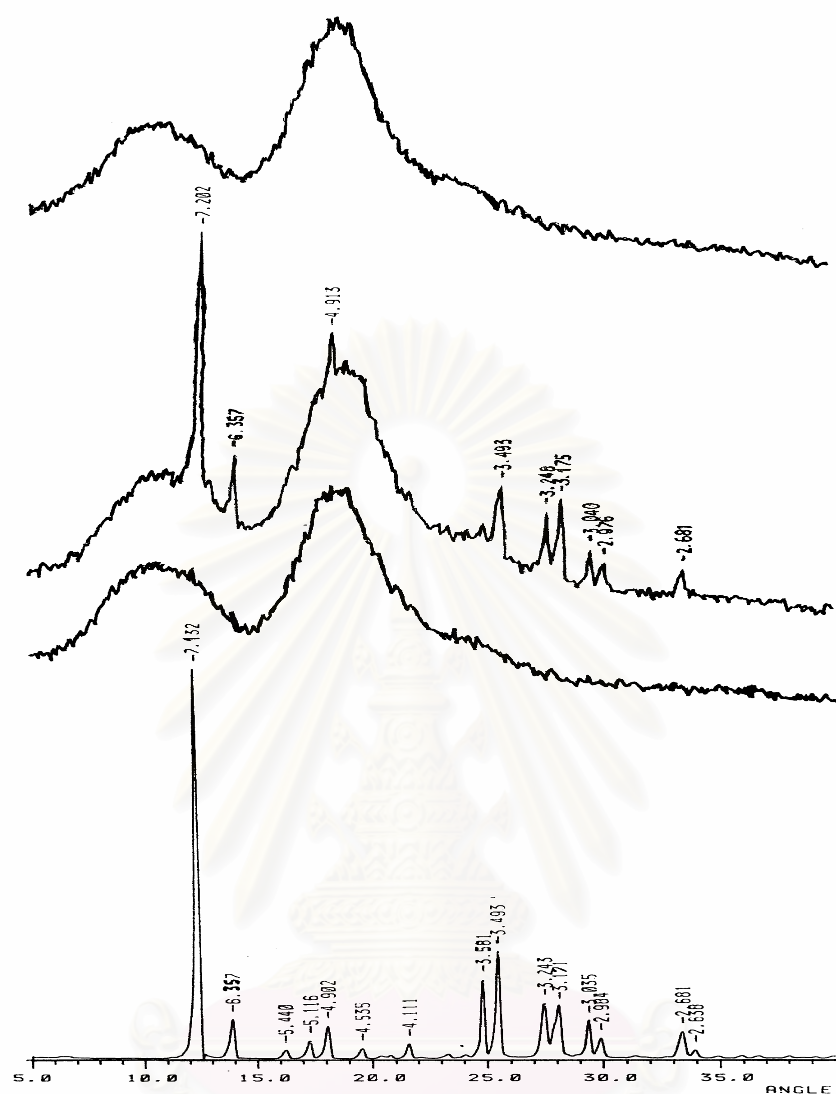

Figure 15 X-ray diffractograms of metronidazole, A; HPBCD, B; physical mixture of metronidazole and HPBCD, C; and metronidazole:HPBCD complex, D.

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externally, between two or more CDs molecules. To clarify the existence of the complexes, ^{1}H-NMR can be used.

3.4 Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H-NMR)

^{1}H-NMR technique was employed to examine the inclusion mode in aqueous solution. The analytical usefulness of this technique resides in the fact that the same types of nuclei, when located in different molecular environments, exhibit different resonance frequencies.

CDs are toroidal molecules with a truncated cone shape having primary and secondary OH groups on the opposite ends of its torus; H-3 and H-5 directed towards the interior, H-6 on the rim and H-1, H-2 and H-4 located to the exterior. It is expected that if inclusion does occurs, protons located within or near the cavity (e.g. H-3, H-5 or H-6) should be strongly shielded (significant upfield shift) due to the anisotropy of the hydrophobic part of the guest molecule. Whereas protons located on the exterior of the torus (H-1, H-2 and H-4) should be relatively unaffected (marginal upfield shifts). Alternatively, if association takes place on the exterior of the torus, H-1, H-2 and H-4 showed be more strongly affected. It is well recognized that upfield shifts are observed in the CDs protons when hydrophobic interactions between drug and CDs occur with downfield shifts observed for the drug protons (Marques et al., 1990; Bekers et al., 1991).

Unfortunately, however, the H-3 and H-5 protons of HPBCD could not be assigned to resonance signals because the signals of the internal protons of this CD are overlapped to those of hydroxypropyl group (Backensfeld et al., 1990; Muller and Albers, 1992; Ventura et al., 1998). Hence, we can only observe the shifts of the drug protons which give information about the existence of an inclusion complex in solution.

The ^{1}H-NMR spectra in D_2O of metronidazole, HPBCD and freeze-dried complex are displayed in Figure16. Table 7 depicts the chemical shifts of drug protons induced by HPBCD. In the presence of HPBCD, all protons were shifted downfield, probably due to steric perturbation through inclusion complex formation

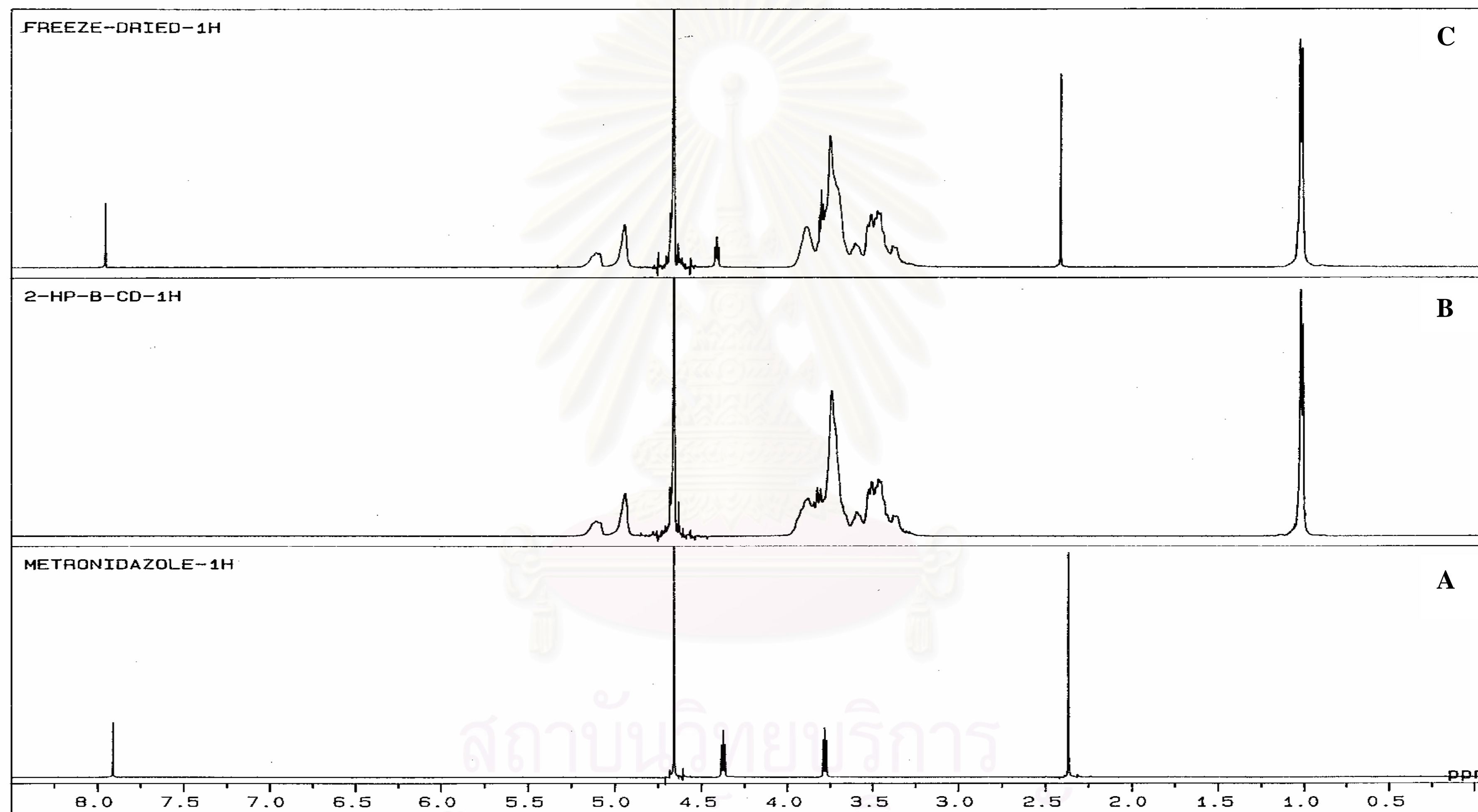

Figure 16 ^{1}H-NMR spectra in D_2O of metronidazole, A; HPBCD, B; and metronidazole:HPBCD complex, C

or be induced by diamagnetic anisotropy of particular bonds or regions of the host (Ueda and Nagai, 1980). Similar small magnitude downfield shifts (0.009-0.016) were observed for papaverine protons when forming complex with HPBCD (Ventura et al., 1998). Therefore, it can be said that an interaction between metronidazole and HPBCD in solution may exist.

Table 7 ^{1}H-NMR chemical shifts (δ) of metronidazole in the presence of HPBCD

Metronidazole	δ_o (ppm)	$\delta_{complex}$ (ppm)	$\Delta\delta(\delta_{complex}-\delta_o$, ppm)
$-C-CH_3$	2.3613	2.4052	0.0439
$CH_2C\mathbf{H}_2OH$	3.7634	3.7842	0.0208
	3.7738	3.7946	0.0208
	3.7842	3.8049	0.0207
$C\mathbf{H}_2CH_2OH$	4.3559	4.3925	0.0366
	4.3663	4.4035	0.0372
	4.3766	4.4133	0.0367
-C-H	7.9095	7.9528	0.0433

(see Appendix IV)

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4. Effect of Temperature on the Stability of Inclusion Complex of Metronidazole:HPBCD in Aqueous Solution

Physical appearances including color and clarity of preparations were studied before and after the stability study for 6 months. The solutions of metronidazole both alone and in inclusion complex with HPBCD were clear and no precipitation throughout the study whereas the color of both solutions changed from colorless to yellow, suggesting the effect of temperature on the physical stability of metronidazole and metronidazole:HPBCD complex. Table 8-9 shows pH values of metronidazole and metronidazole:HPBCD formulations, respectively. It could be noticed that pH values slightly decreased at 70-90°C, whereas it remained unchanged at 60°C.

For chemical stability, Table 10-11 and Figure 17-18 demonstrate percent remaining of metronidazole in both formulations kept at various temperatures.

To determine the order of reaction, graphical method and linear regression (SPSS version 7.5) were used. If a plot of concentration of drug remaining versus time is linear, it means zero-order reaction. When a plot of ln(concentration) against time is a straight line, it is first-order reaction. Linear regression was used to estimate the correlation coefficients (r) of each plot. The order of reaction was decided on which plot gave the higher correlation coefficient (Kendall et al., 1989). Table 12 illustrates the correlation coefficients of metronidazole and metronidazole:HPBCD formulations at different temperatures. The result showed that the higher correlation coefficient were obtained from plotting of ln(concentration) versus time. Thus, the degradation of both formulations followed first-order kinetic. This finding is consistent with a previous study by Wang and Yeh (1993). The presence of HPBCD did not affect this kinetic behaviour. It might be concluded that the degradation mechanism of metronidazole by temperature is still the same even when complexed with HPBCD.

Degradation rate constant is determined by its slope of the plot of ln(concentration) versus time which is best fit to the reaction. The first-order rate constants of both formulations at different temperatures are shown in Table 13.

Table 8 pH values of metronidazole formulation kept at various temperatures.

90°C		80°C		70°C		60°C	
time (day)	pH [a]	time (day)	pH	time (day)	pH	time (day)	pH
0	5.74 ± 0.010	0	5.74 ± 0.010	0	5.74 ± 0.010	0	5.74 ± 0.010
3	5.59 ± 0.005	3	5.74 ± 0.006	3	5.74 ± 0.021	3	5.75 ± 0.013
7	5.50 ± 0.015	14	5.70 ± 0.017	14	5.70 ± 0.037	14	5.73 ± 0.024
14	5.47 ± 0.010	30	5.45 ± 0.035	30	5.59 ± 0.010	30	5.72 ± 0.017
30	5.45 ± 0.014	60	5.58 ± 0.026	60	5.56 ± 0.043	60	5.73 ± 0.018
45	5.41 ± 0.055	90	5.59 ± 0.042	90	5.54 ± 0.008	90	5.74 ± 0.005
60	5.59 ± 0.168	150	5.57 ± 0.021	150	5.56 ± 0.014	150	5.73 ± 0.013
75	5.56 ± 0.200	180	5.46 ± 0.010	180	5.59 ± 0.006	180	5.70 ± 0.010
90	5.45 ± 0.029						

a - mean ± SD, n = 4

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Table 9 pH values of metronidazole : HPBCD formulation kept at various temperatures.

90°C		80°C		70°C		60°C	
time (day)	pH [a]	time (day)	pH	time (day)	pH	time (day)	pH
0	5.76 ± 0.017	0	5.76 ± 0.017	0	5.76 ± 0.017	0	5.76 ± 0.017
3	5.54 ± 0.013	3	5.76 ± 0.037	3	5.77 ± 0.021	3	5.77 ± 0.017
7	5.51 ± 0.013	14	5.76 ± 0.030	14	5.73 ± 0.021	14	5.79 ± 0.010
14	5.41 ± 0.008	30	5.64 ± 0.037	30	5.60 ± 0.006	30	5.72 ± 0.017
30	5.38 ± 0.031	60	5.68 ± 0.013	60	5.54 ± 0.033	60	5.70 ± 0.017
45	5.26 ± 0.008	90	5.63 ± 0.032	90	5.44 ± 0.045	90	5.44 ± 0.045
60	5.10 ± 0.054	150	5.50 ± 0.026	150	5.48 ± 0.031	150	5.65 ± 0.019
75	5.05 ± 0.035	180	5.46 ± 0.008	180	5.48 ± 0.015	180	5.64 ± 0.013
90	5.00 ± 0.069						

a - mean ± SD, n = 4

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Table 10 Percent drug remaining of metronidazole formulation kept at various temperatures.

90°C		80°C		70°C		60°C	
time (day)	% drug remaining [a]	time (day)	% drug remaining	time (day)	% drug remaining	time (day)	% drug remaining
0	100.00 ± 0.00	0	100.00 ± 0.00	0	100.00 ± 0.00	0	100.00 ± 0.00
3	97.76 ± 0.59	3	98.29 ± 1.48	3	98.42 ± 1.21	3	100.60 ± 1.93
7	91.79 ± 0.40	14	92.61 ± 0.42	14	97.81 ± 0.61	14	100.53 ± 1.70
14	88.48 ± 0.50	30	86.39 ± 0.60	30	93.25 ± 0.70	30	99.12 ± 0.85
30	70.75 ± 0.72	60	71.22 ± 1.38	60	86.80 ± 1.16	60	94.36 ± 1.11
45	64.12 ± 1.13	90	58.03 ± 0.85	90	79.11 ± 1.74	90	92.52 ± 0.50
60	56.32 ± 0.72	150	41.52 ± 0.81	150	69.67 ± 0.60	150	87.19 ± 1.17
75	46.97 ± 0.43	180	34.14 ± 0.11	180	60.11 ± 1.35	180	84.22 ± 1.59
90	38.39 ± 0.59						

a - mean ± SD , n = 4

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Table 11 Percent drug remaining of metronidazole : HPBCD formulation kept at various temperatures.

90°C		80°C		70°C		60°C	
time (day)	% drug remaining [a]	time (day)	% drug remaining	time (day)	% drug remaining	time (day)	% drug remaining
0	100.00 ± 0.00	0	100.00 ± 0.00	0	100.00 ± 0.00	0	100.00 ± 0.00
3	94.81 ± 0.27	3	97.77 ± 1.78	3	98.54 ± 1.93	3	100.86 ± 0.79
7	90.15 ± 0.50	14	90.79 ± 1.36	14	98.61 ± 1.37	14	99.17 ± 1.74
14	84.97 ± 0.86	30	86.54 ± 1.30	30	93.98 ± 1.54	30	96.98 ± 1.66
30	72.12 ± 0.63	60	75.01 ± 1.91	60	87.83 ± 1.15	60	94.76 ± 1.04
45	62.34 ± 0.73	90	62.10 ± 1.01	90	83.69 ± 1.31	90	91.81 ± 0.49
60	54.48 ± 0.62	150	46.60 ± 0.86	150	76.83 ± 1.02	150	90.20 ± 1.56
75	46.42 ± 1.19	180	40.26 ± 0.36	180	75.18 ± 0.47	180	86.23 ± 0.88
90	36.06 ± 1.11						

a - mean ± SD , n = 4

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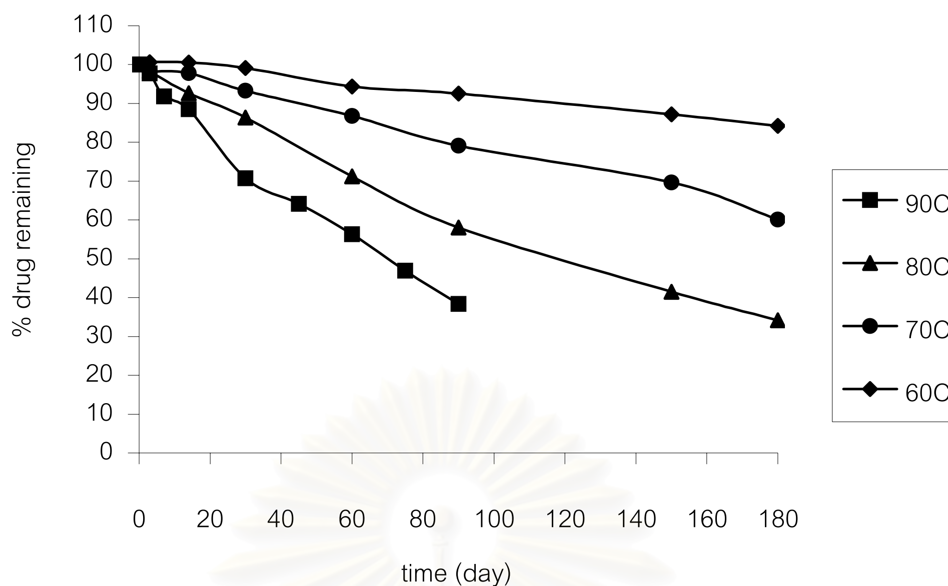

Figure 17 Percent drug remaining of metronidazole formulation kept at various temperatures.

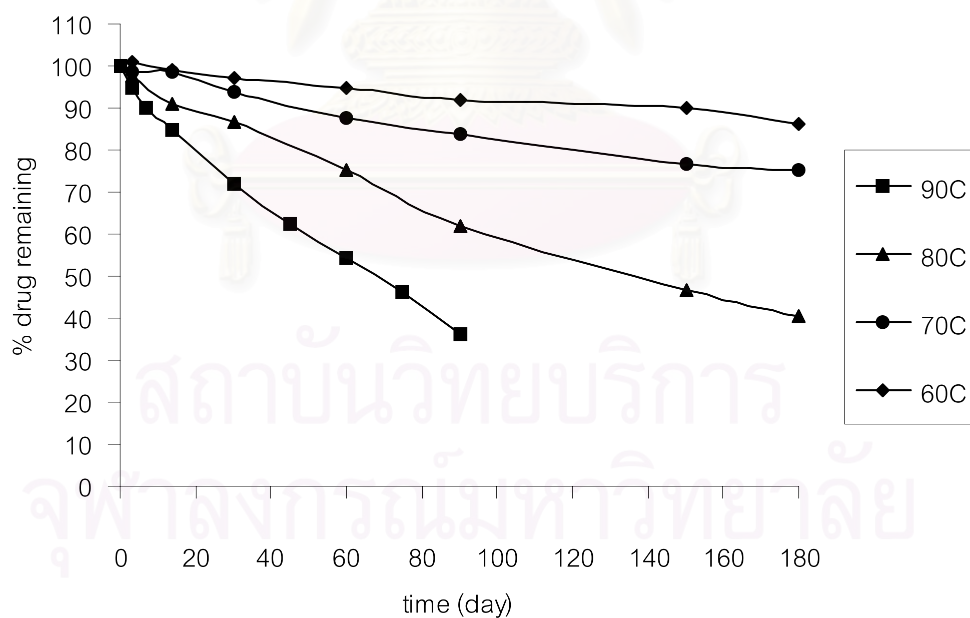

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Figure 18 Percent drug remaining of metronidazole:HPBCD formulation kept at various temperatures.

Table 12 Correlation coefficients of zero and first orders for metronidazole with/without HPBCD formulations

Formula	Temperature (°C)	Correlation coefficient (r)	
		Zero order	First order
Metronidazole	90	0.9922	0.9971
	80	0.9920	0.9995
	70	0.9971	0.9943
	60	0.9936	0.9941
Metronidazole : HPBCD	90	0.9945	0.9966
	80	0.9928	0.9992
	70	0.9852	0.9902
	60	0.9826	0.9840

Table 13 The degradation rate constants of metronidazole kept at various temperatures

Formula	Temperature (°C)	Rate constant, k_1 [a] (day $^{-1}$)
Metronidazole	90	$1.03 \times 10^{-2} \pm 2.98 \times 10^{-4}$ [b]
	80	$5.99 \times 10^{-3} \pm 7.79 \times 10^{-5}$
	70	$2.69 \times 10^{-3} \pm 1.18 \times 10^{-4}$
	60	$1.00 \times 10^{-3} \pm 4.50 \times 10^{-5}$
Metronidazole : HPBCD	90	$1.06 \times 10^{-2} \pm 3.34 \times 10^{-4}$
	80	$5.04 \times 10^{-3} \pm 8.10 \times 10^{-5}$
	70	$1.65 \times 10^{-3} \pm 9.50 \times 10^{-5}$
	60	$7.98 \times 10^{-4} \pm 5.95 \times 10^{-5}$

a – k_1 was obtained from the slope of ln (concentration) vs time curve (First order reaction)

b – mean $\pm$ SE.; Standard error of first order rate constants (SE.) were calculated by using a linear regression

A Drug Stability Analysis Program (DSA program version 2.11) developed by Somchai Mekaroonreung and Churairat Rakwatin was used to compare the differences in degradation rate constant values. The null hypothesis was that a pair of degradation rate constant values was not different from each other, while the alternative hypothesis was that one rate constant value was different from the other at significant level (α) 0.05. Table 14 shows the statistical difference in rate constants of metronidazole formulation with/without HPBCD at the same temperature. It was found that the rate constants of metronidazole formulation were significant higher than those of metronidazole:HPBCD formulation at 60-80°C. Therefore, HPBCD is likely to improve stability of metronidazole. However, at 90°C, the rate constants of metronidazole with/without HPBCD were not significantly different. It is probable that stabilizing effect of HPBCD is not enough to stabilize metronidazole from degradation at high temperature (90°C).

Table 14 Statistical comparison of the degradation rate constants between metronidazole and metronidzole:HPBCD formulation at various temperatures

Temperature (°C)	t-value	Confidence limit	Significant difference
90	0.65	$t_{0.05,df=14} = 2.14$	No
80	8.35	$t_{0.05,df=12} = 2.17$	Yes
70	5.85	$t_{0.05,df=12} = 2.17$	Yes
60	2.74	$t_{0.05,df=12} = 2.17$	Yes

Figure 19-20 depict the Arrhenius plot, i.e. ln(degradation rate constant) versus the reciprocal of temperature, of the degradation rates in the absence and presence of HPBCD over the temperature range 60-90°C. Kinetics parameters of the degradation of metronidazole were calculated using the Arrhenius equation (2) and

$$\ln k = \ln A - (E_a/RT) \quad (2)$$

where k - the rate constant, E_a - the activation energy

A - the frequency factor, R - the molar gas constant

T - the temperature in Kelvins

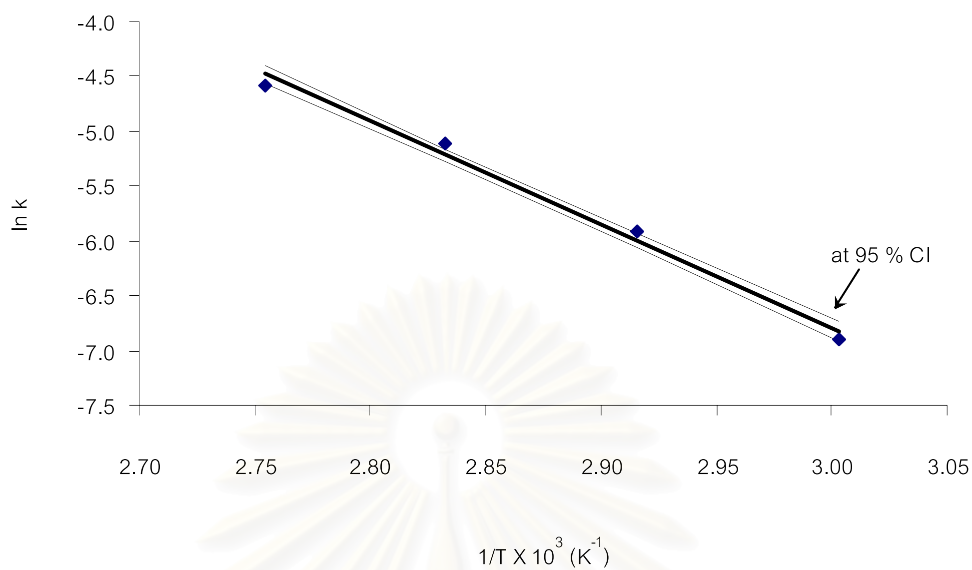

Figure 19 Arrhenius plot of metronidazole formulation

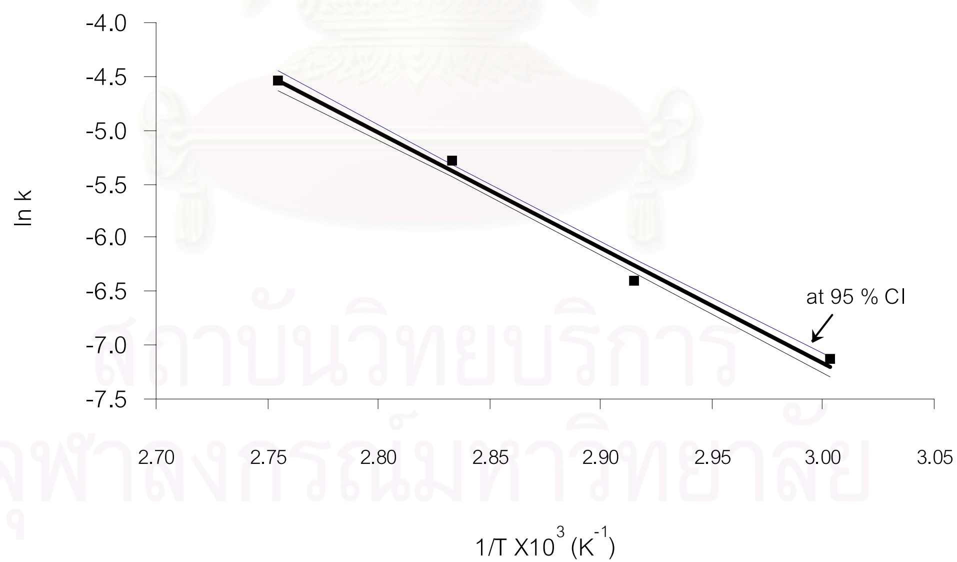

Figure 20 Arrhenius plot of metronidazole : HPBCD formulation

Table 15 Kinetics parameters for the degradation of metronidazole in the absence and presence of HPBCD

	Metronidazole Formulation	Metronidazole : HPBCD Formulation
Arrhenius equation	ln k = 21.543 - 9446.7(1/T)	ln k = 24.998 – 10722.7(1/T)
Correlation of determination, R^2	0.9885	0.9899
Activation energy, E_a (Kcal/mole)	18.77	21.31
E_a at 95 % confidence interval[a] (Kcal/mole)	17.61 –19.93	20.07 – 22.54
$k_{25°C}$ (day^{-1})	3.88 $X10^{-5}$	1.70 $X10^{-5}$
$k_{25°C}$ at 95 % confidence interval (day^{-1})	5.16 X 10^{-5}	2.30 X 10^{-5}
Shelf life at 25°C (day)	2,716.28	6,206.94
Shelf life at 25°C, 95 % confidence interval (day)	2,043.80	4,585.72

a – 95 % confidence interval was calculated by using a DSA program version 2.11

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summarized in Table 15. As can be seen from the coefficient of determination (R^2), the linearity of the regression lines in the plots showed a good indication of invariant activation energy for degradation of metronidazole in the temperature range 60-90°C. Activation energy, calculated from the slope of the regression line, for metronidazole formulation was 18.77 Kcal/mole. It was closely to a previous study by Wang and Yeh (1993) in that the activation energy for degradation of metronidazole at pH 3.1, 0.1 M acetate buffer solution was found to be 15.35 Kcal/mole. Activation energy of metronidazole in the absence of HPBCD was lower than that of metronidazole in the presence of HPBCD. Moreover, it is apparent that temperature affected on the reaction rate because activation energy values were in the range of 10-30 Kcal/mole. By application of the Arrhenius equation, the degradation rate constants at 25°C were extrapolated. If activation energy for degradation remains constant at low temperature, the extrapolated degradation rate constants at 25°C were 3.88 X 10^{-5} and 1.70 X 10^{-5} day^{-1} for metronidazole and metronidazole:HPBCD formulations, respectively. Then the corresponding shelf-life, time that the concentration drops from 100% to 90%, of each formulation was estimated. It was found that the shelf-life of the formulation containing HPBCD was longer than that of the formulation without HPBCD.

From the data obtained, the higher activation energy of the complex indicated that higher energy was needed for the complex to be activated to the excited state. Thus, the degradation of metronidazole in the presence of HPBCD was more difficult than that of metronidazole alone. This suggested some protection against the influence of heat on degradation. It was confirmed by the lower degradation rate constant and the longer shelf-life.

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5. Effect of pH on the Stability of Inclusion Complex of Metronidazole:HPBCD in Aqueous Solution

The pH dependence of metronidazole degradation in the presence of HPBCD was compared with the degradation of the free drug. This study was performed in pH 3.1 (acetate buffer), pH 5.6 (phosphate buffer) and pH 9.2 (borate buffer) at $80\pm1^{\circ}C$. Regarding to Wang and Yeh (1993), the degradation kinetics of metronidazole solutions in pH range 3.1-9.9 (pH 3.1-4.9 acetate buffer, pH 5.4-8.0 phosphate buffer and pH 9.1-9.9 borate buffer) at $90\pm0.2^{\circ}C$ were studied. It was found that no general acid/base catalysis imposed by acetate, phosphate and borate buffer species was responsible for the degradation of metronidazole and only specific acid/base catalysis occurred. Thus, general acid/base catalysis by the involved buffer substances was not taken into account in our study.

Formula A, C and E were 5 mg/ml metronidazole solution at pH 3.1, 5.6 and 9.2, respectively. Whilst formula B, D and F were 5 mg/ml metronidazole:HPBCD solution at pH 3.1, 5.6 and 9.2, respectively.

After kept under accelerated condition, the color of metronidazole solutions in all formulations changed from colorless into yellow regardless of whether solutions containing HPBCD or not. Table 16 displays pH values of all formulation during test period at $80\pm1^{\circ}C$. The pH values of formula C and D did not change throughout the study because pH 5.6 was the most stable pH for aqueous metronidazole solution (Wang and Yeh, 1993). There was a decrease in pH value for formula E and F, while an increase of pH value was found in formula A and B. This is uncommon because the degradation product of metronidazole is likely to be nitrite compound (Barnes and Makohon, 1993; Basly, Duroux and Bernard, 1996) which resulted in reduction of pH values.

Percent remaining of metronidazole in all formulations were shown in Table 17 and Figure 21. The order of reaction was determined by plotting of concentration or ln(concentration) versus time. The correlation coefficients were estimated for each plot by using a linear regression and compared as shown in Table 18. This indicated

Table 16 pH values of metronidazole formulations in different pH values with constant ionic strength (μ=0.5) at 80 ± 1 °C

Time (day)	pH [a]					
	Formula A	Formula B	Formula C	Formula D	Formula E	Formula F
0	3.18 ± 0.013	3.15 ± 0.005	5.59 ± 0.010	5.61 ± 0.015	9.15 ± 0.006	9.19 ± 0.000
0.25	- [b]	-	-	-	9.05 ± 0.010	9.04 ± 0.000
1	3.37 ± 0.067	3.46 ± 0.021	5.54 ± 0.018	5.61 ± 0.010	8.87 ± 0.014	8.85 ± 0.010
3	3.48 ± 0.098	3.70 ± 0.014	5.63 ± 0.018	5.65 ± 0.008	8.74 ± 0.017	8.73 ± 0.005
5	-	-	-	-	8.60 ± 0.000	8.56 ± 0.005
7	3.68 ± 0.134	3.86 ± 0.017	5.60 ± 0.010	5.62 ± 0.008	8.47 ± 0.017	8.43 ± 0.010
10	-	-	-	-	8.33 ± 0.028	8.28 ± 0.013
14	3.89 ± 0.010	3.93 ± 0.017	5.53 ± 0.010	5.60 ± 0.010	8.20 ± 0.010	8.10 ± 0.010
21	3.99 ± 0.022	3.99 ± 0.017	-	-	8.00 ± 0.010	7.83 ± 0.008
28	3.81 ± 0.232	3.99 ± 0.055	5.60 ± 0.026	5.55 ± 0.022	7.92 ± 0.017	7.63 ± 0.014
42	4.08 ± 0.010	4.05 ± 0.020	-	-	7.70 ± 0.008	7.22 ± 0.033
71	4.72 ± 0.074	4.66 ± 0.202	5.53 ± 0.024	5.51 ± 0.025	7.76 ± 0.015	7.39 ± 0.060
84	4.67 ± 0.015	4.63 ± 0.111	5.56 ± 0.013	5.51 ± 0.094	-	-
98	4.70 ± 0.012	4.70 ± 0.024	5.54 ± 0.022	5.54 ± 0.014	7.78 ± 0.042	7.46 ± 0.058
127	4.77 ± 0.010	4.65 ± 0.044	5.57 ± 0.115	5.51 ± 0.024	7.84 ± 0.026	7.47 ± 0.054

a - mean ± SD, n=4

b - not identified

Table 17 Percent remaining of metronidazole in different pH values with constant ionic strength (μ=0.5) at 80 $\pm$ 1 $^{\circ}$C

Time (day)	Percent drug remaining [a]					
	Formula A	Formula B	Formula C	Formula D	Formula E	Formula F
0	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00	100.00 $\pm$ 0.00
0.25	- [b]	-	-	-	96.13 $\pm$ 0.75	96.32 $\pm$ 0.49
1	99.17 $\pm$ 0.21	99.58 $\pm$ 0.37	100.60 $\pm$ 0.53	102.03 $\pm$ 0.22	82.41 $\pm$ 0.69	84.73 $\pm$ 0.16
3	96.60 $\pm$ 0.53	96.22 $\pm$ 0.39	97.77 $\pm$ 0.24	101.21 $\pm$ 0.16	68.15 $\pm$ 0.33	73.35 $\pm$ 0.34
5	-	-	-	-	55.40 $\pm$ 0.23	59.51 $\pm$ 0.28
7	86.92 $\pm$ 0.35	88.87 $\pm$ 0.73	96.82 $\pm$ 0.31	97.11 $\pm$ 0.79	47.37 $\pm$ 0.38	52.23 $\pm$ 0.14
10	-	-	-	-	40.05 $\pm$ 0.10	46.50 $\pm$ 0.05
14	74.23 $\pm$ 0.35	79.83 $\pm$ 0.28	88.62 $\pm$ 0.65	92.97 $\pm$ 0.46	32.14 $\pm$ 0.31	38.69 $\pm$ 0.14
21	64.98 $\pm$ 0.55	73.16 $\pm$ 0.80	-	-	24.44 $\pm$ 0.16	30.69 $\pm$ 0.09
28	59.30 $\pm$ 0.34	67.05 $\pm$ 0.46	83.04 $\pm$ 0.86	89.07 $\pm$ 0.31	18.76 $\pm$ 0.13	24.91 $\pm$ 0.04
42	50.41 $\pm$ 0.60	56.87 $\pm$ 2.67	-	-	12.81 $\pm$ 0.12	18.00 $\pm$ 0.38
71	35.53 $\pm$ 0.60	45.98 $\pm$ 0.33	55.23 $\pm$ 0.75	64.30 $\pm$ 0.11	5.81 $\pm$ 0.02	12.06 $\pm$ 0.28
84	35.99 $\pm$ 0.89	42.16 $\pm$ 1.67	53.74 $\pm$ 0.44	60.51 $\pm$ 0.93	-	-
98	33.90 $\pm$ 1.83	37.98 $\pm$ 0.91	48.75 $\pm$ 0.85	57.35 $\pm$ 0.29	3.19 $\pm$ 0.16	8.04 $\pm$ 0.41
127	27.32 $\pm$ 0.28	30.59 $\pm$ 0.88	41.47 $\pm$ 0.74	47.41 $\pm$ 1.44	1.55 $\pm$ 0.03	4.91 $\pm$ 0.29

a - mean $\pm$ SD, n=4

b - not identified

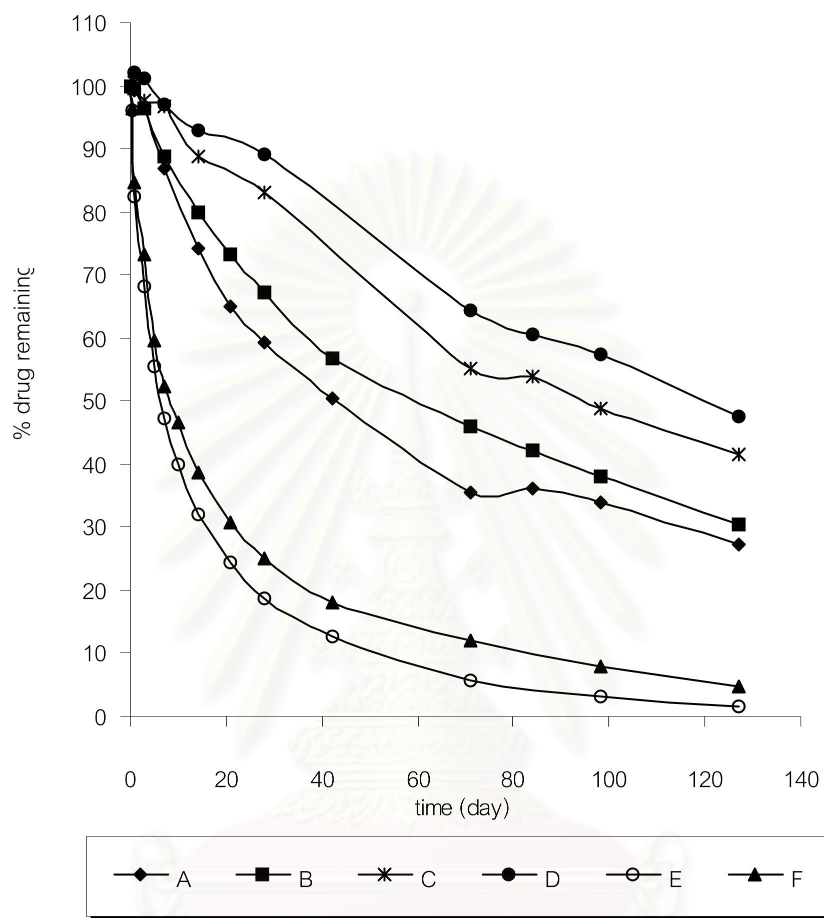

Figure 21 Percent remaining of metronidazole in different pH values with constant ionic strength (μ=0.5) at 80 ± 1 °C

that the degradation reaction of metronidazole for all formulations was said to be first-order kinetic and addition of HPBCD did not affect this kinetic behaviour.

Table 18 shows first-order rate constants of drug degradation at different pH values. The degradation rate constant of metronidazole solution in pH 9.2 was the highest among the three pH studied. This result agreed with a previous study of Wang and Yeh (1993) in that degradation rate of metronidazole in alkaline region (hydroxyl ion) was faster than in an acidic environment (hydrogen ion) as seen in pH rate profile (Figure 1). The statistical difference in rate constant of formulation with/without HPBCD at each pH studied were determined by using a DSA program at significant level (α) 0.05 as presented in Table 19. The difference of rate constants between metronidazole and its complex with HPBCD at pH 3.1 was similar to the rate difference at pH 5.6 (= 0.0012). However, there was no statistically significant difference in rate constants between metronidazole and metronidazole:HPBCD formulations at pH 3.1. It might be due to its higher value of pooled standard error of rate constants which resulted in a lower t-value. At pH 5.6 and 9.2, the rate constants of metronidazole:HPBCD solution were significantly lower than that of metronidazole free drug. It is obvious that HPBCD exert a stabilizing effect. It is probable to conclude that the inclusion of drug into the cyclodextrin cavity protected the drug from hydrolytic environment which resulted in the stabilization of the drug.

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Table 18 Correlation coefficients and rate constants of metronidazole degradation in different pH values with constant ionic strength (μ=0.5) at 80 $\pm$ 1 °C

Formula	Correlation coefficient (r)		Rate constant , k_1[a] (day $^{-1}$)
	Zero order	First order	
A	0.9253	0.9708	0.0106 $\pm$ 8.23 x 10^{-4} [b]
B	0.9568	0.9893	0.0094 $\pm$ 4.39 x 10^{-4}
C	0.9870	0.9957	0.0073 $\pm$ 2.39 x 10^{-4}
D	0.9937	0.9978	0.0061 $\pm$ 1.43 x 10^{-4}
E	0.7672	0.9778	0.0317 $\pm$ 1.96 x 10^{-3}
F	0.7918	0.9603	0.0226 $\pm$ 1.90 x 10^{-3}

a – k_1 was obtained from the slope of ln (concentration) vs time curve (First order reaction)

b – mean $\pm$ SE.; Standard error of first order rate constants (SE.) were calculated by using a linear regression

Table 19 Statistical comparison of the degradation rate constants between metronidazole and metronidzole:HPBCD formulation at different pH values

	t-value	Confidence limit	Significant difference
Formula A vs B	1.24	$t_{0.05,df=20}$ = 2.08	No
Formula C vs D	4.39	$t_{0.05,df=16}$ = 2.12	Yes
Formula E vs F	3.32	$t_{0.05,df=24}$ = 2.06	Yes

6. Effect of Light on the Stability of Inclusion Complex of Metronidazole:HPBCD in Aqueous Solution

On exposure to light, the colourless solution became yellow. This finding is consistent with a previous study by Suwakul (2000) in that metronidazole gel turned into bright yellow when exposed to daylight. Godfrey and Edwards (1991) also reported that a yellow photodegradation product from aqueous metronidazole solution buffered with citrate:phosphate can be further degraded into colourless materials by light, heat or the addition of the nonaqueous solvents. The reaction scheme for the photolytic degradation of metronidazole was proposed by Wilkins and Moore (1988) as shown in Figure 22. The photochemical reaction proceeded through two unstable intermediates, a 4-hydroxyimino 5-ketone (2) and a specie formed by hydrolytic cleavage of the imidazole ring (3) before recyclization to the N-(2-hydroxyethyl)-5-methyl-1,2,4-oxadiazole-3-carboxamide (4), which was the major degradation product of the photolysis of metronidazole (1).

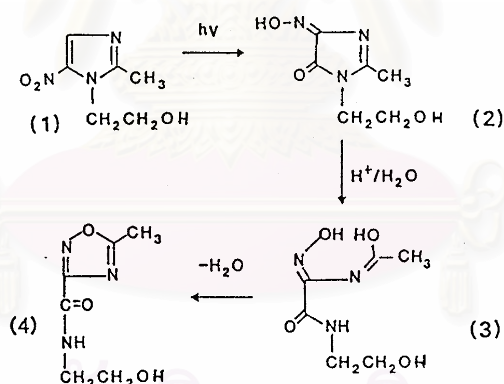

Figure 22 Reaction scheme for the photorearrangement of metronidazole
source : *Photochemistry and Photobiology* 1988, 47: 481-484

Table 20 shows the pH values of metronidazole and metronidazole:HPBCD solutions under light-protected and light-exposed condition. The pH values of both formulations in light-protected condition remained unchanged while the pH values decreased when exposed to light. It is likely to ascribe to the acidic degradation

Table 20 pH values of metronidazole under light-protected and light-exposed condition.

Time (day)	pH [a]			
	Metronidazole formulation		Metronidazole : HPBCD formulation	
	Light-protected	Light-exposed	Light-protected	Light-exposed
0	5.66 ± 0.017	5.66 ± 0.017	5.64 ± 0.000	5.64 ± 0.000
1	- [b]	5.62 ± 0.008	-	5.64 ± 0.012
3	5.63 ± 0.013	5.64 ± 0.032	5.65 ± 0.008	5.62 ± 0.017
5	-	5.57 ± 0.019	-	5.57 ± 0.022
7	5.64 ± 0.010	5.49 ± 0.021	5.66 ± 0.017	5.44 ± 0.094
10	-	5.43 ± 0.066	-	5.45 ± 0.131
14	5.62 ± 0.000	5.33 ± 0.064	5.65 ± 0.013	5.34 ± 0.165
17	-	5.32 ± 0.017	-	5.44 ± 0.095
21	5.67 ± 0.005	5.26 ± 0.157	5.68 ± 0.005	5.36 ± 0.192
28	5.64 ± 0.005	5.29 ± 0.189	5.64 ± 0.000	5.36 ± 0.037
35	5.57 ± 0.005	5.26 ± 0.196	5.58 ± 0.019	5.42 ± 0.122

a – mean ± SD , n = 4
b – not identified

Table 21 Percent remaining of metronidazole under light-protected and light-exposed condition.

Time (day)	Percent drug remaining [a]			
	Metronidazole formulation		Metronidazole : HPBCD formulation	
	Light-protected	Light-exposed	Light-protected	Light-exposed
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
1	- [b]	96.37 ± 0.35	-	95.76 ± 0.54
3	100.19 ± 0.50	87.95 ± 0.91	100.39 ± 0.22	87.48 ± 1.94
5	-	84.51 ± 0.58	-	81.12 ± 1.11
7	100.38 ± 0.90	75.72 ± 0.79	100.19 ± 1.08	72.06 ± 0.79
10	-	70.55 ± 1.25	-	65.51 ± 0.60
14	101.15 ± 0.65	57.36 ± 0.85	101.73 ± 1.45	52.60 ± 0.43
17	-	52.77 ± 2.55	-	43.74 ± 0.33
21	100.96 ± 1.10	43.02 ± 2.64	101.16 ± 0.43	31.79 ± 1.28
28	98.47 ± 0.92	30.40 ± 2.70	98.84 ± 0.42	16.38 ± 0.89
35	100.38 ± 0.71	13.96 ± 1.09	100.77 ± 1.42	4.43 ± 1.13

a – mean ± SD , n = 4
b – not identified

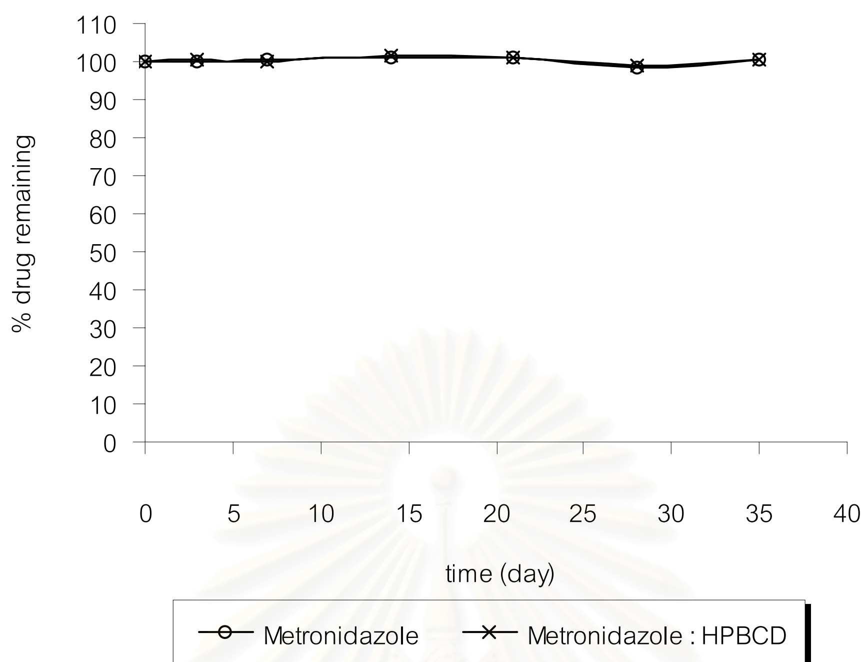

Figure 23 Percent remaining of metronidazole under light-protected condition

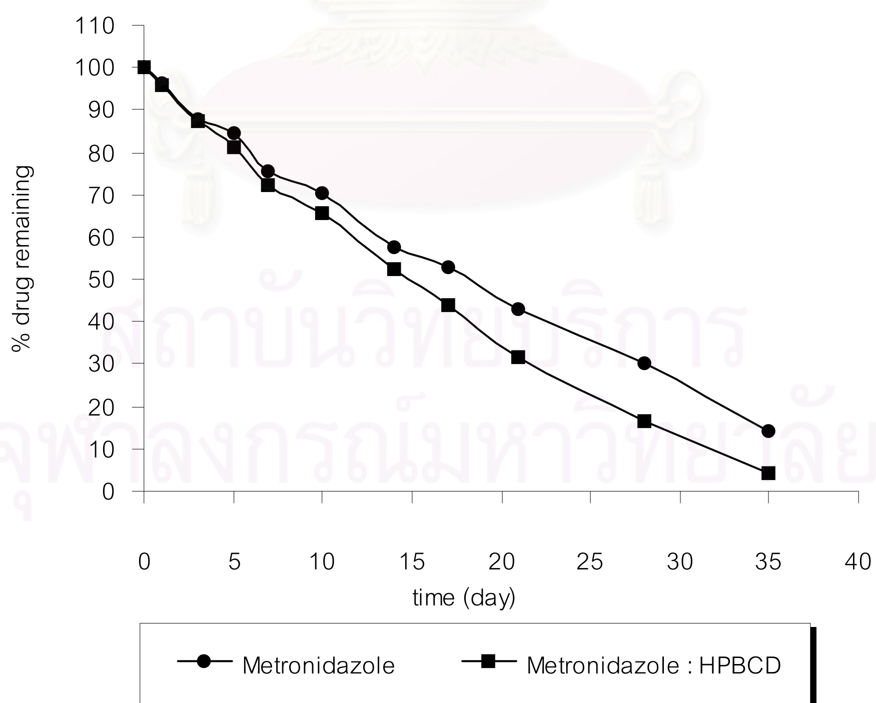

Figure 24 Percent remaining of metronidazole under light-exposed condition.

Table 22 Correlation coefficients of regression lines of metronidazole degradation under light-exposed condition

Formula	Correlation coefficient (r)	
	Zero order	First order
Metronidazole	0.9948	0.9767
Metronidazole : HPBCD	0.9927	0.9592

Table 23 The degradation rate constants of metronidazole under light-protected and light-exposed condition.

Formula	Rate constant, k_0[a] ($mg.ml^{-1}.day^{-1}$)	
	Light-protected	Light-exposed
Metronidazole	$0.0007 \pm 1.52 \times 10^{-3}$ [b]	$0.1265 \pm 4.32 \times 10^{-3}$
Metronidazole : HPBCD	$0.0001 \pm 1.63 \times 10^{-3}$	$0.1448 \pm 5.85 \times 10^{-3}$

a – k_0 was obtained from the slope of concentration vs time curve (Zero order reaction)

b – mean $\pm$ SE.; Standard error of first order rate constants (SE.) were calculated by using a linear regression

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product. Similar result was observed by Godfrey and Edwards (1991), in that the effect of light on metronidazole solution is to cause a fall in pH.

The stability data of metronidazole both free drug and in inclusion complex with HPBCD under photolysis at $60 \pm 1^{\circ}C$ is depicted in Table 21 and Figure 23-24. The plot of concentration versus time gave the higher correlation coefficient (Table 22); therefore, the degradation reaction was concluded to be zero-order. This finding is in agreement with Barnes and Sudgen (1985). However, the result is inconsistent with previous studies by Kendall et al. (1989) and Karim et al. (1991). Kendall et al. (1989) suggested that the degradation reaction of metronidazole in buffer solution (pH 9.2) under irradiation with light 360 nm followed zero order kinetic at $20^{\circ}C$ and first-order kinetic at both 48 and $63^{\circ}C$. Whereas Karim et al. (1991) have revealed that the photodecomposition of metronidazole by sunlight and a photochemical reactor appeared to followed pseudo-first-order kinetic. It might be concluded that the difference in order reaction resulted from differently experimental conditions e.g. light source, temperature.

The zero-order rate constants for both formulations in light-protected and light-exposed condition were calculated and displayed in Table 23. Since no decrease of metronidazole concentration in both formulations under light protected condition and the degradation rate constants (slope) are close to zero, their significance are tested using a simple regression, SPSS version 7.5 (as presented in Appendix V). The null hypothesis is that the slope is equal to zero. While the alternative hypothesis is that the slope is not equal to zero. The result showed that the null hypothesis was accepted ($p>0.05$) so the slope was not statistically different from zero. Consequently, from the statistical point of view, there was no significant degradation of both formulations under light-protected condition. Thus it indicated that wrapping vials with aluminum foil can protect formulations from photodegradation.

As shown in Table 23, the rate constants of both formulations under light-exposed condition were compared by using a DSA program at significant level (α) 0.05. It was found that the rate constant of metronidazole in the presence of HPBCD was significant greater than that of metronidazole alone (t-value = 2.65, $t_{0.05,df=18}$ =

2.10). This meant that HPBCD did not enhance the stability but accelerated the degradation of drug. Early studies have revealed that glucose promoted the decomposition of ampicillin by a nucleophilic reaction (Bundgaard and Larsen, 1979) and acted an accelerated effect on the rate of photolysed reaction of metronidazole at 63°C (Kendall et al., 1989). Thus, referring to structure-like glucose of HPBCD, the possible explanation is probable that hydroxy group of HPBCD may attack the structure of metronidazole by means of a nucleophilic reaction.

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CHAPTER V

CONCLUSIONS

Effects of HPBCD on the solubility and stability of metronidazole in aqueous solution were investigated and then summarized as follows:

1. The solubility of metronidzole increased as a function of HPBCD concentration and the phase solubility diagram can be classified as type A_L. The stability constant of complex was 11.24 M^{-1}, indicating a low stable complex.

2. Freeze-drying can be used to prepare the solid inclusion complex of metronidazole:HPBCD (1:2 molar ratio). From characterization of freeze-dried product, IR spectrum did not show any proof of inclusion complex formation. DSC thermogram and X-ray diffractograms gave some clues indicating that an inclusion complex formation might occur. However, formation of inclusion complex of metronidazole:HPBCD was confirmed by ^{1}H-NMR method.

3. The degradation reaction for metronidazole in the studies of effects of temperature and pH followed first-order kinetic but the photochemical degradation was said to be zero-order kinetic. However, HBCD did not affect the kinetic behaviour of those reactions.

4. The calculated shelf-life of metronidazole in the presence of HPBCD at 25°C was longer than that of metronidazole free drug.

5. HPBCD can protect metronidazole from the hydrolysis degradation by formation of an inclusion complex except at pH 3.1 where there was no significant difference in the degradation rate constant of between metronidazole and metronidazole:HPBCD solutions.

6. HPBCD has a tendency to accelerate the photodecomposition of metronidazole.

7. From this study, metronidazole can form complex with HPBCD and results in increasing solubility of metronidazole. Therefore, it is feasible to prepare a formulation of metronidazole solution having concentration more than 0.5% w/v to reduce volume when administering and also a reconstituted metronidazole powder for injection which can be produced by using HPBCD as solubilizer/stabilizer via freeze-drying technique.

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APPENDICES

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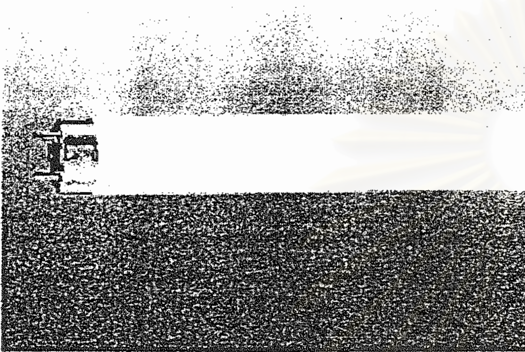
APPENDIX I

TL/05 LAMP

Special fluorescent lamps

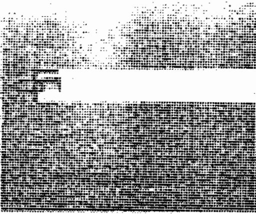
Actinic, colour /05



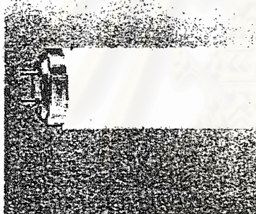
'TL'D Ø 26 mm

Tubular low-pressure mercury-vapour discharge lamps.
The tube wall is internally coated with a white fluorescent powder.
This powder converts the short wave UV radiation, produced by the mercury gas discharge, into long-wave UV radiation.
'TL' /05 lamps emit radiation between 300 and 460 nm with a maximum at 365 nm.
'TL' /05 lamps are highly efficient for use in diazo printing machines.
The spectral energy distribution of 'TL' /05 lamps attracts insects, making these lamps suitable for use in insect traps as well.

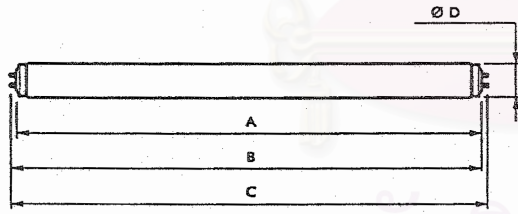
Applications
- Diazo printing machines.
- Insect traps.
- Photochemical processes.



'TL' Miniature



'TL' Ø 38 mm



Dimensions in mm

Type	A max.	B min.	B max.	C max.	D max.
Cap/base G5					
'TL' 4W /05	135.9	140.6	143.0	150.1	16.0
'TL' 6W /05	212.1	216.8	219.2	226.3	16.0
'TL' 8W /05	268.3	293.0	295.4	302.5	16.0
'TL' 11W /05	212.1	216.8	219.2	226.3	16.0
Cap/base G13					
'TL'D 15W /05	437.4	442.1	444.5	451.6	28.0
'TL' 20W /05	589.8	594.5	596.9	604.0	40.5
'TL'DK 30W /05	437.4	442.1	444.5	451.6	28.0
'TL' 40W /05	1199.4	1204.1	1206.5	1213.6	40.5
'TL'K 40W /05	589.8	594.5	596.9	604.0	40.5
'TL' 80W /05	1500.0	1504.7	1507.1	1514.2	40.5
'TL' 140W /05	1500.0	1504.7	1507.1	1514.2	40.5

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Type	Cap/ base	Lamp voltage V	Lamp current A	Diazo watts W	UV-A radiation W	Useful life h	Depreciation 2000 hrs %	Nett weight g	Ordering number	EOC
'TL' 4W /05	G5	29	0.17	0.25	0.20	2000	25	16	9280 000 00500	634801
'TL' 6W /05	G5	42	0.16	0.56	0.70	2000	25	22	9280 005 00500	715616
'TL' 8W /05	G5	56	0.15	0.82	1.00	2000	25	29	9280 010 00500	704436
'TL' 11W /05	G5	37	0.33	0.91	1.15	1000	50	22	9280 006 00500	643063
'TL'D 15W /05	G13	51	0.34	1.70	2.10	3000	20	76	9280 248 00500	704443
'TL' 20W /05	G13	57	0.37	2.20	2.80	3000	15	156	9280 035 00500	704467
'TL'DK 30W /05	G13	45	0.81	3.00	3.70	2000	25	76	9280 195 00500	704450
'TL' 40W /05	G13	107	0.43	5.80	7.00	3000	15	292	9280 060 00500	704481
'TL'K 40W /05	G13	50	0.86	4.00	5.00	2000	30	156	9280 291 00500	704474
'TL' 80W /05	G13	111	0.83	10.90	13.90	2000	30	360	9280 083 00500	704498
'TL' 140W /05	G13	118	1.46	19.00	20.00	2000	35	360	9280 127 00500	643049

PHILIPS

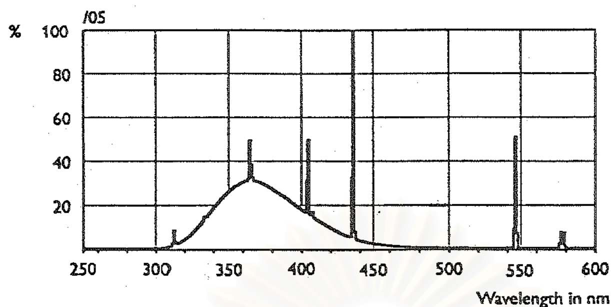

Figure 25 Spectral power distribution of TL/05 lamp

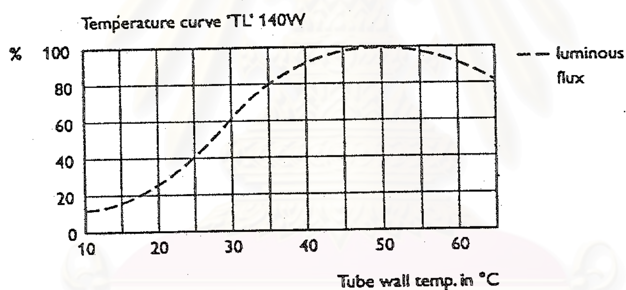

Figure 26 Temperature dependency diagram of TL/05 lamp

APPENDIX II

UV SCAN

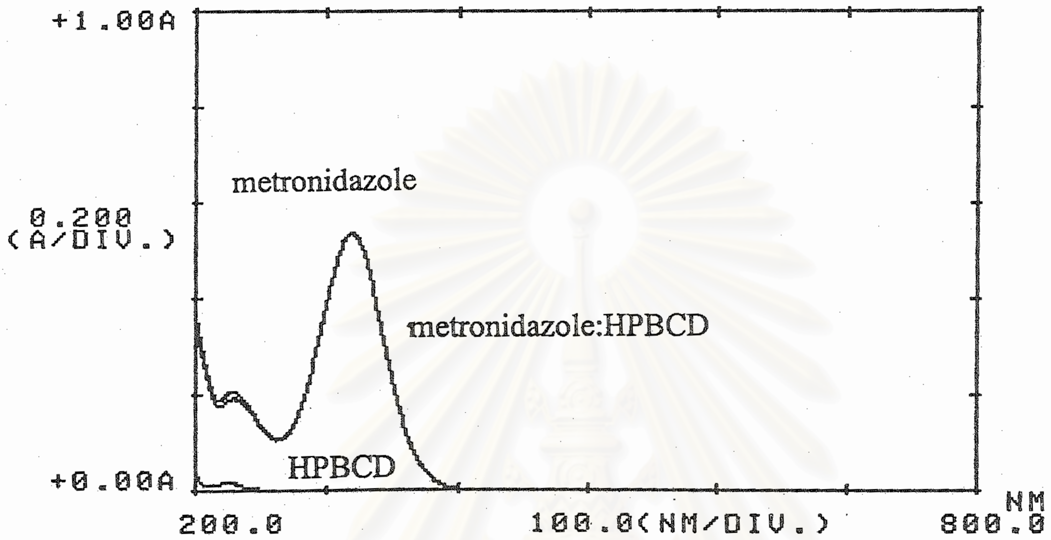

Figure 27 UV scanning spectrum of metronidazole, HPBCD and metronidazole:HPBCD

APPENDIX III

IR SPECTRA

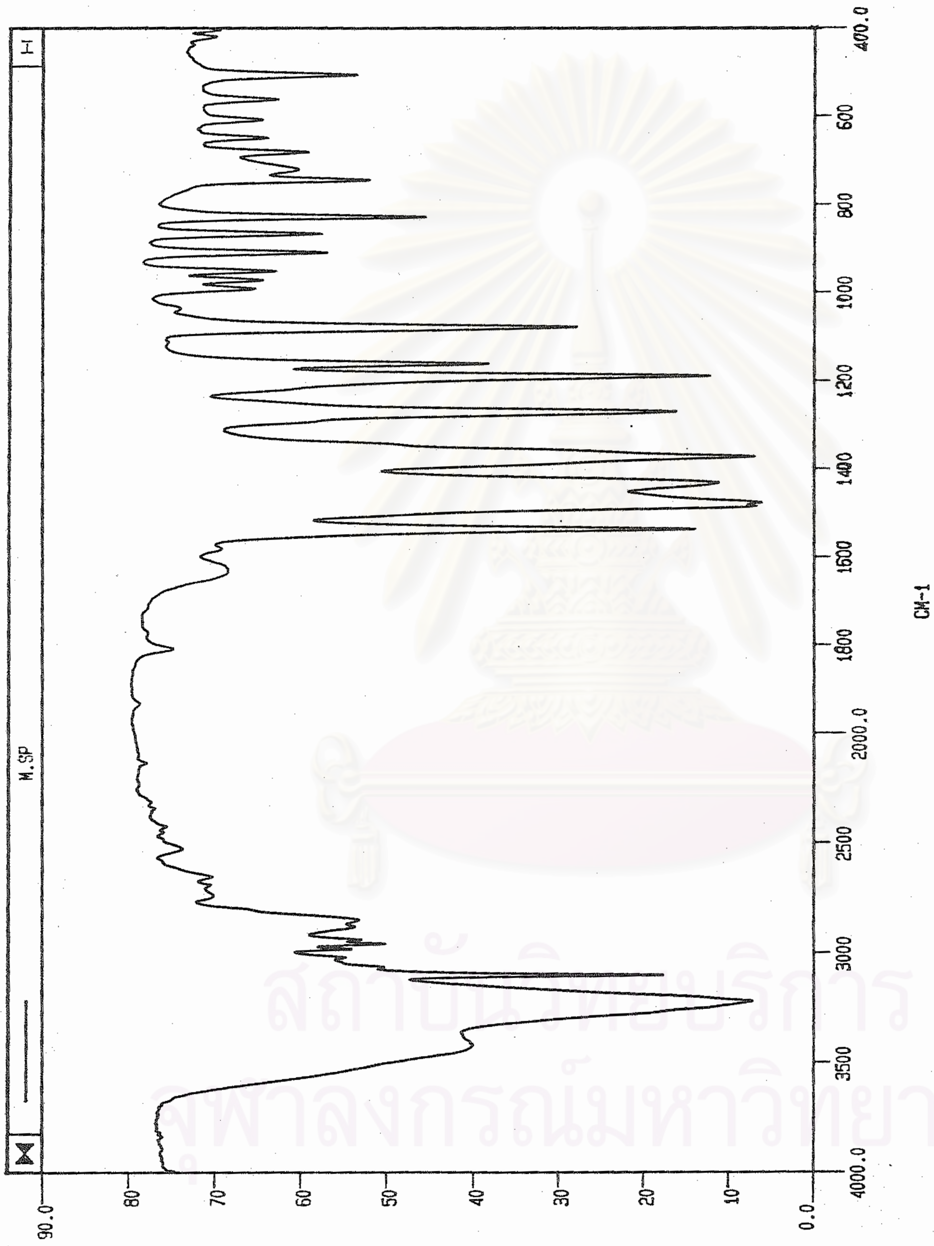

Figure 28 Infrared spectrum of metronidazole

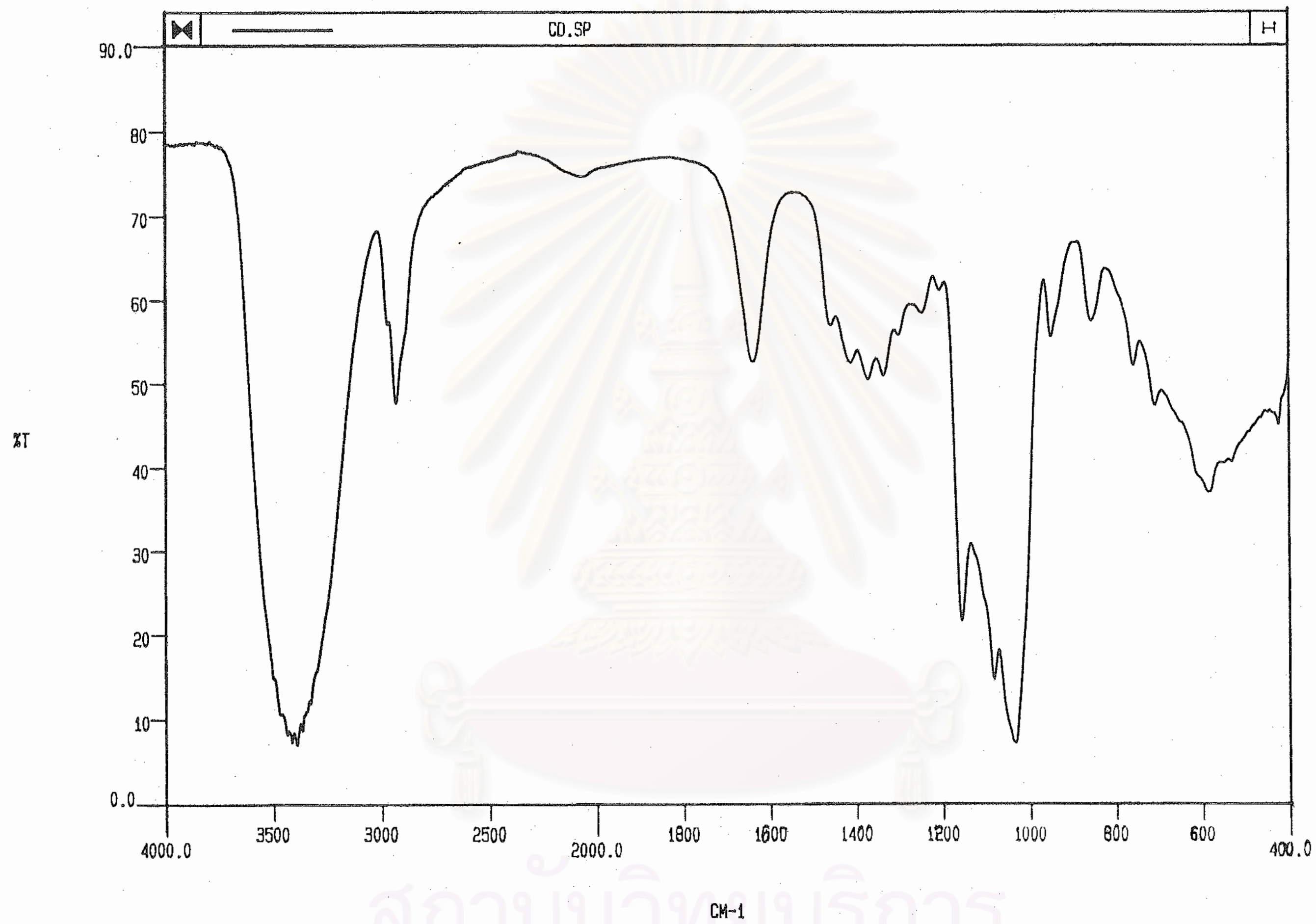

Figure 29 Infrared spectrum of HPBCD

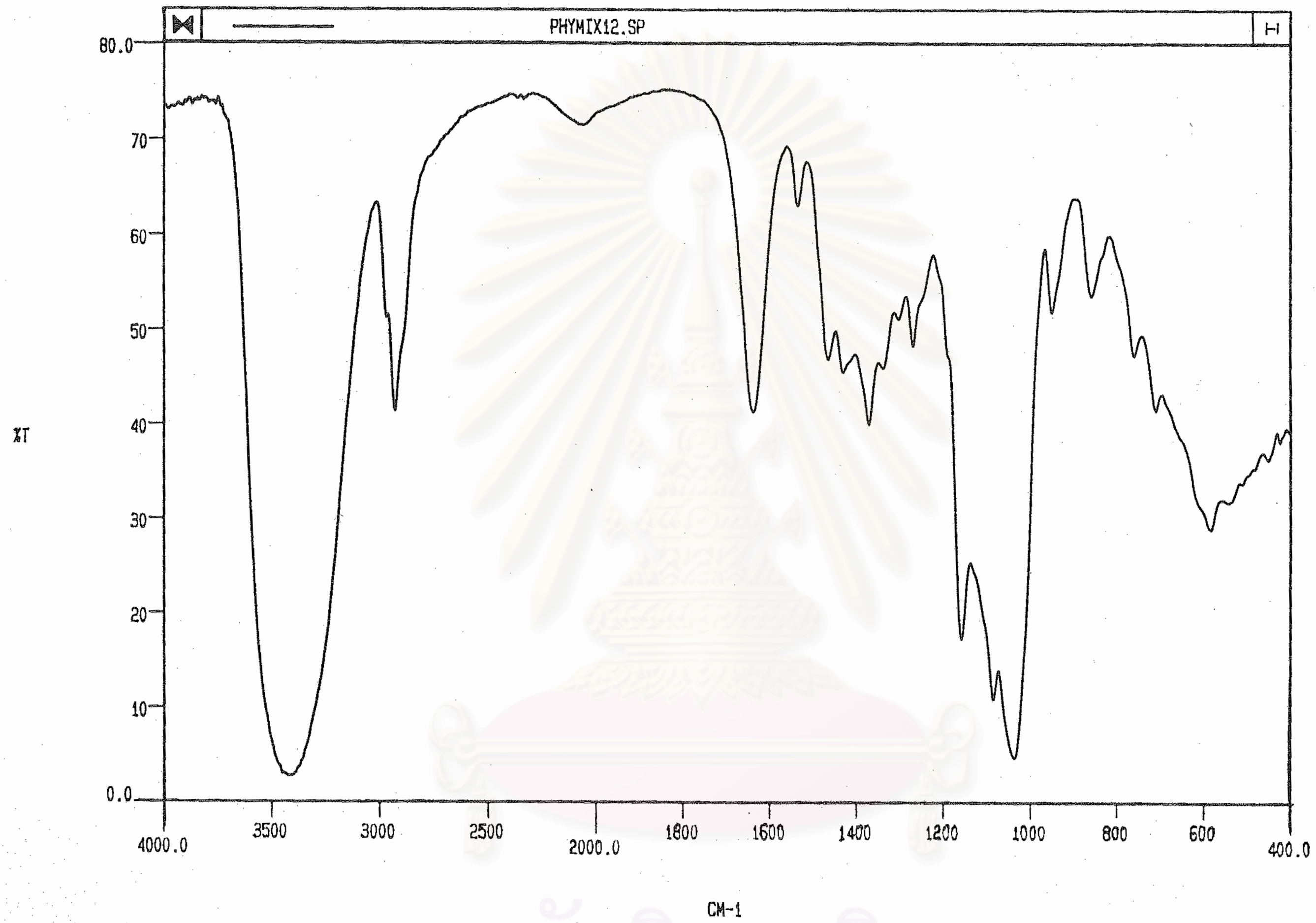

Figure 30 Infrared spectrum of physical mixture of metronidazole and HPBCD

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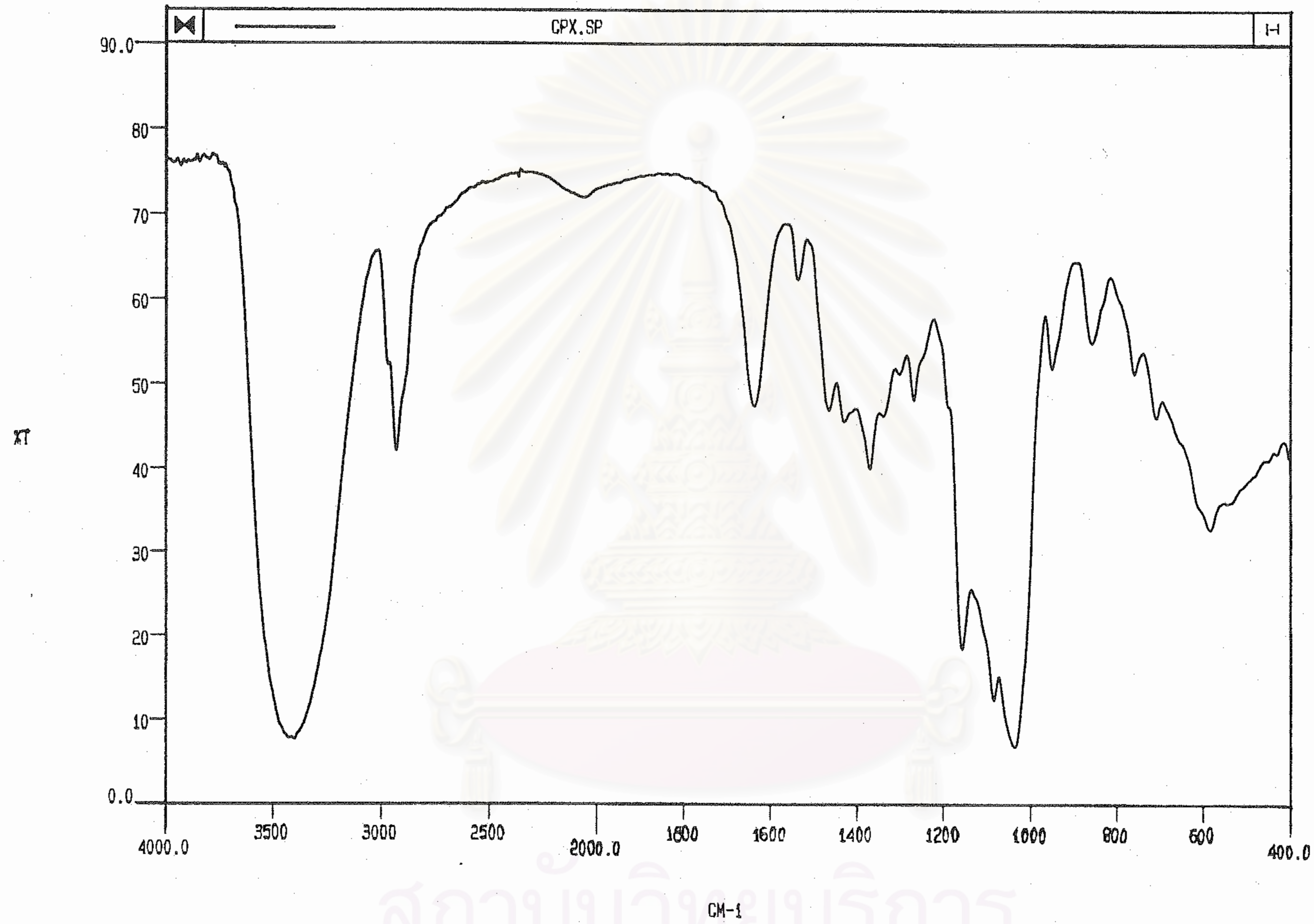

Figure 31 Infrared spectrum of metronidazole:HPBCD complex

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APPENDIX IV

^{1}H-NMR SPECTRA

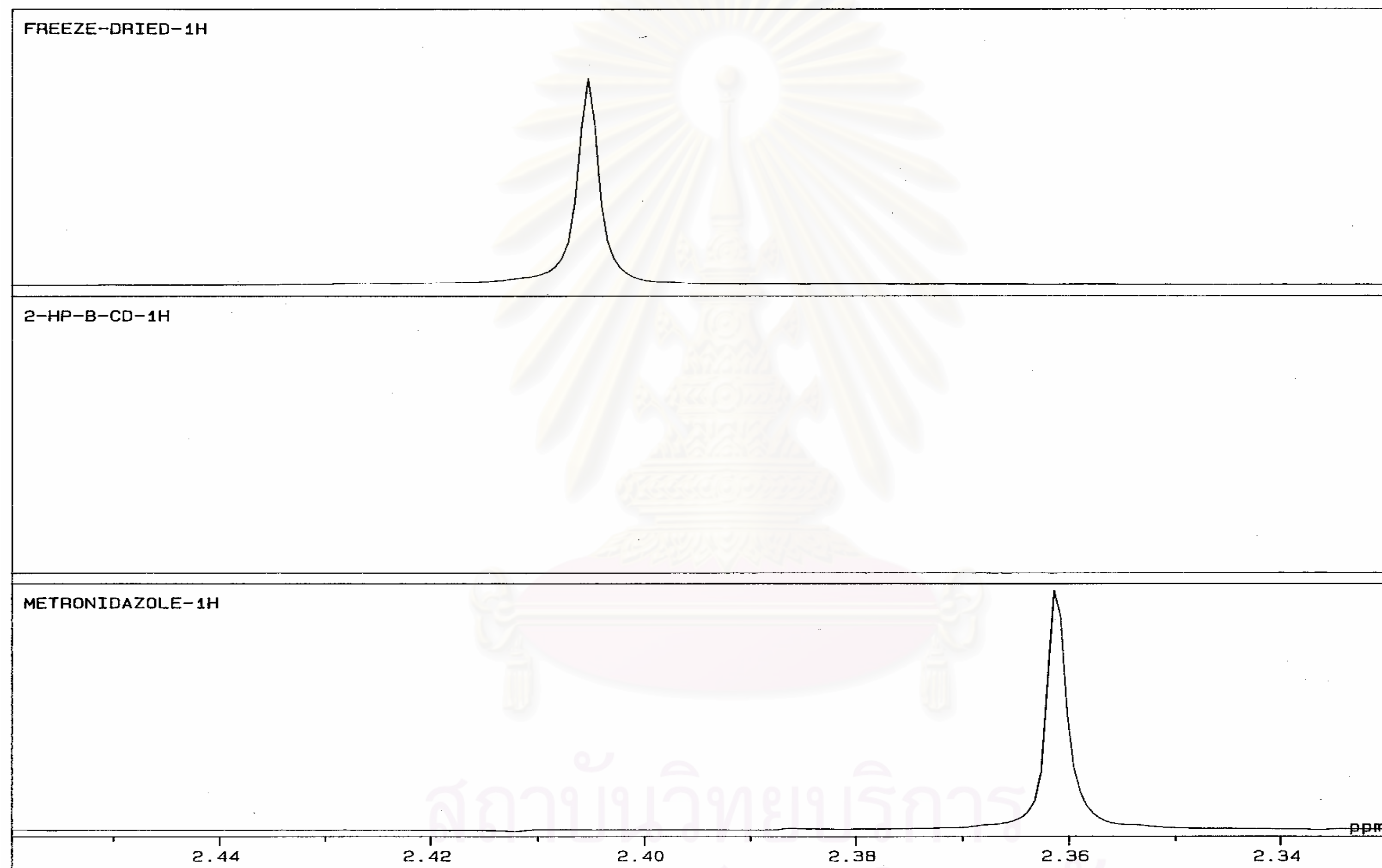

Figure 32 Comparison of ^{1}H-NMR spectra in the range of 2.33-2.46 ppm

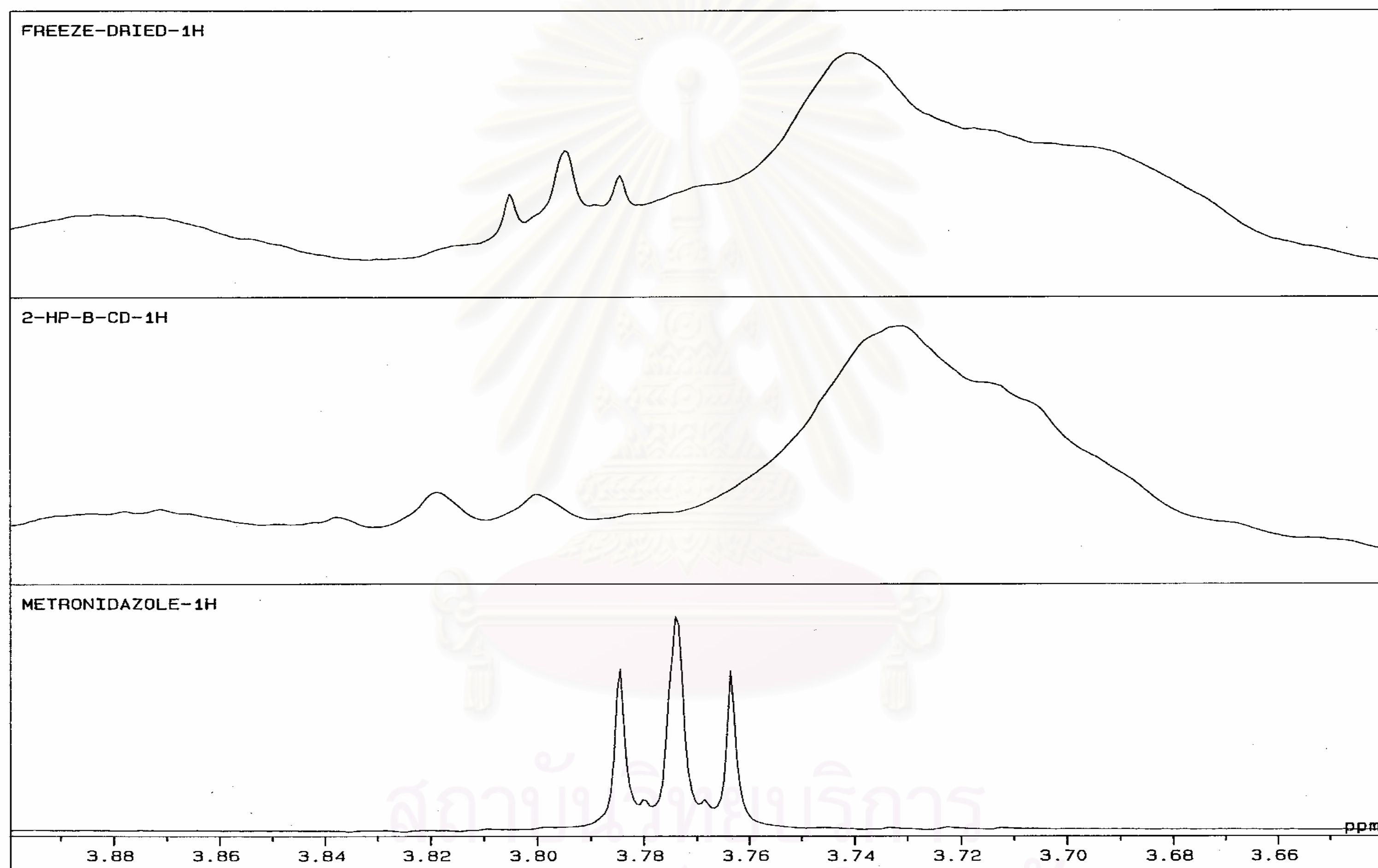

Figure 33 Comparison of ^{1}H-NMR spectra in the range of 3.64-3.90 ppm

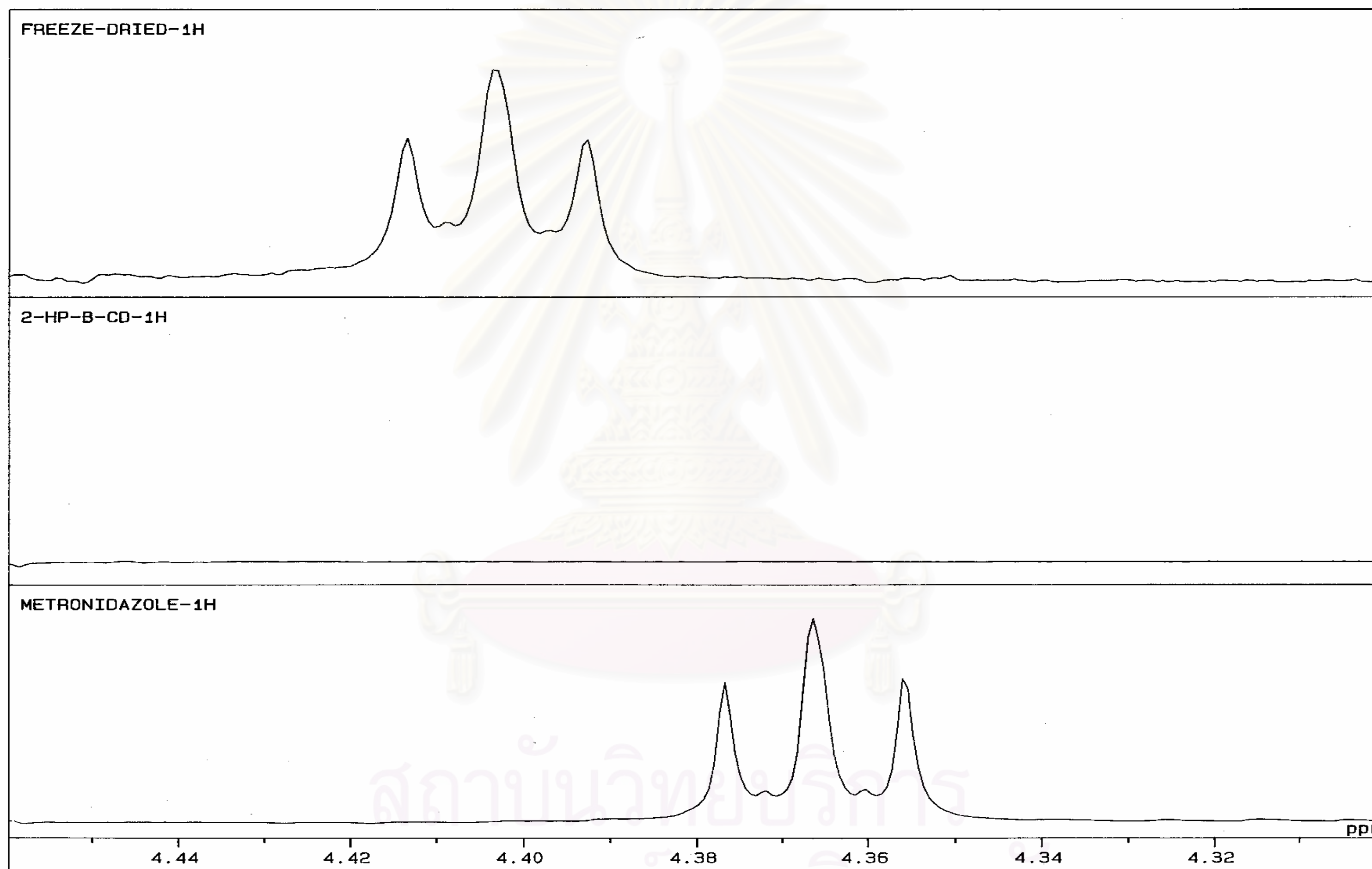

Figure 34 Comparison of ^{1}H-NMR spectra in the range of 4.30-4.46 ppm

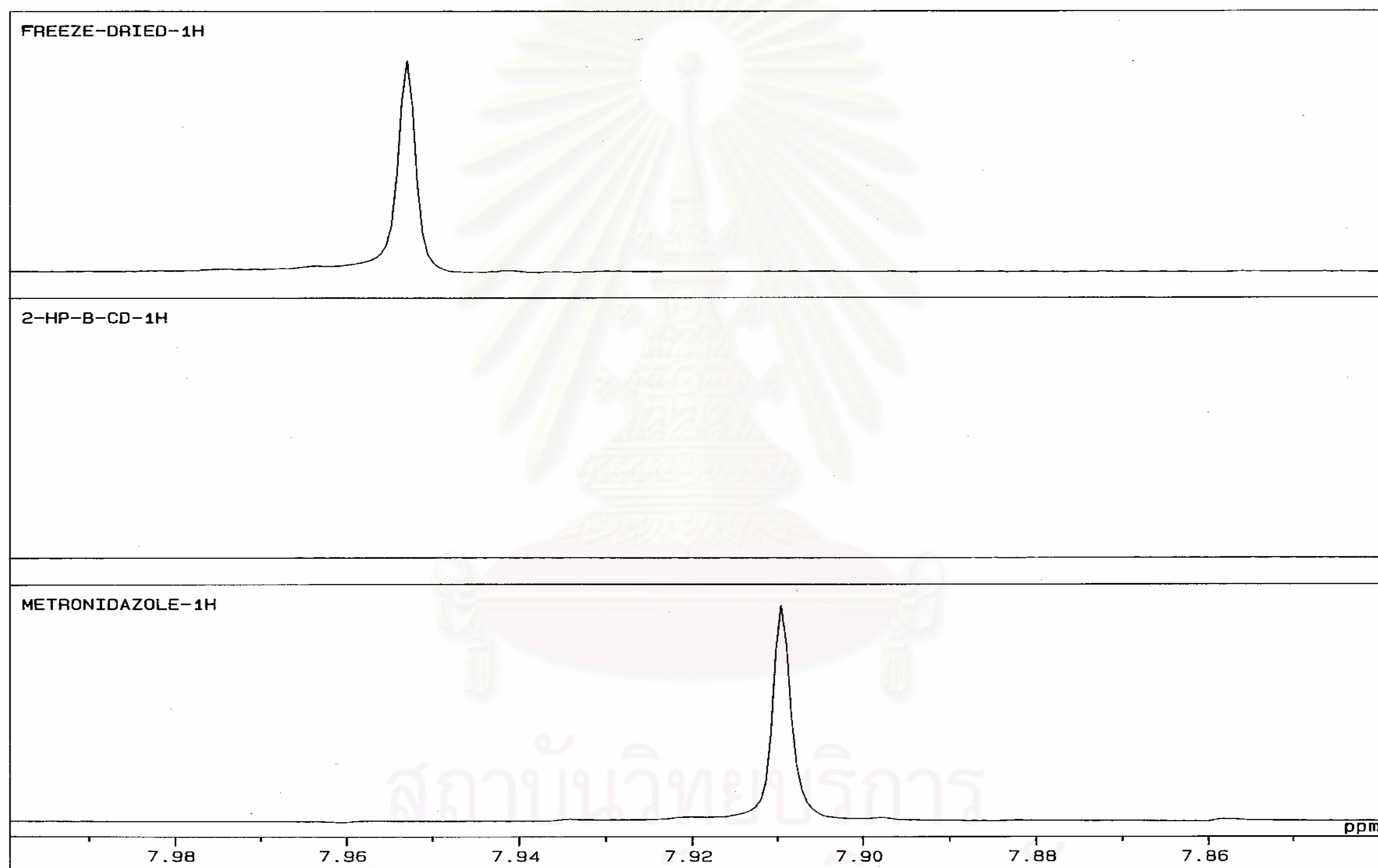

Figure 35 Comparison of ^{1}H-NMR spectra in the range of 7.84-8.00 ppm

APPENDIX V

STATISTICS

Metronidazole formulation in light-protected condition

Curve Fit

Dependent variable.. CONC Method.. LINEAR

Multiple R	.19844
R Square	.03938
Adjusted R Square	-.15275
Standard Error	.04898

Analysis of Variance:

	DF	Sum of Squares	Mean Square
Regression	1	.00049166	.00049166
Residuals	5	.01199405	.00239881

F = .20496 Signif F = .6697

-------------------- Variables in the Equation --------------------

Variable	B	SE B	Beta	T	Sig T
DAY	-.000688	.001520	-.198439	-.453	.6697
(Constant)	5.252048	.029882		175.758	.0000

Metronidazole:HPBCD formulation in light-protected condition

Curve Fit

Dependent variable.. CONCMC Method.. LINEAR

Multiple R	.02610
R Square	.00068
Adjusted R Square	-.19918
Standard Error	.05241

Analysis of Variance:

	DF	Sum of Squares	Mean Square
Regression	1	.00000936	.00000936
Residuals	5	.01373349	.00274670

F = .00341 Signif F = .9557

-------------------- Variables in the Equation --------------------

Variable	B	SE B	Beta	T	Sig T
DAY	-9.49889868E-05	.001627	-.026102	-.058	.9557
(Constant)	5.214323	.031976		163.071	.0000

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VITAE

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