



DEVELOPMENT AND VALIDATION OF A STABILITY-INDICATING ASSAY  
METHOD FOR BROMOCRIPTINE MESYLATE IN BULK AND TABLETS

มหาวิทยาลัยศิลปากร By สงวนลิขสิทธิ์

Phakinee Pukngam

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

MASTER OF PHARMACY

Program of Pharmaceutical Chemistry

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การพัฒนาและการตรวจสอบความถูกต้องของวิธีวิเคราะห์สำหรับทดสอบ  
ความคงสภาพของตัวยาโบรโมคริบทีนมีไซเลทที่เป็นวัตถุขบและยาเม็ด

โดย

นางสาวภาคินี พุกงาม

มหาวิทยาลัยศิลปากร สงวนลิขสิทธิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต

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The Graduate School, Silpakorn University has approved and accredited the thesis title of “Development and Validation of a Stability-Indicating Assay Method for Bromocriptine Mesylate in Bulk and Tablets” submitted by Miss Phakinee Pukngam as a partial fulfillment of the requirements for the degree of Master of Pharmacy in Pharmaceutical Chemistry.

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51360201 : MAJOR : PHARMACEUTICAL CHEMISTRY

KEY WORDS : STABILITY-INDICATING ASSAY METHOD/BROMOCRIPTINE MESYLATE

PHAKINEE PUKNGAM : DEVELOPMENT AND VALIDATION OF A STABILITY-INDICATING ASSAY METHOD FOR BROMOCRIPTINE MESYLATE IN BULK AND TABLETS. THESIS ADVISOR : ASSOC. PROF. JANKANA BURANA-OSOT, Ph.D., 81 pp.

The validated stability-indicating assay method for determination of bromocriptine mesylate (BCT) in bulk and dosage forms. The method was achieved on Zorbax Eclipse XDB column C18 column (Agilent®) with 4.6 x 150 mm dimension. The mobile phase consisted of methanol and 20 mM sodium acetate in the ratio 70:30, v/v, pH 5.0 in isocratic elution at 1.5 ml/min and detected at wavelength 300 nm. In addition, the method was used to resolve the drug from degradation products those obtained under various stress conditions. The drug was subjected to stress conditions of hydrolysis (neutral, acid and alkaline), oxidation, moisture, photolysis and thermal degradation. The drug in solution was found to degrade significantly in alkaline hydrolysis and when exposed to sunlight. The chromatographic method was optimized by using the samples generated from forced degradation studies. Good resolution between the peaks corresponds to the analyte and degradants was observed. The proposed method was validated with respect to specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), stability, robustness and ruggedness as per ICH guideline. The forced degradation studies were suggested the stability-indicating power of the method. Then, the method was applicable to the determination of bromocriptine mesylate in bulk and tablets. Currently, the method can use for stability testing also preparing stability data for pharmaceutical registration.

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คำสำคัญ : วิเคราะห์ที่บ่งชี้ความคงสภาพ/โบรโมคริบทิน มีโซเลท

ภาคินี พุกงาม : การพัฒนาและการตรวจสอบความถูกต้องของวิธีวิเคราะห์สำหรับทดสอบความคงสภาพของตัวยาโบรโมคริบทินมีโซเลทที่เป็นวัตถุคิบบและยาเม็ด. อาจารย์ที่ปรึกษาวิทยานิพนธ์ : ญญ.รศ.ดร.จันคนา บุรณะ โอสถ. 81 หน้า.

วิธีวิเคราะห์หาปริมาณตัวยาโบรโมคริบทินมีโซเลทในวัตถุคิบบและในยาเม็ด ด้วยเทคนิคโครมาโทกราฟีชนิดของเหลวสมรรถนะสูง ได้รับการพัฒนาและตรวจสอบโดยใช้คอลัมน์รีเวอร์สเฟสชนิด C18 Zorbax Eclipse XDB (Agilent®) ขนาด 4.6x150 มิลลิเมตร และใช้สารละลายของเมทานอลผสมกับโซเดียมแอซีเตตความเข้มข้น 20 มิลลิโมลาร์ ในอัตราส่วน 70:30 โดยปริมาตร ค่าความเป็นกรดต่างเท่ากับ 5.0 เป็นวัฏภาคเคลื่อนที่ด้วยอัตราการไหล 1.5 มิลลิลิตรต่อนาทีและตรวจวัดสารที่ความยาวคลื่น 300 นาโนเมตร วิธีวิเคราะห์ดังกล่าวยังใช้ในการศึกษาการสลายตัวของสารภายใต้สภาวะเร่งต่างๆได้แก่ การศึกษาผลของการเกิดปฏิกิริยาไฮโดรไลซิส (ในสภาวะที่เป็นกรด กลางและด่าง) ปฏิกิริยาออกซิเดชัน ผลของความชื้น แสง และความร้อน จากการทดสอบพบว่าในรูปสารละลายสารสลายตัวอย่างรวดเร็วในสภาวะที่เป็นด่าง หรือเมื่อนำไปสัมผัสกับแสงธรรมชาติ จากผลการวิเคราะห์พบว่าวิธีวิเคราะห์สามารถแยกตัวยาสำคัญออกจากสารสลายตัวที่เกิดขึ้นได้ และเมื่อทำการตรวจสอบความถูกต้องของวิธีพบว่าเป็นวิธีวิเคราะห์ที่มีความเฉพาะเจาะจง ความถูกต้องแม่นยำ มีความสัมพันธ์เชิงเส้นตรงระหว่างพื้นที่ใต้พีกกับความเข้มข้นของสาร มีความคงตัวและความคงทนของวิธีวิเคราะห์ สามารถนำวิธีนี้ไปวิเคราะห์หาปริมาณตัวยาโบรโมคริบทินมีโซเลทที่เป็นวัตถุคิบบและยาเม็ด ตลอดจนนำไปประยุกต์ใช้ศึกษาความคงสภาพของสารเพื่อนำไปประกอบการขึ้นทะเบียนยาในปัจจุบันได้

---

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ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์ .....

## ACKNOWLEDGEMENTS

This thesis would never be successfully completed without the kindness supporting and useful advice from many peoples. I will recognize to every moment and every person that contributed me a valuable experience.

Primarily, I would like to express my sincere gratitude to my thesis advisor, Associate Professor Dr. Jankana Burana-osot, for her invaluable advices, guidance, attention and encouragement throughout my study.

Deeply thankfulness to all teachers of the Pharmaceutical Chemistry Department, Faculty of Pharmacy, Silpakorn University for their kindness and giving me a meaningful advice persistently.

I am greatly thanks to Newcharoen Pharmaceutical L.P., and Faculty of Pharmacy, Silpakorn University for the materials and equipments supportive, and also give me a precious opportunity, in particular Mr. Chaiwut Chinsriwongkul and Dr. Akhayachatra Chinsriwongkul from Newcharoen Pharmaceutical L.P. for their excellent recommendation and helpfulness to my study.

I would like to acknowledge the Graduate School, Silpakorn University for the financial support (Annual Government Budget Expenditures for the fiscal year 2010).

I am also grateful to Biolab Co., Ltd. for the kindness supporting of bromocriptine mesylate raw material and working standard.

I would like to sincere thanks to all teachers, follow graduate students, researchers and the staff in Faculty of Pharmacy, Silpakorn University, for giving me the place, equipments, knowledge and friendship.

To all my friends and to other person who have not had specific mention, I would like to record my sincere appreciation for their kind assistance, encouragement and friendship throughout my studying.

Finally, I would like to express my beloved family for constant supportive and encouragement. They give me all worthwhile opportunity, I would like to thanks for their kind understanding, warmth and memorable time. Furthermore, thanks for taking care of me physically and believing in my ability always.

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## CHAPTER 1 INTRODUCTION

### 1. Statement and significance of the research problem

Drug stability is the capacity of a drug substance to remain within established specifications of identity, strength quality, and purity in a specified period of time. The factors that affect drug stability come from the atmosphere such as light, moisture, temperature, acid-base condition and its chemical and physical properties of drug which considered from the quality of raw material such as purity, impurities, crystal or polymorphic form, particle size and residual solvent. Moreover, drug formulation, manufacturing process as well as container of products also have impact on the stability of pharmaceutical products. Therefore, the stability testing was needed to provide evidence how the quality of drug substance or drug product varies with the time under the influence of a variety of environmental factors. (The United State-Food and Drug Administration 2003 : 2)

According to the regulatory definition, a stability-indicating method is one of the numbers of quantitative analytical methods that are based on the characteristic structural, chemical, or biological properties of each active ingredient from its degradation products so that the active ingredient content can be accurately measured (The United State-Food and Drug Administration 1987 : 3). This method also can accurately and precisely quantify the decrease of the active pharmaceutical ingredient content, alone or in the drug product, due to degradation. Consequently, a stability-indicating method is an analytical procedure that can discriminate between the major active pharmaceutical ingredients (API) from the other degradation products at the defined storage conditions. In addition, the method must also be sufficiently sensitive to detect and quantify one or more degradation products (Hong and Shah 2000 : 331).

The stating of Food and Drug administration of Thailand titled ASEAN Harmonization Product on Pharmaceutical Registration for the registration of generic drug, new drug, new generic drug and biological product must be conforms to

ASEAN Harmonization form only, which is issued by the authority for enforcement from January 1, 2009. The stability data must contain the testing condition that follow to ASEAN Stability Study Guideline (Thai-Food and Drug Administration 2008 : 2).

Recently, there is an increased tendency towards the development of stability-indicating assay, using the approach of stress testing as described in the International Conference on Harmonization (ICH) guideline Q1AR(2). This approach is extended to drug combinations, to allow accurate and precise quantitation of multiple drugs, their degradation products, and interaction products. Furthermore, the stability-indicating assay method was used to prepare stability data for the pharmaceutical registration.

## **2. Bromocriptine mesylate and its degradation products**

Bromocriptine mesylate (BCT) is an ergot alkaloid-peptide that acts as a weak partial agonist (or antagonist) at D1-type and a partial agonist at D2-type dopamine receptors, with moderate intrinsic activity. It was the first direct dopamine agonist to be employed in the treatment of Parkinson's disease, after its development as a prolactin inhibitor (Neumeier, Baldessarini and Booth 2005 : 241). As a nonhydrogenated ergot alkaloid, BCT is relatively sensitive to autooxidation both in solid and in dissolved status, but degradation products have not yet been elucidated. (Giron-Forest and Schonleber 1979 : 66). The impurities of BCT were testing in the topic of related substances as mentioned in the compendial pharmacopoeia (British Pharmacopoeial Commission 2009 : 281-283). An analytical method separated bromocriptine mesylate from their degradation products could not be found either in the literature or in an official British Pharmacopoeia (BP). The goal of this study is to establish the analytical method for stability testing of bromocriptine mesylate in finished product through degradation studies under variety of conditions recommended by ICH guideline.

## **3. Objective of this research**

3.1 To develop and optimize the reversed-phase high performance liquid chromatography (RP-HPLC) as the stability-indicating assay method for determination of bromocriptine mesylate in bulk and tablets.

3.2 To perform stress testing studies according to ICH recommendations to resolve the drug from different degradation products obtained under various stress conditions.

#### **4. The research hypothesis**

The developed and validated HPLC stability-indicating assay method could determine bromocriptine mesylate in both bulk and tablets.

มหาวิทยาลัยศิลปากร สงวนลิขสิทธิ์



## CHAPTER 2

### LITERATURE REVIEWS

#### 1. Stability of drugs

Stability is a critical quality attribute of pharmaceutical products, therefore, stability testing play role in the drug development process. The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environment factors, such as temperature, humidity, and light. The stability studies have been used to monitor possible change of drug product or material over time and at different storage conditions. Additionally, the result of stability studies are brought to establish a retest period for the drug substance or a shelf-life for the drug product and use as the recommended storage conditions (International Conference on Harmonization 2003 : 1-9).

The quality of analytical data generated on stability samples is essential to the successful completion of stability studies and to the ability to draw appropriate conclusions regarding the stability of the product under test. It is expected that all analytical methods applied in the study should be stability-indicating and that only those methods that are truly stability-indicating should be used. However, for traditional pharmaceutical products, it has become commonplace to reserve the term to describe the method used to detect chemical degradation of a drug substance or drug product. The development of a stability-indicating method is the access to suitable degraded samples to aid in method development. Obviously, this is unrealistic for several reasons: development timeline, and how stability is affected by batch characteristics such as process parameters, quality of excipients, and environment factors such as humidity and temperature. This is why pharmaceutical chemist has to rely on forced degradation (also called stress studies) samples to

develop stability-indicating assay methods to forecast real-time degradation.

The stability assessment of pharmaceuticals is typically done at three distinct times during development until commercialization: during development, to support the safety and efficacy claims of investigational new drugs; at registration, to ascertain the quality and shelf-life of the marketed product and its ingredients; and finally during the commercialization phase, to ensure the quality of the production and to support site or other changes to the product. Stability information on both drug substance and drug products is required as part of the registration dossier and serves to assign the shelf-life, determine appropriate storage conditions and assure that the quality of the product is unchanged from the time of manufacture to the time of administration to the patient. (Aubry, Tattersall and Ruan 2009 : 140)

## **2. Stability-indicating assay method (SIAM)**

As in the introduction, the accepted definition of a SIAM for a traditional pharmaceutical is a chromatographic (or other separation) method, able to separate the reportable degradants generated upon long-term storage of the product. Traditionally, the stability-indicating quality of the method is demonstrated by using stress samples or long-term stability samples. If a single method is to be used for quality control and stability on an active pharmaceutical ingredient, the method should also be able to separate process-related impurities. Stress testing is not the only avenue available for evaluating the validity of the method for stability determination. When available, naturally aged samples or other degraded samples may be more representative of the product's degradation. The practical steps involved in the development of SIAMs are followed (Bakshi and Singh 2002 : 1011-1040).

### **2.1 The study of the drug structure to assess the likely degradation pathway**

The first thing we have to do is found the informations can simply be gained from the structure, by study of the functional groups and other key components. There are definite functional group categories, like amides, esters, lactams, lactones, etc. that undergo hydrolysis, others like thiols, thioethers, etc. undergo oxidation, and compounds like olefins, aryl halo derivatives, aryl acetic acids, and those with aromatic nitro groups, N-oxides undergo photodecomposition.

Thus one can have a good starting point from the study of degradation behavior of congeners, but critical requirement here is the conduct of an in-depth literature survey.

## **2.2 Collection of information on physicochemical properties**

It is generally important to know various physicochemical parameters like pKa, log P, solubility, absorptivity and maximum absorption wavelength of the drug. For example, the knowledge of pKa is important as most of the pH-related changes in retention occur at pH within  $\pm 1.5$  units of the pKa value, the knowledge of log P for the drug and the identified degradation products provides good insight into the separation behavior likely to be obtained on a stationary phase.

This may be an easy practical step when the degradation products are known and available in the pure form. But when it is a new drug for which degradation pattern has not yet been established, the same might prove to be difficult. In the latter case, the suggested way is to subject the drug to stress studies and the degradation products were observed and changed the spectrum characteristic, first individually in each reaction solution and then in a mixture of all the solutions. If the shifts in wavelength spectra were occurred during the reaction and also guided on the suitable wavelength for analysis. More than one wavelength can be selected for analysis, but taking the benefit of the same requires a multi-wavelength detector. The best choice is using a photodiode array (PDA) detector, which allows recording of ultraviolet (UV)-visible spectrum of the components, as they get resolved on the stationary phase.

## **2.3 Stress (forced) degradation studies**

The ICH guideline Q1A suggests the following conditions to be employed but not provides any details on how hydrolytic, photolytic and oxidative studies have to be actually performed. On the other hand, the information is available in literature but in a staged way, with suggested approaches differing a lot from one another (ICH 2003 : 1).

## **2.4 Preliminary separation studies on stressed samples**

Performing the preliminary separation of the stress samples to study the number and types of degradation products formed under various conditions. To start with a reversed-phase octadecyl column is the simplest way, preferably a new or the one in a good condition. Well-separated and good quality peaks at the outset

provide better confidence because of the unknown nature of products formed during stressing. It should be preferred to use water-methanol or water-acetonitrile as the mobile phase in the initial stages. Between methanol and acetonitrile, the former should be preferred due to its low cost. From the previous studies on the development of assay method for the drug can also be applied here and the organic modifier can be chosen accordingly. The solvent can be changed, if the peak shape or separation problems are seen.

The detection wavelength can be select, based on the study of spectral behavior of degraded samples. The injection volume and the flow rate can be suitably adjusted based on the length of the column. Using these chromatographic conditions, one should follow the changes in all stress samples, at various time periods. The results should be critically compared with the blank solutions injected in the similar manner. It should be observed whether the fall in drug peak is quantitatively followed by a corresponding rise in the degradation product peaks. It should not be taken as a surprise if the peak rise is not in correspondence to fall of the drug. This is because the drug and its products can have very different extinction values. Even there can be situations where no additional peak appears in the chromatogram, other than the drug. Later, during the final method development changes can be made in mobile phase or the sample solvent to have the product shown up in the chromatogram. Even the absence of degradation peak can happen when the product is colored and shows no UV absorption at a particular wavelength at which the analysis has been conducted. This can be verified by simple observation whether any color has developed in the reaction solution. Here also suitable adjustment in the wavelength of analysis can be made for the product to appear in the chromatogram.

### **2.5 Final method development and optimization**

From the preliminary chromatographic studies, the retention time (RT) and relative retention time (RRT) of all products formed should be tabulated for each reaction condition. Special attention is then paid to those components whose RT or RRT is very close. PDA spectra of such components is obtained and critically evaluated to ascertain whether the products are same or different. If PDA results suggested that any of the products are different but are co-eluting, then suitable modification should be done in the chromatographic method to achieve a satisfactory

resolution. In the final step, a mixture of the reaction solutions is prepared, and subjected again to resolution behavior study. While making this mixture, it is not always necessary to add all reaction solutions withdrawn at different time for all conditions. That would make the situation too complex. Rather, only those solutions are mixed where different products are formed in sufficient quantity. Resolution in the mixture is studied closely, to see whether the resolution is similar to that obtained in individual samples. To separate close or co-eluting peaks, the method is optimized, by changing the mobile phase ratio, pH, gradient, flow rate, temperature, solvent type, and the column and its type.

### **2.6 Identification and characterization of degradation products, and preparation of standard**

The identification of degradation products and arrange for their standard are necessary for the establishment of specificity or selectivity of the method. The work on this aspect can even be initiated once an idea on the nature and number of degradation products formed under different degradation conditions is obtained from preliminary separation studies. To identify the resolved products, a conventional way is to isolate them and determine the structure through spectral such as mass spectrometry (MS), nuclear magnetic resonance (NMR), etc. However, this approach is very time consuming when multiple degradation products are formed. So, the modern approach is to use hyphenated liquid chromatography (LC) techniques couple with mass spectrometry. Regarding the product standards, a direct way is to procure them from commercial sources. However, in case they are not available commercially, they have to be either isolated from the degradation reaction solutions or synthesized in the laboratory. To isolate a product, the best way is to identify a reaction condition where it is formed selectively.

### **2.7 Validation of Stability-indicating assay method**

Validation of analytical methods, has been extensively covered in the ICH guidelines Q2A and Q2B , in the Food and Drug Administration (FDA) guidance and by the United State Pharmacopoeia (USP). There are two stages in the validation of a SIAM. First stage is early in the development cycle when drug substance is subjected to forced decomposition studies and the SIAM is established based on the knowledge of drug degradation behavior. The main focus of validation at the stage is

on establishment of specificity/selectivity, followed by other parameters like accuracy, precision, linearity, range, robustness, limit of detection and limit of quantitation. In the second stage, when the developed method is extended to formulations or other matrices, the emphasis gets limited to just prove the pertinence of excipients or other formulation constituents. Here only parameters of critical importance like specificity/selectivity, accuracy and precision are revalidated. If the SIAM is being developed directly for a formulation, without involving the bulk drug route, then all validation parameters are necessary to be established.

The specificity/selectivity of a SIAM can be determined very simply if degradation chemistry of the drug is known and the standards of the product are available. The only effort involved then is the development of a method that separates components from a physical mixture of drug and the degradation products. At this stage, only peak purity becomes crucial. The peak purity can be established by a variety of techniques, like PDA detection, absorbance ratio method, dual wavelength ratio chromatography, second order derivative spectroscopy, spectral suppression, spectral overlay, etc. However, not all these are applicable for on-line peak purity testing. The most popular technique is the PDA analysis, the principle of which is the comparison of the spectra of the analyte peak, and then unslope, at the apex and on the downslope.

The accuracy is usually determined by spiking the known amount of drug to either the placebo or the formulations, and determination of percent recovery of the drug. However, a better method of determining accuracy of a SIAM is by spiking the drug in a mixture of degraded solution. As far as the precision is concerned, there are no special requirements for stability-indicating methods and the same procedure as advocated for normal assay methods can be applied.

The linearity for SIAMs should be established initially in the range of 0-100%, as the drug may fall to very low concentrations during forced decomposition studies. The final validation range, however, can be narrowed based upon the form in which the drug substance or formulation is dispensed.

The detection and quantitation limits are not important for active drug substances, as their concentration is not expected to fall to such a low level in

different formulations during their shelf life. However, these limits should be established for the degradation products.

Robustness can also be established for SIAMs in a similar manner as it is done for conventional methods (Bakshi and Singh 2002 : 1011-1040).

### **3. Stress/forced degradation studies**

Stress testing has long been recognized as an important part of the drug development process. Recent efforts by the ICH with regards to the impurities and stability have brought an increased regulatory scrutiny of impurities, requiring identification and toxicological at very low levels. Moreover, it may help facilitate pharmaceutical development as well in areas such as formulation development, manufacturing, and packaging, in which knowledge of chemical behavior can be used to improve a drug product.

The ICH defined accelerated testing as “studies designed to increase the rate of chemical degradation or physical change of an active drug substance or drug product using exaggerated storage conditions as part of the formal, definitive, storage program. These data, in addition to long-term stability studies, may also be used to assess longer-term chemical effects at non-accelerated conditions and to evaluate the impact of short-term excursions outside the label storage conditions such as might occur during shipping. Results from accelerated testing studies are not always predictive of physical change” (ICH 2003 : 16-17). An important aspect of this definition is that the studies are part of the “formal, definitive, storage program.” In contrast, ICH, in Annex 1 of the revised stability guideline defined stress testing (drug substance) as “studies undertaken to elucidate the intrinsic stability of the drug substance. Such testing is part of the development strategy and is normally carried out under more severe conditions than those used for accelerated testing.”

The degradation products under stress conditions are useful in establishing degradation pathways, developing and validating suitable analytical procedures. However, it may not be necessary to examine specially for certain degradation products if it has been demonstrated that they are not formed under accelerated or long term storage conditions. Result from these studies will form an integral part of the information provided to regulatory authorities.



From the ICH definition, it is cleared that there is now a differentiation between “accelerated testing” and “stress testing.” Stress testing is distinguished by both the severity of the conditions and the focus or intent of the results. Stress testing, which is also often referred to as “forced degradation,” is an investigation of the “intrinsic stability” characteristics of the molecule, providing the foundation for developing and validating analytical methods and for developing stable formulations. Stress testing studies are intended to discover stability issues, and are therefore predictive in nature.

Although the reporting of degradation studies is not required in investigational new drug (IND) applications, preliminary studies may be carried out to facilitate the development of stability-indicating methodology. Studies can be conducted on the drug substance and developmental formulations to test for degradation by thermolysis, hydrolysis, oxidation, and photolysis or to evaluate the potential chemical behavior of the active ingredient. Additionally, the completed studies of the degradation of the drug substance and drug product are required at the new drug application (NDA) stage, including isolation and/or characterization of significant degradation products and a full written account of the degradation studies performed (Reynolds et al. 2002 : 48-50).

#### **4. Regulatory status related to stability-indicating assay method and forced degradation studies**

Many guidelines have been published that addressed directly or indirectly for SIAMs and forced degradation studies. From ICH guideline : Q1A titled on Stability Testing of New Drug Substance and Product had mentioned the stress testing of the drug substance can help identify the likely degradation products, which can in turn help established the degradation pathways and the intrinsic stability of the molecule and validated the stability-indicating power of the analytical procedures used. The nature of the stress testing will depend on the individual drug substance and the type of product involved. Stability studies should include testing of those attributes of the drug substance that are susceptible to change during storage and are likely to influence quality, safety, and efficacy. The testing should cover, as appropriate, the physical, chemical, biological, and microbiological attributes. Fully



validated stability-indicating analytical procedure should be applied (ICH 2003 : 1-9). The ICH guideline Q1B on Stability Testing : Photostability Testing of New Drug Substances and Products emphasized that the forced degradation studies should be designed to provide suitable information to develop and validate test methods for the confirmatory studies. These test methods should be capable of resolving and detecting photolytic degradants that appear during the studies. When evaluating the results of these studies, it is important to recognize that they from part of the stress testing and are not therefore designed to established qualitative or quantitative limit for change. (ICH 1996 : 5). The ICH guideline Q2B entitled Validation of Analytical Procedures : Text and Methodology described in case of the impurities are not available, the test for topic specificity may be demonstrated by comparing the test results of sample containing impurities or degradation products to a second well-characterized procedure, e.g., pharmacopoeial method or other validated analytical procedures. As appropriate, this should include samples stored under relevant stress conditions: light, heat, humidity, acid/base hydrolysis, and oxidation (ICH 2005 : 7). The ICH guideline Q3A on Impurities in New Drug Substance, which provided note for the analytical procedures, the registration application should include documented evidence that the analytical procedures are validated and suitable for the detection and quantification degradation products. It is also required that analytical methods should be validated to demonstrate that impurities unique to the new drug substance do not interfere with or are separated from specified and unspecified degradation product in the products (ICH 2006 : 3). The ICH guideline Q6A entitled Specification: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances also mentioned for the assay, which used a specific, stability-indicating assay to determine strength (content) should be include for all new products. In many cases it is possible to employ the same procedure for both assay of the new drug substance and quantitation of impurities (ICH 1999 : 7).

The United States Pharmacopoeia had a note under “Pharmaceutical Stability”, which said that the stability of manufactured dosage form must be demonstrated by the manufacture, using methods adequate for the purpose. Monograph assays may be used for stability testing if they are stability-indicating, so

they accurately differentiate between the intact drug molecules and their degradation products (USP 2002 : 2213-2214).

### 5. Bromocriptine mesylate

Bromocriptine mesylate, 2-bromo-2 $\beta$ -isopropyl-5 $\alpha$ -isobutyl-ergopeptine methanesulfonate is an amino acid, alkaloid derivative of lysergic acid. It consists of a heterocyclic nucleus to which a single bromine and peptide side chain is attached (Figure 1).

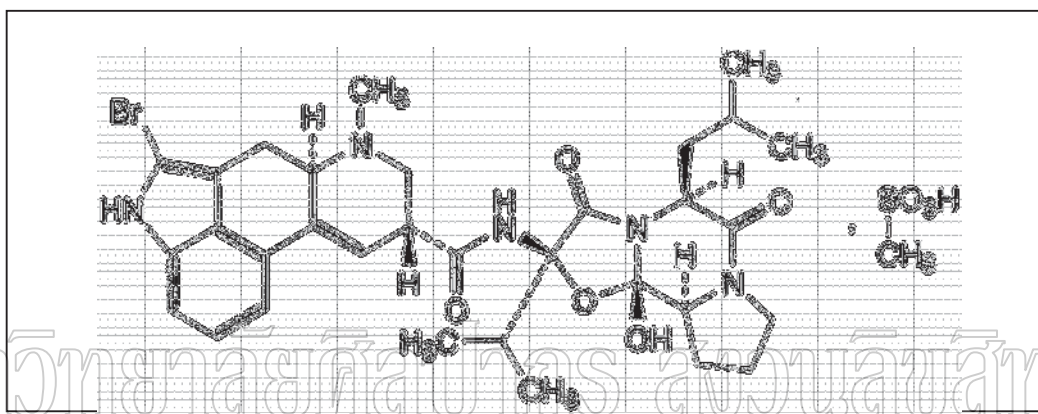


Figure 1 Chemical structure of bromocriptine mesylate

Source: British Pharmacopoeial Commission, The British Pharmacopoeia 2009, Vol.1 (London : The Stationary office, 2009), 281.

The ergot alkaloids and their derivatives are a rich source of catecholaminergic drugs. Ergot (*Claviceps purpurea*) is a parasitic fungus found on grasses and cereals (rye). The long black *sclerotium* (“ergot”) of the fungus is cultivated. Because the fungus is more valuable than the cereal crop, fields are artificially infected and the mixture of indole alkaloid is extracted from the ripe sclerotia. There is a structural correlation between dopamine and ergot alkaloid: the parent tetracyclic indole acid, lysergic acid, can be considered as containing an extended phenylethylamine moiety. (Thomas and Weaver 2005 : 342)

A novel and very interesting group of compounds is represented by bromocriptine, a dopamine receptor agonist that acts as a weak partial agonist at D<sub>1</sub>-type and a partial agonist at D<sub>2</sub>-type dopamine receptor, with moderate intrinsic

activity. Currently, bromocriptine is indicated primarily for the treatment of Parkinson's disease and conditions associated with hyperlactinemia (e.g., amenorrhea, galactorrhea, and infertility). Bromocriptine also is effective in the treatment of acromegaly and neuroleptic malignant syndrome. Bromocriptine mesylate is available as tablets and capsules, containing 2.5 mg (tablets) and 5 mg (capsules) of bromocriptine. (Kulig 2005 : 723-724)

Bromocriptine mesylate exists as a yellowish white crystalline powder that is slightly soluble in water and sparingly soluble in alcohol. It decomposes between 180 and 200°C, thus a melting point or melting range cannot be given. Because of the low solubility of bromocriptine mesylate in water, the pKa value had to be determined in methyl cellosolve/water 8:2 (w/w) and the pKa result was  $4.90 \pm 0.05$ . The partition coefficient between water of pH 1.2 and n-octanol was 1 : 90 and between water of pH 7.5 and n-octanol was 1 : 235 (Giron-Forest and Schonleber 1979 : 63).

As a non-hydrogenated ergot alkaloid, bromocriptine is relatively sensitive to autooxidation both in solid and in dissolved status, but degradation products have not yet been elucidated. Similarity to the oxidative transformation of the parent compounds is to be strongly anticipated (Giron-Forest and Schonleber 1979 : 66). In hydroxyl-containing solvents, nonhydrogenated ergot peptide alkaloids are readily epimerized at C-8 to an equilibrated mixture of the lysergic and iso-lysergic series. Under the same conditions, but at elevated temperatures, the C-2'-center is very likely to be inverted yielding a more acidic isomeric compound. The light-induced addition of water to the 9,10-double bond of bromocriptine yielding with high probability to the lumi-product. Under slightly acidic condition, bromocriptine can formed the corresponding 10 $\alpha$ -methoxy-lumi-derivative by the photo-catalyzed addition of methanol (Giron-Forest and Schonleber 1979 : 66).

Bromocriptine dosage forms, tablets and capsules have been proven stable at least 4 years at ambient temperature when stored in amber glass bottles (Giron-Forest and Schonleber 1979 : 48-67). Bromocriptine mesylate in bulk forms, is sensitive to heat and light but it is stable for up to 3 years at ambient temperature when stored in sealed polyethylene bags contained in twist-off amber glass bottles. In solution, bromocriptine is rather labile in aqueous or aqueous/alcoholic solutions, particularly in the presence of acid, yielding mainly the equilibrated mixture with its

8-epimer and to smaller extent, its hydrolysis products 2-bromo-lysergamide and 2-bromo-lysergic acid and their 8 $\alpha$ -isomers, respectively. 2-Bromo-lysergamide and 2-bromo-lysergic acid are listed by the British Pharmacopoeia as impurities D and E in the monograph (BP 2009 : 281-283). Figure 2 was shown their structures including other five impurities as reported in BP.

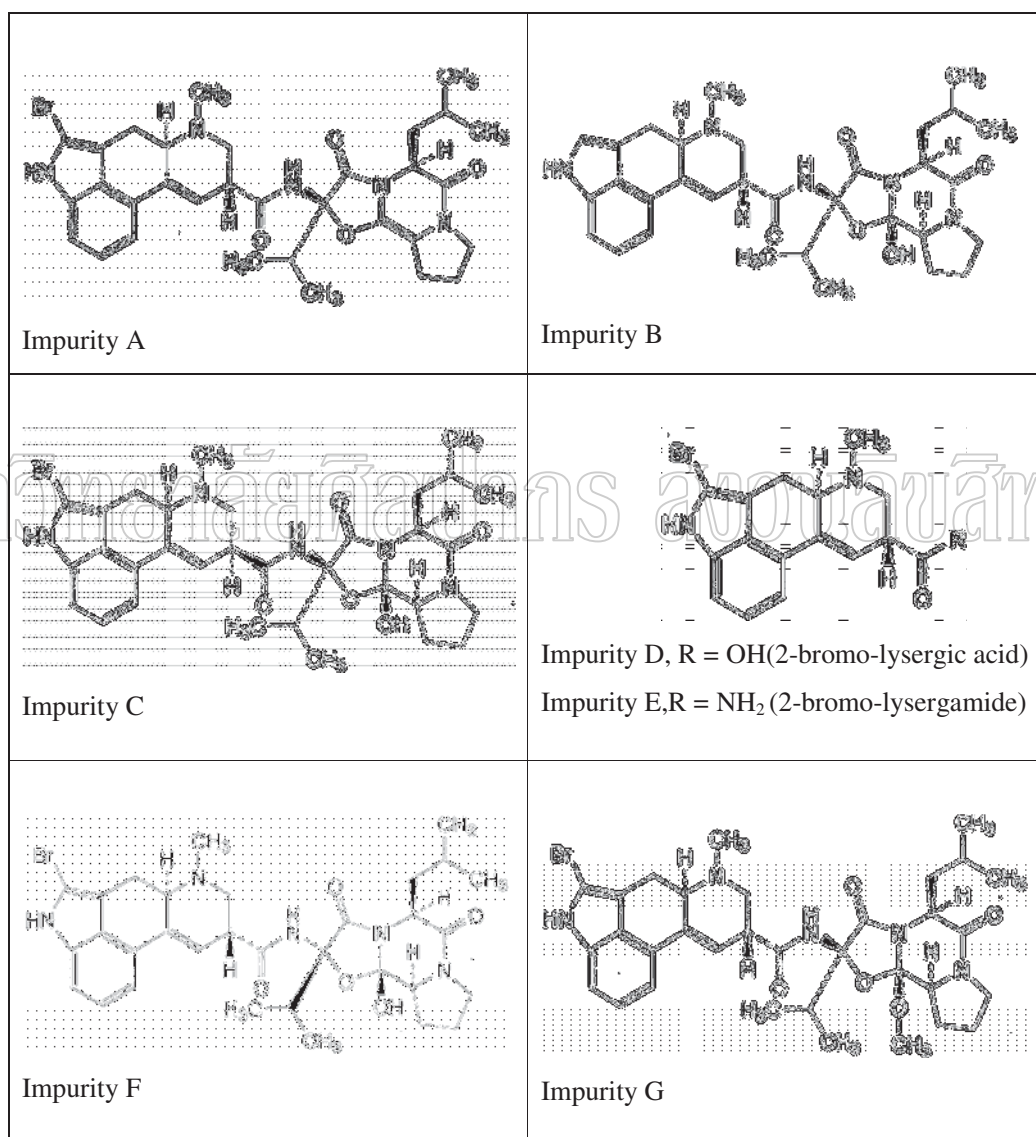


Figure 2 Structures of bromocriptine impurities

Source: British Pharmacopoeial Commission, The British Pharmacopoeia 2009, Vol.1 (London : The Stationary office, 2009), 283.

## 6. Analysis of bromocriptine mesylate

Several high-performance liquid chromatographic methods have been described for the separation and analysis of bromocriptine mesylate and its degradation products in raw material and dosage forms.

Current official methods, USP30 and BP2009, described the assay for bromocriptine mesylate in raw material by potentiometry titration in a mixture of acetic anhydride and glacial acetic acid, with 0.1 N perchloric acid was used as a titrant. Gradient HPLC was used for chromatographic purity test in USP, which used the mixture of citrate buffer, phosphate buffer as a mobile phase and the mixture of methanol and citrate buffer as a diluting solvent. BP2009 has been analysed the related substance by gradient HPLC, which used ammonium carbonate solution and acetonitrile as a mobile phase. The isocratic HPLC was used for analyzing tablets and capsules, with the mixture of acetonitrile and ammonium carbonate as a mobile phase. Since the titration assay cannot differentiate the active product and degradation products in raw material of bromocriptine as well as the method for determination of bromocriptine in dosage forms is not stability-indicated and more complicated in chromatographic purity or related substance analysis. So it cannot be used in stability study for analysis both of bromocriptine and its degradation product simultaneously (BP 2009 : 281-283 ; The United State Pharmacopeial Convention 2007 : 1553-1556).

The chromatographic condition for separating bromocriptine mesylate in tablets using a reversed phase silica gel column, with 0.05 M ammonium acetate and acetonitrile, (40% v/v) pH 5.6 as a mobile phase and detection at 240 nm was reported by Foda and Shafie (Foda and Shafie 1999 : 3201-3209).

The quantitative of bromocriptine in human plasma by liquid chromatography-tandem mass spectrometry was determined. Chromatographic system was performed on a symmetry C18 column using a mobile phase consisting of 25:75:0.1 acetonitrile-water-formic acid with a flow rate of 250  $\mu$ l/min was reported by Dalvador et al. (Salvador et al. 2005 : 237-242).

The comparison of three different methods for the determination of bromocriptine in plasma from patients undergoing ordinary treatment with this drug has been described by Larzen et al. These methods were gas chromatography, mass fragmentography and liquid chromatography. The liquid chromatographic condition

was consisted of a reversed phase column was packed with  $\mu$ Bondapak C18. The mobile phase was methanol-water (65:35) with 0.01 M 1-haptanesulfonic acid. The column flow rate was 1.5 ml/min (Larzen et al. 1979 : 341-349).

Choi et al. have been developed a RP-HPLC for the determination of bromocriptine in plasma and eye tissues by extracting bromocriptine in plasma by a liquid-liquid technique followed by an aqueous back-extraction, allowing injection of an aqueous solvent into a C18 column. The mobile phase was a mixture of acetonitrile and 0.2% triethylamine (pH3), 70:30 v/v, at a flow rate of 1 ml/min with fluorescence detection at an excitation wavelength of 330 nm and an emission wavelength of 405 nm (Choi et al. 1997 : 415-420).

Billups et al. have been developed and validated the RP-HPLC method for simultaneous determination of the dopamine antagonist haloperidol, its diazepam analog, and the dopamine agonist bromocriptine in rat plasma. The sample preparations by protein precipitated with acetonitrile and used droperidol as an internal standard, followed by a double step liquid-liquid extraction with hexane : chloroform (70:30) prior to C18 column separation. The mobile phase was a 0.1% (v/v) trifluoroacetic acid in deionized water, methanol and acetonitrile (45:27.5:27.5, v/v/v). Diode-array detection was used at the maximum wavelength of 245 nm for haloperidol, 254 nm for the diazepam analog and droperidol, and 240 nm for bromocriptine (Billups et al. 2010 : 699-705).

The literature reviews were revealed that the few HPLC methods had been reported for the determination of bromocriptine mesylate both of raw material and in pharmaceutical formulations. None of the previously method was reported as a stability-indicating assay method. Hence, the aim of this study was to develop the stability-indicating assay HPLC method included quantification of bromocriptine mesylate and could separated the drug from its degradation products.

All above described methods from the review literatures were summarized in Table 1.

Table 1 Summaries of published analysis of bromocriptine mesylate

Reference	Sample	HPLC Analytical technique
USP30	Raw material	Chromatographic purity test : in gradient mode <ul style="list-style-type: none"> <li>- HPLC C18 column (300 x 3.9 cm)</li> <li>- Mobile phase was the mixture of phosphate buffer and citrate buffer</li> <li>- UV detector at 300 nm</li> <li>- The flow rate was 2.0 ml/min</li> </ul>
	Capsules	Assay : in isocratic mode <ul style="list-style-type: none"> <li>- HPLC C8 column (250 x 4.0 mm)</li> <li>- the mixture of acetonitrile and ammonium carbonate solution (3 : 2, v/v) as a mobile phase</li> <li>- UV detector at 300 nm</li> <li>- The flow rate was 2.0 ml/min</li> </ul>
	Tablets	Assay : in isocratic mode <ul style="list-style-type: none"> <li>- HPLC C18 column (250 x 4.0 mm)</li> <li>- the mixture of acetonitrile and 0.01 M ammonium carbonate (65 : 35, v/v) as a mobile phase</li> <li>- UV detector at 300 nm</li> <li>- The flow rate was 2.0 ml/min</li> </ul>
BP2009	Raw material	Related substances : in gradient mode <ul style="list-style-type: none"> <li>- HPLC C18 column (120 x 4.0 mm)</li> <li>- The mixture of acetonitrile and ammonium carbonate solution as a mobile phase</li> <li>- UV detector at 300 nm</li> <li>- The flow rate was 2.0 ml/min</li> </ul>



Table 1 (Continue)

Reference	Sample	HPLC Analytical technique
BP2009	Tablets and capsules	Assay : in isocratic mode <ul style="list-style-type: none"> <li>- HPLC C18 column</li> <li>- Mobile phase was consisted of the mixture of acetonitrile and 0.08% ammonium carbonate (55:45, v/v)</li> <li>- UV detector at 300 nm</li> <li>- The flow rate was 1.0 ml/min</li> </ul>
Foda and Shafie, 1996	Tablets	Assay : in isocratic mode <ul style="list-style-type: none"> <li>- HPLC C18 column</li> <li>- Mobile phase was consisted of 0.05 M ammonium acetate and acetonitrile (40% v/v) pH 5.6</li> <li>- UV detector at 240 nm, the flow rate was 0.8 ml/min</li> </ul>
Salvador et al., 2005	Human plasma	Assay : in isocratic mode <ul style="list-style-type: none"> <li>- HPLC C18 column</li> <li>- Mobile phase was consisted of 25 : 75 : 0.1 , v/v of acetonitrile-water-formic acid</li> <li>- Detected by tandem mass spectrometry</li> <li>- The flow rate was 250 µl /min</li> </ul>
Larzen et al., 1979	Human serum or plasma	Assay : in isocratic mode <ul style="list-style-type: none"> <li>- HPLC C18 column</li> <li>- Mobile phase was methanol-water (65:35, v/v) with 0.01 M in 1-heptanesulfonic acid</li> <li>- UV detector at 254 nm and the flow rate was 1.5 ml/min</li> </ul>



Table 1 (Continue)

Reference	Sample	HPLC Analytical technique
Choi et al., 1997	Plasma and eye tissue of rabbit	Assay : in isocratic mode <ul style="list-style-type: none"> <li>- HPLC C18 column</li> <li>- Mobile phase was a mixture of 30 parts of acetonitrile and 70 parts of 0.2% triethylamine (pH 3)</li> <li>- Fluorescence detection was at an excitation wavelength of 330 nm and an emission wavelength of 405 nm</li> <li>- The flow rate was 1.0 ml/min</li> </ul>
Billups et al., 2010	Rat plasma	Assay : in isocratic mode <ul style="list-style-type: none"> <li>- HPLC C18 column</li> <li>- Mobile phase was consisted 0.1% (v/v) trifluoroacetic acid, methanol and acetonitrile (45 : 27.5 : 27.5, v/v)</li> <li>- Diode Array Detector (DAD) detector at 240 nm</li> <li>- The flow rate was 1.0 ml/min</li> </ul>

## CHAPTER 3

### MATERIALS AND METHODS

#### 1. Materials

1. Bromocriptine mesylate (Lot. No.7222100109, Teva Czech Industries, Czech)
2. Bromocriptine mesylate working standard (Teva Czech Industries, Czech)
3. Bromocriptine tablets : Suplac<sup>®</sup> (Lot. No. TA6278, Biolab Co., LTD and Parlodel<sup>®</sup> (Lot. No. T0056, Novartis)
4. Methanol A.R.grade (RCI Labscan Ltd., Thailand)
5. Methanol HPLC grade (RCI Labscan Ltd., Thailand)
6. Acetonitrile A.R. grade (RCI Labscan Ltd., Thailand)
7. Acetonitrile HPLC grade (RCI Labscan Ltd., Thailand)
8. Glacial acetic acid (RCI Labscan Ltd., Thailand)
9. Sodium acetate (Merck, Germany)
10. Sodium hydroxide (Ajax Finechem, Australia)
11. Hydrochloric conc. 37% (RCI Labscan Ltd., Thailand)
12. Ammonium carbonate (Ajax Finechem, Australia)
13. Potassium dihydrogen orthophosphate (Ajax Finechem, Australia)
14. Hydrogen peroxide 30% (Merck, Germany)

#### 2. Equipments

1. Analytical balance (Model AX-205DR, Mettler Toledo, Switzerland)
2. High performance liquid chromatography (HPLC) (Thermo Separation Products, U.S.A) consists with the following
  - a. Degasser (Thermo Separation Products, U.S.A)
  - b. Liquid chromatography pump (P1500, Thermo Separation Products, U.S.A)

- c. Autosampler (AS3000, Thermo Separation Products, U.S.A)
  - d. UV-Visible detector (UV1000, Thermo Separation Products, U.S.A)
  - e. Software ChromQuest (Thermo Separation Products, U.S.A)
3. Agilent HPLC 1100 series (Agilent technologies, U.S.A) consisted with the following
- a. An on-line degasser (G1322A, Agilent technologies, U.S.A)
  - b. Low pressure quaternary pump (G1311A, Agilent technologies, U.S.A)
  - c. Autosampler (G1313A, Agilent technologies, U.S.A)
  - d. Column oven (G1316A, Agilent technologies, U.S.A)
  - e. Photodiode array detector (G13158, Agilent technologies, U.S.A)
4. Zorbax Eclipse XDB-C18 HPLC column, 4.6 x 150 mm, 5  $\mu$ m (Agilent<sup>®</sup>, Part No.993967-902)
5. Syringe filter (Vertipure<sup>™</sup> Nylon syringe filter, 13 mm diameter, 0.45  $\mu$ m pore size, Vertical, Thailand)
- 6. Sonicator (Transsonic T460/H, Elma Hans Schmidbauer, Germany)
  - 7. pH meter (MP220, Mettler Toledo, Switzerland)
  - 8. Indicator paper pH 1.0 – 14.0 Full range (Whatman<sup>®</sup>, U.S.A)
  - 9. Hot air oven (Mettmert, Germany)
  - 10. Water bath (Mettmert, Germany)
  - 11. UVA chamber (with Sylvania<sup>®</sup> 18W, 1000 lumen)

### 3. Methods

#### 3.1 Preliminary studies

The starting method was verified from compendial method (USP 2007 : 1554), C18 HPLC column and the mobile phase consisting of acetonitrile (ACN) and 10 mM ammonium carbonate in the ratio 65 : 35, v/v were used. The proportion of acetonitrile was decreased for satisfactory retention time of bromocriptine until met at 55 percent of acetonitrile. Then, standard solution was used for preliminary studies and the compositions of the mobile phase were varied as follow;

### 3.1.1 Type of inorganic salt

Three kinds of inorganic salts, ammonium carbonate, sodium acetate and potassium dihydrogen orthophosphate were studied and prepared as aqueous solution in mobile phase. The other mobile phase parameters were fixed except the final pH was true value.

### 3.1.2 Concentration of inorganic salt

When the type of salt was selected, the concentrations were investigated at 10, 20 and 50 mM.

### 3.1.3 Determination of the final pH

The final pH of a mobile phase was adjusted according to the buffer capacity range of the selected salts.

### 3.1.4 The proportions of acetonitrile

The run time per injection was extended for separating the degradation products those may occurred before the bromocriptine peak. Hence the proportions of acetonitrile were adjusted.

## 3.2 Method development and optimization

### 3.2.1 Stationary phase selection

Because of non-polar properties of bromocriptine, C18 reversed phase HPLC column was selected in this study. It was Zorbax Eclipse XDB-C18 HPLC Column, 4.6 x 150 mm, 5  $\mu$ m (Agilent®).

### 3.2.2 Wavelength selection

Bromocriptine mesylate in diluting solution was scanned for selecting the maximum wavelength in ultraviolet (UV) range (200 – 400 nm). Furthermore, the automatic program of photodiode array (PDA) detector also used for the detection ability of other degradation products at this wavelength.

### 3.2.3 Mobile phase selection

From the preliminary studies, the mobile phase consisting of acetonitrile and 20 mM sodium acetate in the ratio 45 : 55, v/v, with final pH 5.0 was selected and optimized. In order to provide the best chromatographic separation of bromocriptine mesylate from its forced degradation products, various parameters were investigated as follow;

### **3.2.3.1 Type and proportion of organic solvent**

The separation of bromocriptine was compared between the mobile phase consisting of acetonitrile and methanol. Various proportions of selected organic solvent with 20 mM sodium acetate at pH 5.0 were compared.

### **3.2.3.2 Type and concentration of inorganic salt**

Three kinds of inorganic salts, ammonium carbonate, sodium acetate and potassium dihydrogen orthophosphate were used in the mobile phase. Then, the mobile phase consisting of selected proportion of organic solvents from 3.2.3.1 and selected inorganic salt with final pH 5.0 were prepared by varying the concentrations at salt of 20, 50 and 100 mM.

## **3.2.4 Solution preparation**

### **3.2.4.1 Standard solution**

A standard stock solution was prepared by weighing 500 mg of bromocriptine mesylate to a 100-ml volumetric flask, diluted and dissolved with methanol to obtain the concentration of 5 mg/ml. Then 5.0 ml of this solution was pipetted to a 50-ml volumetric flask, and diluted with methanol. The working standards were prepared spanning the range 100 – 300 µg/ml in 70% methanol for establishing the standard curve.

### **3.2.4.2 Sample solution**

For raw material, transferred accurately weighed 40 mg of bromocriptine mesylate to a 100-ml volumetric flask, dissolved and adjusted to volume by the diluting solvent. Then 5.0 ml of the solution was pipetted to a 10-ml volumetric flask and diluted to volume with the same solvent. Finally, the solution was filtered through 0.45 µm nylon syringe filter before injected to HPLC.

The assay was weighed and finely powdered not less than 20 tablets. An accurately weighed portion of the powder equivalent to about 25 mg of bromocriptine mesylate was transferred to a 50-ml volumetric flask. A 15 ml of diluting solvent was added and sonicated for 15 minutes. The mixture was diluted to volume with the same solvent, mixed and filtered through filter paper, discarded the first portion. A 4.0 ml aliquot of the filtered solution was transferred to a 10-ml volumetric flask, diluted to volume with the same solvent, mixed and filtered through 0.45 µm nylon syringe filter and injected to HPLC.

### 3.2.4.3 Forced degradation sample solutions

The goal of forced degradation studies was achieved about 80 percent of bromocriptine remaining from each forced condition. For neutral, acidic, alkaline hydrolysis and oxidation degradation, the varied parameters were concentrations of each reagent added, temperature and time for reaction. After stressed, subsequently adjusted pH to neutral by sodium hydroxide or hydrochloric acid before the sample was injected to HPLC. These solutions were diluted with the diluting solvent to have the concentration of 200 µg/ml. All degradation studies were performed at an initial concentration of 1 mg/ml of bromocriptine mesylate in methanol. The stock solution was prepared by transferring bromocriptine mesylate 100 mg to a 100-ml volumetric flask, dissolved and adjusted to volume by methanol. Multi-stressed samples were prepared as indicate below. They were chromatographed along with the non-stressed sample.

#### 3.2.4.3.1 Neutral (water) degradation

A 2.0 ml of stock solution was pipetted and transferred to a 25-ml erlenmeyer flask, added 2.0 ml of water. The solution was treated at room temperature or heat to 80°C for 1-10 minutes.

#### 3.2.4.3.2 Acidic hydrolysis

A 2.0 ml of stock solution was pipetted and transferred to a 25-ml erlenmeyer flask, added 1.0 ml of hydrochloric acid (0.1-1.0 N). The solution was treated at room temperature or heat to 80°C for 1-10 minutes.

#### 3.2.4.3.3 Alkaline hydrolysis

A 2.0 ml of stock solution was pipetted and transferred to a 25-ml erlenmeyer flask, added 1.0 ml of sodium hydroxide (0.01-1.0 N). The solution was treated at room temperature or heat to 80°C for 1-10 minutes.

#### 3.2.4.3.4 Oxidative degradation

A 2.0 ml of stock solution was pipetted and transferred to a 25-ml erlenmeyer flask, added 1.0 ml of hydrogen peroxide (0.3-3.0%). The solution was treated at room temperature and heat to 80°C for 1-10 minutes.

#### 3.2.4.3.5 Photolysis by sunlight and electrical light (UVA)

Photolytic studies in solution were performed in methanol and exposed to sunlight and UVA upto 5 days. At solid state, the powder of drug in petri-dish with a thin layer of 1 mm was exposed to the sunlight and UVA upto 5 days. Samples were withdrawn periodically by weighing 10 mg of bromocriptine mesylate to a 10-ml volumetric flask, dissolved and diluted to volume by methanol. A 2.0 ml of the solution was pipetted to a 10-ml volumetric flask, adjusted to volume by 70% methanol, filtered through 0.45  $\mu\text{m}$  nylon syringe filter and injected to HPLC analysis.

#### 3.2.4.3.6 Thermal degradation

At solid state, the pure drug in an opened petri-dish was exposed to dry heat at 80°C for 5 days. A 2.0 ml of the stock solution was pipetted to a 10-ml beaker and kept it on the water bath with the temperature at 80°C for 5 days. Samples were withdrawn at suitable time intervals and subjected to HPLC analysis. The suitable dilution for HPLC analysis was described in 3.2.4.3.5

#### 3.2.4.3.7 Effect of moisture

Each 10-ml volumetric flask contained 10 mg of bromocriptine mesylate. The few drops of water were added as a source of moisture. These flasks were kept at room temperature and protected from light, sampling the sample at suitable time intervals, dissolved and diluted to volume by 70% methanol.

### 3.3 System suitability test

The optimized HPLC condition was tested the suitability of the system according to USP30 (USP 2007 : 251-253) indication. The preparation of forced degradation samples was described in 3.2.4.3. These stressed samples was mixed together and then injected to HPLC by the optimized method. The system suitability was tested by the following parameters.

#### 3.3.1 Tailing factor (T)

Tailing factor was referred to peak asymmetry. Therefore tailing factor should be calculated using the equation (1).

$$T = W_{0.05} / 2f \quad \text{----- (1)}$$

in which  $W_{0.05}$  was the width at 5% of the peak height and  $f$  was the distance between maximum and the leading edge of the peak. A tailing factor of 1 referred to a symmetric peak. The calculated value for the tailing factor for each compound was in the acceptable range of  $0.8 \leq T \leq 1.5$ .

### 3.3.2 Column efficiency (number of theoretical plates ; N)

How well the HPLC column was packed could be represented by the number of the theoretical plates. In this study the number of theoretical plates was calculated by using the equation (2)

$$N=16(t_R/w)^2 \quad \text{----- (2)}$$

in which  $w$  was the width of the peak and  $t_R$  is retention time along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to the component.

### 3.3.3 Resolution (Rs)

The resolution was specified to ensure that bromocriptine peak and closely degradation products were resolved from each other. It was expressed as the equation (3)

$$R=1.18(t_{r,b}-t_{r,a})/(w_{0.5,a}+w_{0.5,b}) \quad \text{----- (3)}$$

in which  $t_{r,a}$  and  $t_{r,b}$  were the retention times or distances along the baseline from the point of injection to the perpendiculars dropped from the maxima of two adjacent peaks, and  $w_{0.5,a}$  and  $w_{0.5,b}$  were the peak widths at half-height. A value of 1.5 for resolution was implied a complete separation of two compounds.

### 3.3.4 Capacity factor (k')

The capacity factor was measured of how much time a bromocriptine was spent in the column. It was defined as the equation (4)

$$k'_{(A)} = (t_A - t_0)/t_0 \quad \text{----- (4)}$$

in which  $t_A$  was the retention time of the compound and  $t_0$  referred to retention time for an unretained peak.  $t_0$  can be calculated by observing the initial baseline deflection of the trace above and below the baseline, caused by the difference in sample solution composition and the mobile phase.



### 3.4 Analytical method validation

Validation was carried out according to ICH guideline with respected to specificity, accuracy, linearity, precision, limit of detection, limit of quantitation, robustness, ruggedness and stability (ICH 2005 : 6-13).

#### 3.4.1 Specificity

The specificity was determined by analyzing the solution contained only bromocriptine mesylate raw material, placebo solution, the mixture of bromocriptine mesylate and placebo, the mixed degradation products from the stressed conditions and bromocriptine tablets. The bromocriptine peak was well separated from other ingredients in the sample.

Additionally, the developed method was proved to be specific to bromocriptine, which was indicated through peak purity threshold, determined by automatic program of PDA detector.

#### 3.4.2 Linearity and range

From ICH guideline, the linearity test was recommended in the range 80 – 120% of the test concentration. In this study, the linearity of system was prepared from bromocriptine mesylate stock solution at five concentration levels from 50-150% of assay analyte concentrations (100, 150, 200, 250 and 300 µg/ml). All solutions were analyzed according to the proposed method. Peak area of bromocriptine mesylate for each individual analysis was calculated and the pattern of linear relationship of the selected response was determined. The peak area versus concentration data were treated by least-squares linear regression analysis.

The linearity of the method was evaluated by plotting the standard curve between concentration added and concentration found from the accuracy test. The degree of linearity was assessed by the correlation coefficient ( $r^2$ ), y-intercept and slope from linear equation.

#### 3.4.3 Accuracy

Accuracy of the assay method was evaluated by preparing the stock solution in the concentration 5 mg/ml in methanol. Placebo was contained lactose 2.3 g, starch 1.0 g, talcum 0.05 g and magnesium stearate 0.05 g. All excipients were mixed homogeneity and dissolved with 50 ml of 70% methanol. The stock solution was pipetted in the subsequent volume of ml to each 50-ml volumetric

flask for preparing the 100, 150, 200, 250 and 300 µg/ml of bromocriptine mesylate respectively, five replicates were done. Then, 2.0 ml of placebo was added to each flask, dissolved and diluted to volume with 70% methanol. The solutions were shaken and sonicated for 15 minutes and filtered through 0.45 µm filter paper. A 5.0 ml of the filtrate was pipetted to each 10-ml volumetric flask and diluted with the same solvent to volume, mixed and filtered through 0.45 µm syringe nylon filter. The amount of bromocriptine mesylate from the calibration curve was calculated.

The percentage of recoveries was calculated by comparing the amount of bromocriptine mesylate found with the amount added, as the equation (5)

$$\% \text{ recovery} = \frac{[\text{concentration found}]}{[\text{concentration added}]} \times 100 \text{ ----- (5)}$$

#### 3.4.4 Precision

System precision was evaluated by repeating ten injections of bromocriptine mesylate standard solution at a test concentration (200 µg/ml). The peak area and retention time of bromocriptine and the relative standard deviation (%RSD) were calculated.

Repeatability or within- run precision was evaluated by analyzing six replicated bromocriptine tablets sample solution within the same day. The within-run precision was reported as %RSD.

Intermediate precision or between-run precision was assessed by analyzing the same concentrations of three replicated samples over three consecutive days.

The sample was prepared by weighed and finely powder not less than 20 tablets. The weighed portion of powder equivalent to 50 mg of bromocriptine mesylate was accurately transferred to a 100 ml volumetric flask, dissolved about 50 ml of 70% methanol, shaken and sonicated for 15 minutes before subsequently diluted with 70% methanol upto volume, filtered through filter paper. Discarded the first portion, 4.0 ml of the filtrate was pipetted to each 10-ml volumetric flask and diluted to volume with the same solvent, mixed and filtered through 0.45 µm syringe filter before injected to the HPLC system.

### 3.4.5 Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ for bromocriptine mesylate was determined at a signal to noise ratio of 3:1 and 10:1, respectively, by injecting a series of diluted solutions with known bromocriptine concentrations. The precision study was also carried at both LOD and LOQ level by injecting ten individual preparations of bromocriptine mesylate and calculating the %RSD of the signal to noise ratio.

### 3.4.6 Robustness

To determine the robustness of the proposed method, experimental conditions were altered such as the components of the mobile phase (ratio of organic solvent, pH, flow rate, temperature etc.) and the resolution between bromocriptine mesylate and its degradation products was evaluated on the combination of degradation products from all stress condition. In this study, the ratio of organic solvent and pH were chosen. The pH value was changed by 0.2 units from 4.8 to 5.2 while the mobile phase components were held constant at methanol and 20 mM sodium acetate in a ratio 70:30, v/v. The effect of the percent of organic solvent on resolution was studied by varying methanol from 68 to 72%. In all the deliberate varied chromatographic conditions (pH values, percentage organic solvent), the retention time, tailing factor, resolution, the number of theoretical plates and capacity factor of bromocriptine peak were illustrated the robustness of the method. The varied parameters for each condition were shown in Table 2.

Table 2 The varied parameters for each conditions in robustness testing

Methanol : 20 mM Sodium acetate (v/v)	pH	Flow rate (ml/min)
68 : 32	5.0	1.0
69 : 31	5.0	1.0
70 : 30	5.0	1.0
71 : 29	5.0	1.0
72 : 28	5.0	1.0
70 : 30	4.8	1.0
70 : 30	4.9	1.0
70 : 30	5.1	1.0
70 : 30	5.2	1.0

### 3.4.7 Ruggedness

The ruggedness was performed by analyzing bromocriptine tablet by using the same chromatographic system but different analysts. Three samples were analyzed by each analyst. The difference between two analysts was compared the percent labeled amount and reported as %RSD.

### 3.4.8 Stability of standard solution

The stability of bromocriptine mesylate standard solution in the diluting solvent was determined at room temperature for in-process analysis. The stability of standard solution in autosampler was studied for 24 hours. The condition was shown in Table 3.

Table 3 The stability study for in-process analyte in the autosampler

Parameters	In process analyte in the autosampler
Concentrations ( $\mu\text{g/ml}$ )	200
Temperature	$25 \pm 2^\circ\text{C}$
Stability condition	In autosampler
Stability checking time	0, 1, 2, 4, 6, 8, 12, 16, 20 and 24 hrs

### 3.5 Quantitative analysis of raw material and tablets

The proposed HPLC method was used for analysis of bromocriptine mesylate in bulk and tablets. Two commercially available tablet formulations were analyzed. The amounts of bromocriptine in samples were calculated using peak area obtained from assay preparation compared with those obtained from standard solution. The preparation of raw material and tablets were described in 3.2.4.2.

Bromocriptine mesylate raw material (Lot.No.7222100109) was kindly supported from Biolab Co., LTD. Two commercially available bromocriptine tablets, Suplac<sup>®</sup>(Lot.No. TA6278) and Parlodel<sup>®</sup>(Lot.No. T0056) were bought from drugstore.

## CHAPTER 4

### RESULTS AND DISCUSSION

#### 1. Preliminary studies

The starting method was consisted of acetonitrile and 10 mM ammonium carbonate in the ratio of 55:45, v/v with flow rate 1.0 ml/min. The diluting solvent was 55% acetonitrile. Other parameters in the mobile phase were varied as followed;

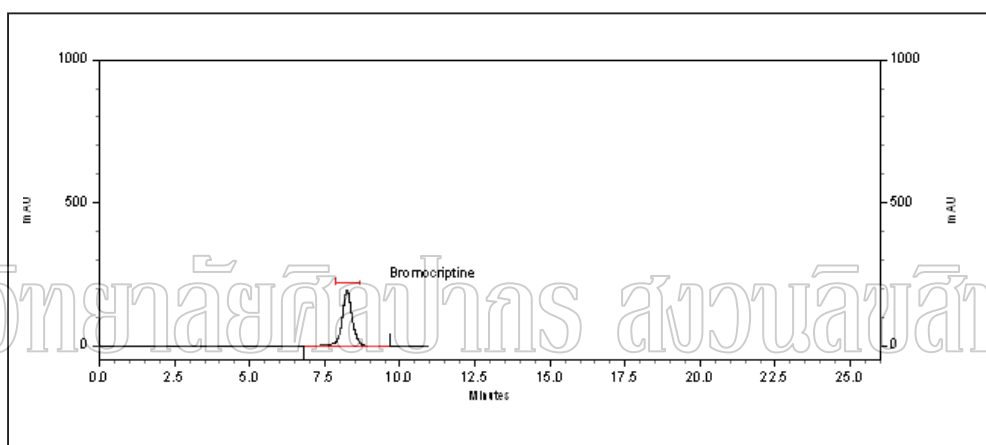


Figure 3 Chromatogram of bromocriptine from HPLC condition consisted of acetonitrile : 10 mM ammonium carbonate = 55:45, flow rate 1.0 ml/min, detected at 300 nm

#### 1.1 Type of inorganic salt

The aqueous solution in the mobile phase was prepared from ammonium carbonate, sodium acetate and potassium dihydrogen orthophosphate at the concentration of 10 mM. The proportion of acetonitrile was fixed at 55 percent. The results were found the mobile phase consisting of ammonium carbonate and sodium acetate were given a symmetry peak better than potassium dihydrogen orthophosphate. However, ammonium carbonate was made the mobile phase at high pH that may caused the HPLC column deteriorated in the short time so sodium acetate

was chosen for this method. The result and effect of each salt on retention time, tailing factor, theoretical plates, resolution and capacity factor of bromocriptine were reported in Table 4. The chromatograms from each salt were shown in Figure 4.

Table 4 Effect of salt in the mobile phase on system suitability parameters

Mobile phase composition (55 : 45, v/v)	pH	RT (min)	T	N	Rs	k'
ACN : 10 mM Ammonium carbonate	8.61	8.44	1.04	5325	-	6.21
ACN : 10 mM Sodium acetate	7.77	8.44	1.05	5276	-	6.21
ACN : 10 mM Potassium dihydrogen orthophosphate	5.23	6.61	0.71	2246	-	4.65

### 1.2 Concentration of inorganic salt

In order to determine the effect of concentration of sodium acetate on separation of bromocriptine, three concentrations of salt (10, 20 and 50 mM) were prepared with constant organic solvent proportion. The influence of salt concentrations on system suitability parameters were shown in Table 5 and Figure 5.

The variation of salt concentrations did not affect the system suitability parameters of bromocriptine, but at concentration of 20 mM was given the better symmetry peak than 10 and 50 mM. Therefore, 20 mM of sodium acetate was selected for the aqueous solution of the mobile phase.

Table 5 Effect of sodium acetate concentrations in the mobile phase on system suitability parameters

Mobile phase composition (55 : 45, v/v)	pH	RT (min)	T	N	Rs	k'
ACN : 10 mM Sodium acetate	7.77	8.44	1.05	5276	-	6.21
ACN : 20 mM Sodium acetate	7.87	8.35	0.97	4828	-	6.14
ACN : 50 mM Sodium acetate	8.22	8.39	0.94	4884	-	6.17

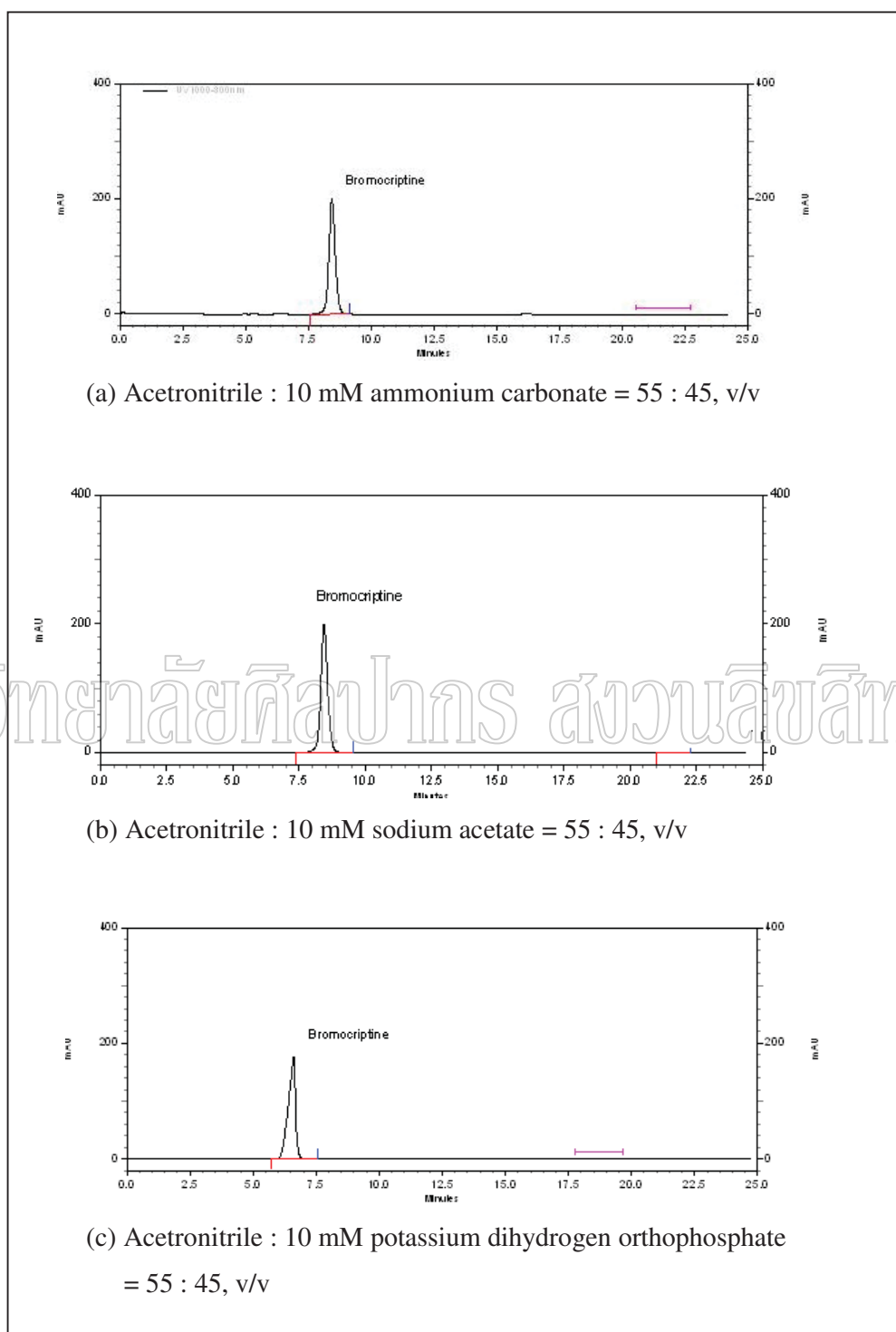


Figure 4 Chromatograms of bromocriptine from each salt in the composition of the mobile phase, flow rate 1.0 ml/min, detected at 300 nm

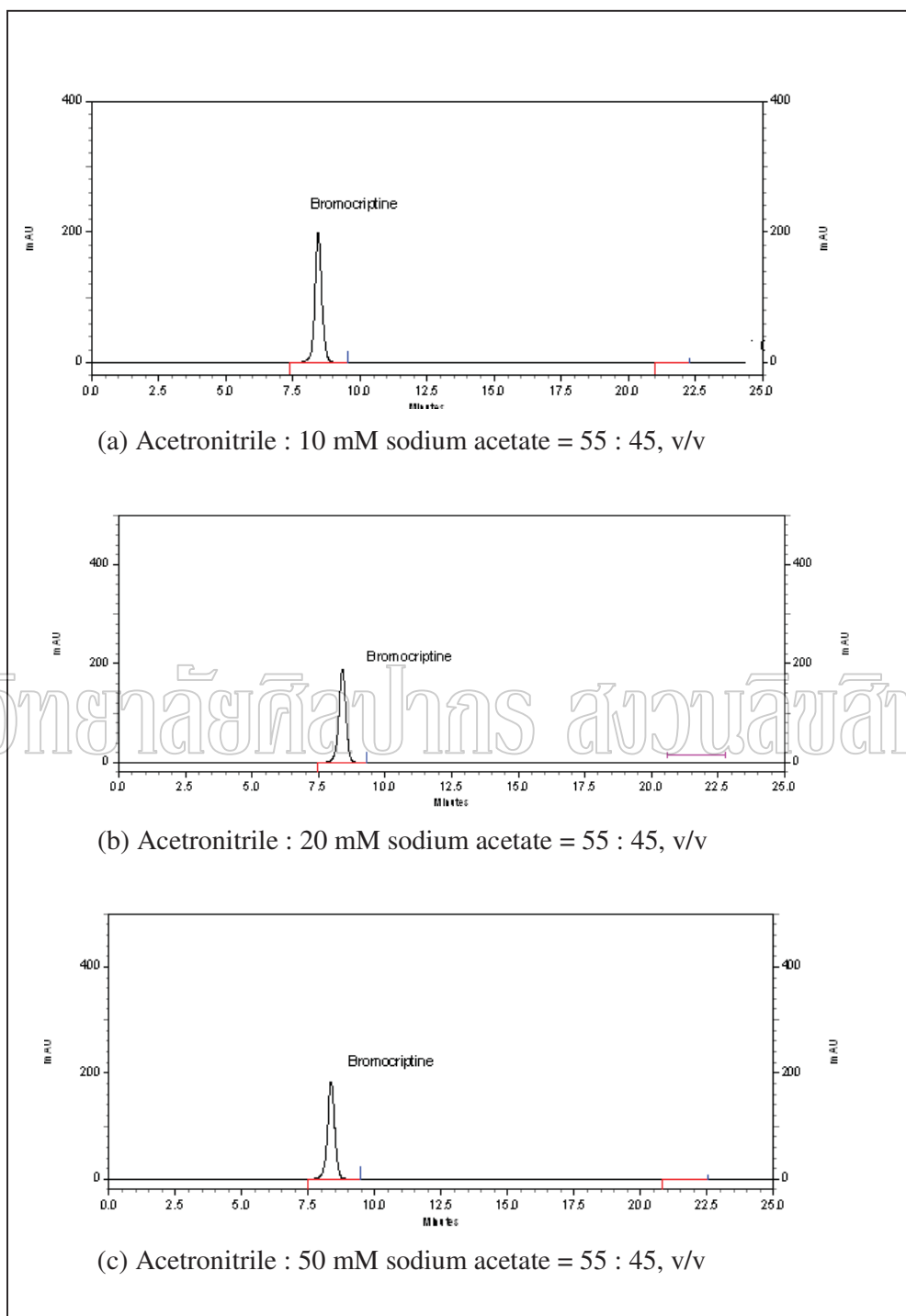


Figure 5 Chromatograms of bromocriptine from various concentrations of sodium acetate in the composition of the mobile phase, flow rate 1.0 ml/min, detected at 300 nm



### 1.3 Determination of final pH

For selected inorganic salt, 20 mM of sodium acetate was mixed with acetonitrile gave the pH still high so the pH was adjusted down by glacial acetic acid. At pH 5.0 was given the retention time shorter than other pH. At low pH bromocriptine was ionized and shown polar properties that made bromocriptine eluted fastly. Additionally, the buffer capacity of sodium acetate buffer was covered this pH. Therefore pH 5.0 was considered the suitable pH of the mobile phase.

### 1.4 The proportion of acetonitrile

The retention time of bromocriptine was prolonged by decreasing the proportion of acetonitrile. At the ratio 45:55, v/v, bromocriptine peak was found satisfactory retention time but coeluted peak was appeared when analyzing with the degradation sample. From the experiment, the effect of the various proportions of acetonitrile in the mobile phase was shown in Table 6 and Figure 6.

Table 6 Effect of acetonitrile proportions in the mobile phase on system suitability parameters

Mobile phase compositions (ACN : 20 mM Sodium acetate, v/v, pH 5.0)	RT (min)	T	N	Rs	k'
55 : 45	4.11	1.47	3015	-	2.52
50 : 50	5.43	1.08	4546	-	3.64
45 : 55	8.48	1.01	4266	-	6.25

Then the preliminary studies was ended with the HPLC condition that the mobile phase consisting of acetonitrile and 20 mM sodium acetate in the ratio 45:55, v/v with final pH 5.0 and flow rate 1.0 ml/min.

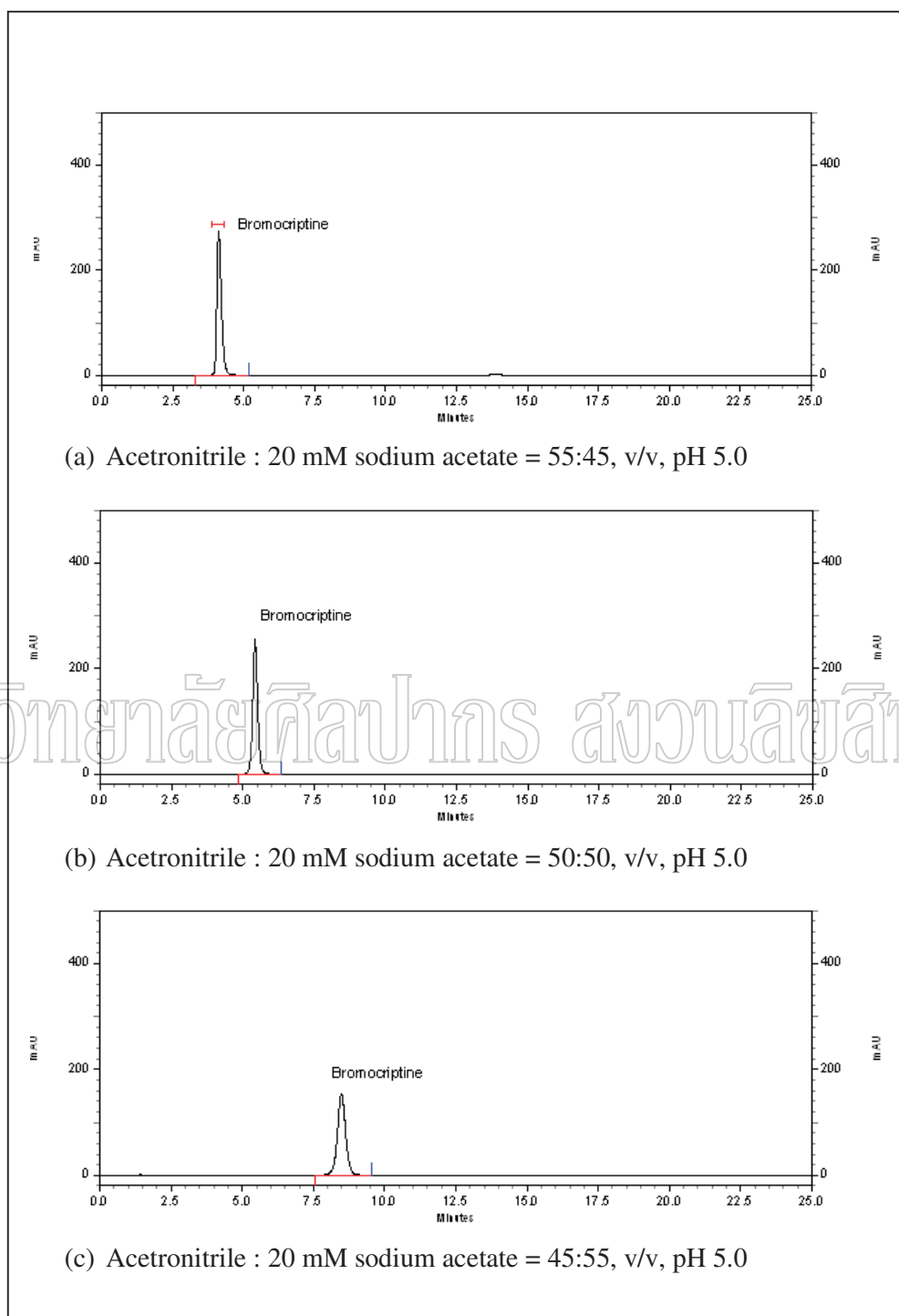


Figure 6 Chromatograms of bromocriptine from various proportions of acetonitrile in the composition of the mobile phase, flow rate 1.0 ml/min, detected at 300 nm

## 2. Method development and optimization

The C18 column and UV detector were selected for this study, other HPLC parameters appropriate for analyzing bromocriptine mesylate was determined according to the follow physicochemical properties of drug.

The UV spectrum of bromocriptine mesylate in 70% methanol was shown in Figure 7. The maximum wavelength was found at 300 nm. The detection ability of other degradation products was proved by PDA detector as shown in Figure 8. Therefore this wavelength could be detected both of bromocriptine and its degradation products simultaneously. So, wavelength 300 nm was selected for the method development.

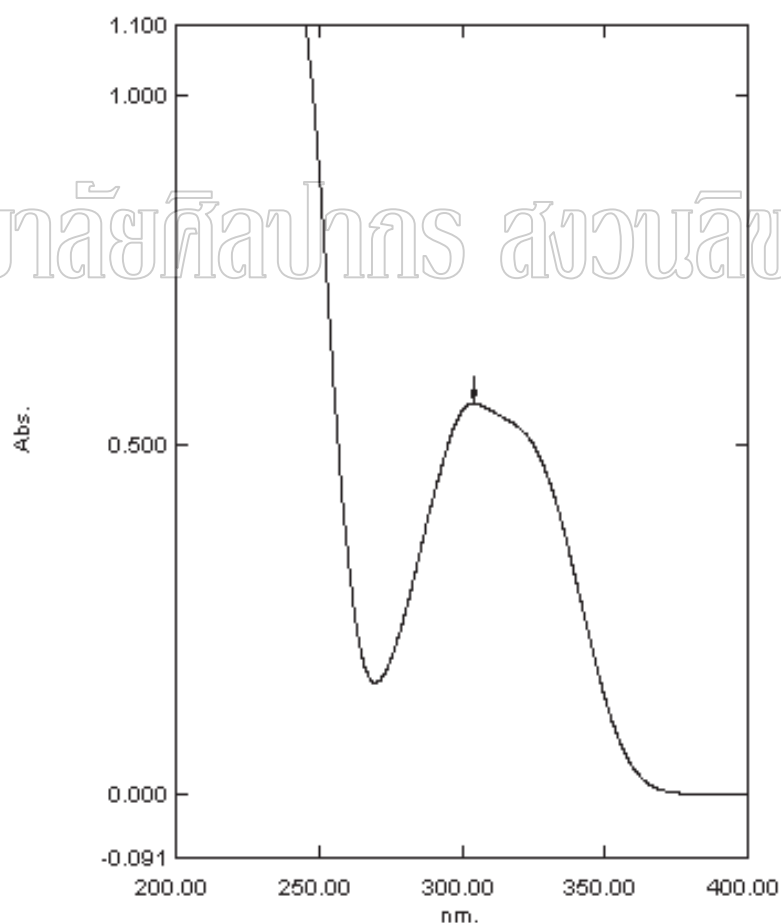


Figure 7 UV absorption spectrum of bromocriptine (0.04 mg/ml)

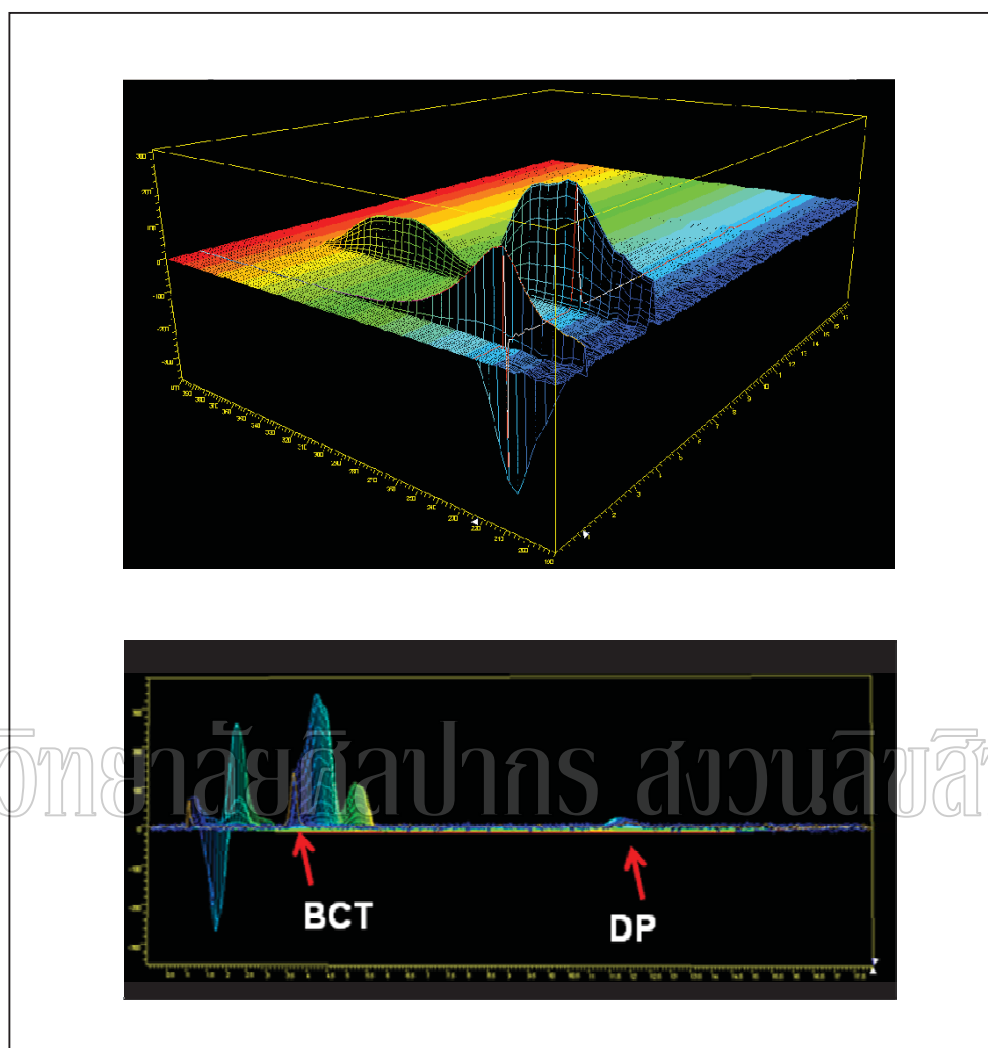


Figure 8 3D-PDA Chromatogram of bromocriptine from mixed degradation samples (HPLC condition consisting of methanol:20 mM sodium acetate = 70 : 30, v/v, pH 5.0, flow rate 1.5 ml/min, detected at 300 nm)

Remarks: BCT = Bromocriptine and DP = main degradation product

## 2.1 Mobile phase selection

From preliminary studies condition, the degraded sample was investigated as follow;

### 2.1.1 Type and proportion of organic solvent

When the stressed samples were analyzed by the starting method from the preliminary studies, it was found that bromocriptine could not resolved from

the closely degradation product and the coeluted peak was found. The chromatogram of bromocriptine from this method was shown in Figure 9.

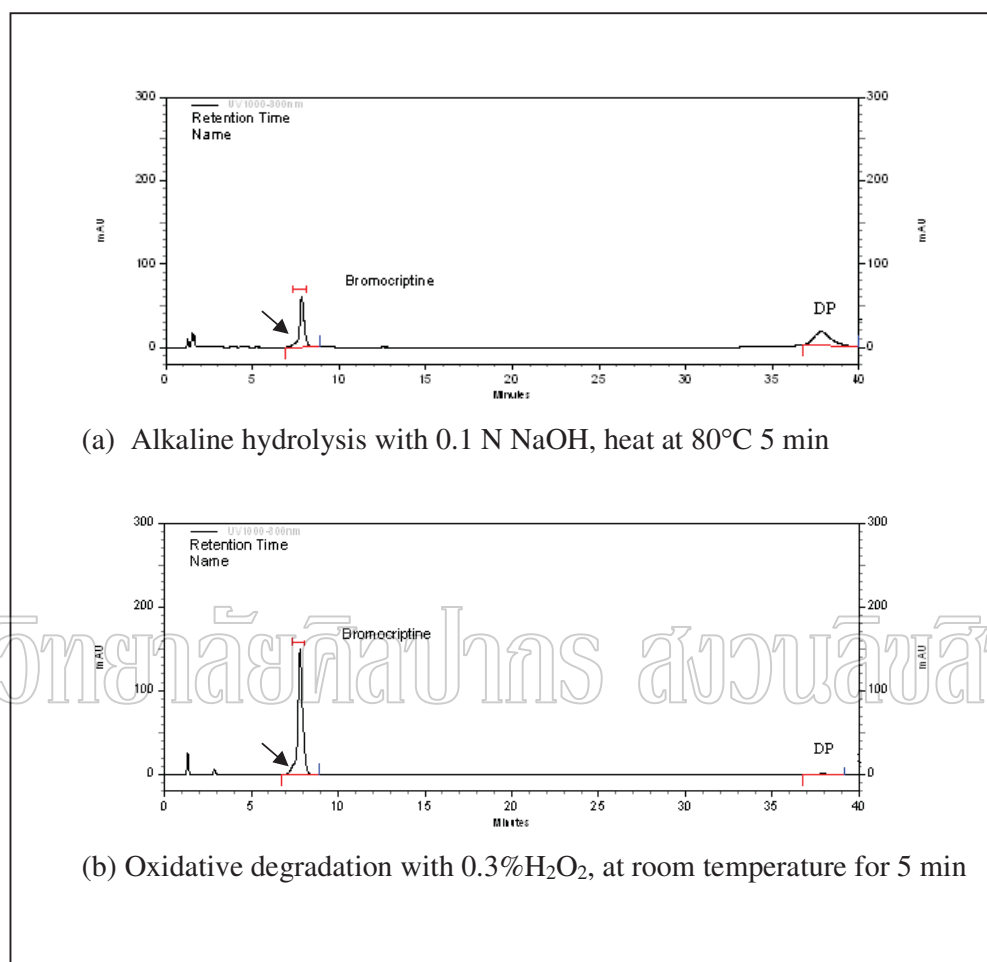


Figure 9 Chromatograms of bromocriptine (HPLC condition consisting of acetonitrile : 20 mM sodium acetate = 45:55, v/v, pH 5.0, flow rate 1.0 ml/min, detected at 300 nm)

Normally, the coeluted or fronting peak may occur when the compositions in diluting solvent and mobile phase were incompatible. In this condition, the diluting solvent was acetonitrile in water so it could not cause coeluted/fronting peaks from this reason. The preparation of acetonitrile in mobile phase was studied again but coeluted peak still occurred. The organic part was then switched from acetonitrile to methanol. It was found that, methanol had more

selectivity than acetonitrile as shown in Figure 10. Additionally, methanol was less toxicity and less cost than acetonitrile so acetonitrile was selected for the HPLC mobile phase.

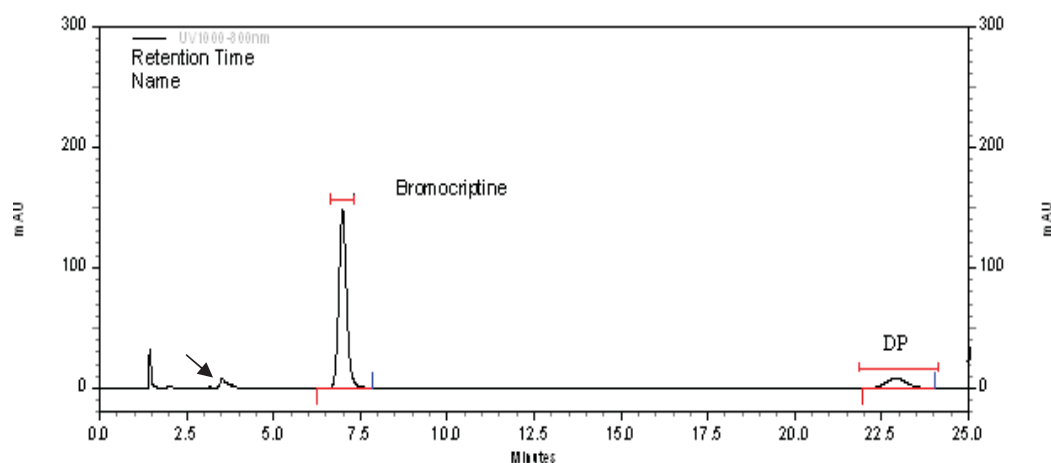


Figure 10 Chromatograms of bromocriptine from oxidative degradation (HPLC condition consisting of methanol : 20 mM sodium acetate = 70:30, v/v, pH 5.0, flow rate 1.0 ml/min, detected at 300 nm)

When HPLC condition was substituted with methanol, the retention time of bromocriptine was increased so the ratio of methanol was tried to increase. Consequently, the proportions of methanol were varied from 73:27 to 75:25 ratios with 20 mM of sodium acetate. First, the ratio of 74:26 was chosen to varied types and concentration of salt but the resolution of bromocriptine and its degradants was less than 2. So, the proportions of methanol were studied again at the ratio 70:30 to 72:28 ratios. The result showed that, the selected optimum composition was the mixture of methanol and 20 mM sodium acetate in the ratio of 70:30, v/v, pH 5.0, and the flow rate was increased from 1.0 ml/min to 1.5 ml/min. The system suitability parameters were satisfactory achieved. The efficiency of bromocriptine separation from the mixture of various methanol proportions and 20 mM sodium acetate as a mobile phase was shown in Table 7 and Figure 11, 12 and 13.

Table 7 Effect of methanol proportions to the separation of bromocriptine

Methanol : 20 mM sodium acetate (v/v)	Flow rate (ml/min)	RT (minutes)	T	Rs	k'	Run time (minutes)
75 : 25	1.00	4.08	1.17	1.31	1.85	13
74 : 26	1.00	4.73	1.16	1.85	2.36	15
73 : 27	1.00	5.18	1.15	1.75	2.62	17
72 : 28	1.00	5.58	1.08	2.02	2.90	18
71 : 29	1.00	6.10	1.08	2.12	3.27	21
70 : 30	1.00	7.00	1.15	2.25	3.82	25
70 : 30	1.50	4.54	1.06	2.20	3.61	18

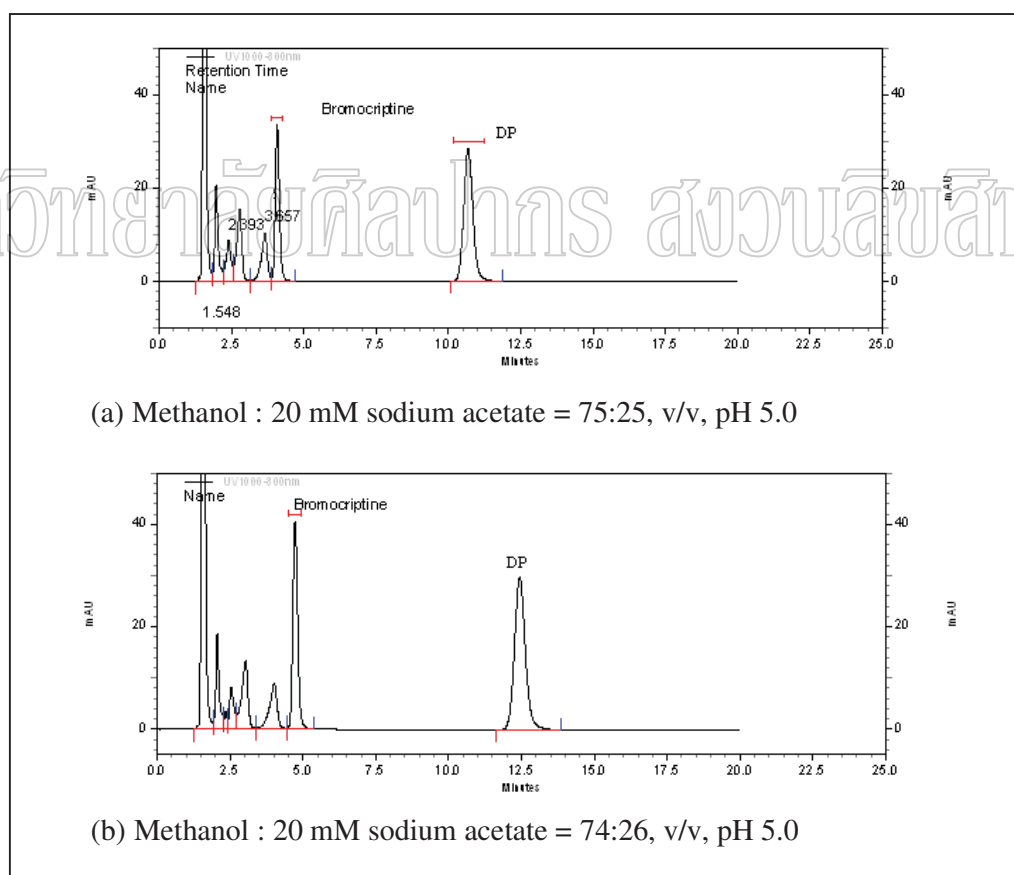


Figure 11 Chromatograms of bromocriptine in degradation samples (HPLC condition consisting of methanol :20 mM sodium acetate = 75:25 and 74:26, v/v, pH 5.0, flow rate 1.0 ml/min, detected at 300 nm)

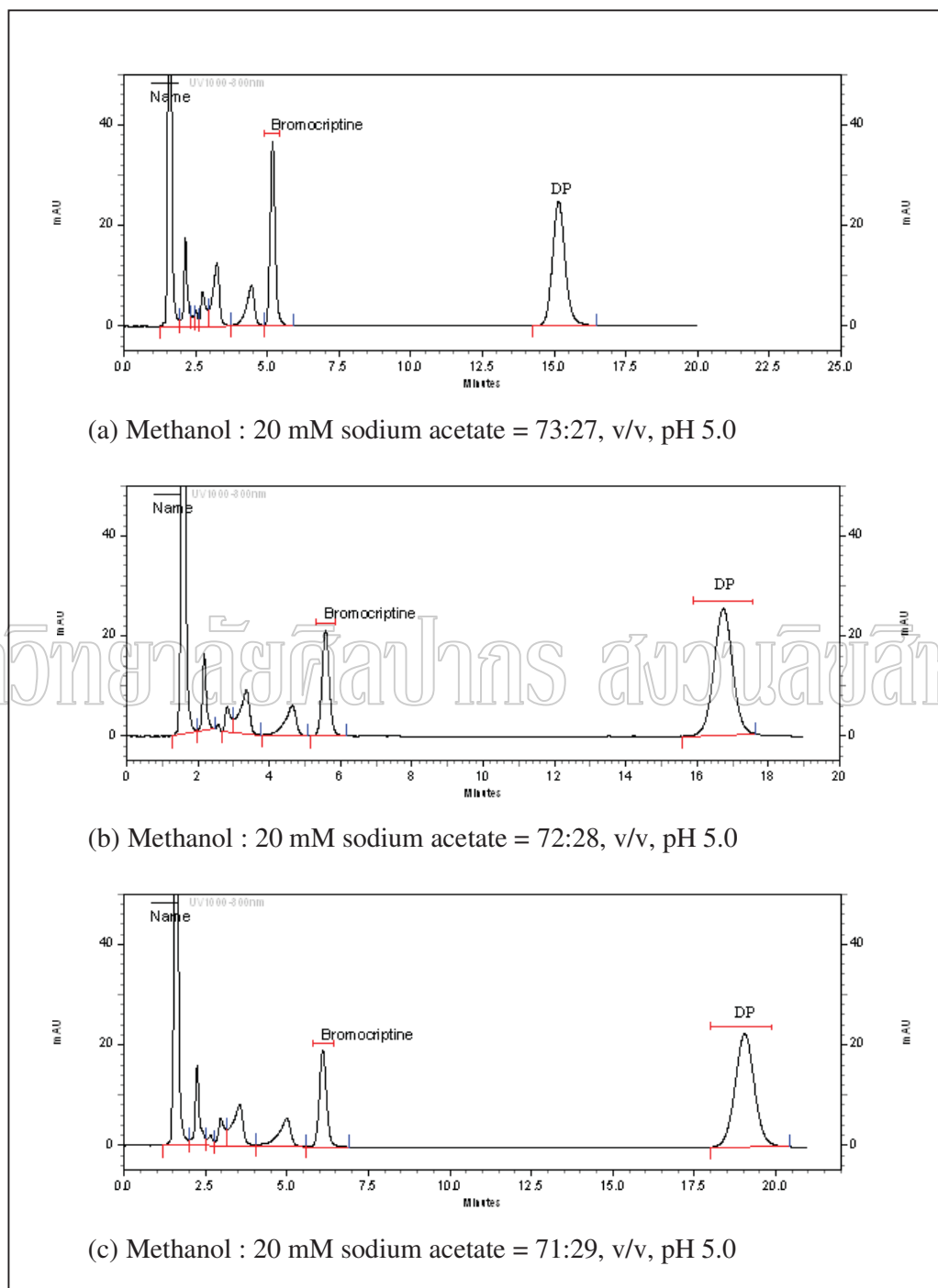


Figure 12 Chromatograms of bromocriptine in degradation samples (HPLC condition consisting of methanol : 20 mM sodium acetate = 73:27 to 71:29, v/v, pH 5.0, flow rate 1.0 ml/min, detected at 300 nm)



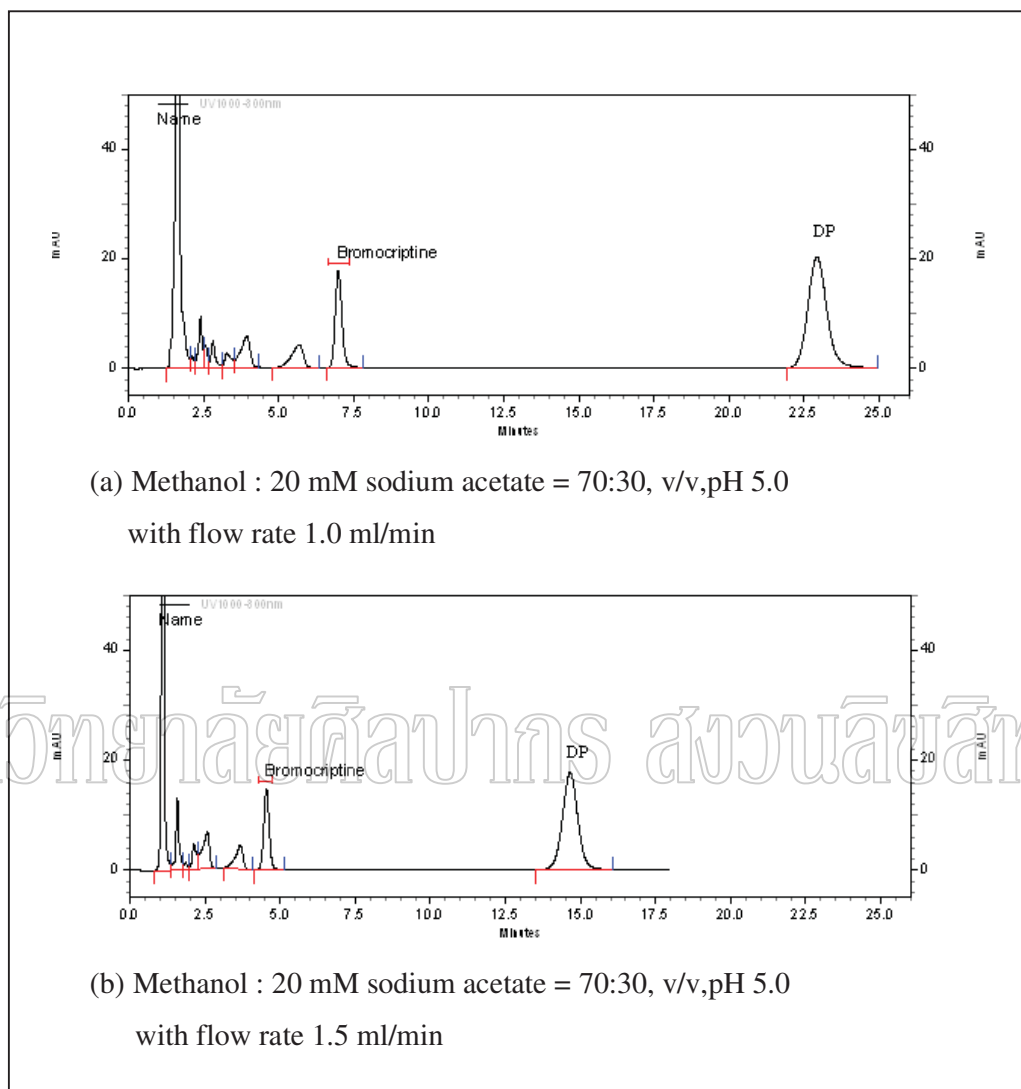


Figure 13 Chromatograms of bromocriptine in degradation samples (HPLC condition consisting of methanol : 20 mM sodium acetate = 70:30, v/v, pH 5.0, flow rate 1.0 ml/min and 1.5 ml/min, detected at 300 nm)

### 2.1.2 Type and concentration of salt

The aqueous solution preparing from ammonium carbonate, sodium acetate and potassium dihydrogen orthophosphate at the initial concentration of 20 mM and final pH 5.0 as in the preliminary studies were determined for the influence of the salt type. Fixed methanol compositions in the mobile phase were

evaluated. The mobile phase consisted of ammonium carbonate could not separate the drug and irregular peak was appeared on the chromatogram because its capacity buffer was not effective at this pH. The retention time of bromocriptine with the mobile phase consisting of potassium dihydrogen orthophosphate was similar to sodium acetate but the bromocriptine peak did not resolved from closely degradants. Because of sodium acetate was made acidic condition that correlated to pH 5.0, so the retention time of bromocriptine was decreased as a basic compound was easily eluted in their ionized form. The good separation was obtained when sodium acetate was used in aqueous solution. Therefore sodium acetate was selected to develop for optimum concentration. Three levels of sodium acetate concentrations were studied (20, 50 and 100 mM). When the concentration of sodium acetate was increased the resolution of bromocriptine was become lower and the nearest degradant did not resolved from bromocriptine completely. The effect of types and concentrations of salt on retention time, tailing factor, resolution and capacity factor of bromocriptine were reported in Table 8 and 9 and Figure 14 and 15.

Table 8 Effect of salt types on system suitability parameters

Type	RT (minutes)	T	N	Rs	k'
Sodium acetate	4.59	1.15	3783	1.60	2.25
Potassium dihydrogen phosphate	4.34	0.93	3765	0.99	2.06
Ammonium carbonate	ND	ND	ND	ND	ND

Remark: ND = Not detected

Table 9 Effect of sodium acetate concentrations on system suitability parameters

Concentration (mM)	RT (minutes)	T	N	Rs	k'
20	4.76	1.13	3197	1.62	2.35
50	4.61	1.14	3247	1.34	2.22
100	4.47	1.09	3254	1.07	2.13

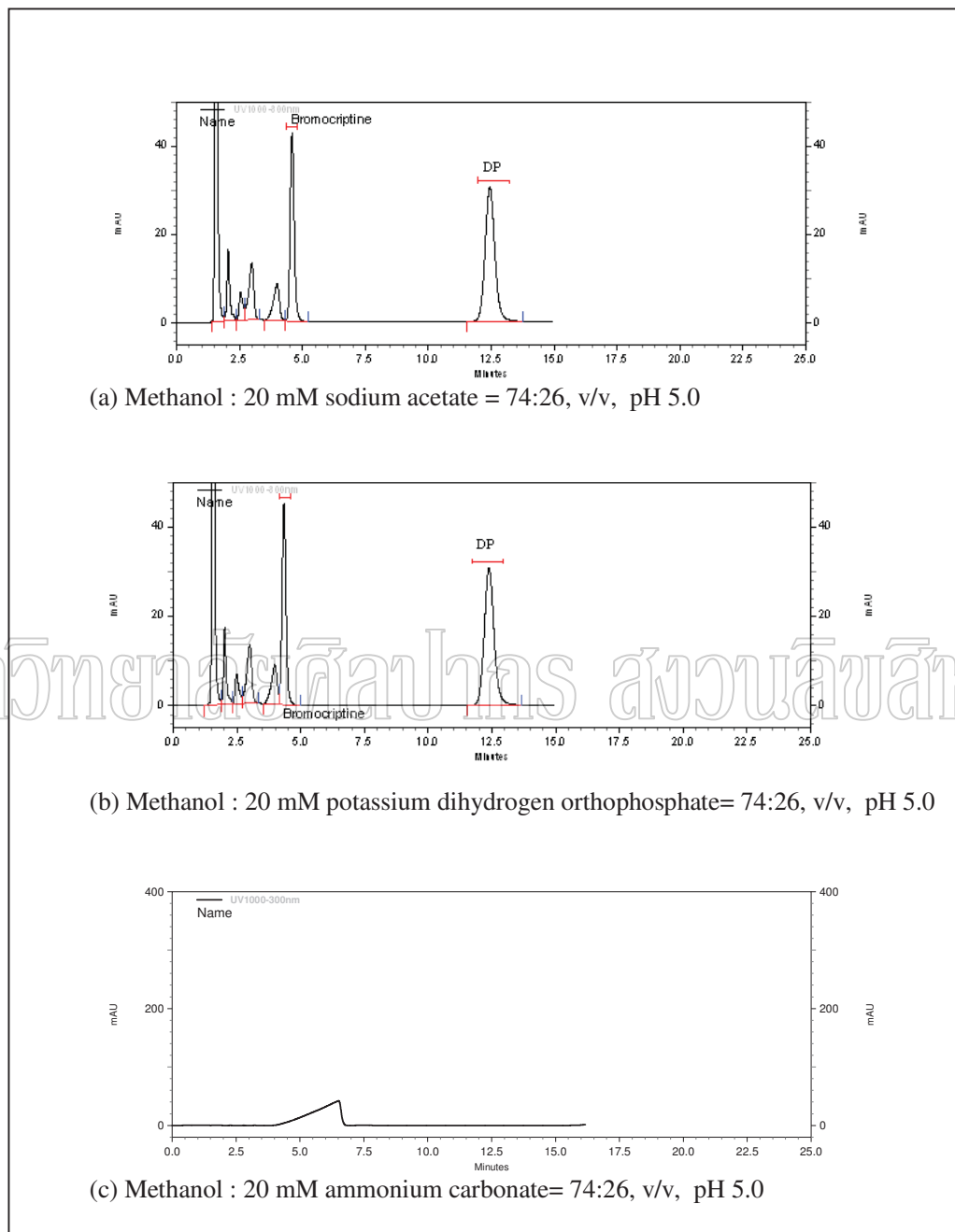


Figure 14 Chromatograms of bromocriptine in degradation samples (HPLC condition consisting of methanol:20 mM of various salts = 74:26, v/v, pH 5.0, flow rate 1.0 ml/min, detected at 300 nm)

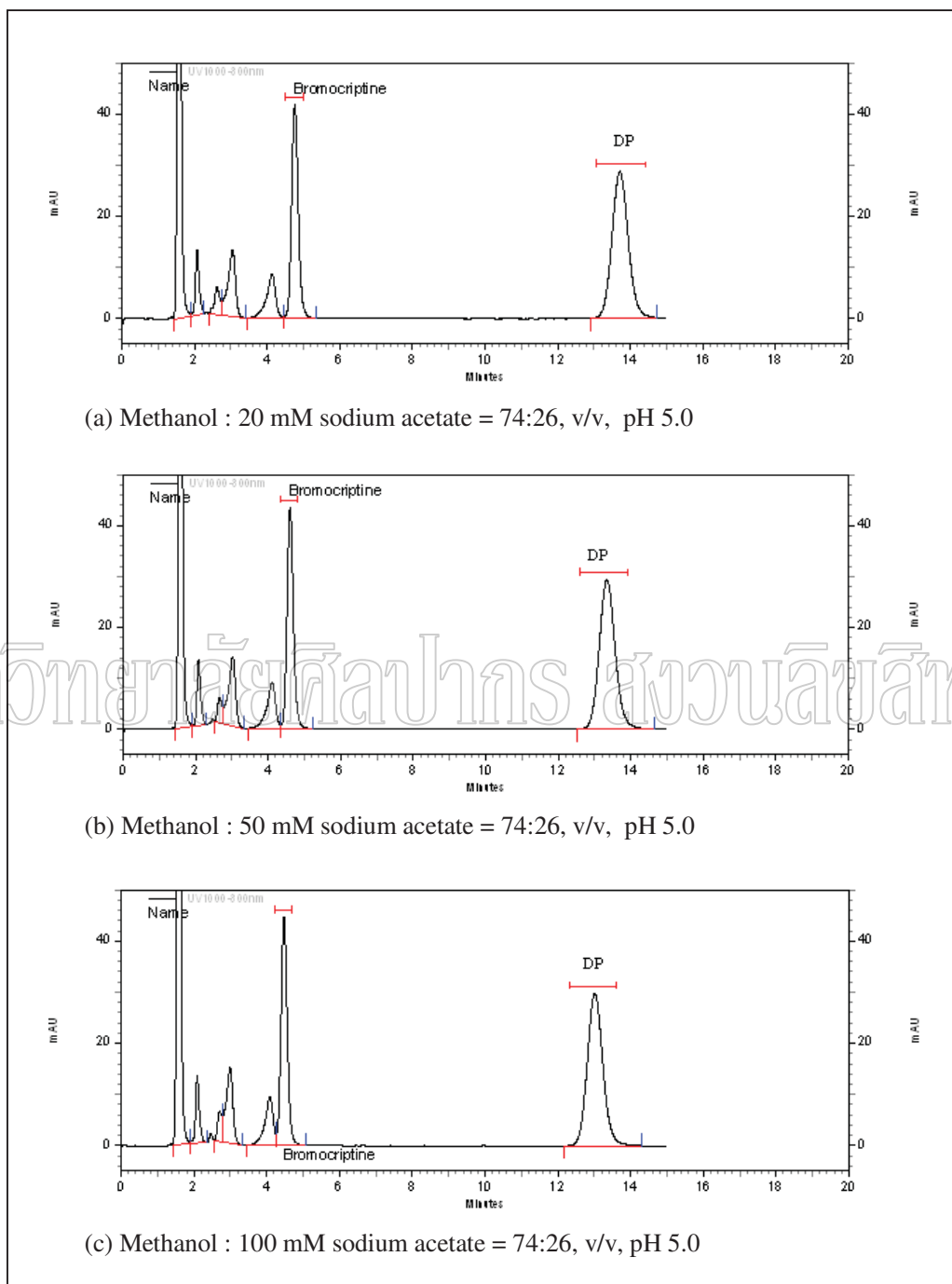


Figure 15 Chromatograms of bromocriptine in degradation samples (HPLC condition consisting of methanol : various concentrations (mM) of sodium acetate = 74:26, v/v, pH 5.0, flow rate 1.0 ml/min, detected at 300 nm)

The concentration of sodium acetate had a small considerable effect on the chromatographic behavior of bromocriptine. When the concentration was increased the resolution was decreased, the best resolution was found at concentration of 20 mM. Hence, the sodium acetate at concentration of 20 mM was selected because it was given the better resolution than others also symmetry peak shape of bromocriptine.

## **2.2 Forced degradation studies**

Degradation studies were carried out following an ICH guideline which established the requirements of stability indicating method. HPLC studies on the drug under different stress conditions indicated the following degradation behavior as illustrated in Figure 16, 17, and 18 and various stress conditions were shown in Table 10 and 11. The system suitability parameters of bromocriptine from each sufficient condition were summarized in Table 12.

The degradation rate of the drug was defined as severe degradation means after the stressed condition the active drug was remained less than 50% within 5 minutes, moderate degradation means the drug was remained more than 50% but not more than 80% and mildly degradation means the drug was remained about 80% within the studied time. The drug was reported as stable when more than 95% of bromocriptine remained.

### **2.2.1 Neutral (water) degradation**

Sufficient degradation was observed when heating at 80°C for 5 minutes. The degradation product was appeared at the retention time of 13.84 minutes.

### **2.2.2 Acidic hydrolysis**

The drug was gradually decreased with time when heating at 80°C in 0.1 N hydrochloric acid for 5 minutes. The degradation products were formed at the retention times of 1.80, 2.05, 5.70 and 13.74 minutes. The rate of hydrolysis in acid was slower than alkaline or neutral hydrolysis.

### **2.2.3 Alkaline hydrolysis**

The drug was found to be severe degradation to alkaline hydrolysis. The reaction in sodium hydroxide at 80°C was so fast that most of the drug was degraded in only 5 minutes. Subsequently, the studies were performed in 0.1

N sodium hydroxide at ambient temperature for 5 minutes, the drug was sufficient degraded to 80% of bromocriptine remaining. The drug degradation was associated with the appearance of the major degradation product at the retention time approximately 13.72 minutes.

#### **2.2.4 Oxidative degradation**

The drug was showed sufficient degradation when the drug treated in 3% hydrogen peroxide and heat at 80°C for 5 minutes. The degradants were appeared at the retention times of 1.99, 2.30, 2.85 and 13.69 minutes.

#### **2.2.5 Photolysis by sunlight and electrical light (UVA)**

Bromocriptine powder was stable to the UVA light upto 5 days. On the other hand, when the drug exposed to sunlight for 16 hours, minor degradation products were found.

The sample solution was degraded rapidly under sunlight in only 30 minutes and formed a major degradant at the retention times of 13.66 minutes. When exposed the solution to UVA light, mildly degradation products were occurred in 4 days at the retention times of 2.25, 3.19, 5.58, 6.07 and 13.65 minutes.

#### **2.2.6 Thermal degradation**

At solid state studies, the result was showed that the drug was stable to dry heat at 80°C for 5 days as the same as in solution state, mildly degraded was occurred when the solution put on the water bath at temperature 80°C for 5 days.

#### **2.2.7 Effect of moisture**

No major degradation product was observed after exposure the drug powder to moisture at room temperature for 5 days.

In this study, the inherent stability of bromocriptine mesylate was established based on test utilising ICH recommended stress conditions. The drug was severe degraded in alkaline hydrolysis, moderate degraded in neutral hydrolysis and oxidative condition. The mildly unstable was found in acidic hydrolysis. In solution state, the drug was rapidly degraded under sunlight and mildly degradation was observed when the drug exposed to UVA light. In solid state, the drug was quite stable under moisture, photolytic and thermal stress condition.

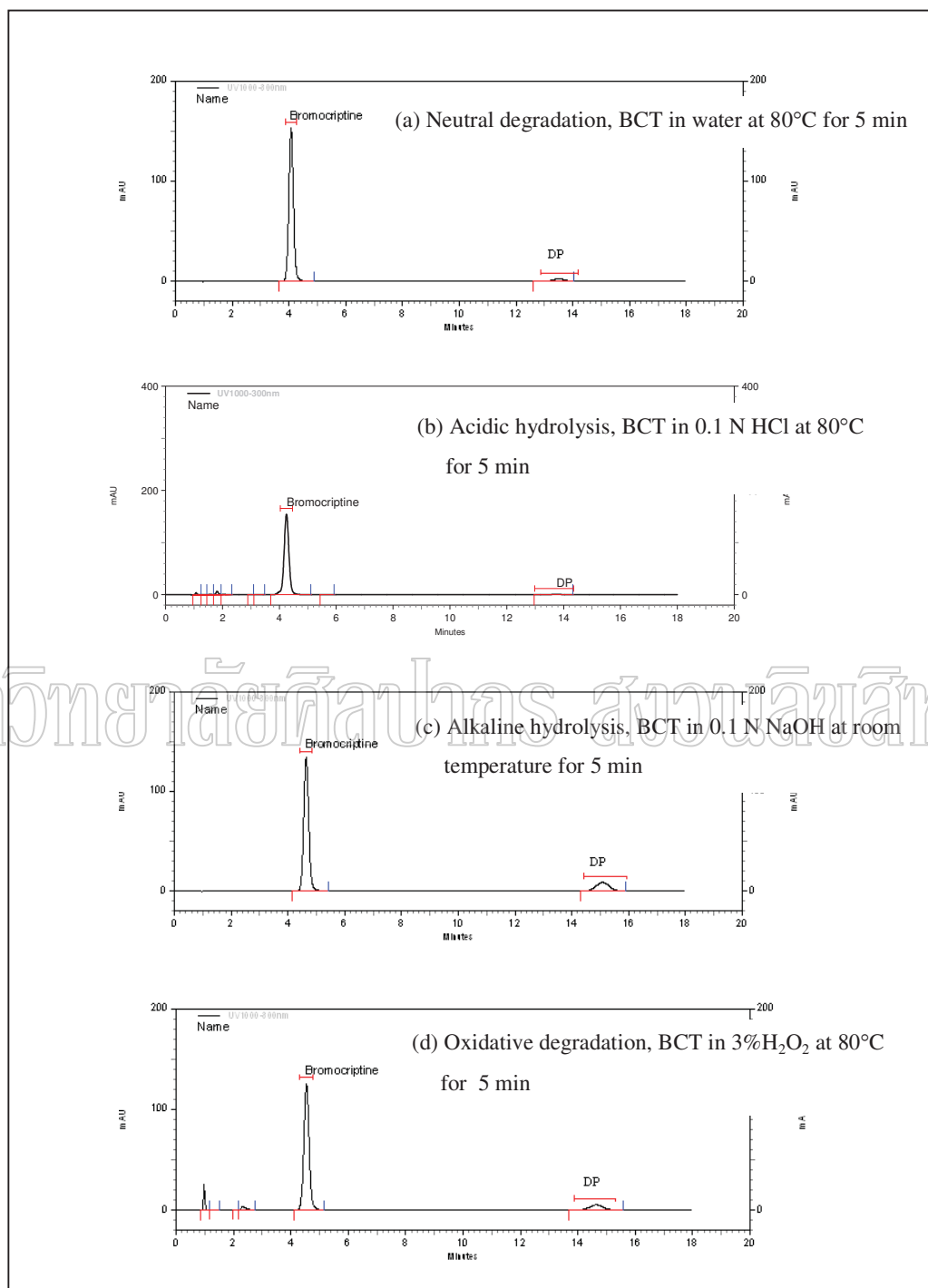


Figure 16 Chromatograms showing decomposition of bromocriptine mesylate in various conditions (HPLC condition consisting of methanol : 20 mM sodium acetate = 70:30, v/v, pH 5.0, flow rate 1.5 ml/min, detected at 300 nm)

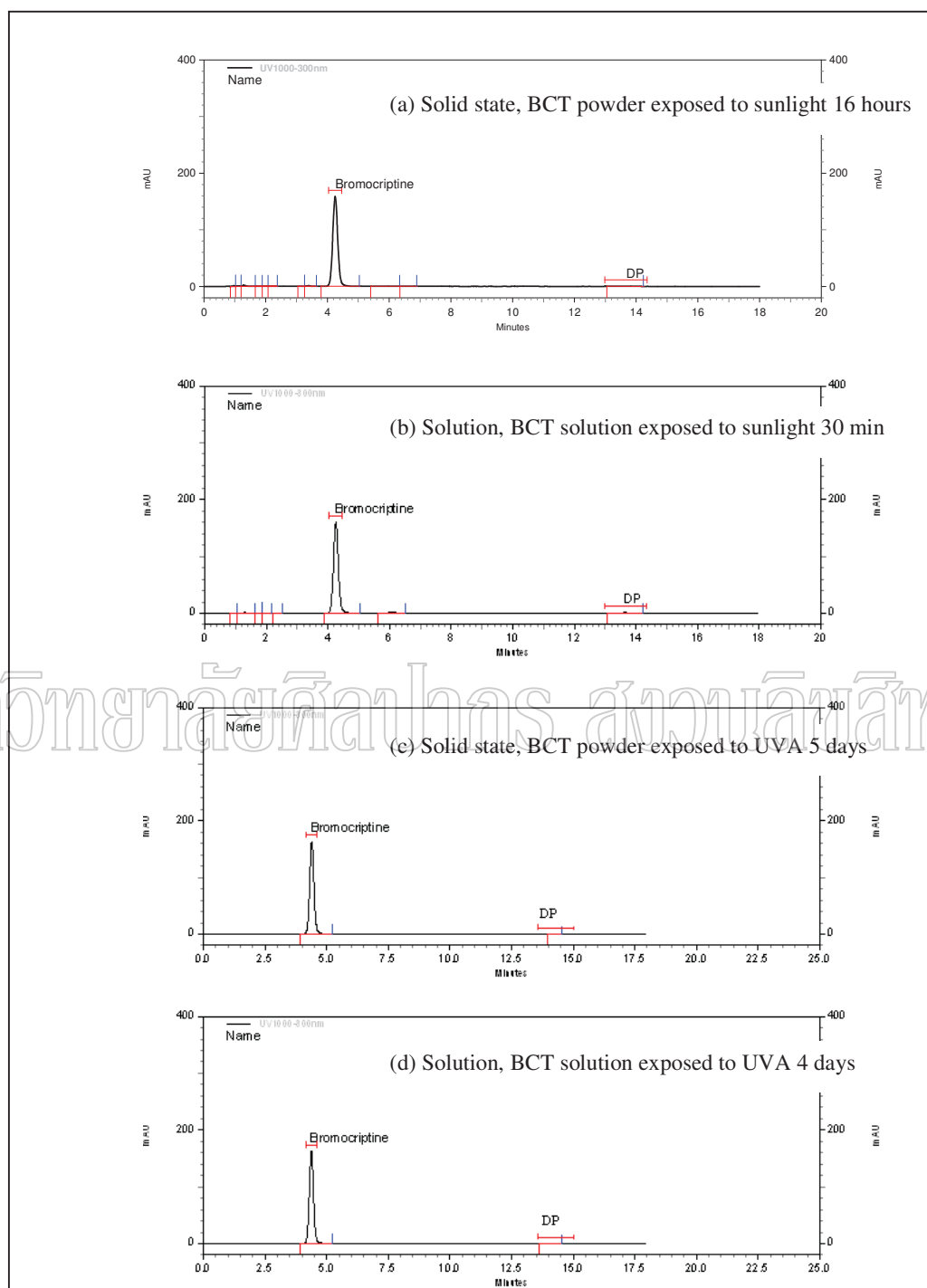


Figure 17 Chromatograms showing decomposition of bromocriptine mesylate in Photolysis by sunlight and UVA (HPLC condition consisting of methanol : 20 mM sodium acetate = 70:30, v/v, pH 5.0, flow rate 1.5 ml/min, detected at 300 nm)



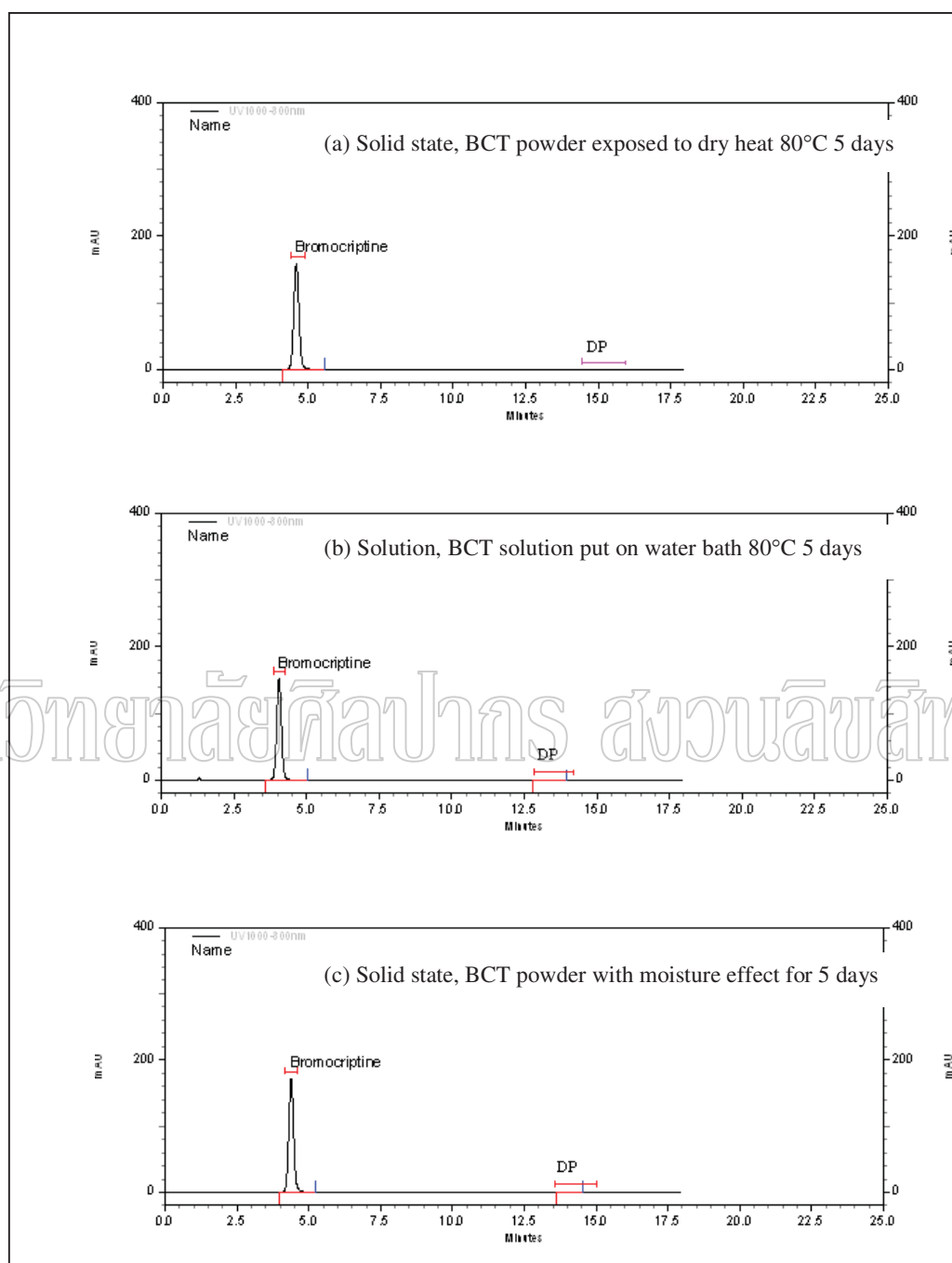


Figure 18 Chromatograms showing decomposition of bromocriptine mesylate with thermal effect and moisture effect (HPLC condition consisting of methanol : 20 mM sodium acetate = 70:30, v/v, pH 5.0, flow rate 1.5 ml/min, detected at 300 nm)

Table 10 Degradation data for bromocriptine mesylate under hydrolysis (acid, neutral and alkaline) and oxidation

Stress condition	Reagent added			Treated condition		Active drug present after degradation (%)
	Type	Concentration	Amount (ml)	Temperature (°C)	Time (min)	
Neutral degradation	water	-	2.0	RT	5	98.34
	water	-	2.0	80	1	96.12
	water	-	2.0	80	5	87.88
	water	-	2.0	RT	8	71.12
	water	-	2.0	80	6	70.02
Acidic hydrolysis	HCl	0.1 N	1.0	80	5	81.02
	HCl	1 N	1.0	80	1	99.91
	HCl	1 N	1.0	80	2	89.76
Alkaline hydrolysis	NaOH	0.01 N	1.0	RT	0	94.63
	NaOH	0.1 N	1.0	RT	0	88.14
	NaOH	0.1 N	1.0	RT	5	82.84
	NaOH	0.1 N	1.0	RT	10	75.27
	NaOH	0.1 N	1.0	80	1	36.28
	NaOH	0.1 N	1.0	80	5	9.13
Oxidative degradation	H <sub>2</sub> O <sub>2</sub>	0.3%	1.0	RT	5	85.69
	H <sub>2</sub> O <sub>2</sub>	3%	1.0	80	5	78.02

RT = Room temperature

Table 11 Degradation data for bromocriptine mesylate stored with moisture, thermal and photolysis conditions

Stress condition	BCT form	Degradation time (hr)	Active drug present after degradation (%)
Photolysis by sunlight	Solid	4	95.99
		16	80.59
Photolysis by UVA	Solution	0.5	84.02
		8	28.25
		48	99.32
Photolysis by UVA	Solid	120	97.68
		48	88.18
		96	85.23
		12	99.12
Thermal (80°C)	Solid	120	98.14
		12	96.12
		120	87.83
Moisture	Solution	1	99.12
		24	98.87
		48	98.82
		120	97.50

### 3. System suitability test

System suitability test parameters were checked by analyzing a mixture of forced samples to ensure that the system is working correctly during the analysis. The HPLC condition consisting of methanol and 20 mM sodium acetate in the ratio 70:30, v/v, pH 5.0 with flow rate 1.5 ml/min and detected at 300 nm. Method performance data including resolution, tailing factor, theoretical plate and capacity factor are listed in Table 12 and the chromatogram of bromocriptine in mixed forced sample was shown in Figure 19. Bromocriptine peak was separated from other degradants completely. All stress conditions were satisfactory and indicative the good specificity of the method for assessment the stability of bromocriptine.

Table 12 The system suitability test results for bromocriptine in mixed forced samples

Stress condition / Peak found	RT (min)	T	N	Rs	k'
Neutral degradation					
BCT	4.30	1.06	3192		3.42
DP	13.84	1.02	4704	17.27	13.34
Acidic hydrolysis					
A1	1.80	-	2161	1.22	0.88
A2	2.05	-	1512	1.28	1.12
BCT	4.25	1.01	3047	8.51	3.40
A3	5.70	0.92	3104	4.04	4.91
DP	13.74	0.89	5206	13.74	13.24
Alkaline hydrolysis					
BCT	4.26	1.06	3270	-	3.41
DP	13.72	1.02	4708	17.24	13.22
Oxidation					
D1	1.99	-	1078	0.65	1.06
D2	2.30	1.68	906	1.11	1.38
D3	2.85	-	2156	2.02	1.95
BCT	4.26	1.06	3283	5.18	3.41
DP	13.69	1.03	4700	17.21	13.18

A1-A3 = degradation product from acid hydrolysi, and DP =major degradation product

D1-D3= degradation products from oxidative degradation

Table 12 (Continue)

Stress condition / Peak found	RT (min)	T	N	Rs	k'
Solid exposed to sunlight					
S1	1.29	-	613	0.86	0.34
S2	2.26	-	876	1.03	1.34
S3	3.21	-	727	2.44	2.32
BCT	4.25	1.06	3232	3.06	3.41
S4	6.60	1.08	4180	3.65	5.84
DP	13.67	0.95	5308	12.21	13.17
Solution exposed to sunlight					
L1	1.788	-	530	1.92	0.85
L2	2.32	1.26	1703	1.24	1.41
BCT	4.25	1.07	3278	7.39	3.41
L3	6.07	1.04	4106	5.38	5.29
DP	13.66	0.91	5055	13.22	13.15
Solution exposed to UVA					
V1	2.25	1.22	1582	1.14	1.33
V2	3.19	2.20	810	2.80	2.31
BCT	4.25	1.07	3231	2.84	3.41
V3	5.58	-	1786	3.22	4.78
V4	6.07	-	3238	5.29	1.02
DP	13.65	0.97	5072	12.39	13.14
Mixed forced sample					
M1-M7	-	-	-	-	-
M8	3.39	-	2105	0.66	2.52
BCT	4.26	1.06	3285	2.92	3.41
M9	6.06	-	4580	5.52	5.28
DP	13.64	0.99	4933	13.35	13.14

S1-S4 = degradation products from solid exposed to sunlight, L1-L3= Degradation products from solution exposed to sunlight, V1-V4= degradation products from solid exposed to UVA and M1-M9 = degradation products in mixed forced samples

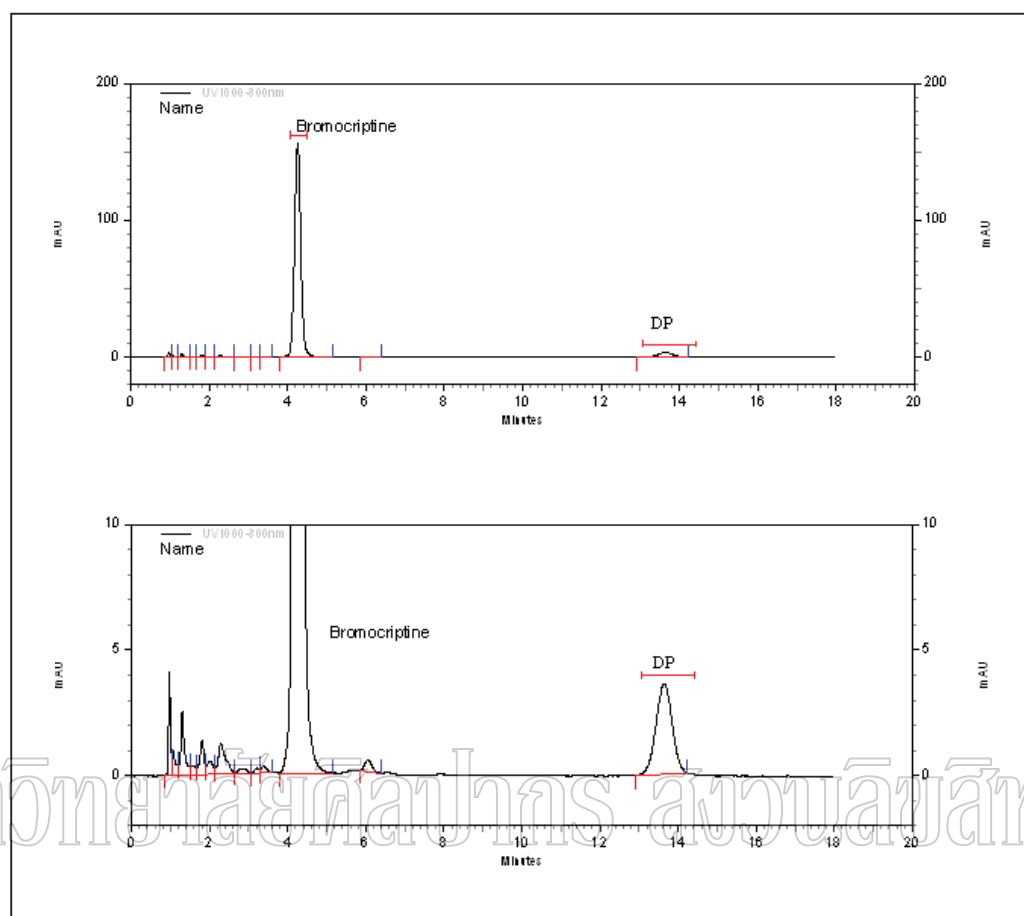


Figure 19 Chromatograms showing of bromocriptine peak in mixed forced degradation samples from various stress conditions (HPLC condition consisting of methanol : 20 mM sodium acetate = 70:30, v/v, pH 5.0, flow rate 1.5 ml/min, detected at 300 nm)

#### 4. Analytical method validation

##### 4.1 Specificity

The specificity of method is the extent to which it can be used for analysis of a particular analyte in a mixture or matrix without interference from other components. In this assay, specificity was tested by the HPLC chromatograms recorded for bromocriptine mesylate raw material, placebo solution, the mixture of bromocriptine mesylate and placebo at the concentration 200 µg/ml, the mixed degradation products from the stress conditions and bromocriptine tablets, all chromatograms were indicated the method enabled specific analysis of the drug, well

resolved peaks of bromocriptine from its degradants were observed in the Figure 20 and 21.

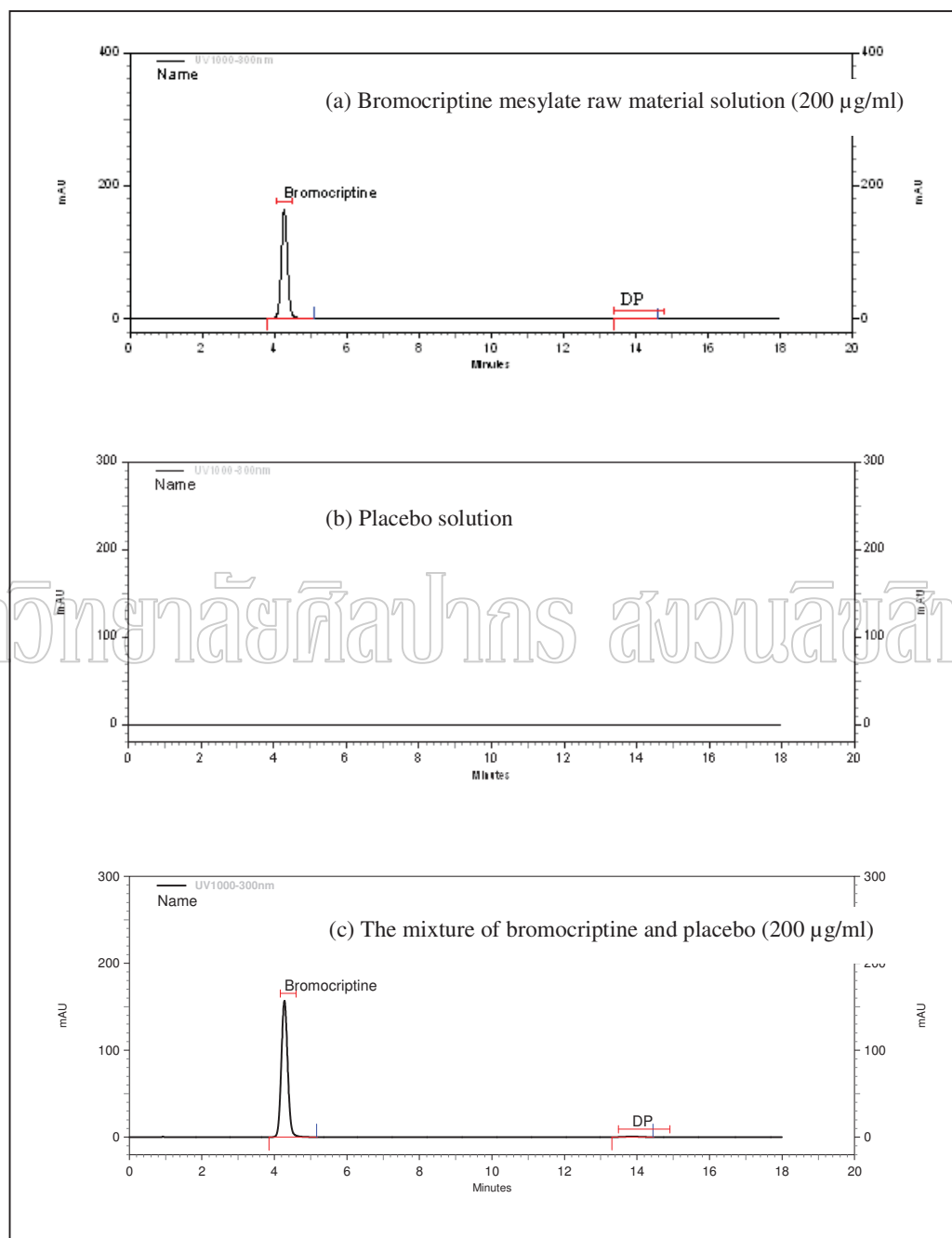


Figure 20 Chromatograms showing specificity of bromocriptine mesylate (HPLC condition consisting of methanol : 20 mM sodium acetate = 70:30, v/v, pH 5.0, flow rate 1.5 ml/min, detected at 300 nm)

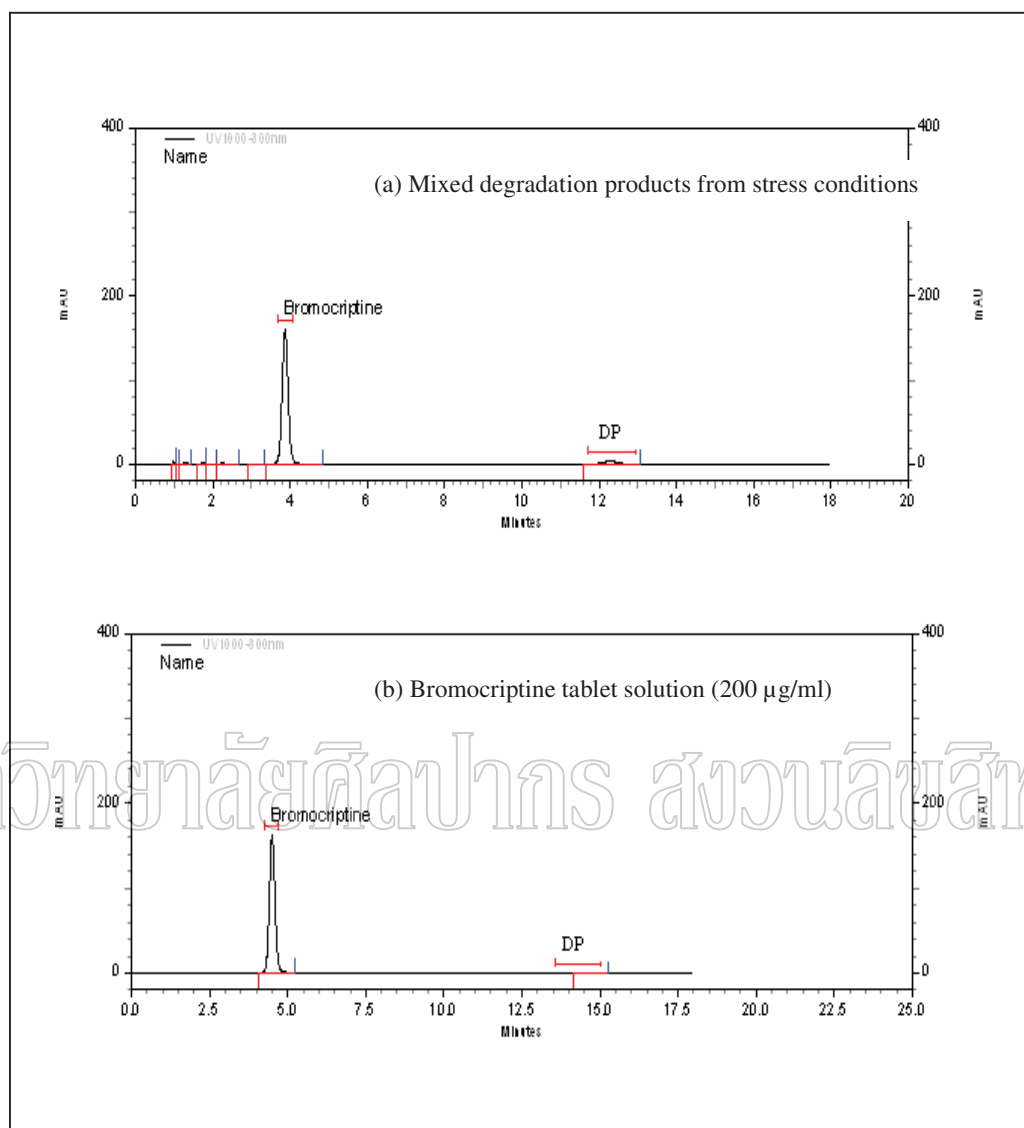


Figure 21 Chromatograms showing specificity of bromocriptine mesylate (HPLC condition consisting of methanol : 20 mM sodium acetate = 70:30, v/v, pH 5.0, flow rate 1.5 ml/min, detected at 300 nm)

Additionally, the peak purity has been done by analyzing the mixture of forced samples through PDA detector. The method proved to be specific to bromocriptine which was indicated through purity threshold values, were reported in Table 13.



Table 13 PDA peak purity parameter for bromocriptine under various forced conditions

Condition	Peak purity
Neutral degradation	998.532
Acidic hydrolysis	999.494
Alkaline hydrolysis	996.637
Oxidative degradation	999.548
Solid state to sunlight-16 hours	998.634
Solution to sunlight-30 minutes	998.610
Solution to UVA-96 hours	999.070
Mixture of forced samples	995.430

#### 4.2 Linearity and range

The linearity of system was found a linear correlation between peak area and concentration in range of 100 to 300  $\mu\text{g/ml}$ , the correlation coefficient was 0.9999 as shown in Figure 22 and Table 14.

The linearity of the method was revealed an excellent linear correlation between the concentration added and concentration found from the accuracy test. The calibration curve was shown in Figure 23 and the regression parameters of the linearity of the method were reported in Table 15.

Table 14 Regression parameters of the linearity of system

Regression parameters	Day 1	Day 2	Day 3	Mean $\pm$ SD
Slope	10234.25	10213.26	10231.50	10226.34 $\pm$ 11.41
Y-intercept	-23906.76	-19328.67	-18120.40	-20451.24 $\pm$ 3052.34
Correlation coefficient ( $r^2$ )	0.9999	0.9999	1.0000	0.9999 $\pm$ 0.00

Table 15 Regression parameters of the linearity of the method

Regression parameters	Values
Slope	1.0016
Y-intercept	1.05
Correlation coefficient ( $r^2$ )	0.9999

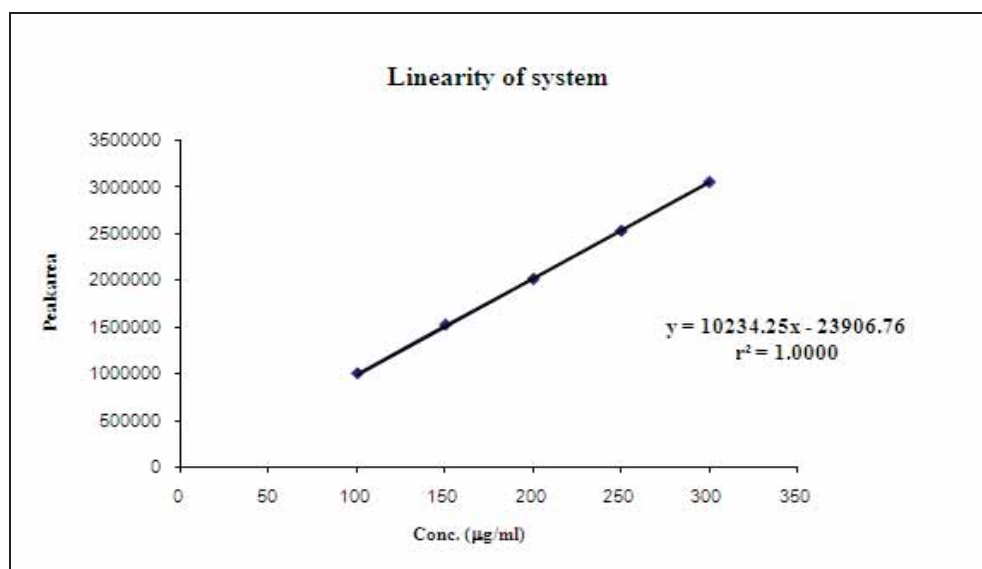


Figure 22 The calibration curve was shown the linearity of system on the range of concentration 100 – 300 µg/ml of bromocriptine mesylate (HPLC condition consisting of methanol : 20 mM sodium acetate = 70:30, v/v, pH 5.0, flow rate 1.5 ml/min, detected at 300 nm)

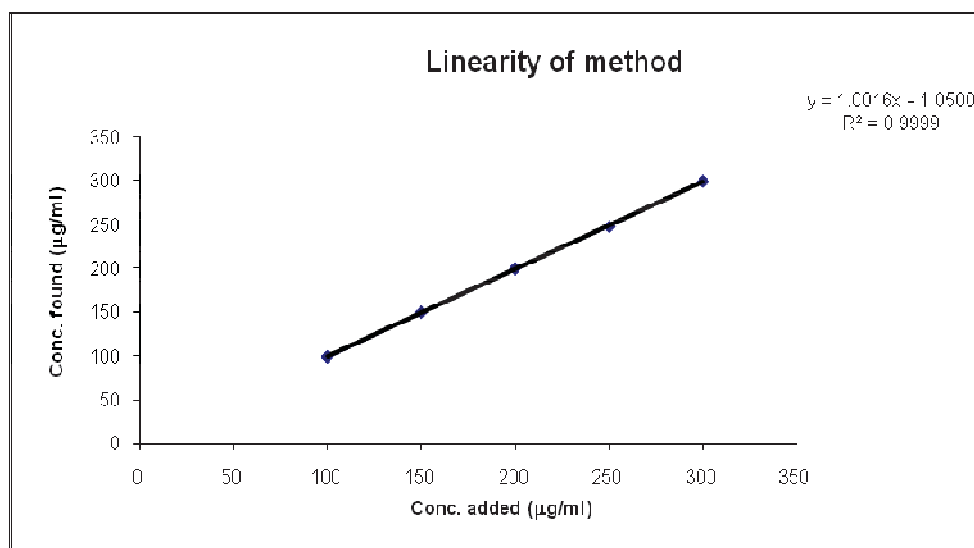


Figure 23 The calibration curve was shown the linearity of the method (HPLC condition consisting of methanol : 20 mM sodium acetate = 70:30, v/v, pH 5.0, flow rate 1.5 ml/min, detected at 300 nm)

### 4.3 Accuracy

The accuracy study has been done by analyzing placebo solutions spiked with known amount of bromocriptine. The recovery results were within the acceptance criteria (98 – 102%). The results for the accuracy were shown in Table 16. The excellent recoveries was suggested the good accuracy of the proposed HPLC method.

Table 16 Recovery of bromocriptine mesylate from accuracy test

Actual concentration ( $\mu\text{g/ml}$ )	Calculated concentration ( $\mu\text{g/ml}$ ) $\pm$ SD ; %RSD (n=3)	%Recovery
100	98.75 $\pm$ 0.15 ; 0.15	98.75
150	149.8 $\pm$ 0.20 ; 0.13	99.86
200	199.8 $\pm$ 0.62 ; 0.31	99.90
250	248.0 $\pm$ 0.74 ; 0.30	99.18
300	300.1 $\pm$ 0.92 ; 0.31	100.0
Average		99.43 $\pm$ 0.55

### 4.4 Precision

Three levels of precision: system precision, repeatability and intermediate precision were performed. The precision results were described below;

System precision, as one of the precision characteristics, the proposed analytical method was performed for ten injections of the standard solution. The result was met the requirement  $\text{RSD} \leq 2.0\%$  (Table 17).

Repeatability or within-run precision was expressed in terms of %RSD and found to be 0.15 for six replicated determinations of commercial sample solution within one day (Table 18).

The %RSD for inter-day or between run precision of peak area is 0.14 as shown in Table 19. The results showed that %RSD from each precision type less than 2.0%, indicated that the method was sufficient precise.

Table 17 System precision data of bromocriptine mesylate (n=10)

Trial	Retention time (minutes)	Peak area
1	4.23	1962869
2	4.23	1965644
3	4.23	1963434
4	4.23	1961160
5	4.23	1966931
6	4.24	1964210
7	4.23	1969799
8	4.24	1963512
9	4.23	1963754
10	4.24	1958897
Average	4.23	1964021
SD	0.00	2999.37
%RSD	0.11	0.15

Table 18 Repeatability or within run precision data (n=6)

Trial	Concentration found ( $\mu\text{g/ml}$ )	Content (mg/tablet)	%Labeled amount
1	231.10	2.90	101.20
2	229.77	2.89	100.62
3	227.04	2.85	99.42
4	228.13	2.87	99.90
5	227.94	2.86	99.81
6	226.46	2.85	99.16
Average			100.02
SD			0.76
%RSD			0.76

Table 19 Inter-day or between-run precision data (n=9)

Trial	Peak area	Average Peak area	Concentration found ( $\mu\text{g/ml}$ )	Content (mg/tablet)	% Labeled amount	Average %LA $\pm$ SD (n=3)	%RSD (n=3)
Day 1	2187590	2190718	227.04	2.85	99.42	99.47 $\pm$ 0.33	0.33
	2193845						
	2204266						
	2194688						
	2183103						
	2186887						
Day 2	2176261	2179213	225.87	2.84	98.90	99.46 $\pm$ 0.79	0.79
	2182164						
	2209229						
	2213986						
	2179235						
	2188380						
Day 3	2224913	2220026	230.05	2.89	100.73	100.4 $\pm$ 0.55	0.55
	2215138						
	2213601						
	2224117						
	2204850						
	2191185						
Total (n=9)					99.77 $\pm$ 0.70	0.70	

#### 4.5 Limit of detection and limit of quantitation

The detection limit (LOD) of this method was 0.8  $\mu\text{g/ml}$  based on peaks that could be easily manual measured signal to noise ratio was about 3 : 1. The limit of quantitation (LOQ) was 2.5  $\mu\text{g/ml}$  which was established for the analyte concentration that gave signal to noise ratio about 10 : 1. Precision data were obtained by the repeated analysis and reported in %RSD value, as shown in Table 20.

Table 20 Limit of detection and limit of quantitation of bromocriptine mesylate

Trial	Signal to noise ratio	
	LOD (0.8 $\mu\text{g/ml}$ )	LOQ (2.5 $\mu\text{g/ml}$ )
1	3.000	9.000
2	3.000	9.000
3	2.938	8.800
4	3.000	9.000
5	2.938	8.800
6	2.938	8.800
7	2.875	8.800
8	2.875	8.800
9	3.000	9.000
10	2.875	9.000
Average	2.944	8.900
%RSD	1.86	1.18

#### 4.6 Robustness

For the evaluation of the method robustness, one chromatographic parameter was changed while the other parameters were kept unchanged. A mixture of degradants from all forced conditions was injected to each changed condition. The method robustness was tested after changing the final pH of the mobile phase (4.8 to 5.2), the result revealed that the proposed method was robust for these small changes in final pH. In the addition, the effect of the percent of organic strength on resolution was studied by varying methanol proportions from 68 to 72%. The resolution between

bromocriptine and its degradation products was not significantly altered. The chromatographic parameters including retention time, tailing factor, theoretical plate, resolution and capacity factor were listed in Table 21 and 22. The results showed that the variation of the pH of the mobile phase by  $\pm 2$  units and organic strength of the mobile phase by  $\pm 2\%$  did not have a significant effect on retention time and system suitability parameters, illustrating the robustness of the method.

Table 21 The robustness test results of the developed method by varied pH

Final pH of Mobile phase	T	N	Rs	k'	RT (min)	Peak area
4.80	1.06	2695	2.28	2.58	3.45	1726164
4.90	1.06	2756	2.43	2.69	3.54	1742859
5.00	1.06	2891	2.89	3.01	3.87	1763958
5.10	1.06	3019	3.25	3.42	4.31	1779572
5.20	1.05	3124	3.82	2.91	4.70	1785637
%RSD						1.42

Table 22 The robustness test results of the developed method by varied the methanol proportions in the mobile phase

Methanol proportion (v/v)	T	N	Rs	k'	RT (min)	Peak area
68	1.05	3082	5.18	4.15	5.08	1738729
69	1.05	2960	2.66	3.43	4.34	1763603
70	1.06	2891	2.89	3.01	3.87	1763958
71	1.06	2833	2.83	2.68	3.73	1766613
72	1.06	2792	2.61	2.41	3.34	1750173
%RSD						0.68

#### 4.7 Ruggedness

The ruggedness of the proposed method was established by determining bromocriptine with the same chromatographic system and the same column between two analysts. The mean standard deviation and %RSD for two sets of

data was shown in Table 23. Ruggedness of the method was shown by the overall RSD value of 0.83% between the two sets of data. The result was indicated that the method had a high precision.

Table 23 The results for ruggedness test by two analysts

Sample No.	% Labeled amount	
	Analyst A	Analyst B
1	99.87	99.01
2	100.26	98.48
3	99.27	98.07
Average	99.80	98.52
SD	0.50	0.47
%RSD	0.50	0.48
Average(n=6)	99.16	
SD (n=6)	0.82	
%RSD (n=6)	0.83	

#### 4.8 Stability of standard solution

The stability of bromocriptine solution was established by keeping the solution in HPLC autosampler at room temperature for 24 hours. The result was showed no significant changed in amount of bromocriptine. The stability of drug was represented by %different between the initial concentration and the final concentration at the end of time. The %different was found to be 0.74% and met the requirement (not more than 2.0%). It was concluded that bromocriptine mesylate was stable in standard solution for at least 24 hours (Table 24).

The results of each validation parameter and acceptance criteria were summarized in the Table 25. The proposed HPLC method was satisfied the regulatory requirement of specificity, accuracy, linearity, precision, limit of detection, limit of quantitation, robustness, ruggedness and stability. Hence the method was used for the quantitative analysis of bromocriptine mesylate in raw material and tablets.



Table 24 Stability data of bromocriptine mesylate standard solution in autosampler for 24 hours

Time (hours)	Peak area	Concentration ( $\mu\text{g/ml}$ )	Calculated in %
Initial	1962869	200.34	100.17
1	1969000	200.95	100.48
2	1962869	200.34	100.17
4	1966934	200.74	100.37
6	1955301	199.58	99.79
8	1955537	199.60	99.80
12	1956598	199.71	99.85
16	1948014	198.85	99.42
20	1947846	198.83	99.41
24	1948134	198.86	99.43
% different			0.74

Table 25 Summary of validation parameters

Parameter	Result	Acceptance criteria
Specificity	Specific	Specific
Linearity		
Correlation coefficient ( $r^2$ )	0.9999	$\geq 0.999$
Accuracy (%Recovery)	$99.43 \pm 0.55$	98 – 102
Precision (%RSD)		
System precision	0.15	1.0
Intra-day precision	0.76	2.0
Inter-day precision	0.70	2.0
Robustness	Robust	Robust
Ruggedness	0.83	$\leq 2\%$
Stability	Stable at 24 hours	$\leq 2\%$

### 5. Quantitative analysis of raw material and tablets by proposed method.

With the proposed method, the content of bromocriptine obtained by analysis of bromocriptine mesylate raw material (Lot. No. 7222100109) was 100.01% calculated on the dried basis. The %labeled amount of two commercially available tablets in Thailand were shown in Table 26 and Table 27 respectively. The chromatograms from the determination of bromocriptine mesylate in bulk and tablets were shown in Figure 24.

Table 26 Determination of bromocriptine mesylate in raw material

Sample No.	Peak area	
	Sample 1	Sample 2
1	1904521	1908335
2	1918161	1927261
3	1943460	1923861
Mean $\pm$ SD	1922049 $\pm$ 19761.52	1919819 $\pm$ 10089.68
%RSD	1.03	0.53
%Assay (as is)	99.67	99.70
%Assay (on dried basis)	100.0	

Table 27 Quantitative analysis of bromocriptine tablets

Sample NO.	Amount found			
	Brand 1 (Suplac <sup>®</sup> )		Brand 2 (Parlodel <sup>®</sup> )	
	(mg/tablet)	%LA	(mg/tablet)	%LA
1	2.89	100.7	2.84	98.95
2	2.90	101.1	2.84	98.95
3	2.87	100.0	2.85	99.30
Mean $\pm$ SD	-	100.6 $\pm$ 0.53	-	99.07 $\pm$ 0.20
%RSD	-	0.53	-	0.20

Remark : Sample 1 tablet contains bromocriptine mesylate 2.87 mg (equivalent to bromocriptine 2.5 mg)

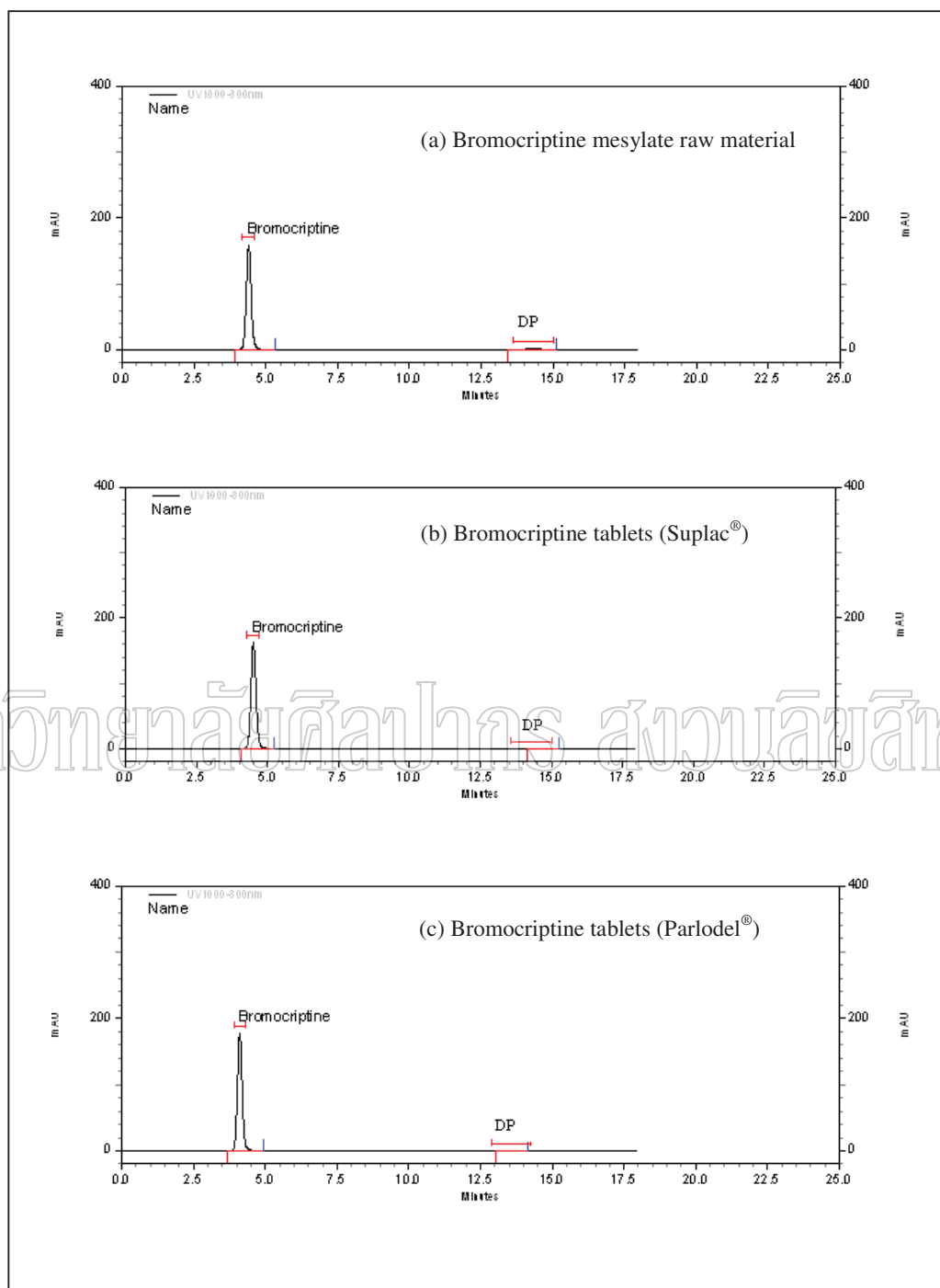


Figure 24 Chromatograms of bromocriptine mesylate from the determination in raw material and two commercially tablets (HPLC condition consisting of methanol : 20 mM sodium acetate = 70:30, v/v, pH 5.0, flow rate 1.5 ml/min, detected at 300 nm)

Both of the results in bromocriptine raw material and tablets were complied with the requirement of the compendial method. The potency of bromocriptine in USP30 was defined as, the raw material was contained not less than 98.0% and not more than 102.0% of bromocriptine mesylate, calculated on the dried basis and the %labeled amount contained bromocriptine mesylate equivalent to not less than 90.0% and not more than 110.0% of the labeled amount of bromocriptine (USP 2007 : 1553,1555). Additionally, the degradation product was detected from raw material and both brands of tablets. It was proved that the proposed method can be employed as a stability-indicating assay method.

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## CHAPTER 5

### CONCLUSIONS

In this study, the HPLC method condition for separation bromocriptine and its degradation products was optimized during the method of development phase when it was investigated the influence of different parameters on separation such as mobile phase pH, type and proportion of organic solvent, type and concentrations of salt. The best results were achieved under isocratic elution with mobile phase containing methanol and 20 mM sodium acetate in the ratio of 70:30 (v/v), pH 5.0 with the flow rate 1.5 ml/min. Wavelength used for the detection was 300 nm.

Bromocriptine mesylate was subjected to stress conditions under various ICH recommended conditions. The drug was found severe degradation in alkaline hydrolysis, moderately degraded in neutral hydrolysis and oxidative condition. The mildly degradation was found in acid hydrolysis. In solution, the drug had severe degraded when exposed to sunlight and mildly degraded was performed when exposed to UVA light or put on the water bath at temperature 80°C. However, the drug remained stable under moisture, photolytic and thermal stress in solid state.

The proposed method is enough specific, precise and accurate for quantitative analysis of bromocriptine mesylate in both bulk drug and tablets. Satisfactory results were obtained by validation of the method as well as robust with minor variation in chromatographic parameters. No attempt was made to quantify the degradation products; quantitation is possible after isolation of the degradation products in pure form. This method can be used for the determination of bromocriptine mesylate in the presence of degradation products obtained under different forced conditions. The quantitative analysis was proved that the proposed method was specific for the estimation of bromocriptine mesylate in bulk and tablet formulations. Recently, this method can use for submission as part of registration applications, in case of the impurity or degradation product standards are not

available, specificity may be demonstrated by comparing the test results of sample containing impurities or degradation product and the sample stored under relevant stress conditions (ICH 2005 : 7-8).

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**APPENDIX**

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Table 28 List of abbreviations

Symbol	Definition
k'	capacity factor
r <sup>2</sup>	correlation coefficient
°C	degree Celsius
µg/ml	microgram per milliliter
µl/ml	microliter per minute
µm	micrometer
pKa	minus logarithm base 10 of Ka, -log Ka
mg	milligram
pH	the negative logarithm of the hydrogen ion concentration
log P	Partition coefficient
%	percent
v/v	volume by volume
w/w	weight by weight
%	percent
<	less than
>	more than
≤	less than or equal to
API	Active Pharmaceutical Ingredient
A.R.	Analytical Reagent
BCT	Bromocriptine
BP	British Pharmacopoeia
DP	Degradation product
FDA	Food and Drug Administration
HPLC	High Performance Liquid Chromatography

Table 28 (continue)

Symbol	Definition
hr	hour
ICH	International Conference on Harmonization
IND	Investigational New Drugs
LA	Labeled Amount
LC	Liquid Chromatography
LOD	Limit of detection
LOQ	Limit of quantitation
M	Molarity
mg	milligram
mg/ml	milligram per milliter
min	minute
ml	milliliter
ml/min	milliliter per minute
mM	millimolar
NMR	Nuclear Magnetic Resonance
MS	Mass spectrometry
N	Number of Theoretical Plates
NDA	New Drug Application
nm	nanometer
PDA	Photodiode Array Detector
SD	standard deviation
RSD	Relative Standard Deviation
RT	Retention time, Room Temperature
RRT	Relative Retention Time
Rs	Resolution

Table 28 (continue)

Symbol	Definition
SIAM	Stability-indicating Assay Method
T	tailing factor
UV	Ultraviolet
UVA	Ultraviolet A
US	United State
USP	United State Pharmacopoeia

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