วิธีไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโตกราฟีเพื่อวิเคราะห์หาปริมาณของยาฆ่าเชื้อรากลุ่มอิมิดาโซล เมื่อมีสารสลายตัวในครีมชนิดน้ำมันในน้ำ

นางสาวสิริวรรณ ทิวทอง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF IMIDAZOLE ANTIMYCOTIC DRUGS IN THE PRESENCE OF DEGRADED PRODUCTS IN OIL-IN-WATER CREAM

Miss Siriwan Thiwthong

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By	Miss Siriwan Thiwthong
Field of study	Pharmaceutical Chemistry
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Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master 's Degree

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สิริวรรณ ทิวทอง : วิธีไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโตกราฟีเพื่อวิเคราะห์หาปริมาณของยาฆ่าเชื้อรากลุ่มอิมิดาโซลเมื่อมีสารสลายตัวในครีมชนิดน้ำมันในน้ำ.
(HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF IMIDAZOLE ANTIMYCOTIC DRUGS IN THE PRESENCE OF DEGRADED PRODUCTS IN OIL-IN-WATER CREAM)
อ. ที่ปรึกษา : ผศ.ดร. อุษา กล้ากสิกิจ, 67 หน้า. ISBN 974-17-4304-1.

วิธีไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโตกราฟี(เอชพีแอลซี) ซึ่งเป็นวิธีที่ง่ายและใช้บ่งถึงความคงตัวของสารได้รับการพัฒนาขึ้นมาเพื่อวิเคราะห์แยกยาฆ่าเชื้อรากลุ่มอิมิดาโซล โคลไตรมาโซล และคีโตโคนาโซล ออกจากสารสลายตัว สารอื่นๆ ในสูตร สิ่งปนปลอม ในตัวยาและครีม ส่วนประกอบที่เหมาะสมของโมบายเฟสที่ใช้คือ 2.5 มิลลิโมลาร์ แอมโมเนียมไดไฮโดรเจนฟอสเฟต พีเอช 7.0 ผสมกับเมธานอลในอัตราส่วน 3 ต่อ 7 โดยปริมาตร สเตชันเนรีเฟสเป็นซี 18 รีเวอร์สเฟสคอลัมน์โดยตรวจวัดที่ความยาวคลื่น 230 นาโนเมตร วิธีเอชพีแอลซีที่นำเสนอถูกนำมาใช้ในการวิเคราะห์สารตัวอย่างซึ่งประกอบด้วยตัวยาฆ่าเชื้อราบริสุทธิ์และสารสลายตัวโดยเปรียบเทียบกับวิธีของบีพี 2002 และยูเอสพี 26 กับ เอนเอฟ 21 จากการศึกษาพบว่าวิธีเอชพีแอลซีที่นำเสนอให้ผลการทดลองที่ดีกว่าวิธีมาตรฐานจากเภสัชตำรับ ในการวิเคราะห์ยาฆ่าเชื้อราในครีมพบว่าเป็นสิ่งจำเป็นที่ต้องแยกโคลไตรมาโซลและคีโตโคนาโซลออกจากสารอื่นในครีมก่อนการฉีดสารเข้าเครื่องมือเอชพีแอลซีโดยใช้เวลาในการให้คลื่นความถี่สูงและเวลาในการทำให้ครีมแข็งตัวที่เหมาะสม จากการศึกษาความคงตัวของยาฆ่าเชื้อราในครีมซึ่งมีความเป็นกรดด่างต่างกันพบว่าโคลไตรมาโซลให้ความคงตัวดีในพีเอชระหว่าง 4 ถึง 8 และคีโตโคนาโซลจากพีเอช 3 ถึง 8 จากการทำวาลิเดชันของวิธีเอชพีแอลซีพบว่าให้ความสัมพันธ์เชิงเส้นตรงในช่วงความเข้มข้นระหว่าง 50 ถึง 150 ไมโครกรัมต่อมิลลิลิตรสำหรับโคลไตรมาโซล และ 100 ถึง 300 ไมโครกรัมต่อมิลลิลิตรสำหรับคีโตโคนาโซล และให้ความแม่นยำของวิธีวิเคราะห์สูงโดยมีค่าเบี่ยงเบนมาตรฐานสัมพัทธ์ของยาทั้งคู่ไม่เกิน 1 เปอร์เซ็นต์ ไม่พบสารสลายตัวในโคลไตรมาโซลและคีโตโคนาโซลครีมซึ่งเก็บไว้ที่อุณหภูมิ 40 ± 1 องศาเซลเซียส ความชื้นสัมพัทธ์ 75 ± 5 เปอร์เซ็นต์ เป็นเวลานาน 6 เดือน วิธีการเอชพีแอลซีที่นำเสนออาจสามารถนำไปใช้โดยให้ผลเป็นที่น่าพอใจในการวิเคราะห์หาปริมาณของยาตัวอื่นในกลุ่มอิมิดาโซล

ภาควิชา เภสัชเคมี	ลายมือชื่อนิสิต
สาขาวิชา เภสัชเคมี	ลายมือชื่ออาจารย์ที่ปรึกษา
ปีการศึกษา 2546	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม -

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SIRIWAN THIWTHONG : HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF IMIDAZOLE ANTIMYCOTIC DRUGS IN THE PRESENCE OF DEGRADED PRODUCTS IN OIL-IN-WATER CREAM.
THESIS ADVISOR : USA GLAGASIGIJ, Ph. D., 67 pp. ISBN 974-17-4304-1.

A simple stability-indicating high performance liquid chromatographic (HPLC) method has been developed which separates imidazole antimycotic drug, clotrimazole and ketoconazole from decomposition products, other ingredients, impurities in bulk drug and cream. The suitable composition of mobile phase was found to be 2.5 mM ammonium dihydrogen phosphate pH 7.0 in methanol (3:7, v/v) with C_{18} reversed-phase column as a stationary phase and UV detection at 230 nm. The proposed HPLC assay methods was used to analyze sample composed of pure antimycotic drugs and their degraded products, in comparison with BP 2002, USP 26 and NF 21. From the study obtained, the developed HPLC assay method exhibited better results than official pharmacopeial methods. In analysis of antimycotic drugs in cream, it was necessary to separate clotrimazole and ketoconazole from other ingredients in cream base before injection to HPLC instrument by using suitable sonication and freezing time. The stability of antimycotic drugs in cream was studied as a function of pH and was found to be stable at pH 4 to 8 for clotrimazole and pH 3 to 8 for ketoconazole. The HPLC method was validated and linearity was found in range 50 - 150 μg/ml (clotrimazole) and 100 – 300 μg/ml (ketoconazole) with good precision, not more than 1 % RSD for both drugs. No degradation product was found in clotrimazole and ketoconazole cream stored at 40 ± 1 °C, 75 ± 5 % RH for six months. The proposed HPLC method might be applied satisfactorily in quantitative analysis of another drugs in imidazole group.

Department Pharmaceutical Chemistry . Student's signature .....
Field of study Pharmaceutical Chemistry . Advisor's signature .....
Acadamic year 2003 . Co-advisor's signature - .

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LIST OF ABBREVIATIONS

%	percentage
μm	micron or micrometer
μl	microliter
°C	degree Celcius
cm	centrimeter
CV	coefficient of variation
g	gram
HPLC	high-performance liquid chromatography
hr	hour
mM	millimolar
M	molar
mg	milligram
min	minute
ml	milliliter
mm	millimeter
nm	nanometer
o/w	oil-in-water
r^2	coefficient of correlation
RH	relative humidity
RSD	relative standard deviation
TLC	thin layer chromatography
t_r	retention time
UV	ultraviolet
vol.	volume
v/v	volume by volume
w/o	water-in-oil

CHAPTER I

INTRODUCTION

Imidazole antimycotic drugs constitute an important class of drugs that continues to expand. These antimycotics are currently used in a variety of pharmaceutical formulation such as tablets, creams, lotions, etc. at relatively low concentrations (1-2 %)(Di Pietra et al., 1992). Imidazole derivatives, such as clotrimazole and ketoconazole are widely used in the treatment of fungal infections and their antifungal activity are the alteration of cellular membrane, resulting in increased membrane permeability, secondary metabolic effects and finally following by growth inhibition (Heel et al., 1982; McEvoy, ed., 1989). Besides the fungistatic activity of the drug may result from interference with ergosterol synthesis (McEvoy, ed., 1989).

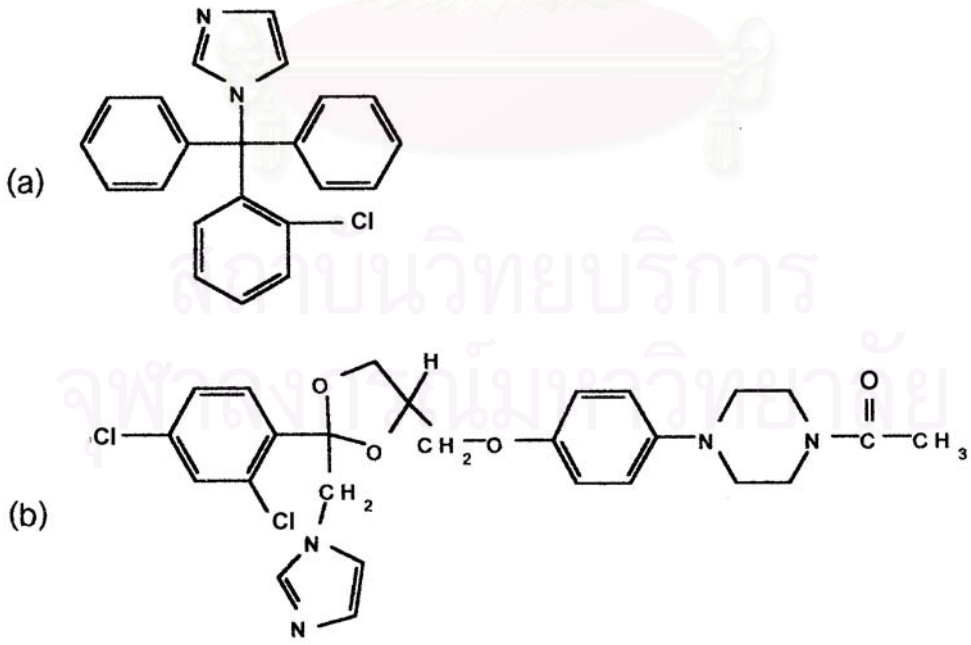

Figure 1 Structure of clotrimazole (a) and ketoconazole (b).

Clotrimazole is a broad-spectrum antimycotic drug effective against pathogenic dermatophytes, yeasts, and several species of Candida, Trichophytor, Microsporum, Epidermophyton and Malassezia. Preparations of the drug are used both in the topical treatment of dermal infections and to combat candidiasis (Hoogerheide and Wyka, 1982).

Ketoconazole is active against most pathogenic fungi, including dermatophytes and yeasts so it is used to treat a wide variety of superficial or systemic fungal infection. It is effective after oral administration against superficial mycoses e.g., dermatophyte or yeast skin infection, oral vaginal candidosis and systemic mycoses e.g., systemic candidosis, paracoccidioidomycoses, histoplasmosis (Heel et al., 1982; Borger and Bossche, 1982).

In field of medical, pharmaceutical and cosmetic sciences, cream is the semisolid emulsion which can be prepared either oil-in-water (o/w) or water-in-oil (w/o) emulsions. Because of complex composition of cream formulations, effectively and specifically quantitative analytical method of active ingredient is necessary and essential to discriminate each active ingredient from cream base. Since some drugs may decompose in process or after formulation due to interaction with some ingredients in product or by method of preparation. Thus, the assay method should be clearly distinguish active ingredients from their degraded products if exist (Carstensen and Rhodes, 2000).

The major mechanisms of the decomposition of drugs are summarised in Table 1 (Stewart and Tucker, 1984; 1985).

Table 1 Mechanism of drug decomposition in solution.

Mechanism	Comment
Hydrolysis	Involves the interaction of drugs with water; includes drugs which are esters, amides, lactones or lactams; catalyzed by hydrogen or hydroxyl ions.
Oxidation	Involves chain reactions under the influence of molecular oxygen or the reversible loss of electrons; catalyzed by oxygen, heavy metal ions and light.
Isomerisation	Involves conversion of a drug into its optical isomer which may be biologically inactive or possess reduced activity.
Photolysis	Involves drug decomposition under the influence of ultraviolet light often resulting in discolouration of formulations.
Polymerisation	Involves the composition of two or more drug molecules to produce a complex molecule.

From literature survey, it suggests that the chromatographic technique is one of the favourite approach for the quality control of the active ingredients since chromatography under appropriate selection can be effectively used to analyze required drugs from other ingredients and their degraded products (Fong and Lam, 1991; Di Pietra et al., 1992). High-performance liquid chromatographic (HPLC) method is one of primarily separation and analytical method to detect and quantify analytes in less or more complex mixtures and matrices (Neue, 1997). Moreover, HPLC is also used as stability-indicating method, identification and quantitative analytical method for impurity and

degraded products (Fong and Lam, 1991; Grim, 1987; Ahuja, 1998; Golden et al., 1996; Carstensen and Rhodes, 2000).

Clotrimazole is a weak base with pKa 4.7. It is stable in the solid state under normal storage conditions or by heat (up to 70 degree Celsius) or by exposure to daylight (up to two weeks). The primary degradation products of clotrimazole results from hydrolysis of imidazole group to 2-chlorotritanol (Hoogerheide et al., 1981).

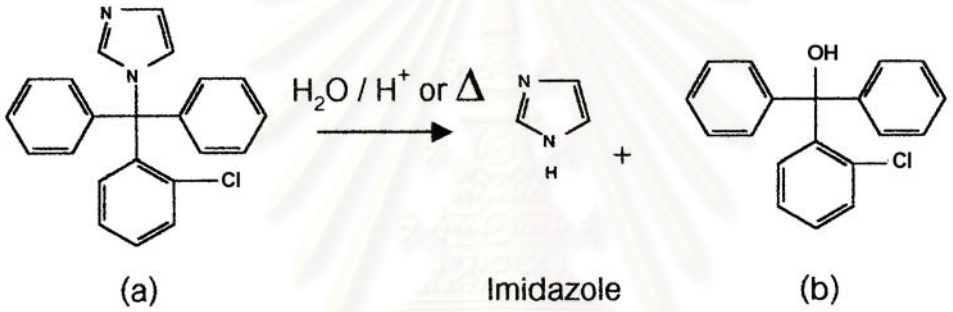

Figure 2 Degradation pathway of clotrimazole: (a) clotrimazole, (b) 2-chlorotritanol.

Ketoconazole is a weak base with two pKa, 2.9 and 6.5. It may undergo degradation including acid-catalysis hydrolysis (Skiba, 2000; Kumer, 1991), oxidation (Skiba et al., 2000) or photolysis (Thoma and Kubler, 1996). The major of photodegradation product of ketoconazole is 1- acetyl-4-[4-[(1H-imidazo[2,1-a]3,4-dihydro-7-chloro-isoquinolyl)-6-spiro-2′-(1,3-dioxan-4-yl)]methoxy]phenyl]piperazine (Nuntanakorn, 1996).

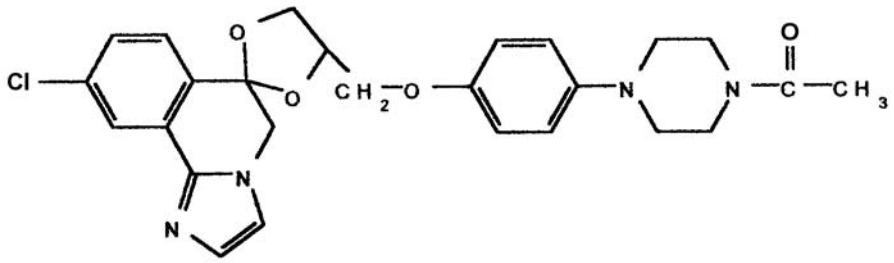

Figure 3 The structure of 1- acetyl-4-[4-[(1H-imidazo[2,1-a]3,4-dihydro-7-chloro-isoquinolyl)-6-spiro-2′-(1,3-dioxan-4-yl)]methoxy] phenyl]piperazine.

From the literature reviews of both drugs, there were various HPLC investigation methods of clotrimazole and ketoconazole in many dosage froms e.g., solution, tablet but with a few in cream (Allen and Martin, 1996; Cavrini et al., 1982; Di Pietra et al., 1992; Xu, 1999).

In pharmacopieal method (BP 2002), clotrimazole is analyzed separately from its degraded product, 2-chlorotritanol by HPLC method using Lichrosorb-RP18 or Spherisorb ODS as a stationary phase and 0.02 M orthophosphoric acid-methanol (3:7, by vol.) adjusted to pH 7.5 with 10 % v/v triethylamine in methanol as a mobile phase, however, their resolution is quite low. In USP 26 and NF 21, 10 μm C_{18} column and 0.025 M dibasic potassium phosphate and methanol (1:3) are used as a stationary phase and mobile phase, respectively. For ketoconazole, the analytical method in USP26 and NF 21 is applied only to solution and tablet with none in BP 2002. Moreover, there is no pharmacopeial HPLC method (BP 2002, USP 26 and NF 21) to analyze ketoconazole quantitatively from its degraded product, therefore, it is interesting to investigate whether the proposed HPLC method can analyzed ketoconazole from its degraded products in cream preparation.

Thus, it is necessary to find appropriate separation method and suitable HPLC systems which can be effectively used in analysis both clotrimazole and ketoconazole separately from their degraded products and also from another interferences in drug formulations. Since cream is a favourite dosage form for antimycotic drugs, however, many ingredients in cream base generally interfere quantitative assay of active ingredient. In this research, the effect of ingredients in cream base is studied by incorporating clotrimazole and ketoconazole to oil-in-water cream base of known compositions with concentration by weight, 1 and 2 percent, respectively. Consequently, the pH of preparation is varied to study stability of drug and amount of active ingredients is analyzed by using developed method described earlier. The HPLC method proposed here should be used effectively to quantitative analysis of clotrimazole and ketoconazole, separately from their degraded product for satisfactorily therapeutic effect. The method may be applied to used in quality control of another imidazole antimycotic drugs. Since the proposed method can be used in quantitative analysis both clotrimazole and ketoconazole, thus it saves a lot of time in preparation of mobile phase and regeneration the column after finishing assay of one drug, following by the other and also reduces a cost of analysis.

CHAPTER II

EXPERIMENTS

Apparatus

1. High-performance liquid chromatograph (Waters Association, Milford, MA, U.S.A.) compose of
    a. Model 600E Multisolvent delivery system
    b. Model 484 Tunable absorbance detector (2.0 a.u.f.s.)
    c. Model 746 Integrator
    d. Waters intelligent sample processor (WISPTM) (autosampler, 20 μl injection)
2. Analytical column, 25 cm x 4.6 mm i.d., 5 μm C_{18} reversed-phase column (Apollo, Alltech Associates, Inc.)
3. Guard column, 2 cm x 2 mm, 40μm C_{18} pellicular particle (Upchurch, UK)
4. Ultraviolet-visible spectrophotometer (Milton Roy Spectronic 3000 array)
5. pH meter (Consort C231)
6. Sonicator bath (Transsonic digitals, ELMA, Germany)
7. Thin layer chromatographic plate (Silica gel GF_{254}, 0.25 mm, 3 x 7 cm, Merck, Germany)
8. Incubator with humidity controller (Model 435314, Hotpack, U.S.A.)

Meterials

1. Clotrimazole was kindly donated by Government Pharmaceutical Organization, Thailand)
2. Ketoconazole was a gift from Pharmasant Laboratories Co., Ltd
3. 2-Chlorotritanol (Bristish Pharmacopoeia reference, UK)

4. Cetyl alcohol
5. Stearyl alcohol
6. Glyceryl monostearate
7. Span 60
8. Methyl paraben
9. Propyl paraben
10. Polyethylene glycol 400
11. Liquid paraffin
12. Glycerin
13. Tween 60

Reagents from 4. to 13. were of pharmaceutical grade and kindly donated by Government Pharmaceutical Organization, Thailand. Other reagents used in the experiments were of analytical grade or better

14. Methanol (Lab Scan, Thailand)
15. Ammonium dihydrogen phosphate (Merck, Germany)

Methods

The experiments were performed in the following sequences

1. Physicochemical effects on degradation of active ingredients
2. Suitable HPLC system and sample pretreatment
3. Method validation
4. Stability test on oil-in-water cream
5. Assay application

1. Physicochemical effects on degradation of active ingredients

1.1. Effect of acid-base catalysis on clotrimazole and ketoconazole degradation

1.1.1. Effect on clotrimazole

Acid catalysis was studied by modification of the method by Skiba et al., 2000. The 5 % w/v clotrimazole was prepared in 10 ml 1 N sulfuric acid and the solution was warmed on boilng water bath for 24 hours. Thereafter, the solution was neutralized with 1 N sodium hydroxide to basic to litmus paper and the final solution was evaporated to dryness on boiling water bath.

Base catalysis was performed with the same procedure but in 1 N sodium hydroxide.

1.1.2. Effect on ketoconazole

The experiments were done as earlier described in clotrimazole.

1.2. Effect of photolysis on clotrimazole and ketoconazole degradation

1.2.1. Effect on clotrimazole

Photodegraded product was prepared by modification of the method by Kumer et al., 1991. Methanolic solution of clotrimazole (5 mg/ml) was irradiated under UV lamp (254 nm) at room temperature for 120 hours. Then, the solvent was removed by using rotary evaporator under reduced pressure at room temperture until dried residue was obtained. Degraded product obtained was analyzed by the thin layer chromatography (Figure 4 and Figure 5) using silica gel GF_{254} as stationary phase and iodine detection.

1.2.2. Effect on ketoconazole

The experiments were done as earlier described in clotrimazole.

2. Suitable HPLC system and sample pretreatment

Reagent solution :

A. 0.01 M Phosphate buffer solution

Accurately weighed 1.15 g of ammonium dihydrogen phosphate [$(NH_4)H_2PO_4$] to a 1000-ml volumetric flask and adjusted to volume with water.

B. Stock solution of standard clotrimazole

Accurately weighed 0.125 g of standard clotrimazole to a 100-ml volumetric flask and adjusted to volume with methanol.

C. Stock solution of standard ketoconazole

Accurately weighed 0.125 g of standard ketoconazole to a 100-ml volumetric flask and adjusted to volume with methanol.

D. Stock solution of methyl paraben

Accurately weighed 0.113 g of methyl paraben to a 100-ml volumetric flask and adjusted to volume with methanol.

E. Stock solution of propyl paraben

Accurately weighed 0.230 g of propyl paraben to a 100-ml volumetric flask and adjusted to volume with methanol.

F. Stock solution of 2-chlorotritanol

Accurately weighed 0.010 g 2-chlorotritanol to a 100-ml volumetric flask and adjusted to volume with methanol.

2.1. Optimization of mobile phase

Standard solution :

- Solution 1 (clotrimazole) :

Pipetted 5.0, 2.0, 2.0 and 5.0 ml of solution B, D, E and F, respectively, to a 25-ml volumetric flask and adjusted to volume with methanol.

- Solution 2 (ketoconazole) :

Pipetted 5.0, 2.0 and 2.0 ml of solution C, D and E, respectively, to a 25-ml volumetric flask and adjusted to volume with methanol.

2.1.1. The percentage of organic modifier in the mobile phase

Methanol, an organic modifier in mobile phase was varied from 70 to 100 % in water (by volume) for finding suitable analytical retention time. At least three replicate injections (20 μl) of solution 1 (clotrimazole) were made by setting flow rate of mobile phase at 1 ml/min with UV detection at 230 nm and the results obtained were demonstrated in Figure 6, 7 and Table 2. The same procedure was also performed with solution 2 (ketoconazole) and the results presented in Figure 8.

2.1.2. The pH of mobile phase

pH of mobile phase is also affected retention of drug on stationary phase due to its polarity. Thus, the pH of appropriate mobile phase (solution A) was varied from 3 to 8 by using 1 N phosphoric acid (H_3PO_4) or 1 N sodium hydroxide (NaOH) and the resulted solution was mixed with appropriate amount of methanol from 2.1.1. Figure 10 demonstrated effect of pH of mobile phase on retention time of ketoconazole and clotrimazole, respectively.

2.1.3. The concentration of salt in mobile phase

Amount of salt [$(NH_4)H_2PO_4$] in mobile phase was varied by using appropriate percentage methanol and pH selected from 2.1.1. and 2.1.2., respectively as follows:

a. 0.0025 M Phosphate solution

Accurately weighed 0.2876 g of $(NH_4)H_2PO_4$ to a 1000-ml volumetric flask and adjusted to volume with water. Thereafter, the solution was adjusted to desired pH selected from 2.1.2. with 1 N H_3PO_4 or 1 N NaOH and the resulted solution was mixed with appropriate amount of methanol from 2.1.1.

b. 0.005 M Phosphate solution

Prepared the mobile phase as described above in a. but with 0.5752 g of $(NH_4)H_2PO_4$.

c. 0.02 M Phosphate solution

Prepared the mobile phase as described above in a. but with 2.3006 g of $(NH_4)H_2PO_4$.

Figure 11 and Table 3 demonstrated the effect of salt concentration on retention time of ketoconazole and clotrimazole, respectively.

2.2. Sample pretreatment

Cream preparation

Cream base composed of cetyl alcohol, stearyl alcohol, glyceryl monostearate, span 60, methyl paraben, propyl paraben, polyethylene glycol 400, liquid paraffin, glycerin, tween 60 and water was prepared and contained 1 % by weight of clotrimazole. Ketoconazole in the same composition of cream base was also prepared but with 2 % concentration. Both imidazole drugs were separated from other ingredients in cream by using appropriate procedure e.g., sonication and freezing time.

Reagent solution :

Stock standard solution of clotrimazole

Accurately weighed 0.02 g of standard clotrimazole to a 50-ml volumetric flask and adjusted to volume with methanol.

Internal standard solution of clotrimazole

Accurately weighed 0.018 g of standard diltiazem to a 100-ml volumetric flask and adjusted to volume with methanol.

Stock standard solution of ketoconazole

Accurately weighed 0.04 g of standard ketoconazole to a 50-ml volumetric flask and adjusted to volume with methanol.

Internal standard solution of ketoconazole

Accurately weighed 0.06 g of standard clotrimazole to a 50-ml volumetric flask and adjusted to volume with methanol.

Standard solution of clotrimazole

Mixed well 5.0 ml of stock standard solution of clotrimazole with 5.0 ml of internal standard solution and 10.0 ml methanol in 125-ml glass-stoppered Erlenmyer flask.

Standard solution of ketoconazole

Mixed well 5.0 ml of stock standard solution of ketoconazole with 5.0 ml of internal standard solution and 10.0 ml methanol in 125-ml glass-stoppered Erlenmyer flask.

Sample solution of clotrimazole

Mixed 0.2 g of 1% clotrimazole cream with 5.0 ml of internal standard solution and 10.0 ml methanol in 125-ml glass-stoppered Erlenmyer flask.

Sample solution of ketoconazole

Mixed 0.2 g of 2% ketoconazole cream with 5.0 ml of internal standard solution and 10.0 ml methanol in 125-ml glass-stoppered Erlenmyer flask.

2.2.1. Optimal sonication time

Sonication is one of extraction method to liberate drug from its dosage form, thus, an appropriate sonication time is necessary to ensure that all of interesting drug is completely released from its matrix. Thus, the flasks of sample solution of clotrimazole were sonicated for 5, 10, 20 and 30 minutes,respectively. After filtration through 0.45 μm nylon membrane, the filtrate of each sonication time (made up of three test solutions) was injected in duplicates to high-performance liquid chromatograph by using developed HPLC system obtained from 2.1., comparing with clotrimazole standard solution. The same procedure

was also performed with ketoconazole cream and both results were demonstrated in Figure 12.

2.2.2. Optimal freezing time

Solubility of compound generally reduces when temperature decreases. In cream preparation, many ingredients in cream base change from liquid form to semisolid or solid form when the temperature of cream base solution decreases. Thus, interferences caused by the ingredients of the cream base to quantitative analysis of active ingredient may be reduced or eliminated. In present study, after sonication sample solution of clotrimazole with appropriate time obtained from 2.2.1., the sample solution was placed in freezer of refrigerator for a period of 0,10, 20 and 30 minutes, respectively. Thereafter, the procedure was perform with the same procedure described above in 2.2.1., beginning with “After filtration…”. Ketoconazole in cream was also analyzed in the same manner as clotrimazole.

2.3. Comparison between developed HPLC method and pharmacopeial method.

Reagent solution :

i. Solution of clotrimazole and 2-chlorotritanol

Accurately weighed 0.05 g and 0.005 g of clotrimazole and 2-chlorotritanol to a 50-ml volumetric flask and adjusted to volume with methanol.

ii. Solution of ketoconazole and degraded products

Accurately weighed 0.04 g of ketoconazole to a 50-ml volumetric flask and adjusted to volume with methanol, kept in room temperature and exposed with light for 8 weeks. Mixed well 5.0 ml of this solution with 15.0 ml of methanol.

Pharmacopeial assay method

A. Clotrimazole

Clotrimazole and its degraded product, 2-chlorotritanol were assayed and compared by using developed method, BP 2002 method and USP 26 and NF 21 method.

BP 2002 method

The mobile phase was prepared by mixing 300 ml of 0.02 M orthophosphoric acid adjusted to pH 7.5 with 10 % v/v solution of triethylamine in methanol with 700 ml of methanol. The flow rate was set at 1.5 ml/min and detection at 215 nm.

USP 26 and NF 21 method

The mobile phase was prepared by mixing 250 ml of 0.025 M dibasic potassium phosphate solution with 750 ml of methanol. The flow rate was set at 1 ml/min and detection at 254 nm.

B. Ketoconazole

Ketoconazole and degraded products also were assayed and the results were compared by using developed method, BP 2002 method and USP 26 and NF 21 method.

BP 2002 (modified from analysis of ketoconazole raw material)

The mobile phase was prepared by mixing 500 ml of 3.4 g/l of tetrabutylammonium hydrogen sulphate with 500 ml of acetonitrile. The flow rate was set at 2 ml/min and detection at 220 nm.

USP 26 and NF 21

The mobile phase was prepared by mixing 300 ml of ammonium acetate solution (1 in 200) with 700 ml of diisopropylamine in methanol (1 in 500). The pharmacopeia's flow rate was 3 ml/min, but in this experiment was set at 2.5 ml/min to minimize overloading on analytical column and pump device of instrument. The detection wavelength was 225 nm.

The solution i and ii was injected by using the three HPLC system of each drug described above. The analytical column was used in comparative systems was Apollo C_{18}, 25 cm x 4.6 mm, 5 μm and guard column was C_{18}, 2 cm x 2 mm, 40 μm. The chromatograms of each drug obtained from three analytical methods were shown in Figure 14 and 15.

3. Method validation

To ascertain the suitability and effectiveness of the proposed HPLC method, it should be subjected to test for method validation (USP 26 and NF 21) whose essential parameters are accuracy, precision, specificity, linearity and range.

Reagent solution :

I. Stock solution of standard clotrimazole

Accurately weighed 0.5 g of standard clotrimazole to a 100-ml volumetric flask and adjusted to volume with methanol.

II. Stock solution of internal standard of clotrimazole

Accurately weighed 0.045 g of standard diltiazem to a 250-ml volumetric flask and adjusted to volume with methanol.

III. Stock solution of standard ketoconazole

Accurately weighed 1.0 g of standard ketoconazole to a 100-ml volumetric flask and adjusted to volume with methanol.

IV. Stock solution of internal standard of ketoconazole

Accurately weighed 0.3 g of standard clotrimazole to a 250-ml volumetric flask and adjusted to volume with methanol.

Standard solution of clotrimazole

Pipetted 2.0, 3.0, 4.0, 5.0, and 6.0 ml of the solution I into separate five 50-ml volumetric flasks and adjusted to volume with methanol; The solution were labelled C1, C2, C3, C4 and C5, respectively. Each solution (5.0 ml) was pipetted separately to 125-ml glass-stoppered Erlenmyer flask containing 5.0 ml of the internal standard (II) and 10.0 ml of methanol, mixed thoroughly.

Sample preparation of clotrimazole cream

Weighed 0.2 g of cream base (without drug) into separate five 125-ml glass-stoppered Erlenmyer flasks. Then, solution C1 was added to one flask, followed by 5.0 ml of the internal standard solution (II) and 10.0 ml of methanol, mixed well. The final solution was analyzed by proposed HPLC method, using appropriate sonication time, freezing time as described in 2.2.1. and 2.2.2. The other solution (C2, C3, C4 and C5) were also assayed with the same manner as solution C1.

Standard solution of ketoconazole

Standard solution of ketoconazole (solution K1, K2, K3, K4 and K5) were prepared and mixed well with internal standard solution (IV) and methanol as mentioned above in clotrimazole.

Sample preparation of ketoconazole cream

Follow the same procedure as described under sample preparation of clotrimazole cream.

Method validation parameters

3.1. Accuracy

The standard solution and filtrated sample solution of both drugs were injected in six replications by developed HPLC system. The accuracy of the proposed method was evaluated as the percentage of recovery from the true value and demonstrated in Figure 16, 17, Table 4 and 5, respectively.

3.2. Precision

The precision of the method was presented in the term of the percentage of relative standard deviation (% RSD) and demonstrated in Table 6 and 7, respectively.

3.3. Specificity

The specificity of the method was presented by the comparison of chromatogram between standard solution and cream base without drug in Figure 18.

3.4. Linearity and range

Standard clotrimazole solutions were prepared at five concentrations (C1, C2, C3, C4 and C5). Each concentration was made up of three test solutions and injected in duplicates to high-performance liquid chromatograph in concentration range from 50 to 150 μg/ml of clotrimazole. For standard ketoconazole solution (solution K1, K2, K3, K4 and K5), its concentration range was 100 to 300 μg/ml. The results of peak area ratios and concentrations of each drug was plotted and the relationship between these variables were mathmatically calculated and expressed by regression analysis in Figure 19 and 20.

4. Stability test on oil-in-water cream

On prolong storage, the pharmaceutical preparations may be deteriorated due to temperature, humidity and also pH of product themselve. These may be affected to stability of active ingredients and also to its appearance. For evaluate this, the stability of clotrimazole and ketoconazole in

cream base (1 % and 2 %, respectively) by various accelerated conditions were studied and compared.

4.1. Effect of temperature and humidity

Each drug preparation was divided in 5 g x 120 aluminium tubes and stored in incubator at 40 ± 1 °C and 75 ± 5 % RH. Three sample tubes of each drug were assayed in duplicates on days 0, 7, 14, 21, 28, 35, 42, 49, 56, 70, 84, 98, 112, 126, 140, 156, 168 and 182 by using the developed method, as demonstrated in Figure 21.

4.2. Effect of pH

The pH of 1 % clotrimazole and 2 % ketoconazole cream were adjusted in ranges, 3 to 8, with 1 N H_3PO_4 and 1 N NaOH and kept them in room temperature (about 30 °C) for three weeks. The content of clotrimazole and ketoconazole in cream were assayed on weeks 0, 1 and 3. The results were shown in Figure 22 and 23.

5. Assay application

The four commercial products of 1 % clotrimazole and 2 % ketoconazole cream were assayed by using developed sample pretreatment and proposed HPLC system to evaluate whether the developed method was able to assay the content of drugs in finished products.

CHAPTER III

RESULTS AND DISCUSSION

1. Physicochemical effects on degradation of active ingredients

1.1. Effect of acid-base catalysis on clotrimazole and ketoconazole degradation

1.1.1. Effect on clotrimazole

Clotrimazole, an imidazole antimycotic drug is a weak base with pKa, 4.7 (Figure 1). Then it may undergo degradation including oxidation and hydrolysis, especially in aqueous media. Upon studying acid-base catalysis hydrolysis, it was found that clotrimazole underwent complete acid catalysis hydrolysis as revealed by thin layer chromatography (Figure 4). A new spot formed was found in various developing solvent systems on silica gel GF_{254} with iodine detection while unobserved new spot was found in base catalysis hydrolysis. Since clotrimazole is a weak base, preferably acid catalysis should be established. The results corresponded well with previously reported by Hoogerheide et al.,1981.

1.1.2. Effect on ketoconazole

Ketoconazole is also a weak base with two pKa, 2.9 and 6.5. Acid catalysis hydrolysis of ketoconazole exhibited incomplete hydrolysis products whereas no degradation observed in base

catalysis hydrolysis (Figure 5). Since complete degradation of clotrimazole by acid catalysis hydrolysis was obtained, in contrast with ketoconazole, suggesting better intrinsic stability of ketoconazole in aqueous media and may provide better stable pharmaceutical products.

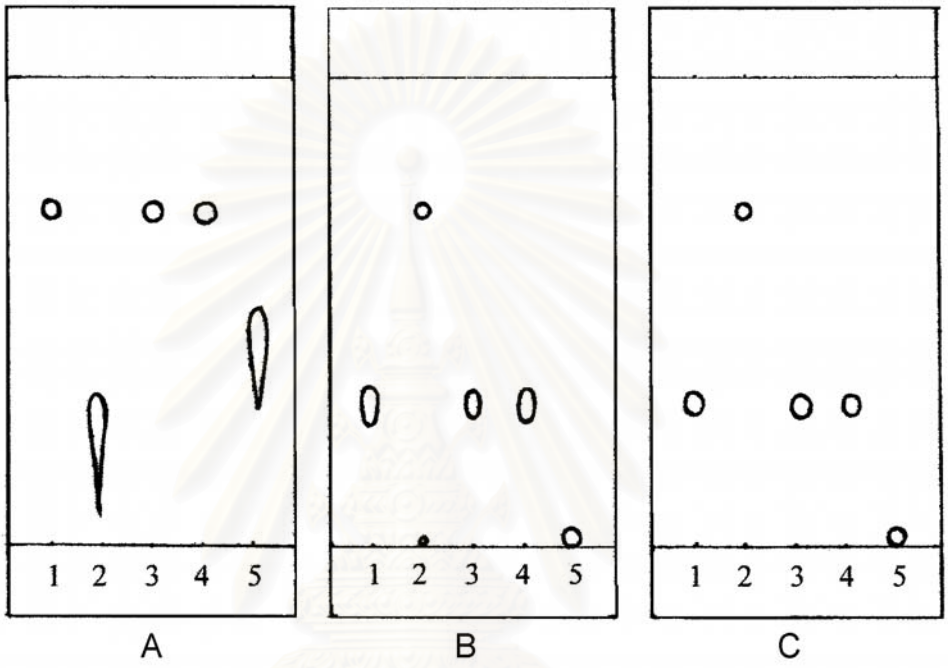

Figure 4 TLC of hydrolysis and photolysis products of clotrimazole on 0.25 mm, 3 x 7 cm Silica gel GF_{254} plates with iodine detection.
Developing solvent systems ; A = methanol:chloroform (1:1, by vol.),
B = benzene:n-hexane (1:1, by vol.),
C = n-hexane:ethyl acetate (1:1, by vol.).
1 = clotrimazole, 2 = acid-catalyzed clotrimazole,
3 = base-catalyzed clotrimazole, 4 = light-catalyzed clotrimazole (120 hours under 254 nm UV light), 5 = imidazole.

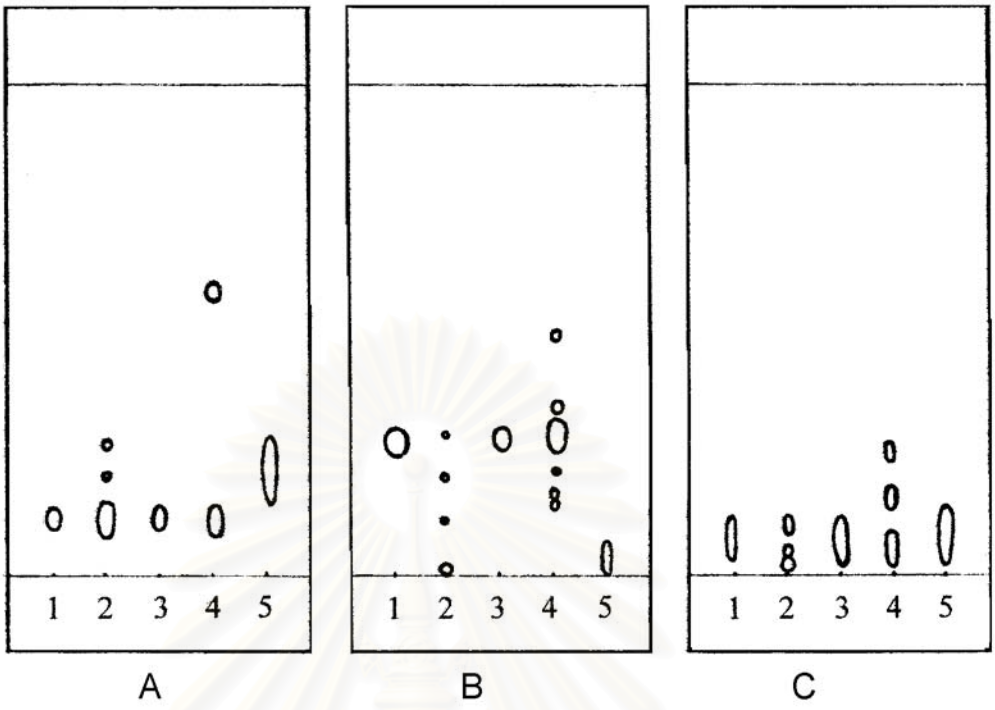

Figure 5 TLC of hydrolysis and photolysis products of ketoconazole on 0.25 mm, 3 x 7 cm Silica gel GF_{254} plates with iodine detection. Developing solvent systems ; A = 100 % acetonitrile, B = methanol:chloroform (5:95, by vol.), C = acetone:chloroform (1:1, by vol.). 1 = ketoconazole, 2 = acid-catalyzed ketoconazole, 3 = base-catalyzed ketoconazole, 4 = light-catalyzed ketoconazole (120 hours under 254 nm UV light), 5 = imidazole.

1.2. Effect of photolysis on clotrimazole and ketoconazole degradation

1.2.1. Effect on clotrimazole

No photolysis product was observed upon irradiation clotrimazole under 254 nm UV light for 120 hours as illustrated by TLC,

single spot with same R_f value as clotrimazole was obtained in various developing solvent systems (Figure 4). Thus, clotrimazole showed good stability to photolysis under investigation.

1.2.2. Effect on ketoconazole

On the contrary, ketoconazole kept under UV light for 120 hours showed many spots on TLC plates in various developing solvent systems (Figure 5). Thus, the drug was decomposed by UV light and produced many degraded products, as well as reported by Nuntanakorn, 1996.

Since physicochemical parameters such as hydrolysis and photolysis effected decomposition of clotrimazole and ketoconazole in different ways, carefully restricted control amount of drugs in pharmaceutical dosage form is essential and necessary. Therefore, suitable stability indicating assay method for quantitation the amount of drugs in pharmaceutical preparation is developed and modified.

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2. Suitable HPLC system and sample pretreatment

2.1. Optimization of mobile phase

2.1.1. The percentage of organic modifier in the mobile phase

In reversed-phase chromatography, organic modifiers e.g., methanol, acetonitrile or tetrahydrofuran are usually added to the

mobile phase to modify the eluent strength and to increase the efficiency of the chromatographic peaks. Its action is competitive binding with active site on stationary phase, thus retention time of interesting compound is able to regulate depending on the polarity of compound. Since clotrimazole and ketoconazole are fairly soluble in methanol, therefore, it was selected as organic modifier in mobile phase [0.01 M $(NH_4)H_2PO_4$ pH 7.0].

Upon varying percentage of methanol as illustrated in Figure 6 and Table 2, it was found that the best separation of clotrimazole from its degraded product, 2-chlorotritanol and frequently used preservatives in imidazole antimycotic preparation (methyl paraben and propyl paraben) was obtained from 70 % methanol although long retention time of clotrimazole (t_r = 41.80 min) was observed. However, at higher percentage of methanol (80 and 90 %), overlapping of clotrimazole and its degraded product was shown with shorter retention time (t_r = 13.53 and 6.57 min for 80 and 90 %, respectively). This might be due to higher competitive binding of methanol to active site on stationary phase and also higher drug solubility obtained at higher percentage methanol. Thus 70 % methanol was used as the suitable organic modifier in the following experiment.

The similar results was also achieved with ketoconazole as demonstrated in Figure 7 and 8. Therefore, 70 % methanol was selected as organic modifier in analysis of ketoconazole. Since clotrimazole was well separated from ketoconazole as illustrated in Figure 6 and 7, thus it was able to used as internal standard in

determination of ketoconazole. Figure 9 demonstrated chromatographic peaks of mixture of clotrimazole, ketoconazole and preservatives (methyl paraben and propyl paraben) in 100 % acetonitrile (Figure 9a) and 100% methanol. It was shown that the compounds could not be separated from each other, thus, neither pure acetonitrile nor pure methanol could be selected as the mobile phase.

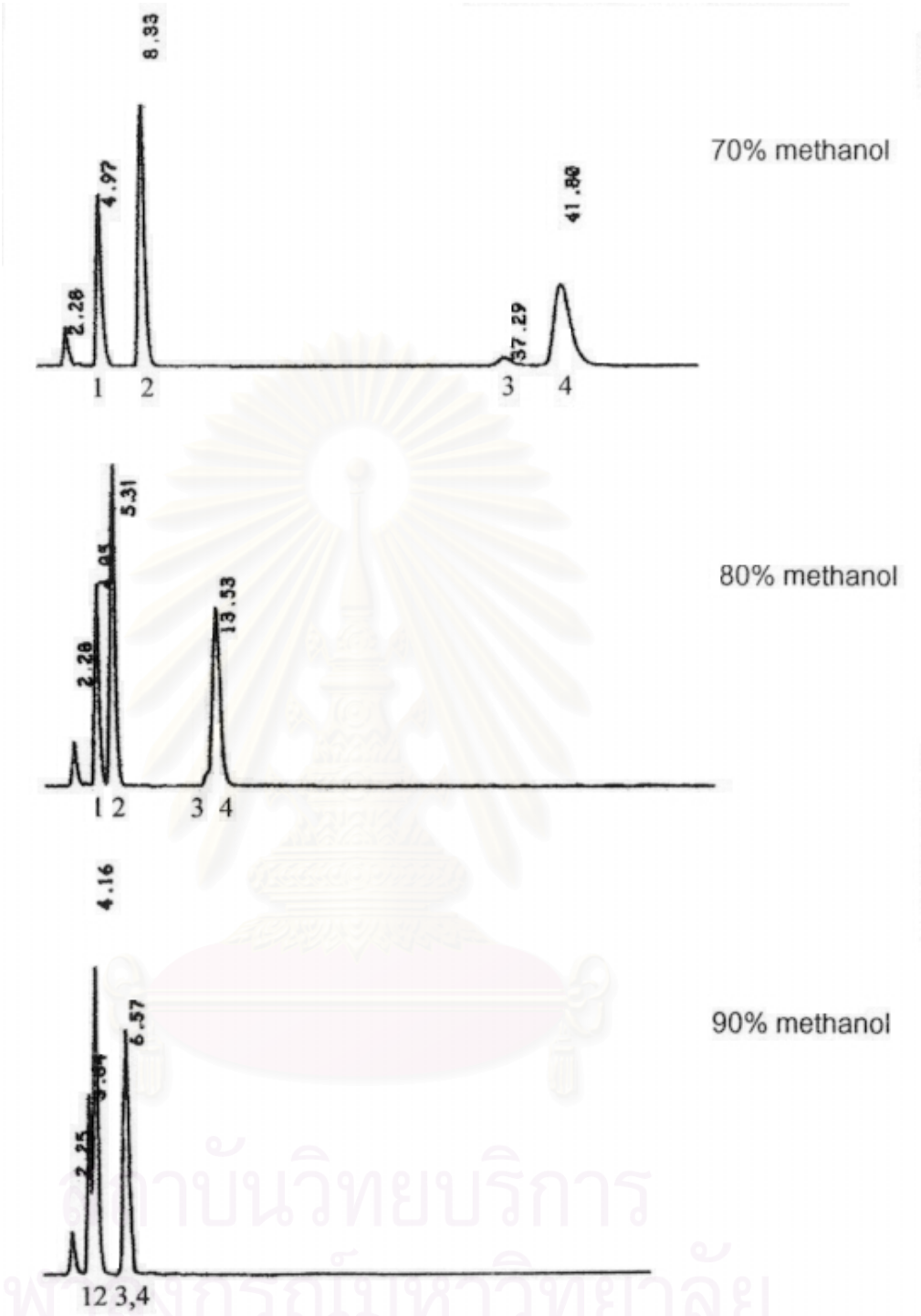

Figure 6 HPLC separation of clotrimazole (4) from its degraded product 2-chlorotritanol (3) and preservative [methyl paraben (1) and propyl paraben (2)] at the various percentage of methanol in mobile phase. Column : Apollo C_{18} (5 μm); Mobile phase: methanol in water at a flow rate 1 ml/min. Detection at 230 nm.

Table 2 Effect of the percentage of organic modifier (methanol) in mobile phase on separation of clotrimazole from its degraded product and preservative.

Substance	Retention time , min.*			
	Percentage of methanol			
	70 %	80 %	90 %	100 %
1. Clotrimazole	41.80 ± 0.37	13.53 ± 0.05	6.57 ± 0.00	3.70 ± 0.00
2. Degraded product:				
2-Chlorotritanol	37.29 ± 0.27	13.53 ± 0.05	6.57 ± 0.00	3.70 ± 0.00
3. Preservative				
a. Methyl paraben	4.97 ± 0.03	4.05 ± 0.01	3.64 ± 0.00	2.17 ± 0.00
b. Propyl paraben	8.33 ± 0.01	5.31 ± 0.04	3.64 ± 0.00	2.17 ± 0.00

* Mean ± S.D. of at least three determinations.

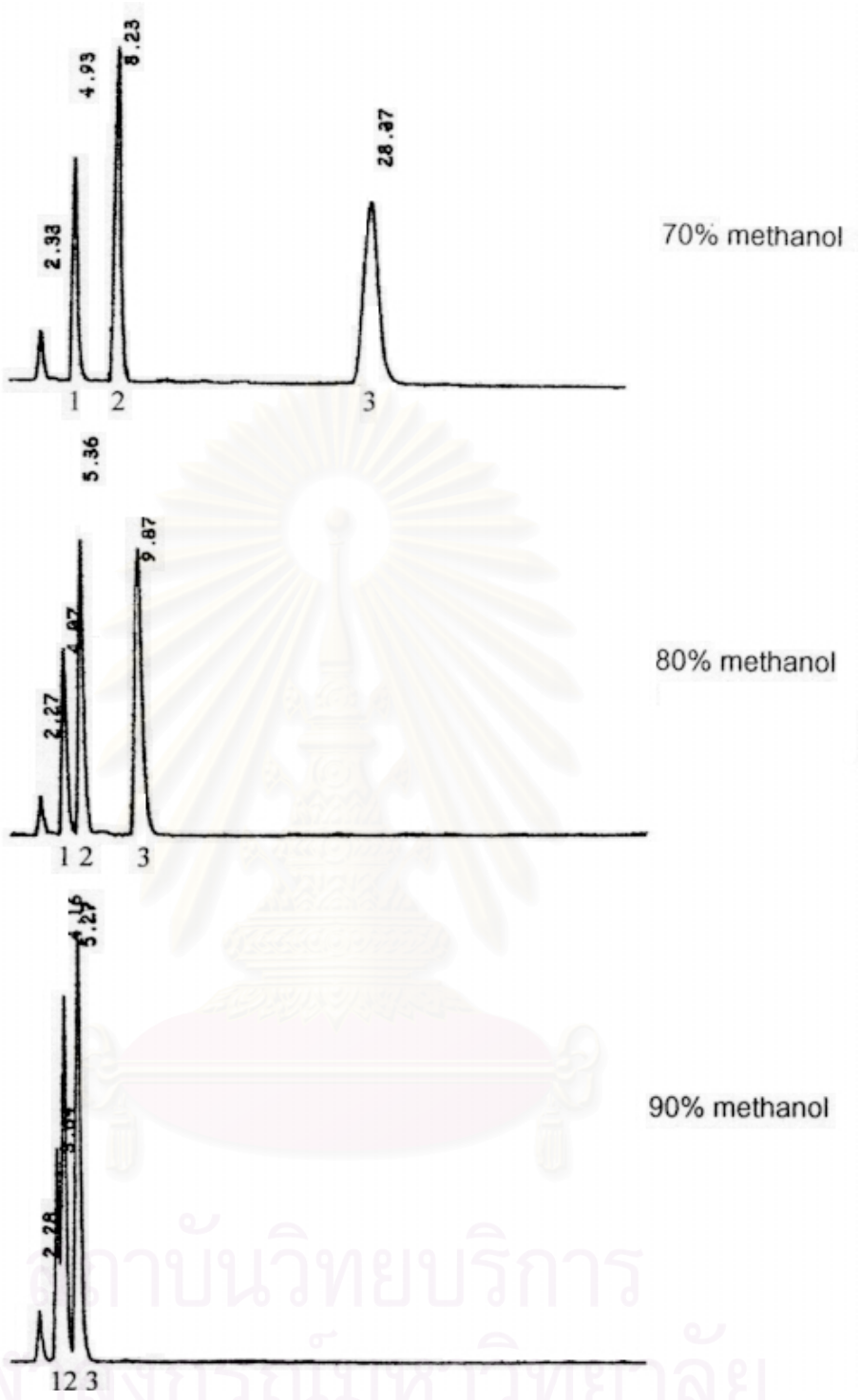

Figure 7 HPLC separation of ketoconazole (3) from preservative [methyl paraben (1) and propyl paraben (2)] at the various percentage of methanol in mobile phase.

Column : Apollo C_{18} (5 μm); mobile phase: methanol in water at a flow rate 1 ml/min. Detection at 230 nm.

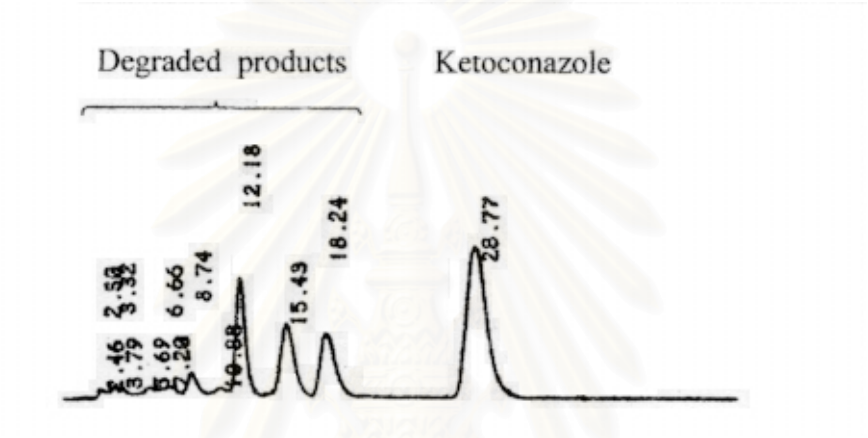

Figure 8 HPLC separation of ketoconazole from its degraded products at 70 % methanol in mobile phase.
Column : Apollo C_{18} (5 μm); mobile phase: 70 % methanol in water at a flow rate 1 ml/min. Detection at 230 nm.

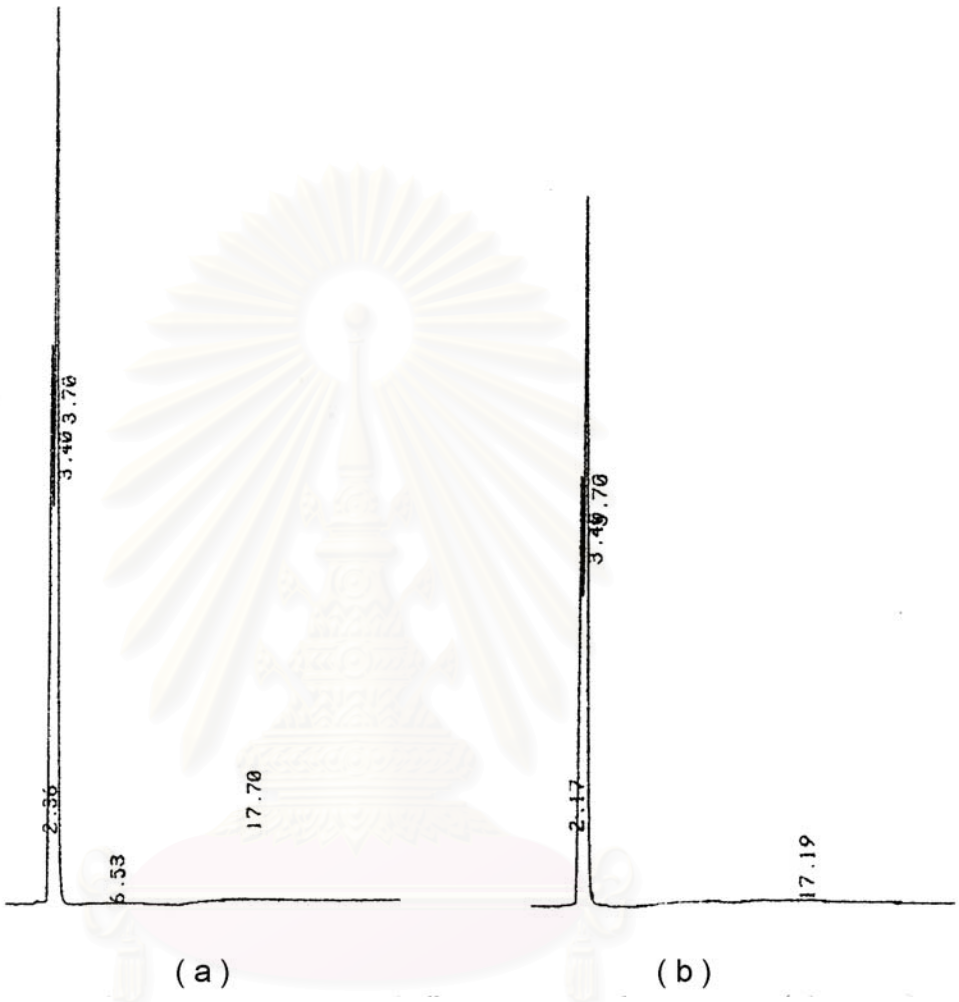

Figure 9 HPLC chromatograms of mixture of clotrimazole, ketoconazole and preservative (methyl paraben and propyl paraben).
Mobile phase (a) 100 % acetonitrile (b) 100 % methanol.

2.1.2. The pH of mobile phase

pH is one of important factor that affects selectivity of the separation. In C_{18} reversed-phase column, stationary phase is long hydrocarbon chain, thus, nonionized form of drug prefers partition in stationary phase. As a consequence, a longer retention time is achieved. Clotrimazole (pKa 4.7) and ketoconazole (pKa 2.9 and 6.5) are weakly basic drug, thus they ionized in acidic solution. As illustrated in Figure 10, their retention time increased slightly upon increasing pH, however, decreasing of pH less than 5 resulted in sharply decreasing of retention time due to ionization of clotrimazole and ketoconazole at low pH. 2-Chlorotritanol (degraded product of clotrimazole) and preservative (methyl paraben and propyl paraben) are nonionized compounds, then no significant changes in their retention time were observed upon changing of pH. Since pH 7.0 of mobile phase gave symmetrical peak shape with appropriate separation of compounds and also high stability to acid catalysis of both drugs, then it was selected as appropriate pH of mobile phase in the following experiment.

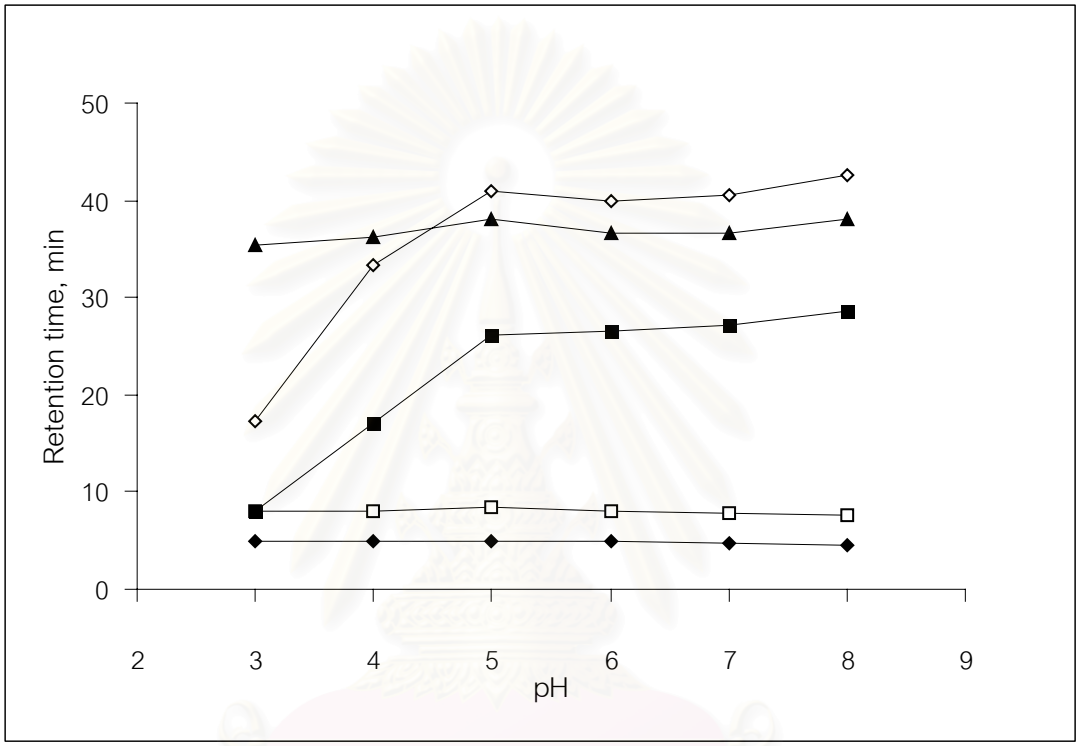

Figure 10 Effect of pH of mobile phase on retention time of clotrimazole (—◇—), ketoconazole (—■—), 2-chlorotritanol (—▲—), methyl paraben (—◆—) and propyl paraben (—□—).
Mobile phase is 0.01 M. $(NH_4)H_2PO_4$ with methanol (3:7, by vol.).

2.1.3. The concentration of salt in mobile phase

For ionizable compounds, the ionic strength of buffer is an important parameter to consider at an early stage of the optimization. Effect of increasing ionic strength is to increase in number of counter ions attracted towards silanol group on C_{18} stationary phase. Therefore, interaction between analyte and silanol group is blocked, as a consequence peak sharps and retention time reduces. In experiment, the concentration of $(NH_4)H_2PO_4$ was varied from 0.0025 to 0.02 M (Figure 11). It was found that no significant differences in peak shape and retention time of clotrimazole and ketoconazole with various salt concentrations and also with 70 % methanol (Figure 7, 8). However, higher signal responses were observed with mobile phase contained 0.0025 M $(NH_4)H_2PO_4$ leading to higher sensitivity in analysis and also for minimizing acid hydrolysis at low pH, buffering capacity of mobile phase was controlled at pH 7 by using 0.0025 M $(NH_4)H_2PO_4$. Another advantage obtained from using salt in mobile phase was to decrease or compress diffusion of substances in low viscosity mobile phase, thus, sharp chromatograms were achieved, presenting overlap of closely chromatograms. Higher salt concentration was avoided for prevention of obstruction in HPLC pump device.

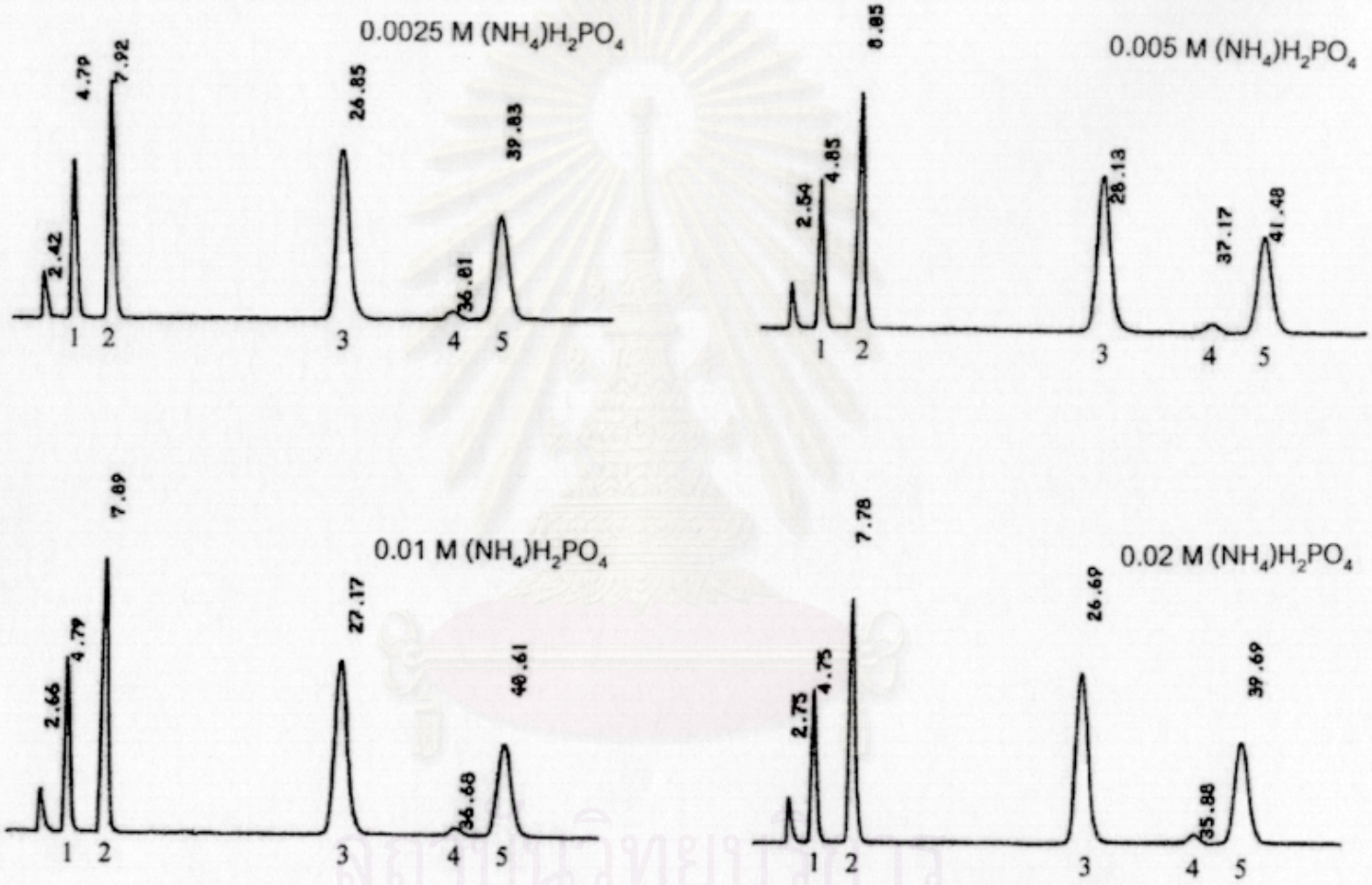

Figure 11 Effect of salt concentration in mobile phase on separation of clotrimazole (5), ketoconazole (3), 2-chlorotritanol (4), methyl paraben (1) and propyl paraben (2).

Mobile phase is $(NH_4)H_2PO_4$ solution pH 7.0 with methanol (3:7, by vol.).

Table 3 Effect of salt concentration in water pH 7.0 with methanol (3:7, by vol.) on separation of clotrimazole, ketoconazole, 2-chlorotritanol, methyl paraben and propyl paraben.

Substance	Retention time, min*			
	Concentration of $(NH_4)H_2PO_4$, M			
	0.0025	0.005	0.01	0.02
1. Clotrimazole	39.83 ± 0.20	41.48 ± 0.26	40.67 ± 0.30	39.69 ± 0.20
2. Ketoconazole	26.85 ± 0.09	28.13 ± 0.20	27.17 ± 0.21	26.69 ± 0.22
3. 2-Chlorotritanol	36.01 ± 0.10	37.17 ± 0.10	36.68 ± 0.11	35.88 ± 0.29
4. Methyl paraben	4.79 ± 0.01	4.85 ± 0.02	4.79 ± 0.10	4.75 ± 0.01
5. Propyl paraben	7.92 ± 0.02	8.05 ± 0.06	7.89 ± 0.11	7.78 ± 0.02

* Mean ± S.D. of at least three determinations.

2.2. Sample pretreatment

Sample preparation is a crucial step in overall chromatographic process and can affect the chromatogram if not develop or treat properly. The purpose of this step is to prepare the sample so that the drug substance can be readily chromatographed, separated from other materials. Various interferences in pharmaceutical preparation are removed and eliminated, enhancing sensitivity of drug detection and prolonging life of the analytical column. In this investigation, the sample pretreatment involved sonication and freezing step, respectively.

2.2.1. Optimal sonication time

After diluting sample with methanol, it was sonicated for the purpose of dispersing drug substance into the solvent. Sonication time was varied at 5, 10, 20 and 30 minutes. It was found that there were no difference between short and long sonication time to quantify clotrimazole. Then clotrimazole was easily extracted from cream preparation, thus, the optimal sonication time 5 minutes was selected for clotrimazole. For ketoconazole, constant amount of drug was obtained after sonication for 10 minutes, thus 10 minutes was chosen for appropriate sonication time to extract ketoconazole from pharmaceutical preparation.

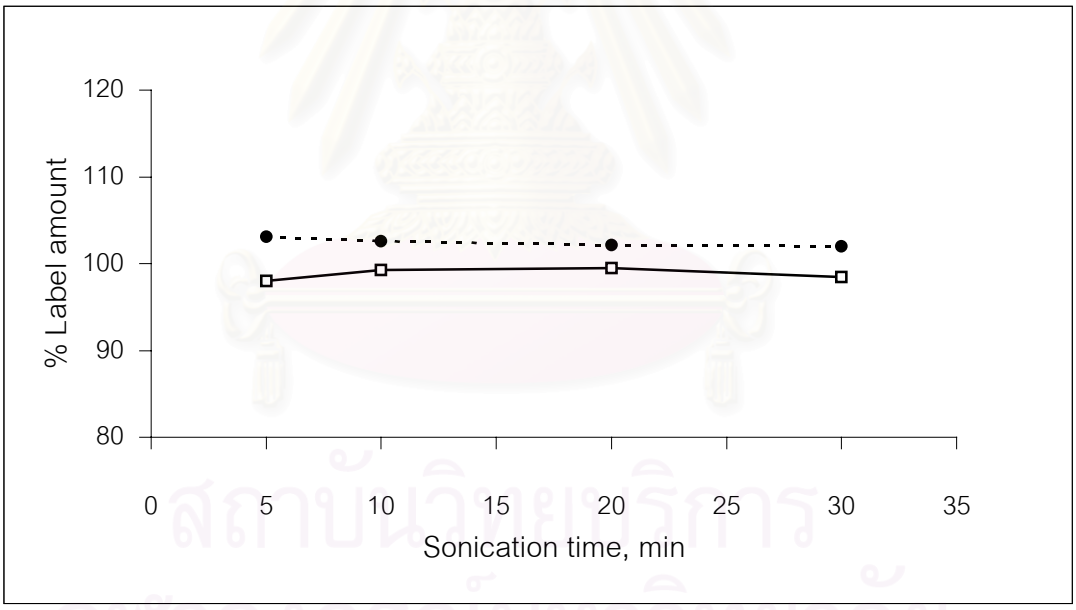

Figure 12 Effect of sonication time on quantitative analysis of clotrimazole (---●---) and ketoconazole (—▫—). Each point represents mean ± S.D. of six determinations.

2.2.2. Optimal freezing time

Some ingredients in cream base were solidified after decreasing temperature, thus interferences in quantitative analysis of drug was decreased or diminished. Freezing time was varied at 0, 10, 20 and 30 minutes. However, it was found that some oil globules appeared in the filtrate at the bottom of container after sonication and fitration but without freezing. These oil globules might contaminate in injected solution and obstruct stationary phase. Thus, the sample solution after sonication should be frozen and filtrated before injection. It was found that there were not difference between short and long time to quantify clotrimazole and ketoconazole. Therefore, the optimal freezing time, 20 minutes, was selected for both drugs to ensure adequate time for separation of ingredients of cream base from drug of interest.

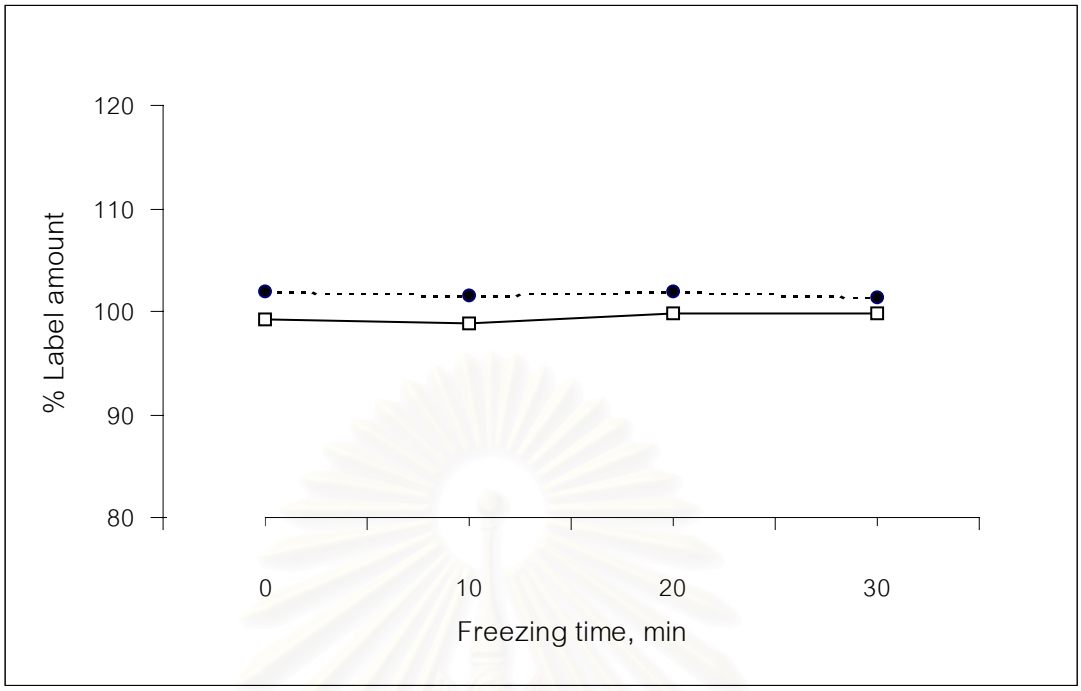

Figure 13 Effect of freezing time on quantitative analysis of clotrimazole (---●---) and ketoconazole (—▫—). Each point represents mean ± S.D. of six determinations.

Optimal condition for quantitative analysis of clotrimazole and ketoconazole in cream preparation

Appropriate amount of cream was diluted with methanol using the sonication time 5 minutes for clotrimazole cream and 10 minutes for ketoconazole. After that the sample was freezed in refrigerator for 20 minutes and filtrated for both drugs. The filtrated sample solution was injected to high pressure liquid chromatograph, using Apollo C_{18}, 5 μm, 25 cm x 4.6 mm, i.d., as analytical column. Mobile phase was 0.0025 M $(NH_4)H_2PO_4$ pH 7.0 : methanol (3:7, by vol.) with flow rate 1.0 ml/min and UV detection at 230 nm.

2.3 Comparison between developed HPLC method and pharmacopeial method.

Separation of clotrimazole and ketoconazole from their degraded products by developed and pharmacopeial methods was demonstrated in Figure 14 and 15. Amount of methanol in mobile phase of developed method and BP 2002 method for analysis of clotrimazole was similar (about 70 %) with slightly more (75 %) in USP 26 and NF 21 method. Differences among three analytical methods were kind of buffer salts and another reagents, flow rate of mobile phase and detection wavelength. As illustrated in Figure 14, The developed method showed better resolution between clotrimazole and its degraded product (2-chlorotritanol), comparing with BP method. On the contrary, overlap of clotrimazole and 2-chlorotritanol into one peak was observed with USP method. This might be due to more partition of clotrimazole and 2-chlorotritanol in mobile phase, caused by high percentage of methanol. Thus, two compounds came out from column too rapid, then separation could not be obtained.

Satisfactory separation of ketoconazole from its degraded products was achieved by developed method (Figure 15). USP 26 and NF 21 method also exhibited separation but with lower resolution. However, its procedure uses high flow rate which may not be suitable for stationary phase of small particle size since high back pressure will produce and deteriorate analytical column and also pumping device of high pressure liquid chromatograph. No separation of ketoconazole from its degraded products was shown in BP 2002 method.

The data presented here, illustrated that the developed method could be used to separate clotrimazole and ketoconazole from their degraded products with high efficiency and satisfactory results comparing with pharmacopeial methods.

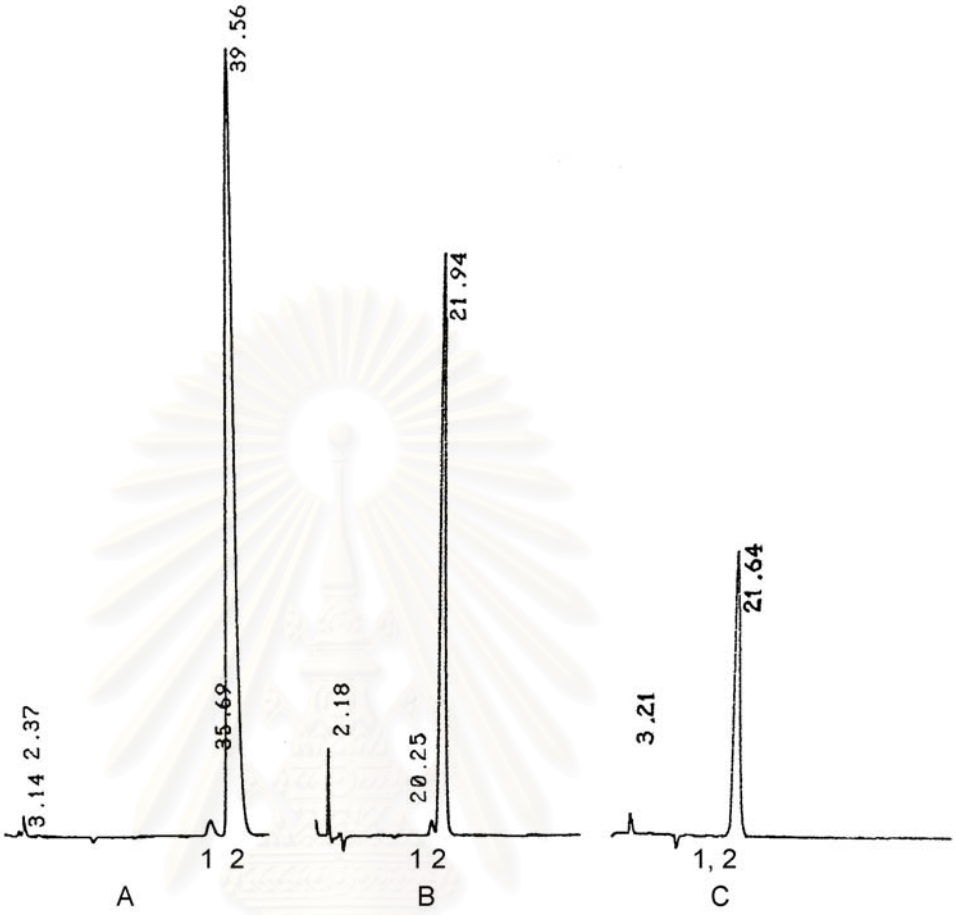

Figure 14 Comparative analytical methods for separation of clotrimazole (2) and 2-chlorotritanol (1).

HPLC systems as follow;

(A) Developed method : Mobile phase was 0.0025 M $(NH_4)H_2PO_4$ pH 7 with methanol (3:7, by vol.), flow rate 1 ml/min and detection at 230 nm.

(B) BP 2002 method : Mobile phase was 0.02 M orthophosphoric acid adjusted to pH 7.5 with 10 %v/v solution of triethylamine in methanol with methanol (3:7, by vol.), flow rate 1.5 ml/min and detection at 215 nm.

(C) USP 26 and NF 21 method : Mobile phase was 0.025 M dibasic potassium phosphate with methanol (1:3, by vol.), flow rate 1 ml/min and detection at 254 nm.

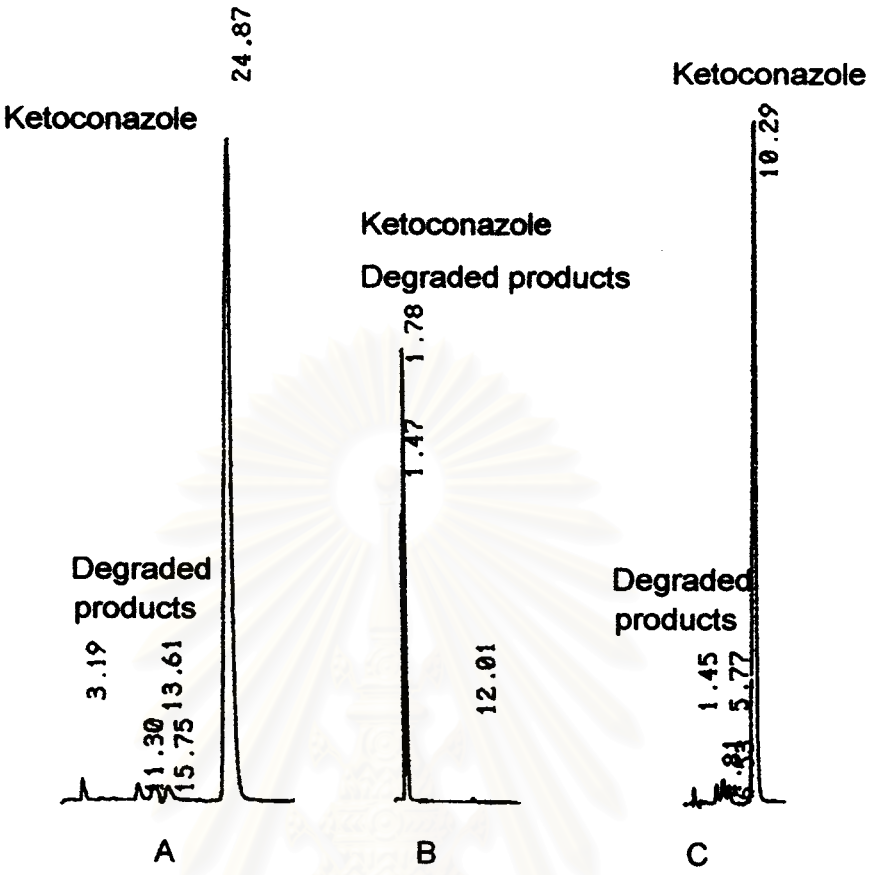

Figure 15 Comparative analytical methods for separation of ketoconazole and its degraded products.

HPLC systems as follow;

(A) Developed method : Mobile phase was 0.0025 M $(NH_4)H_2PO_4$ pH 7 with methanol (3:7, by vol.), flow rate 1 ml/min and detection at 230 nm.

(B) BP 2002 method : Mobile phase was 3.4 g/l solution of tetrabutylammonium hydrogen sulphate with acetonitrile (1:1, by vol.), flow rate 2 ml/min and detection at 220 nm.

(C) USP 26 and NF 21 method : Mobile phase was 1 in 500 of diisopropylamine in methanol with 1 in 200 of ammonium acetate solution in water (7:3, by vol.), flow rate 2.5 ml/min and detection at 225 nm.

3. Method validation

A validation of analytical method is used to generate reliable and accurate data during assay development and post approval of drug products. The procedure, in general, includes the acceptance of raw materials and in process control and releasing of finished products, in process testing. In this experiment, the parameters essential to the validation e.g., accuracy, precision, specificity, linearity and range were studied and evaluated according to USP 26 and NF 21, as follow:

3.1. Accuracy

Accuracy is the measurement of how close the experimental values to the true value. It is expressed as the percent of analyte recovered from the matrix or from spiking samples in a blind study, mathematically calculated in confidence intervals. In this study, the experiment was performed by analyzing synthetic mixtures (placebo) spiked with known quantities of drug, as demonstrated in Figure 16 and 17 for clotrimazole and ketoconazole, respectively.

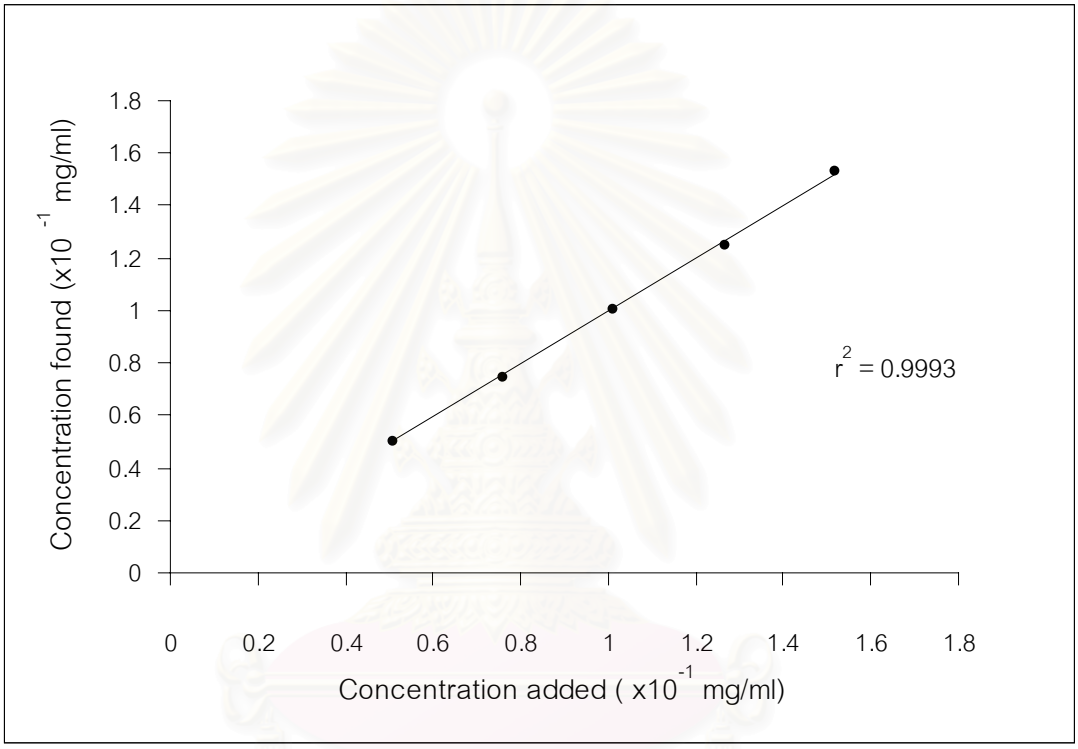

Figure 16 Correlationship between concentration added and concentration found of clotrimazole. Each point represents mean ± S.D. of six determinations.

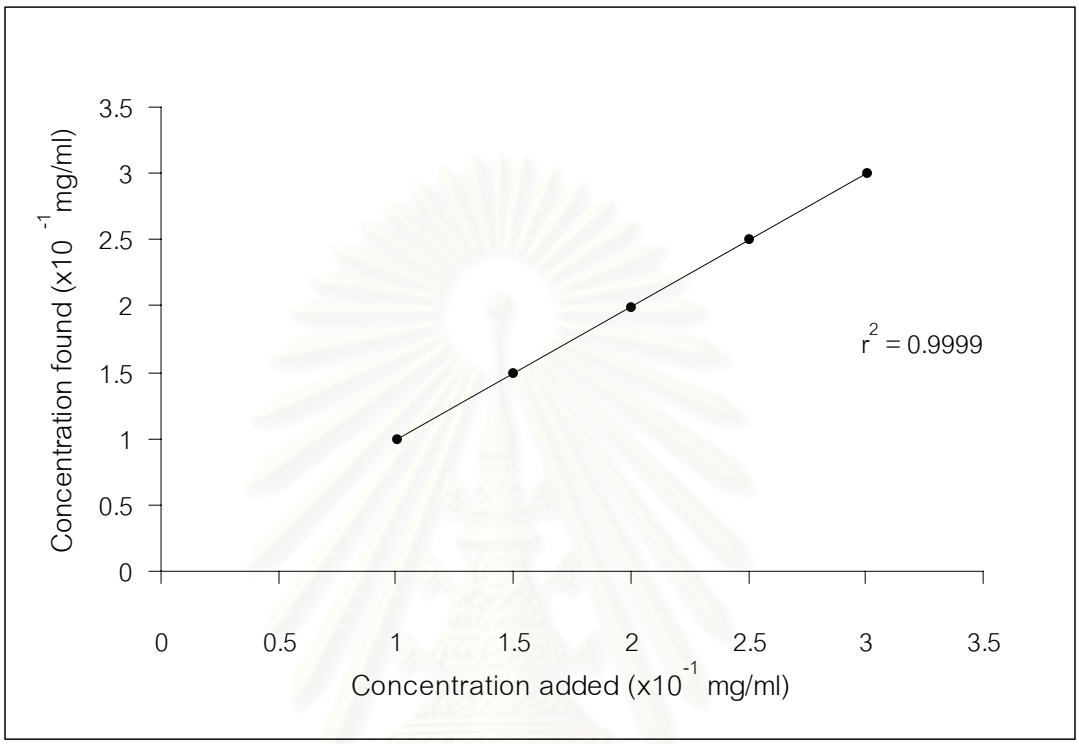

Figure 17 Correlationship between concentration added and concentration found of ketoconazole. Each point represents mean ± S.D. of six determinations.

The accuracy, expressed as percentage recovery of clotrimazole and ketoconazole were shown in Table 4 and 5, respectively. The accuracy were recovered in 95 % confidence intervals at all concentration levels of the drugs used. Therefore, the accuracy of the proposed method was within the acceptance criteria.

Table 4 Percentage recovery of clotrimazole analysis.

Concentration($x10^{-1}$ mg/ml)	% Recovery *	% RSD
0.5057	100.20	0.50
0.7586	98.95	0.56
1.0114	99.81	0.34
1.2642	98.79	0.72
1.5171	100.90	0.75

* Mean of six determinations.

Table 5 Percentage recovery of ketoconazole analysis.

Concentration($x10^{-1}$ mg/ml)	% Recovery *	% RSD
1.0007	99.42	0.74
1.5015	99.79	0.56
2.0014	99.83	0.61
2.5018	99.94	0.16
3.0021	99.86	0.14

* Mean of six determinations.

3.2. Precision

Precision is the measurement of how close the data values are to each other for a number of measurements under the same analytical conditions. The precision of an analytical method is usually expessed as the relative standard deviation (RSD) or coefficient of variation (CV). Precision is further subdivided into repeatability and reproducibility. For most purposes, repeatability is the criterion of concern in USP analytical procedures, although reproducibility between laboratories or intermediate precision may well be considered during the standardization of a procedure before it is submitted to the pharmacopeia.

Table 6 The precision of clotrimazole in spiked cream base sample.

Concentration($x10^{-1}$ mg/ml)	% RSD	
	Within-run (n=6)	Between-run (n=3)
0.5	0.50	0.93
1.0	0.34	0.54
1.5	0.75	0.53

Table 7 The precision of ketoconazole in spiked cream base sample.

Concentration($x10^{-1}$ mg/ml)	% RSD	
	Within-run (n=6)	Between-run (n=3)
1.0	0.74	0.88
2.0	0.61	0.52
3.0	0.14	0.32

The precision of clotrimazole was determined by analyzing six replications of cream sample spiked with 0.05, 0.075, 0.10, 0.125 and 0.15 mg/ml, while for ketoconazole, it was 0.10, 0.15, 0.20, 0.25 and 0.30 mg/ml, respectively. As shown is Table 6 and 7, % RSD of repeatability and reproducibility were less than 1 % for all concentrations presented. Therefore, the precision of proposed method was acceptable according to the criteria "the % RSD obtained at each concentration level should not exceed 1 %".

3.3. Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurity, degradation products and matrix components. As shown in Figure 18A and 18B, clotrimazole and its internal standard (diltiazem) were separately clearly from other components in cream base as well as ketoconazole and its internal standard (clotrimazole) in Figure 18A and 18D. Figure 18C and 18E demonstrated quite well separation of clotrimazole and ketoconazole from their degraded products, respectively.

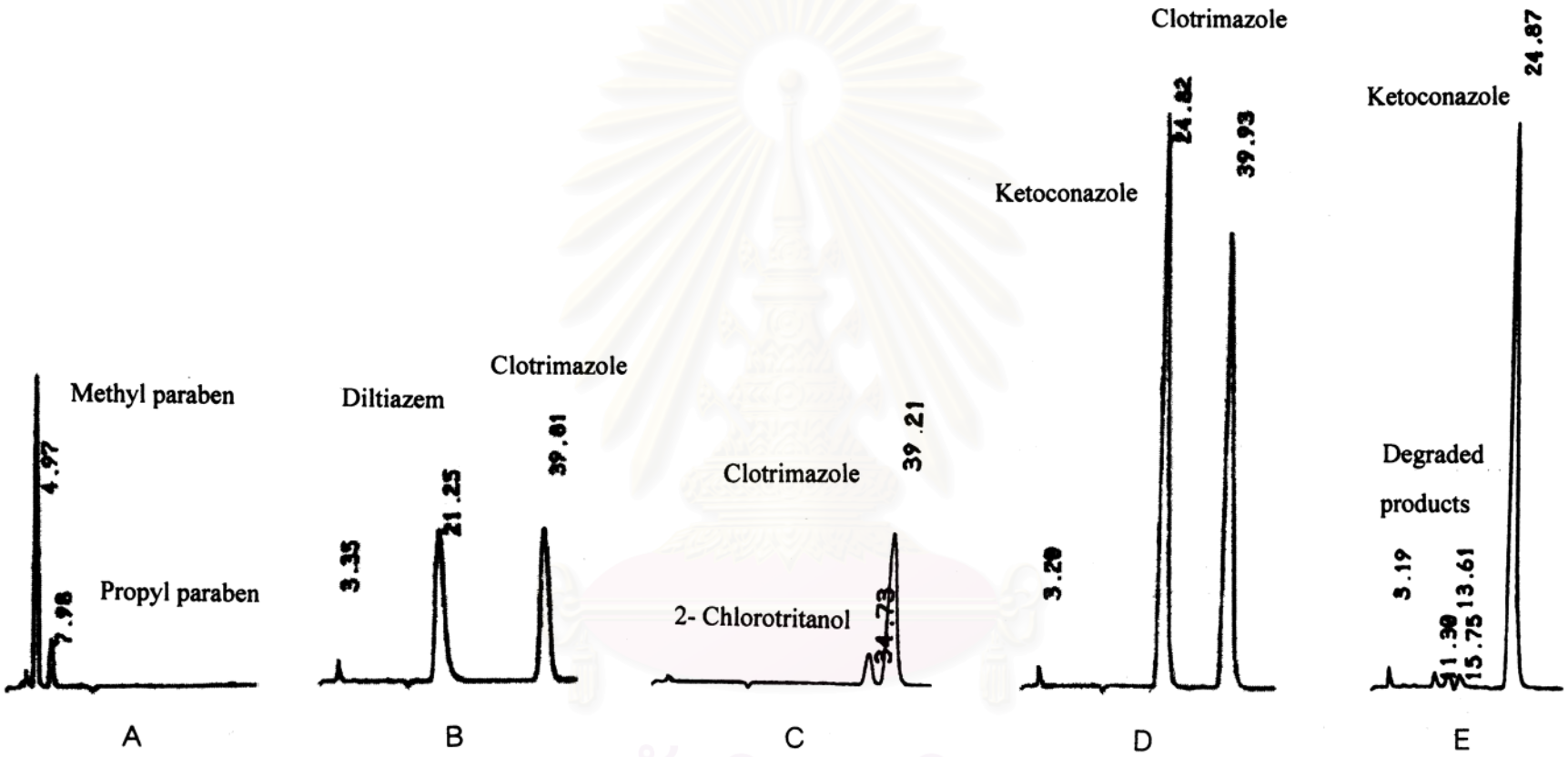

Figure 18 HPLC chromatogram of (A) cream base, (B) diltiazem and clotrimazole, (C) clotrimazole and 2- chlorotritanol, (D) ketoconazole and clotrimazole, (E) ketoconazole and its degraded products. Column: Apollo C18 (5 μm); mobile phase: 0.0025 M $(NH_4)H_2PO_4$ solution pH 7.0 with methanol (3:7, by vol.) at a flow rate 1 ml/min. Detection at 230 nm.

3.4. Linearity and range

The linearity of an analytical procedure is its ability to obtain test results that are directly proportional to the concentration of analyte in the sample within a given range. Linearity is generally reported as the variance of slope of regression line calculated according to an established mathematical relationship from test results obtained by the analysis of samples with varying concentrations of analyte.

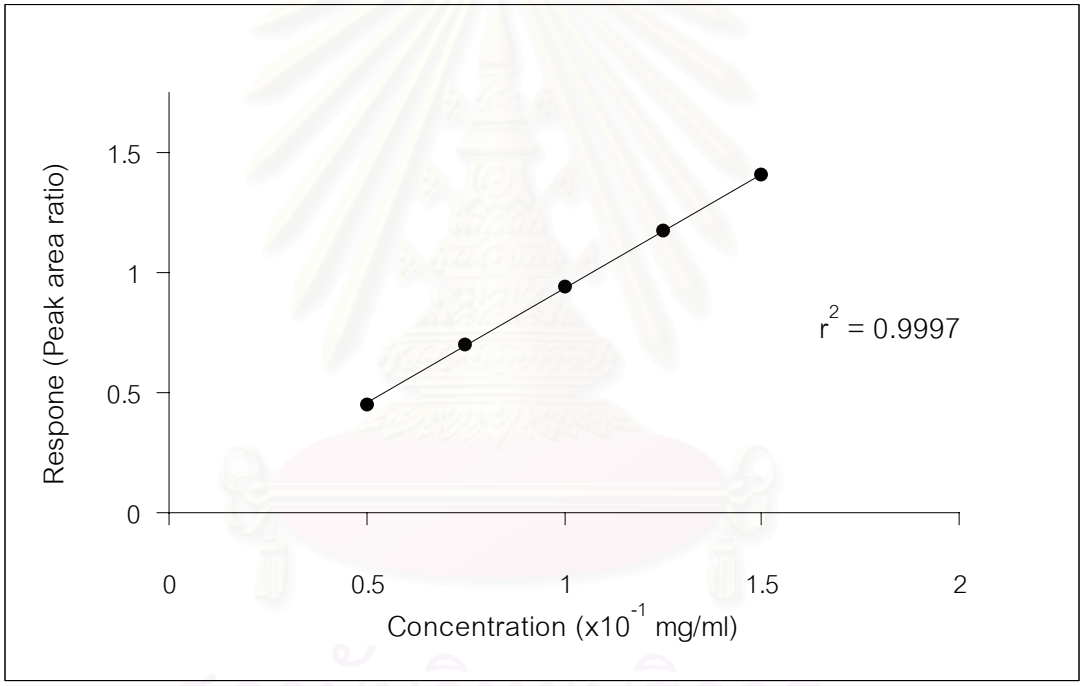

Figure 19 Calibration curve of clotrimazole.

Each point represents mean ± S.D. of six determinations.

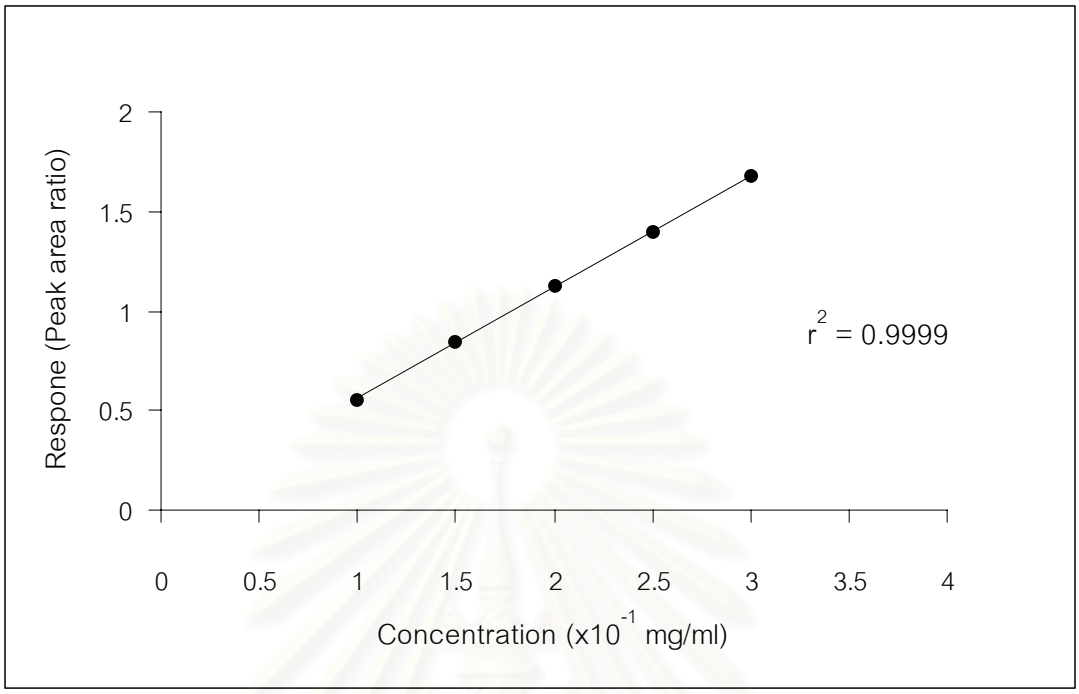

Figure 20 Calibration curve of ketoconazole.
Each point represents mean ± S.D. of six determinations.

In this study, linearity was performed between 50-150 % of target concentration as shown in Figure 19 and 20 for clotrimazole and ketoconazole, respectively. A coefficient of correlation (r^2) value, an intercept, and a slope were measured as follow.

For clotrimazole:

$$Y = 0.9539X - 0.0201 , r^2 = 0.9997$$

For ketoconazole:

$$Y = 0.5604X - 0.0015 , r^2 = 0.9999$$

Range of an analytical method is the interval between the upper and lower concentration levels of analyte, covering usually used concentration.

The proposed method should be shown in good precision, accuracy and linearity. According to USP, the range of method is validated by verifying that acceptable precision and accuracy obtained by the analytical method when the actual analysis of samples containing analyte is performed throughout the intervals of the range.

In this experiment, linearity was found in drugs concentration range with good precision 0.05 – 0.15 and 0.1 – 0.3 mg/ml for clotrimazole and ketoconazole, respectively.

4. Stability test on oil-in-water cream

4.1. Effect of temperature and humidity

The cream preparation was accelerated at 40 ± 1 °C with 75 ± 5 % RH for 6 months and sampling to assay the amount of drug at various interval of time. As illustrated in Figure 21, the amount of clotrimazole and ketoconazole in cream remained nearly constant over the period of study, thus, the cream products showed good stability under accelerated condition.

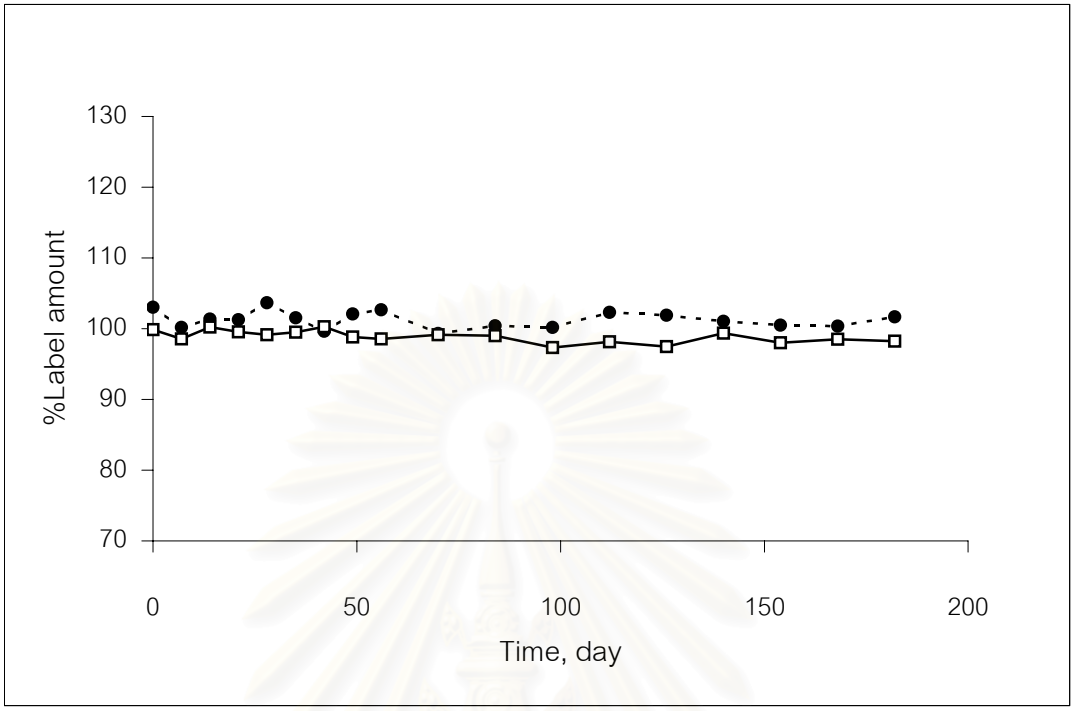

Figure 21 Stability of clotrimazole (---•---) and ketoconazole (—▫—) cream at 40 ± 1 °C, 75 ± 5 % RH.
Each point represents mean ± S.D. of six determinations.

4.2. Effect of pH

pH of 1 % clotrimazole and 2 % ketoconazole cream were adjusted within ranges from 3 to 8 with 1 N H_3PO_4 and 1 N NaOH and kept them for 3 weeks. The cream were assayed for amount of drugs left on weeks 0, 1 and 3. As demonstrated in Figure 22, concentration of clotrimazole in cream base pH 3 reduced significantly from 100.81 to 81.22 % on week 3 with no significant difference in quantity of clotrimazole in cream base, adjusted to pH between 4 and 8. Since clotrimazole is a weak base with pKa 4.7, it was ionized at pH 3 and its ionized form was easily catalyzed to 2-chlorotritanol at low pH

(Hoogerheide et al., 1981). Then, pH of clotrimazole cream should be adjusted to pH more than 3 for maintenance satisfactorily therapeutic amount of drug.

Slightly reduction in concentration of ketoconazole was found in cream base pH 3 as illustrated in Figure 23, Thus, ketoconazole was more stable in prepared cream base more than clotrimazole. However, ketoconazole is a weak base with pKa 2.9 and 6.5, thus, it may easily undergo acid-catalyzed hydrolysis (Skiba, 2000; Kumer, 1991). Therefore, pH of cream preparation should be kept at neutral for prevention of drug degradation and maintenance efficiency of drug.

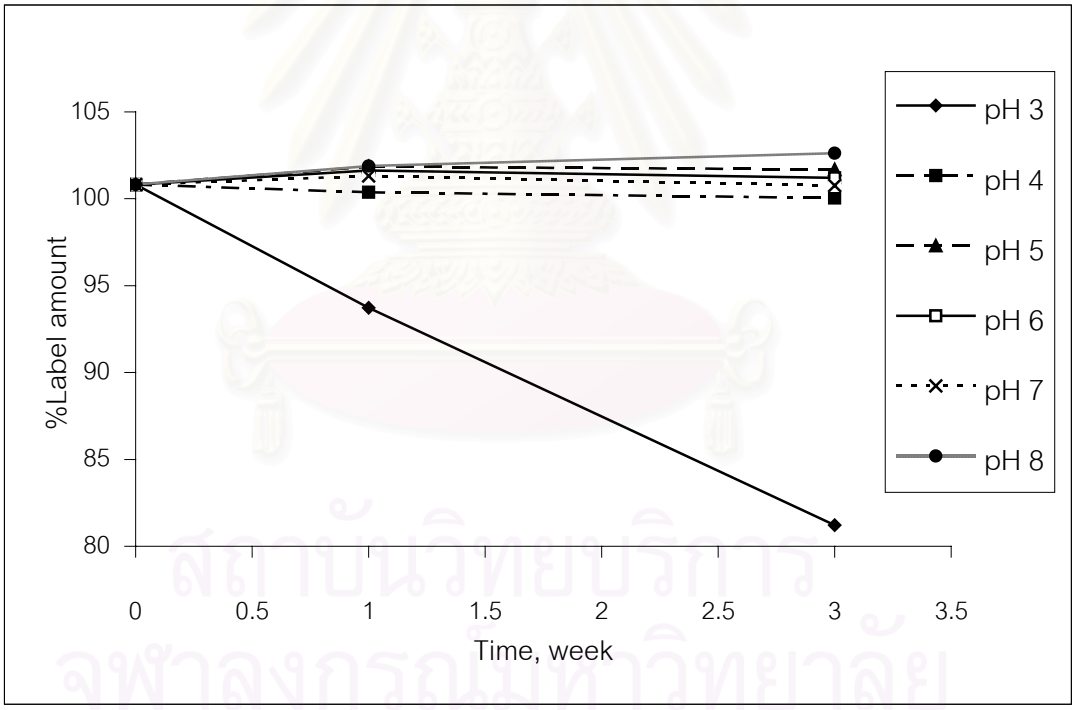

Figure 22 Stability of clotrimazole in cream base of various pH values. Each point represents mean ± S.D. of six determinations.

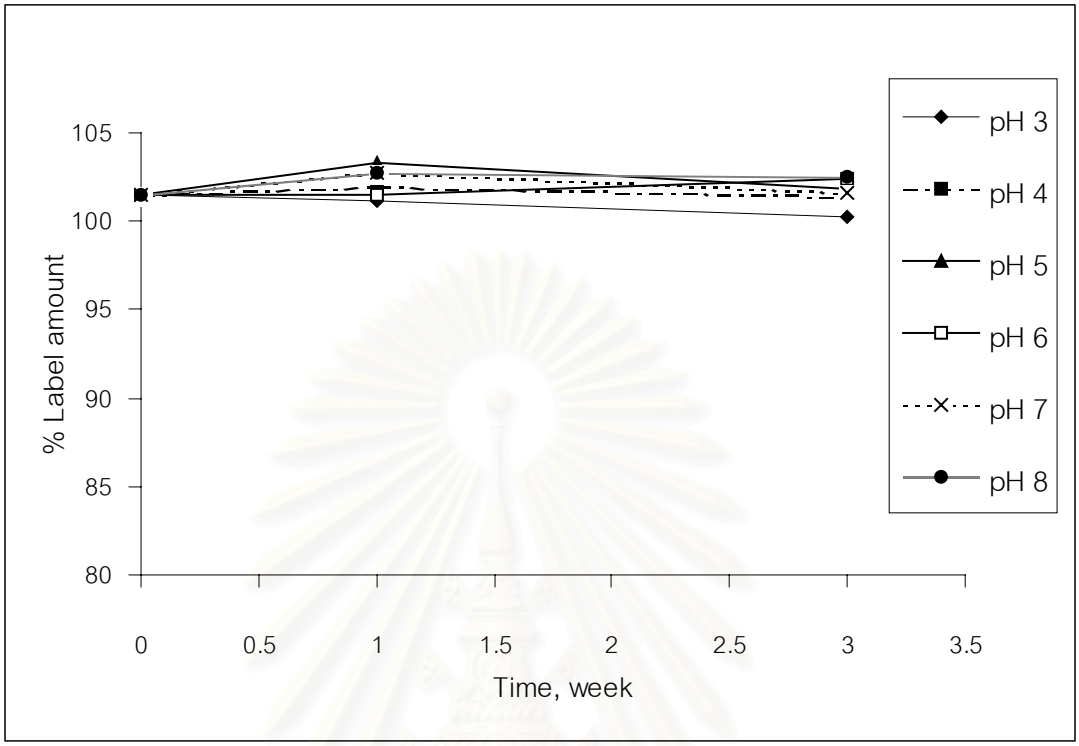

Figure 23 Stability of ketoconazole in cream base of various pH values. Each point represents mean ± S.D. of six determinations.

5. Assay application

The developed HPLC method was used to determine amount of clotrimazole and ketoconazole in commercial cream preparations which their cream bases contained methyl paraben and propyl paraben similar to studied cream base.

Table 8 Assay results of HPLC analysis of clotrimazole cream.

Product	% Label amount (± % RSD, n=6)
A	101.01 (± 0.66)
B	99.44 (± 0.78)
C	100.33 (± 0.83)
D	89.65 (± 0.96)*

* Appearance of cream is hard when sonication.

Table 9 Assay results of HPLC analysis of ketoconazole cream.

Product	% Label amount (± % RSD, n=6)
E	101.59 (± 1.68)
F	100.20 (± 078)
G	101.48 (± 0.15)
H	85.69 (± 0.41)*

* Appearance of cream is hard when sonication.

As shown in Table 8 and 9, almost tested commercial products, except products D and H, had assay content nearly 100 % label amount and there was no any degraded product detected in chromatograms in all products. Assay content of clotrimazole in product D was found to be 89.65 % with ketoconazole in product H 85.69 %. These low assay value in product D and H might be due to inapplicable removal of all active ingredients from their cream bases since the appearance of cream base seem hard in sonication step. From the results obtained, it was demonstrated that the developed method was applicable to use satisfactorily in analysis of clotrimazole and

ketoconazole separately from their degraded products, if existed in cream products.

CHAPTER IV

CONCLUSION

Clotrimazole and ketoconazole are imidazole antimycotic drug and are used widely in fungal topical infection. These drugs degrade easily by acid-catalyzed hydrolysis and ketoconazole easily photo-induced catalysis and their degraded products are inactive, thus, the restricted quality control of amount of drugs is important and necessary. There were many investigations of HPLC methods for quantitative assay of both drugs in various dosage forms but a few in cream. The method in pharmacopieas (USP and BP) mentioned the assay method of clotrimazole cream separately from its degraded products, but no official method for ketoconazole cream. However, the USP and BP methods have low resolution for assay clotrimazole separately from its degraded product (2-chlorotritanol). These pharmacopeial procedures used a large amount of cream which wax in cream, if incomplete eliminated, could obstruct analytical column. The assay method for ketoconazole in USP used high flow rate (3 ml/min) which would established high back pressure in stationary phase of small particle size, resulted in deterioration of analytical column and pumping system of high pressure liquid chromatograph on prolong usage.

In addition, pH of mobile phase for analysis of ketoconazole (BP 2002) was 2.04 which might cause cleavage of organic groups from their solid supports, consequently by reducing column efficiency. Some mobile phase used diisopropylamine and triethylamine for blocking free silanol group on stationary phase, however, these chemicals have pungent odor and may

harmful to operator. Tetrabutylammonium hydrogen sulphate, used in mobile phase as buffering salt, also acts as ion-pairing agent. Thus, it will change physicochemical property of analytical column on prolong usage, and reduce column life.

In this study, another HPLC method was developed for assay clotrimazole and ketoconazole separated from their degraded products, especially in cream preparation. This proposed method was simple, using common chemical reagents and nontoxic for preparing mobile phase. Pretreatment steps for separation ingredients in cream base from active ingredients and HPLC procedure were easy to perform, although longer retention time was obtained. The HPLC method presented here could separate clotrimazole and ketoconazole from their degraded products clearly with high resolution, thus, this developed HPLC method may be used as stability-indicating assay method. Moreover, the developed HPLC method may be used in quality control of another imidazole antimycotic drugs with proper modification, depending on physicochemical property of each drug.

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VITAE

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