การวิเคราะห์ปริมาณแมนนิดีปีนไฮโดรคลอไรด์ในวัตถุดิบและยาเม็ด ด้วยวิธีไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโตกราฟี

นาย จันทร์สะพา ปามะณีวงค์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชเคมี ภาควิชาอาหารและเภสัชเคมี
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DETERMINATION OF MANIDIPINE HYDROCHLORIDE IN BULK DRUG AND TABLETS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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จันทร์สะพา ปามะณีวงค์ : การวิเคราะห์ปริมาณแมนนิดีปีนไฮโดรคลอไรด์ในวัตถุดิบและยาเม็ดด้วยวิธีไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโตกราฟี (DETERMINATION OF MANIDIPINE HYDROCHLORIDE IN BULK DRUG AND TABLETS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : อ. ดร.วัลลภา ทาทอง, 83 หน้า.

การศึกษานี้ได้พัฒนาวิธีไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโตกราฟีสำหรับวิเคราะห์ปริมาณแมนนิดีปีนไฮโดรคลอไรด์ในวัตถุดิบและยาเม็ด โดยเป็นวิธีทดสอบที่ผ่านการตรวจสอบความถูกต้องของวิธี และเป็นวิธีทดสอบที่บ่งชี้ถึงความคงตัวของตัวยา ระบบเครื่องประกอบด้วยเฟสคงที่คือคอลัมน์ชนิดซี -18 ซึ่งมีขนาดเส้นผ่านศูนย์กลางภายใน 4.6 มิลลิเมตร มีความยาว 150 มิลลิเมตร และพาร์ติเคิ้ลมีขนาด 3.5 ไมโครเมตร เฟสเคลื่อนที่เป็นสารละลายผสมของอะซิโตไนไตรล์และแอมโมเนียมฟอร์เมตบัฟเฟอร์ซึ่งมีความเข้มข้น 25 มิลลิโมล และมีพีเอช 3.1 ในอัตราส่วน 55:45 อัตราการไหลมีค่า 0.7 มิลลิลิตรต่อนาที กำหนดอุณหภูมิของคอลัมน์เป็น 30 องศาเซลเซียส และตรวจวัดสารที่ความยาวคลื่น 230 นาโนเมตร ทำการศึกษาวิธีทดสอบในสภาวะเร่งให้เกิดการสลายตัวของตัวยา ภายใต้สภาวะการเกิดไฮโดรไลซิส ออกซิเดชัน โฟโตไลซีส และความร้อน

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CHANSAPHA PAMANIVONG : DETERMINATION OF MANIDIPINE HYDROCHLORIDE IN BULK DRUG AND TABLETS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY. THESIS ADVISOR : WALAPA TATONG, Ph.D., 83 pp.

A validated stability-indicating HPLC method was developed for determination of Manidipine hydrochloride in bulk drug and tablets. The system comprised of a Symmetry C-18 (4.6 x 150 mm, 3.5 µm) as stationary phase, a 45 : 55 mixture of ammonium formate buffer (25 mM, pH 3.1) as mobile phase, flow rate of 0.7 ml/min, column temperature at 30°C and detection wavelength at 230 nm. The stress-testing was carried out under the conditions of hydrolysis, oxidation, photolysis and thermal degradation.

The developed method was validated for specificity, linearity and range, precision, accuracy, limit of detection, limit of quantification, stability of solutions, and system suitability. The linearity of the proposed method was in the range of 50 - 150 µg/ml. The system precision and method precision expressed as the relative standard deviation were less than 2%. The accuracy expressed as the percentage recovery was 99.4. The limit of detection and quantification were 0.141 and 0.427 µg/ml, respectively. The purposed method was successfully applied to the direct determination of Manidipine hydrochloride in tablet formulations.

Department : Food and Pharmaceutical Chemistry Student's Signature Chansapha Pamanivong

Field of Study : Pharmaceutical Chemistry Advisor's Signature Walapa Tatong

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LIST OF ABBREVIATIONS

α	=	alpha
β	=	beta
µg/ml	=	microgram per milliliter
µl	=	microlitre
µm	=	micrometer
σ	=	standard deviation of y-intercept
ACN	=	Acetonitrile
cm	=	centimeter
°C	=	degree Celsius
DHP	=	dihyropyridine
HPLC	=	high-performance liquid chromatography
hr	=	hour
ICH	=	International Conference on Harmonization
i.d.	=	internal diameter
LC	=	Liquid Chromatography
LOD	=	limit of detection

LOQ = limit of quantification

m = meter

mg/ml = milligram per milliliter

min = minute

ml = milliliter

ml/min = milliliter per minute

mm = millimeter

mM = millimolar

MND = Manidipine hydrochloride

MW = molecular weight

nm = nanometer

N = number of theoretical plate

r^2 = coefficient of determination

R = Resolution

RH = Relative humidity

RSD = relative standard deviation

Rt = Retention time

S = slope of regression line

SD	=	standard deviation
T	=	tailing factor
UV	=	ultraviolet
v/v	=	volume per volume

CHAPTER I

INTRODUCTION

The drugs classified as calcium antagonists (CAs), or calcium-channel blockers (CCBs) are a diverse group of antihypertensive medications introduced into clinical medicine in the 1960s. They possess the property of blocking the transmembrane flow of calcium ions into the cell through voltage-gated channels, thus inhibiting the activation of the actin-myosin complex and muscular contraction [1]

Calcium channel antagonists, particularly agents in the long-acting dihydropyridine (DHP) group, have well established antihypertensive efficacy and safety and have demonstrated a reduction in morbidity and mortality from cardiovascular disease and stroke [2,3]. Current attention has shifted to the newer long-acting calcium channel antagonists (second- and third-generation), which are less likely than older, short-acting agents (first-generation) to induce significant reflex tachycardia or sympathetic activation by virtue of their slow onset and prolonged duration of activity (table I).

Manidipine (MND) a third-generation calcium channel antagonist, because of its highly lipophilic nature, strong membrane binding and slow release to calcium channels, shows a long duration of action (Table 1.1). The drug has antihypertensive efficacy comparable with that of amlodipine and other second-generation DHPs, but an improved tolerability profile [4]. It does not significantly affect norepinephrine levels, suggesting a lack of sympathetic activation.

Table 1.1 Classification of dihydropyridine calcium channel antagonists

Generation	Property
First	Short-acting compounds, commonly used in special galenic preparations (e.g. nifedipine gastrointestinal therapeutic systems [GITS])
Second	Compound with a long plasma half-life (e.g. amlodipine)
Third	Highly lipophilic compound with strong membrane binding and slow release to calcium channels (e.g.manidipine, lacidipine, lercanidipine)

MND has gradual onset of action and long duration of action enabling once daily administration. Furthermore, it dilates both the efferent renal arterioles and appears to have beneficial renal effects unrelated to its antihypertensive effect. This drug is used in the treatment of angina pectoris, hypertension and congestive heart failure [5].

MND hydrochloride, 2-[4-(diphenylmethyl)-1-piperazinyl]ethylmethyl-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate dihydrochloride (Figure 1.1), was synthesized by Meguro and co-workers of the Central Research Division, Takeda Chemical Industries Ltd, Japan [6].

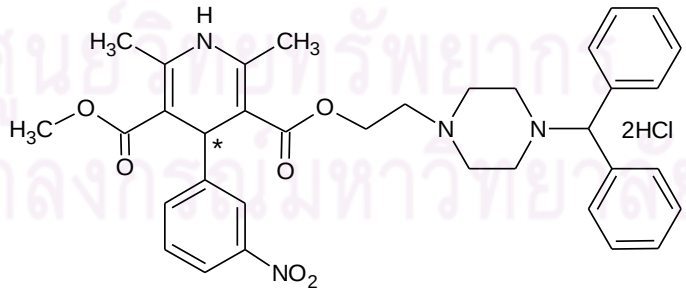

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Figure 1.1 Structure of Manidipine hydrochloride, the stereogenic centre is marked with an asterisk.

The structural characteristic is the 1,4-DHP moiety exhibiting phenyl substitution in position 4. The vascular selectivity of 1,4-DHPs is apparently coupled to the chemistry of the substitution in 2-position, the phenyl substituents in 4-position of the DHP-ring and the kind of substituents. The optimal interaction and therapeutic efficacy of the compound depends on its chemical structure. The most undesirable property of the 1,4-DHPs from pharmaceutical point of view is their high photochemical sensitivity, which can involve molecular changes leading to decrease of therapeutic effect and even some toxic effects after administration.

Physically, MND hydrochloride ($C_{35}H_{38}N_4O_6.2HCl$) is pale yellow crystalline material with a molecular weight of 683.63. It exists in two crystalline forms, α-form and β-form. The α-form is yellow crystal with a melting point of 157°C to 163°C and the β-form is light yellow, fine crystal with a melting point of 174°C to 180°C. It is soluble in dimethyl sulfoxide but insoluble in water, ethyl acetate, and ethyl ether. The compound has an asymmetric carbon at the 4-position of the DHP ring and therefore has two enantiomeric forms. Like most other calcium blocker agents of the DHP type, MND is therapeutically used as racemic mixture of R(-)- and S(+)-MND and commercially available in tablets [7,8]. It has been reported that daylight (400-600 nm) very rapidly dehydrates MND to the nitroso-pyridine species giving the parent drug with short half life in an organic solvent or plasma. Furthermore, UV light (254 nm) oxidizes MND to dehydromanidipine, devoid of any pharmacological activity which is also metabolic [9,10]. Accordingly, the control of the drug and its by-product, as such or in pharmaceutical formulations, constitutes a noteworthy analytical problem.

The purpose of this study is to develop and validate a stability-indicating high-performance liquid chromatographic (HPLC) method for determination of MND hydrochloride and to apply the proposed chromatographic method to the quantitative determination of MND hydrochloride in bulk drug and commercial tablets.

The expected benefit of this study is to obtain a rapid, sensitive and accurate HPLC method for determination of MND hydrochloride, which is useful for quality control of both drug substance and finished products. The method is also useful for stability

testing of MND hydrochloride Tablets. Besides, knowledge obtained form this study can be applied for developing HPLC methods for determination of other 1,4-DHP derivatives.

CHAPTER II

LITERATURE REVIEW

A thorough literature search has revealed that, most of the HPLC methods reported for determination of MND are in biological samples [11-15]. T. Miyabayashi et al. [11] quantitatively determined MND and its pyridine metabolite in human serum by HPLC with ultraviolet detection at 230 nm and column switching by two ODS columns, Develosil ODS-3k (3 μm, 100 x 2.1 mm) for column 1 and Develosil ODS-5k (100 x 2.1 mm, 5 μm) for column 2. The mobile phase for column 1 was 0.02 M potassium dihydrogenphosphate : acetonitrile (54:46, v/v) containing 5 mM sodium nonane-sulphonate adjusted to pH 3.0 with 85% (w/v) orthophosphoric acid and the mobile phase for column 2 was 0.02 M potassium dihydrogenphosphate : acetonitrile (54:46, v/v) adjusted to pH 3.0 with 85% (w/v) orthophosphoric acid. The temperature and flow rate for both columns were 40^{o}C and 0.3 ml/min, respectively.

T. Ohkubo et al. [12] reported the determination of MND in serum by an isocratic HPLC using column switching with electrochemical detection. The mobile phase consisted of 0.1 M disodium hydrogenphosphate (pH 4.5, adjusted with 50% phosphoric acid) : acetonitrile (51:49, v/v) and the flow rate was 1.0 ml/min at ambient temperature. MND was separated from an endogenous interference peak in serum and concentrated on a pre-column (C_{18}) by column switching and then the corresponding fractions were introduced to an analytical column with a C_8 stationary phase and detected by high conversion efficiency amperometric detection at +0.7 V.

J. Jing et al. [13] reported the determination of MND in human plasma and pharmacokinetics study. After basified plasma with ammonia, MND and the internal standard felodipine were extracted with n-hexane and separated on a Hypersil ODS2 column with a mobile phase of methanol : 5 mM ammonium acetate solution containing 0.1% acetic acid (85:15, v/v). The HPLC/ESI-MS was performed in positive ion selected

ion monitoring (SIM) mode using target ions at m/z of 611.4 for MND and m/z of 384 for the internal standard. The detector voltage was set at 1.75 kV and the flow-rate was 0.7 ml/min with an effective injected volume of 10 µl.

Successful application of chiral stationary phase-HPLC combined with sensitive column-switching HPLC to the stereospecific determination of MND in human serum was reported by M. Yamaguchi et al. [14]. The human serum sample obtained after ingestion of MND was extracted twice with a mixture of n-hexane diethyl ether under alkaline conditions. The enantiomers in the extract were separated on a chiral stationary phase column (Chiralcel OJ) (250 x 4.6 mm, 10 µm); the mobile phase was n-hexane : ethanol : methanol (80:15:5, v/v). The operating temperature and the flow rate were 50°C and 1.0 ml/min, respectively; ultraviolet detection was carried out at 230 nm.

T. Uno et al. [15] developed enantioselective HPLC determination of MND in human plasma. The method involved a rapid and simple extraction of human plasma based on C8 bonded-phase extraction, and extraction samples were purified and concentrated on the pre-column, a trimethyl-silylated silica stationary phase (10 x 4.6 mm, 5 µm), by using the column-switching technique. (+)-MND, (-)-MND and (+)-barnidipine as an internal standard were detected by ultraviolet detection at 254 nm, and enantiomers of MND were quantitatively separated by HPLC on a Pirkle-type chiral column (SUMICHIRAL OA-4500) with a non-aqueous mobile phase (n-hexane : 1,2-dichloroethane : methanol : trifluoroacetic acid, 250:140:12:1, v/v).

Few spectrophotometric methods for determination of MND in commercial tablets have been described [16-18]. Among them, N. De Laurentis et al. [16] reported the method based on the formation of charge transfer complex between MND as n-donor and iodine the s-acceptor. The iodine was found to form charge-transfer complex in a 1:1 stoichiometry with absorption bands at 290 and 353 nm. Conformity to Beer's law enabled the assay of dosage forms of MND, the concentration range for the best accuracy is 3-11 micrograms/ml. The method can be applied successfully to the analysis of commercially available MND dihydrochloride tablets. G. Ragno et al. [17] investigated simultaneous quantitative assay of MND and its main photodegradation by-

product using a multivariate calibration on UV spectra based on a classical least squares regression.

K. Javidnia et al. [18] has been studied the photodegradation kinetics of Nitrendipine and Felodipne on application of a self-modeling curve resolution method. The radiation tests were employed utilizing a 254 nm UV lamp for nitrendipine and natural sunlight for felodipine. The UV-vis absorbance spectra of the methanolic solution of nitrendipine expose to the UV lamp were recorded between 220 and 400 nm in 24 hr intervals for 15 days. I the case of felodipne, the photodegradation was monitored in sequential sunny days in the time duration of 10 a.m. till 4 p.m. At the end of the day, the resulting solution was protected by aluminum foil and stored in the refrigerator to prevent further degradation. The absorption spectra of the irradiated solitions were recorded between 230 and 400 nm in 2 hr intervals for 4 days. Since each day the drug was exposed to sunlight for 6 hr, the total lighting time was 24 hr and 14 absorption spectra were collected throughout.

Ana B. Baranda et al. [19] studied the stability of calcium channel antagonists by analyzing with LC-MS-MS, using Luna RP-C18 (2) analytical column (150 x 2 mm i.d., 3.0 µm), heated at 40^{o}C. Gradient and isocratic methods were run for the quantification of the degradation process and the identification of degradation products, respectively. The mobile phase consisted of solvent A (0.1% formic acid, 1 mM ammonium formate, pH 2.7) and solvent B (acetonitrile / 0.1% formic acid, 1 mM ammonium formate pH 2.7, 95:5, v/v). The gradient method: 0-3 min: 20% B, 3-11 min: 20-40% B (linear), 11-12 min: 40-70% B (linear), 12-12.5 min: 70-95% B (linear), 13.5-15.5 min: 95-20% B (linear). The isocratic chromatographic conditions were obtained a good separation; the same solvent A and B as for the gradient method were used with the following compositions (A:B, v/v) depending on the studied compound: Nicardipine (75:25), Lercanidipine, Nifedipine (60:40), Nitrendipine (57:43), Nifedipine, Nimodipine, Nisoldipine and Isradipine (50:50), Nilvadipine (48:52) and Lacidipine (40:60), respectively.

Nevertheless, J. Mielcarek et al. [20] has been studied the photodegradation products of new DHP derivatives using Liquid Chromatography, which was performed under isocratic conditions at flow rate of 0.7 ml/min, injection volume was 20 µl and the run time set to 35 min. Irradiated sample was chromatographed on a 4.0 x 250 mm Lichrospher RP-18 column (Merck) with phosphate buffer (pH 4.5) : acetonitrile : methanol (35.7 : 34.3 : 30.0, v/v) as mobile phase.

The photodegradation of pharmaceutical components and their degradation products of some DHP calcium channel blockers such as amlodipine and nifedipine were monitored by HPLC using a reversed phase column with UV detection. Y. Kawabe et al. [21] investigated using a column of 4.6 x 250 mm and detected by an absorption at 234 nm. Photoexposed sample of Nifedipine was eluted using aqueous 65% (v/v) methanol at a 0.45 ml/min flow rate. Amlodipine was eluted by using 50 mM ammonium formate (pH 4.4) : acetonitrile (6:4, v/v) at 0.5 ml/min flow rate. Nilvadipine was analyzed using 50 mM ammonium formate (pH 4.4) : acetonitrile (4:6, v/v) at flow rate of 0.8 ml/min. The photochemical reactions involved in the photodegradation of these pharmaceuticals including aromatization of the DHP moiety and conversion to nitroso group from nitro group in the benzene rings.

G. Ragno et al. [22] developed the analysis of photodegradation DHP product by derivative spectrophotometry and gas chromatography. DHP derivative was prepared by oxidation of the drug by irradiating an ethanolic solution of 1 mg/ml with UV light (lamp of 350 nm, 50 W, at a distance of 30 cm) about 12 hr. The UV method was recorded in the wavelength region 500 – 190 nm in 10 mm silica quartz cells. Gas chromatograph was equipped with an “on column” injector and a flame ionization detector, performed on a 30 m x 0.53 mm i.d. phenylmethyl silicone fused-silica wide-bore column, with a film thickness of 0.88 µm, the oven temperature was from 230^{o}C rising to 260^{o}C (5^{o}C/min), the detector temperature was 300^{o}C. Nitrogen was used as carrier gas at a pressure of 280 kPa. All assay procedures were carried out in a dark room provided with a red lamp of 60 W, maintained at a distance of about 2 m.

J. Mielcarek et al. [23] studied the course of the photodegradation of 1,4- DHP ring by means of UV-visible spectrophotometry and HPLC method. The methanol solution of 1,4 DHP product (nisoldipine) with the concentration of 7.77 x 10^{-6} M was monitored and recorded the absorption in the range of 200 – 500 nm. The HPLC method analyzing was employed RP-18 analytical column 250 x 4.6 mm with a column temperature of 20°C. The analysis was carried out under isocratic conditions with mobile phase consisting of methanol : water : acetonitrile (5:4:1, v/v) at the flow rate 0.6 ml/min.

Stressed degradation studies are designed to produce potential degradation products which may be encountered in real-world scenarios (Table 2.1). Degradants generated may or may not be what is seen during stability studies. [24]

Table 2.1 Recommended degradation conditions

Degradation Reaction	Typical Condition
Acid hydrolysis	Sample in aqueous acid or acidified solvent at ~0.5 N up to 24 hr (or) Heat/reflux or UV radiation in ~0.5 N HCl up to 24 hr
Base hydrolysis	Sample in aqueous base or basic solvent at ~0.5 N up to 24 hr (or) Heat/reflux or UV radiation in ~0.5 N NaOH up to 24 hr
Oxidation	Treat with ~30% H_2O_2 up to 24 hr (or) UV radiation in ~30% H_2O_2 up to 24 hr
Light decomposition (photolysis)	Expose to high-intensity UV light in suitable increment, up to 24 hr
Thermal decomposition (pyrolysis)	Expose to ~100°C heat in suitable increments, up to 24 hr

Performing the actual degradation is not an exact science and may require modifying conditions to obtain the desired 10% - 30% degradation. However, if the maximum conditions listed above do not produce degradation, then it is not necessary

to continue the experiments until degradation occurs. A statement is then added to the report confirming the stable nature of the molecules. For multicomponent formulations (e.g., with more than one active), individual active solutions should be made for each component. For product families that utilize the same excipients, forced degradation should be formed on only on formulation. The concentration of the acid, base, and peroxide solutions may be reduced or the sample concentration may be lowered with subsequent increase of the injection volume to maintain the appropriate amount of material on column. If forced degradation demonstrates lack of specificity, analysis of expired finished product may be used to prove that the forced degradation conditions are not producing real degradation peaks. The run time for forced degradation samples should be sufficiently long to observe the retention time of the latest eluting active or degradant.

M. Basaki et al. [25] has been studied degradation of 1,4-DHP (Felodipine) under different ICH guideline on stability testing of new drug substances and products (Q2B) [26]; prescribed stress conditions (hydrolysis, oxidation and photolysis), and establishment of a stability-indicating reversed-phase HPLC assay. Degradation was found to occur in alkaline medium, under high acid conditions, under oxidative stress, and also in the presence of light in acid condition. Separation of drug and the degradation product under various conditions were successfully achieved on an analytical column of C-18, 250 x 4.6 mm, utilizing water : acetonitrile in the ratio of 86:14, v/v with a detection wavelength of 310 nm. The method was validated with respect to linearity, precision, accuracy, selectivity and ruggedness. The response was linear in the drug concentration range of 5 – 500 µg/ml. The RSD values for intra- and inter-day precision studies were < 1 and <2.6%, respectively. The recovery of the drug ranged between 100 - 103 % from a mixture of degradation products.

The investigation of forced degradation study of DHP products (Nimodipine) was carried out according to ICH guideline (Q1A) [27] by S. P. Bhardwaj et al. [28]. The drug was subjected to acid (0.1 N HCl) and alkaline (0.1N NaOH) hydrolytic conditions at 80^{o}C, as well as to oxidize decomposition at room temperature. Photolysis was carried

out in 0.1N HCl, water and 0.1N NaOH at 40°C. Additionally, the solid drug was subjected to 50°C for 60 days in a dri-bath, and to the combined effect of temperature and humidity, with and without light, at 40°C/75% RH. The products formed under different stress conditions were investigated by LC and LC-MS. The LC method that could separate all degradation products performed under various stress conditions involved a C-18 column (250 x 4.6 mm) and a mobile phase comprising of acetonitrile and phosphate buffer (pH 3.0), which was run in a gradient mode. The flow rate was 1ml/min and the detection wavelength was 210 nm, respectively. The developed method was found to be precise, accurate, specific and selective. It was suitability modified for LC-MS studies by replacing phosphate buffer with water, where the pH was adjusted to 3.0 with formic acid. The drug showed instability solution state (under acidic, neutral, alkaline and photolytic stress conditions), but was relatively stable in the solid-state, except formation of minor products under accelerated conditions. Primarily, maximum degradation products were formed in acid conditions, though the same were also produced variably under other stress conditions.

Analytical test method validation is performed to ensure that an analytical methodology is accurate, specific, reproducible and robust over the specified range that an analyte will be analyzed. Method validation provides an assurance of reliability during normal use, and is sometime referred to as the process of providing documented evidence that the method does what it is intended to do. For pharmaceutical HPLC methods validation, guidelines from the US Food and Drug Administration (FDA), the United States Pharmacopeia (USP) and the International Conference on Harmonization (ICH) provides a framework for performing such validation. This would require the pharmaceutical manufacturers to establish and document the accuracy, sensitivity, specificity, reproducibility and any other attribute necessary to validate test methods. Validation is customized by choosing necessary tests and acceptance criteria for a given method. The comprehensiveness of this kind of validation is based upon the type of method and requirements.

The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components.

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

The quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantification limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as different laboratories, analysts, instruments, lots of reagents, elapsed assay times, assay temperatures, or days.

A. Elshanawane et al. [30] has been studied the application of a validated and stability-indicating LC method to establish for analysis of 1,4-DHP such as Nitrendipine and Nicardipine in the presence of the degradation products generated in studies of forced decomposition. The drug substances were subjected to stress by hydrolysis (0.1 M NaOH and 0.1 M HCl), oxidation (30% H_2O_2), photolysis (254 nm), and the thermal treatment (80^{o}C). The drugs were degraded under basic and acidic conditions and thermal treatment but were stable under other stress conditions investigated. Successful separation of the drugs from the degradation products was achieved on a 250 x 4.6 mm i.d., 5 µm, cyanopropyl column, with 40 : 60 (v/v) aqueous 0.01 M ammonium acetate buffer (pH 6.0 adjusted by acetic acid) : methanol as mobile phase at a flow rate of 1 ml/min. The volume injected was 20 µl and the detection used UV absorption at 210 nm. The method was validated for specificity, selectivity, solution stability, accuracy, and precision, respectively.

A simple stability-indicating LC method has been developed for the quantitative determination of DHP product (Amlodipine) in bulk drug samples and in pharmaceutical dosage forms in the presence of degradation products by D. V. Subba Rao et al. [31]. The drug was subjected to stress conditions of hydrolysis (1N HCl and 1 N NaOH), oxidation (6% H_2O_2), photolysis (254 nm) and thermal degradation (60^{o}C). Successful separation of the drugs from the degradation products was achieved on an analytical column of RP-18 100 x 4.6 mm i.d., 3 µm, with 40 : 60 (v/v) buffer (pH 3.0) : acetonitrile as mobile phase. The buffer consisted of 10 mM sodium dihydrogen phosphate monohydrate (pH 3.0 adjusted by diluted phosphoric acid). The flow rate of 1 ml/min

and the column temperature was maintained at 27°C. The injection volume was 20 µl and the eluent was monitored at a wavelength of 210 nm. The developed method was validated with respect to linearity, accuracy, precision, and ruggedness, respectively.

MND hydrochloride is not official in the United States Pharmacopeia (USP), British Pharmacopoeia (BP), and European Pharmacopoeia (EP), but MND is recently official in Japanese Pharmacopoeia (JP) XV, Supplement I. Consequently, the implementation of an analytical methodology to determine MND hydrochloride in pharmaceuticals is a pending challenge of the pharmaceutical analysis. In the present work, a simple, rapid, precise and accurate isocratic reversed-phase stability-indicating HPLC method with UV detection for the simultaneous determination of MND hydrochloride and degradation products in bulk drug and commercial tablets is described. The proposed HPLC method is helpful for pharmaceutical quality assurance and the investigation of drug-stability.

CHAPTER III

EXPERIMENTATION

Material and method

Material

1. Apparatus and Instrument

- High-Performance Liquid Chromatograph (SHIMADZU 10 AVP, Japan) equipped with an Auto-Sampler SIL-10 ADVP, an UV-Vis Detector SPD-10 AVP, a Column Oven CTO-10 AVP, a Pump LC-10 ADVP, a Degasser DGU-14 A and a System Controller SCL-10 AVP.
- Mass Spectrophotometer (Shimadzu, Japan)
- Analytical Column, Symmetry RP18, 3.5 µm, 4.6 x 150 mm i.d. (Waters Corporation, Ireland)
- Analytical balance, Mettler AG 245 (Mettler-Toledo, Switzerland)
- Hot air oven (Binder, Germany)
- Magnetic stirrer, Heidolph MR3001 (Heidolph, Germany)
- pH meter, Beckman ϕ50 (Beckman, USA)
- Ultrasonic bath, Cavitator ME 4.6 (Mettler, Switzerland)

2. Chemicals and Reagents

- Acetonitrile, HPLC grade (Burdick & Jackson, USA)
- Ammonium formate (Biotech, China)
- Ammonium hydroxide, 90% (May & Baker, Dagenham, England)
- Formic acid, 90% (Merck, Germany)
- Hydrogen peroxide, 30% (BDH, England)

- Hydrochloric acid, 37% (Merck, Germany)
- Methanol , HPLC grade (Burdick & Jackson, USA)
- Manidipine Hydrochloride working standard, 98.5% purity on as is basis, Lot No.070330 (Xiamen Shinbon Chemicals, China)
- Sodium hydroxide, pellets (Mallinckrodt, Mexico)

3. Test samples

Manidipine hydrochloride Tablets, Madiplot (Takeda, Japan), Lot No.0994, Exp.30.09.2011, were purchased from the Community Pharmacy Laboratory (OSOT SALA), Faculty of Pharmaceutical Sciences, Chulalongkorn University. Each tablet contains manidipine hydrochloride 10 mg.

Method

This study composed of four experimental steps as follows:

1. HPLC method development
2. Optimization of HPLC conditions
3. Analytical Method Validation
4. Assay application

1. HPLC method development

1.1 Buffer preparation (25 mM ammonium formate, pH 3.1)

- 1M formic acid: 4 ml of formic acid was diluted with water to 100 ml.
- A 0.2382-g of ammonium formate (NH_4HCO_2) was dissolved in 700 ml of water, add 20 ml of 1M formic acid, and mix thoroughly. Adjust to pH 3.1 with ammonium hydroxide (NH_4OH), dilute with water to 1000 ml and mix. Then filter the solution through a filter paper pore size 0.45 µm and use the filtrate as a buffer for mobile phase preparation.
- Diluent: a mixture of water and acetonitrile (1:1)

1.2 Stock sample solution

Weigh and finely powder not less than 20 tablets of MND hydrochloride Tablets. Transfer an accurately weighed portion of the powder, equivalent

to about 10 mg of MND hydrochloride, to a 100-ml volumetric flask, add 10 ml of methanol and sonicate for 5 minutes. Allow the solution to cool to room temperature, dilute with diluent to volume, and mix. Pass a portion of this solution through a filter with a pore size of 0.45 µm, discarding the first 4 ml of the filtrate. Subsequent filtrate obtained is used as the stock sample solution.

1.3. Stock standard solution

Accurately weigh and transfer 100 mg of manidipine hydrochloride standard to a 100-ml volumetric flask, add 10-ml of methanol and sonicate for 5 minutes. Allow the solution to cool at room temperature then dilute with methanol to volume and mix. The solution concentration is 100 µg/ml, kept in a refrigerator at 4^{o}C and protected from light.

1.3.1 Standard solution for linearity and range

Accurately weigh and transfer 62.5 mg of manidipine hydrochloride standard to a 25-ml volumetric flask, add 10 ml of methanol and sonicate for 5 minutes. Allow the solution to cool at room temperature then dilute with methanol to volume and mix (2.5 mg/ml). Transfer 1, 2, 3 ml of this stock standard solution to each of 50-ml volumetric flasks then dilute diluent to make a concentration of 50, 100 and 150 µg/ml, respectively. Transfer 10 ml of this stock standard solution to a 20-ml volumetric flask add diluent to volume (1.25 mg/ml), then transfer 3 and 5 ml of this solution to each of 50-ml volumetric flasks and dilute with diluent to volume to obtain solutions of concentration of 75 and 125 µg/ml, respectively.

1.3.2 Standard solution for LOD and LOQ

Transfer 25 ml 0f MND HCl stock standard solution to 100-ml volumetric flask, add diluent to volume, and mix. Then transfer 10 ml of the solution to a 50-ml volumetric flask, add diluent to volume, and mix (50 µg/ml). Transfer 6, 7, 8, 9 and

10 ml of the resulting solution to each of 100-ml volumetric flasks, add diluent to volume, and mix to obtain solutions of concentration of 3, 3.5, 4, 4.5 and 5 µg/ml, respectively.

1.3.3 Standard solution for Accuracy

Accurately weighed 60 mg of MND hydrochloride standard to a 100-ml volumetric flask, add 10 ml of methanol and sonicate for 5 minutes. Allow the solution to cool at room temperature then dilute with methanol to volume and mix; the solution concentration is 0.6 mg/ml (Stock standard solution A).

Accurately weighed 62.5 mg of MND hydrochloride standard to a 25-ml volumetric flask, add 10 ml of methanol and sonicate for 5 minutes. Allow the solution to cool at room temperature then dilute with methanol to volume and mix; the solution concentration is 2.5 mg/ml (Stock standard solution B)

- Transfer 5ml of sample stock solution to a 50-ml volumetric flask add diluent to volume, and mix to make a concentration of 10 µg/ml.

- Transfer 5ml of sample stock solution to two 50-ml volumetric flasks, add exactly 5 and 10ml of stock standard solution A, add diluent to volume, and mix to make a concentration of 70 and 130 µg/ml, respectively.

- Transfer 5ml of sample stock solution to a 50-ml volumetric flasks, add exactly 2ml of stock standard solution B, add diluent to volume, and mix to make a concentration of 110 µg/ml.

1.4 Stressed sample solutions

Stress studies were carried out under the conditions of hydrolysis, oxidation, photolysis and thermal degradation as mentioned in ICH guidelines (Q1A).

1.4.1 Effect of hydrolysis degradation

Acid-base catalyst was studied using the stress condition with the addition of 1N hydrochloric acid and 1N sodium hydroxide.

Acid catalysis on standard and sample solution was studied by adding 10 ml of 1N hydrochloric acid to 10 ml of methanolic containing 100 µg/ml of MND HCl and mixing thoroughly, the final concentration is 10 µg/ml. The solution was then kept in the hot air oven at 60°C for 3 hr. Thereafter, the solution was neutralized with 1N sodium hydroxide. Degraded product obtained was analyzed by HPLC system as mentioned in 1.5.

Base catalysis was performed with the same procedure as acid catalysis, but replacing of 1N hydrochloric acid with 1N sodium hydroxide.

1.4.2 Effect of oxidation degradation

Oxidative degradation on standard and sample solution was studied by adding six drops of H_2O_2 to 10 ml of methanolic containing 100 µg/ml of MND and mixing thoroughly, the final concentration is 10 µg/ml. The solution was then kept in the hot air oven at 60°C for 3 hr. Thereafter, degraded product obtained was analyzed by HPLC with the chromatographic condition as described in 1.5.

1.4.3 Effect of photolysis degradation

The methanolic standard and sample solution containing 100 µg/ml of MND was prepared. The solution was then exposed to the UV at 254 nm for 3 hr. Thereafter, degraded product obtained was analyzed by HPLC system with the chromatographic condition as described in 1.5.

1.4.4 Effect of thermal degradation

The methanolic standard and sample solution containing 100 µg/ml of MND was prepared. The solution was then kept in the hot air oven at 60°C for 3 hr. Thereafter, degraded product obtained was analyzed by HPLC system with the chromatographic condition as described in 1.5.

1.5 HPLC condition

Final chromatographic conditions were an isocratic elution as follow:

Column	Symmetry RP-18, 4.6 x 150 mm, 3.5 µm
Mobile phase	25 mM Ammonium formate, pH 3.1 : Acetonitrile (45 : 55)
Flow rate	0.7 ml/min
Detector	UV-Vis at 230-nm
Injection volume	20 µl

The mobile phase was a pre-mixed preparation by degas in ultrasonic bath for 45 min.

2. Optimization of HPLC conditions

The effects of ammonium formate buffers (pH and concentration), composition of the mobile phase (percent of acetonitrile), detection wavelength and column temperature on HPLC separation were investigated.

2.1 Effect of buffer pH

Various ammonium formate buffer systems in the acidic at pH 2.8, 3.0, 3.1, 3.3 and 3.4 were studied. These buffers were prepared at constant ionic strength of 25 mM. Base catalysis of standard solution of MND hydrochloride 10 µg/ml was analyzed.

2.2 Effect of buffer concentration

The ammonium formate buffer concentration in the range of 20, 25 and 30 mM at pH 3.1 were investigated. Base catalysis of standard solution of MND hydrochloride 10 µg/ml was analyzed.

2.3 Effect of mobile phase composition

The percentage of acetonitrile in the mobile phase at 54, 55 and 56 were investigated. Base catalysis of standard solution of MND hydrochloride 10 µg/ml was analyzed.

2.4 Effect of detection wavelength

The detection wavelengths at 225, 230 and 235-nm were studied. Base catalysis of standard solution of MND hydrochloride 10 µg/ml was analyzed.

2.5 Effect of column temperature

The column temperatures studied were set at 27, 30 and 33°C. Base catalysis of standard solution of MND hydrochloride 10 µg/ml was analyzed.

3. Analytical Method Validation

The validation was performed according to the procedure recommended by the International Conference on Harmonization (ICH, 1996). The following measures of method performance were assessed: specificity, linearity and range, precision, accuracy, determination of detection (LOD), and quantification (LOQ), stability of standard solution and system suitability.

3.1 Specificity

The standard and sample solutions (10 µg/ml) of MND hydrochloride from forced degradation procedure studies of hydrolysis (1N HCl and 1N NaOH at 60°C for 3 hr), oxidation (30% H_2O_2 at 60°C for 3 hr), photolysis (UV 254-nm for 3 hr) and thermal degradation (60°C for 3 hr) were triplicate injected into the HPLC system. The chromatograms of all analyte peaks in sample solution were compared with chromatogram of standard solution.

3.2 Linearity and range

Linearity and range was obtained with calibration curves using five standard solutions prepared over the range of 50 – 150 µg/ml from the stock standard solution. Each solution was injected in triplicate; least square linear regression analysis was performed by plotting peak areas versus concentrations. The coefficient of determination (r^2) was calculated and should be at least 0.99

3.3 Precision

Precision was accessed from the % relative standard deviation (%RSD), which was determined from the following formula:

%RSD = (SD x 100) /

Where SD is the standard deviation of the observed data. $\bar{x}$ is the average peak area value. The %RSD should be less than 2.0 %

The precision was assessed by the following methods:

(1) System precision

The system precision determined by six replicate injections of standard solution with a concentration of 100 µg/ml.

(2) Method precision

Method precision was determined by triplicate injections of six determinations of sample solution at concentration of 100 µg/ml on intra-day and inter-day precision for three non-consecutive days.

3.4 Accuracy

The accuracy was calculated from percent recovery using standard addition method. Three different amounts of MND hydrochloride reference standard were added to the sample solution (Solution of Madiplot). The three concentrations covered a 60 – 120 % interval of the expected assay concentrations. The solutions were prepared in three determinations at each concentration. Percent recovery was calculated from the ratio of the amount found and the amount added. The mean recoveries should be within in the acceptance criteria, 98 – 102 %, of MND hydrochloride.

3.5 Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were obtained from a calibration curve constructed by analyzing standard solutions. Five point calibrations were performed over the range 3 to 5 µg/ml; the solutions were injected in triplicate. Peak areas of standard solution were plotted against concentrations. Least square linear regression analysis was performed to determine slope, intercept and the standard deviation of the y-intercept.

The LOD and LOQ were calculated by following equations:

$$LOD = 3.3\,\sigma/S$$

$$LOQ = 10\,\sigma/S$$

Where σ is the standard deviation of the y-intercept and S is the slope of the regression line. At the LOQ, the %RSD of the within-day precision should be less than 10%.

3.6 Stability of standard solution

The stability of standard and sample solution was determined at concentration of 100 µg/ml. The solution was injected in triplicate on 0, 1, 2, 3 and 4 hr to analyzing for set criteria value.

3.7 System suitability

The system suitability testing was evaluated by six replicate injections of base catalysis of standard solution of MND hydrochloride 10 µg/ml. The percent of relative standard deviation (%RSD) of peak area was determined. The %RSD should be less than 2, the number of theoretical plate should be not less than 2000 and

tailing factor should be less than 2 and the resolution must not less than 2. The tailing factor, number of theoretical plate and resolution are calculated as follows:

- Tailing factor (T)

$$T = \frac{W_{0.05}}{2f}$$

Where $W_{0.05}$ is the peak width at 5% height and f is the distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline as shown in Figure 3.1

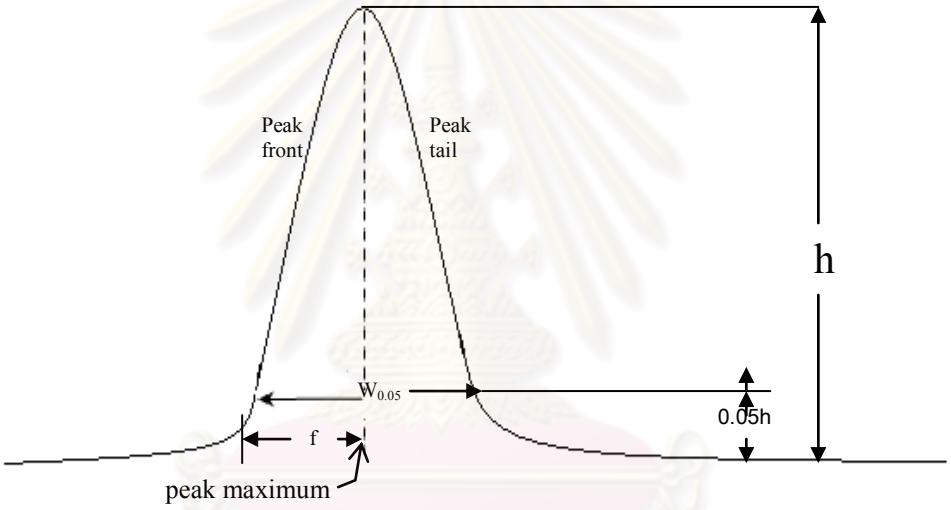

Figure 3.1 Chromatogram for calculation of tailing factor

- Number of theoretical plate (N)

$$N = 5.54\left(\frac{t}{W_{h/2}}\right)^2$$

Where t is the retention time of the substance and $W_{h/2}$ is the peak width at half-height as shown in Figure 3.2

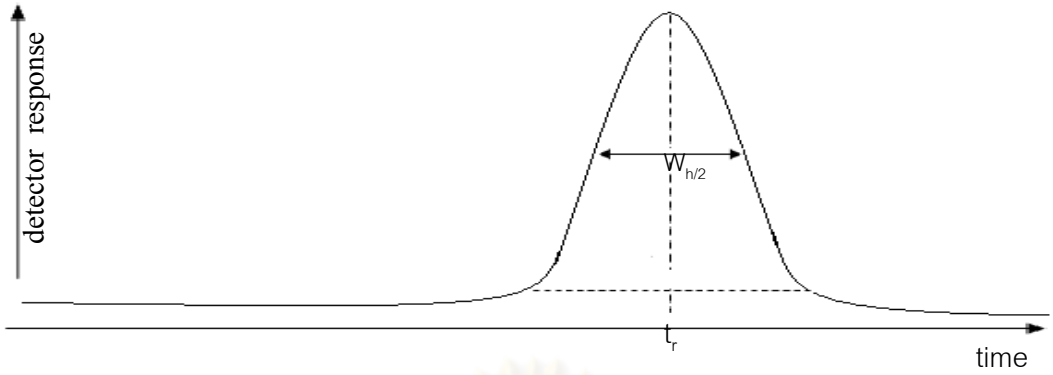

Figure 3.2 Chromatogram for calculation of the number of theoretical plates

$$R = \frac{2(t_2 - t_1)}{W1 + W2}$$

Where

t_1 and t_2 are the retention times of the first and second adjacent peaks W_1 and W_2 are their baseline peak widths.

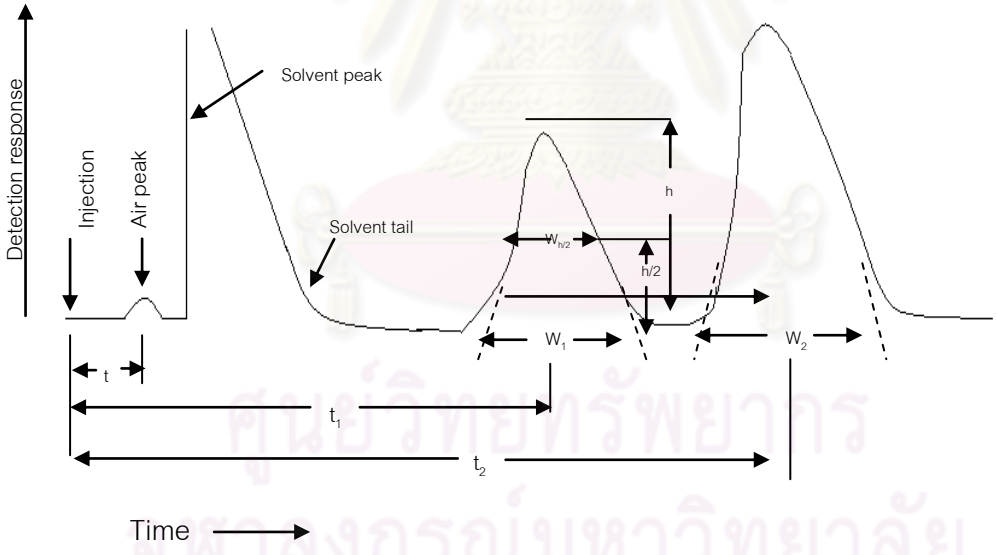

Figure 3.3. Chromatogram for calculation of resolution

4. Assay application

Weigh and finely powder not less than 20 tablets of MND hydrochloride Tablets. Transfer an accurately weighed portion of the powder, equivalent to about 10 mg of MND hydrochloride, to a 100 ml volumetric flask, add 10 ml of methanol and

sonicate for 5 minutes. Allow the solution to cool to room temperature, dilute with a mixture of water and acetonitrile (1:1) to volume, and mix. Pass a portion of this solution through a filter with a pore size of 0.45 µm, discarding the first 4 ml of the filtrate.

CHAPTER IV

RESULTS AND DISCUSSION

The current International Conference on Harmonisation (ICH) guidelines suggest that quantitative analysis of pharmaceuticals should be done by using stability-indicating assay (SIA) method. For this study, in order to obtain the HPLC SIA method, alkaline stress standard solution of 10 μg/ml was used in the analytical method development due to the lack of standards of impurities and degradation products. The alkaline stress standard solution was selected because many peaks were observed in the chromatogram.

1. HPLC method development

The UV absorption spectrum of the methanolic standard MND hydrochloride solution of 1 μg/ml exhibited three absorption maxima at wavelength of 228 and 353-nm as shown in Figure 4.1. The absorption wavelength at 230-nm was selected as the detection wavelength in this study.

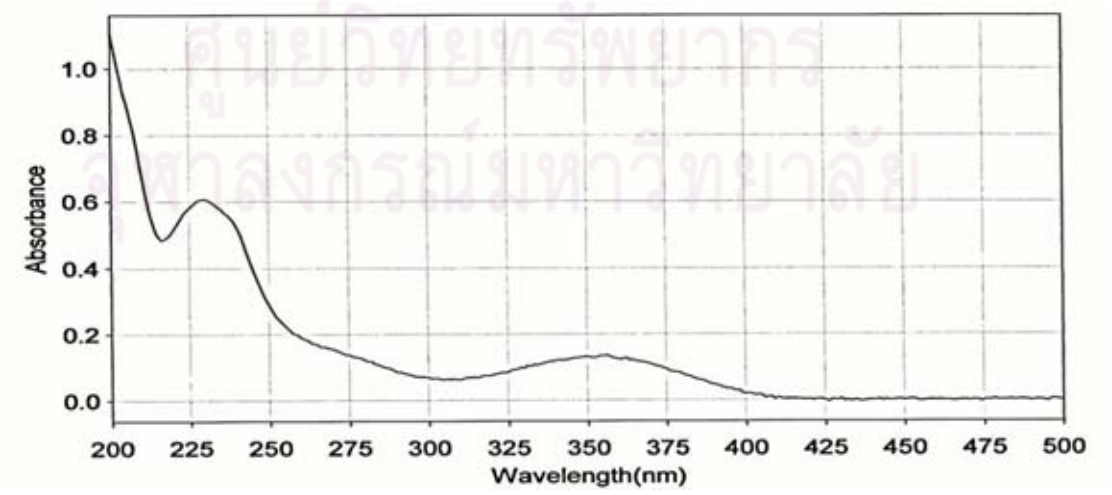

Figure 4.1 Absorption spectrum of MND hydrochloride (1 μg/ml, in methanol)

MND is a weak base with a pKa value of 9.4 and fully protonated in acidic pH medium. In this study, MND was selected to analyze in an acidic mobile phase in order to obtain an appropriate analytical run time. Ammonium formate buffer with optimal buffer capacity in the pH range of 2.8 – 4.8 was selected as an aqueous phase of the mobile phase. Besides, ammonium formate is suitable for buffer mobile phase for LC/MS as it is volatile in nature.

In initial studies, the separation of analytes was carried out using gradient elution mode. Mobile phase was a variable mixture of Solution A and Solution B as shown in Table 4.1 Solution A was a filtered and degassed mixture of 200 mM ammonium formate pH 3.0, water, and acetonitrile (10:40:50) and Solvent B was a filtered and degassed mixture of 200 mM ammonium formate pH 3.0, water, and acetonitrile (10:10:80). The liquid chromatograph is equipped with a 230-nm detector and 4.6-mm x 15-cm Symmetry column C18, 3.5 μm and was maintained at a temperature of 30°C. The flow rate was about 0.7 ml/min. The chromatograph was programmed as follows.

Table 4.1 Mobile phase composition of gradient elution mode

Time (minutes)	Solution A (%)	Solution B (%)	Elution
0-10	50	50	isocratic
10-13	50 → 5	50 → 95	Linear gradient
13-15	5	95	isocratic
15-18	5 → 50	95 → 50	Linear gradient
18-25	50	50	equilibration

Figure 4.2 represented the HPLC chromatogram of alkaline stress MND hydrochloride solution with the retention time of MND was 3.7 min and degradant peaks were detected at 2.7, 3.1 and 4.7 min, respectively. The first degradant peak at 2.7 min was too close to the solvent front.

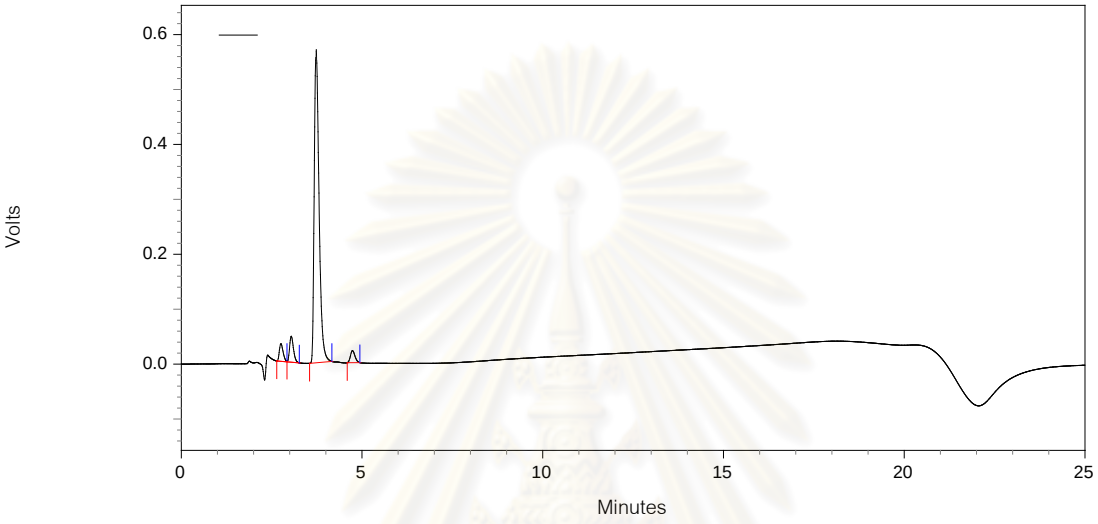

Figure 4.2 Chromatogram of alkaline stress MND hydrochloride standard solution under gradient elution mode with a mobile phase of variable mixtures of 200 mM ammonium formate pH 3.0, water, and acetonitrile in Solution A (10:40:50) and Solution B (10:10:80).

As expected, decreasing percentage of acetonitrile in Solvent A to 40% and 20%, while maintained composition ratio of Solvent B and the rest of chromatographic system, MND and degradant peaks of the alkaline stress standard solution were shifted to longer retention times, as shown in Figure 4.3 and Figure 4.4.

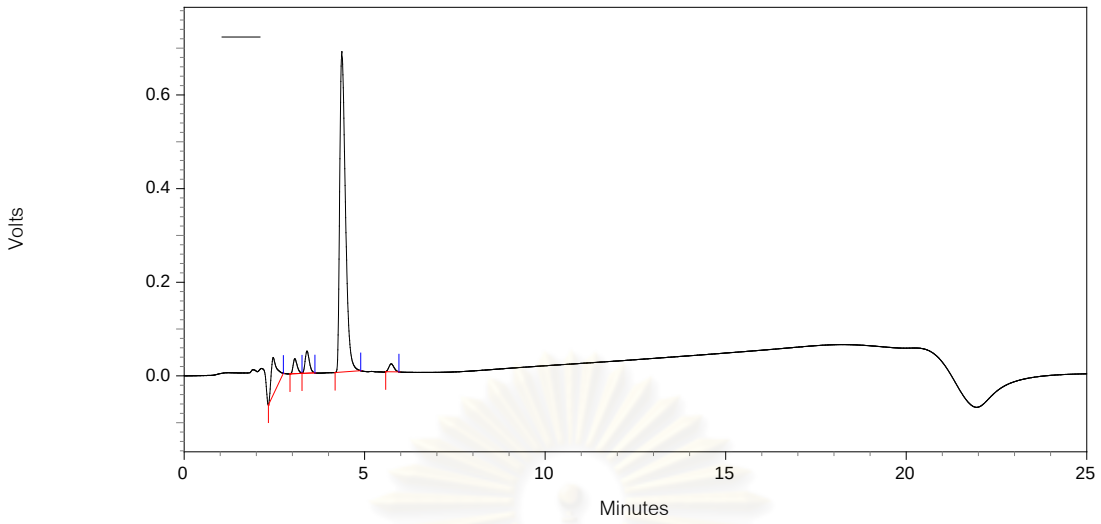

Figure 4.3 Chromatogram of alkaline stress MND hydrochloride standard solution under gradient elution mode with a mobile phase of variable mixtures of 200 mM ammonium formate pH 3.0, water, and acetonitrile in Solution A (10:50:40) and Solution B (10:10:80).

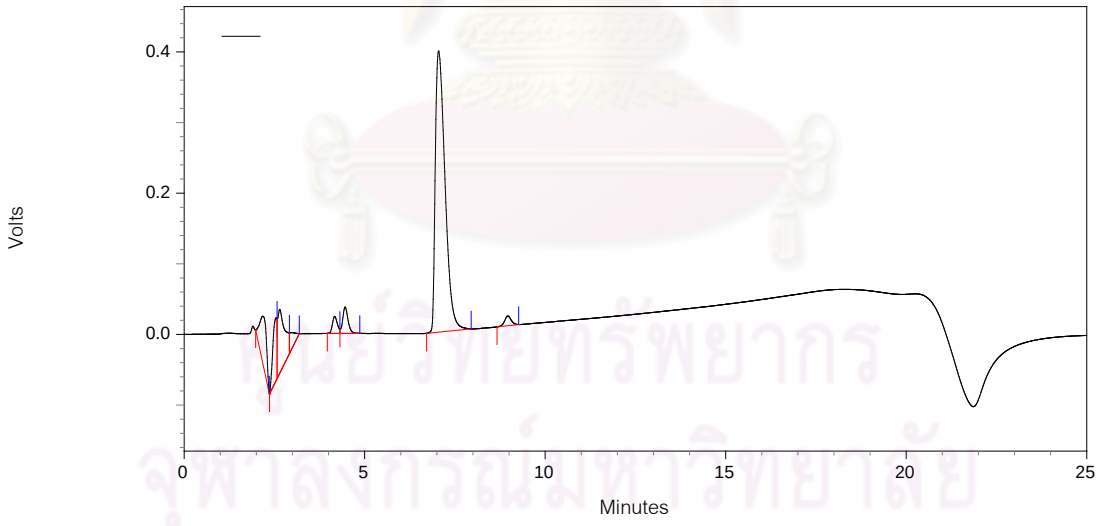

Figure 4.4 Chromatogram of alkaline stress MND hydrochloride standard solution under gradient elution mode with a mobile phase of variable mixtures of 200 mM ammonium formate pH 3.0, water, and acetonitrile in Solution A (10:70:20) and Solution B (10:10:80).

Chromatograms in Figures 4.2 - 4.4 showed that there were eluted peaks just during the first 10 minutes of the run, indicating unnecessary for gradient elution mode. Thus, the isocratic analysis method was developed and a suitable mobile phase was a mixture of 25 mM ammonium formate, pH 3.1 and acetonitrile (45:55).

2. HPLC method optimisation

Optimisation of HPLC condition was performed using alkaline stress MND standard solution. The following parameters were consecutively optimized: concentration and pH of the buffer solution, composition of the mobile phase (percentage of acetonitrile), detection wavelength, and column temperature. Retention time (R_t) and resolution (R) for each condition were compared.

2.1 Effect of buffer pH

The buffer pH is one of the most important parameters for improving resolution in HPLC. The pH of the mobile phase is usually taken by convention to be the same as that of the aqueous fraction, this implies an obviously false assumption. The pKa values of the acids used to prepare the buffer change with the solvent composition (and each in a different degree), and so does the pH of the buffer. Sometimes the pH is measured after mixing the buffer with the organic modifier; but even in this instance, the potentiometric system is usually calibrated with aqueous standards and the measured pH is not the true pH of the mobile phase (Lambert et al [32]).

Ammonium formate buffer with pH range of 2.8 – 3.4 was investigated by analyzing the alkaline stress standard sample solution (Figure 4.5 – 4.9).

At pH 2.8 (Figure 4.5), MND was eluted with a retention time of 4.2 min and four degradant peaks were detected at retention times of 2.9, 3.8, 5.6 and 7.2 min, respectively. MND was not completely resolved from degradant peak of 3.8 min.

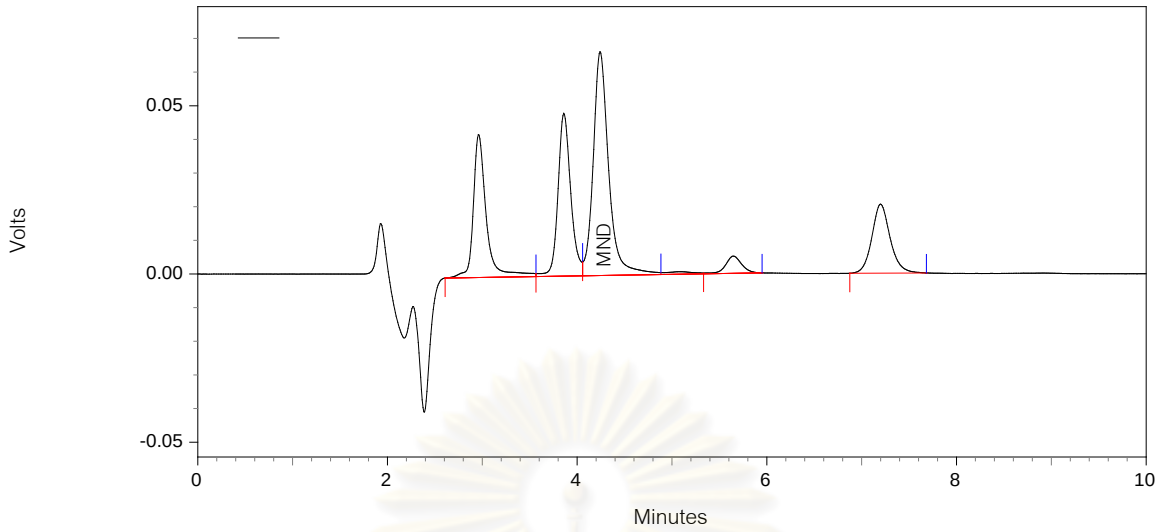

Figure 4.5 Effect of buffer pH on alkaline stress MND standard solution under condition of 25 mM ammonium formate pH 2.8 : ACN (45:55), detection wavelength of 230-nm and column temperature at 30°C

At pH 3.0 (Figure 4.6), the retention time of MND peak was shifted to 4.9 min and the retention time of four degradant peaks were detected at 3.3, 3.9, 5.5 and 7.3 min, respectively. Interestingly, increasing pH from 2.8 to 3.0 affected elution time of just MND and the first degradant. The MND peak was not completely resolved from degradant peak of 5.5 min.

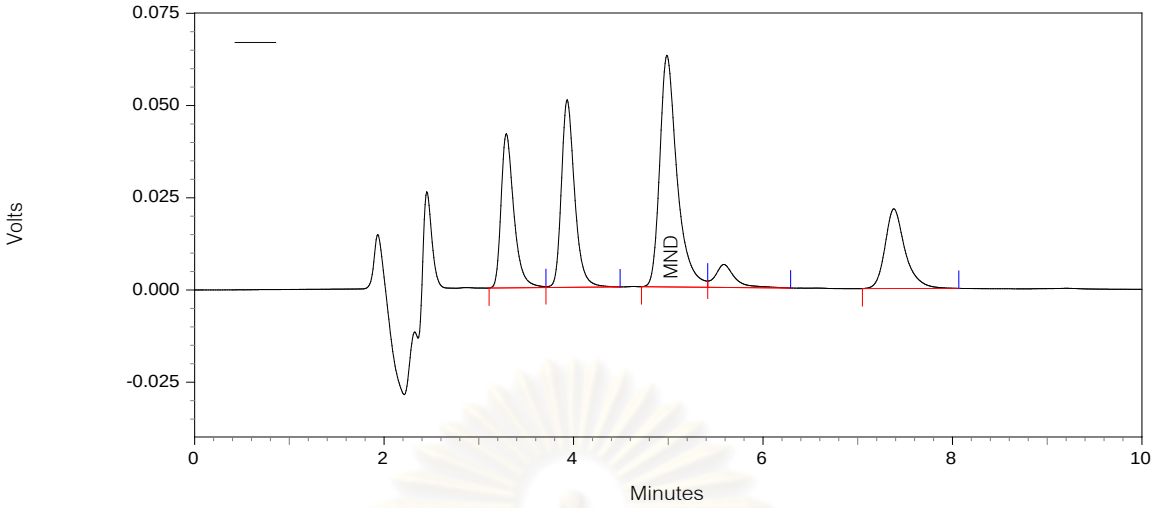

Figure 4.6 Effect of buffer pH on alkaline stress MND standard solution under condition of 25 mM ammonium formate pH 3.0 : ACN (45:55), detection wavelength of UV 230-nm and column temperature at 30°C

At pH 3.1 (Figure 4.7), The MND peak was observed at retention time of 4.9 min and four degradant peaks were eluted at retention times of 3.3, 4.1, 5.8 and 7.7 min, respectively. MND was completely resolved from adjacent degradant peaks of 4.1 and 5.8 min.

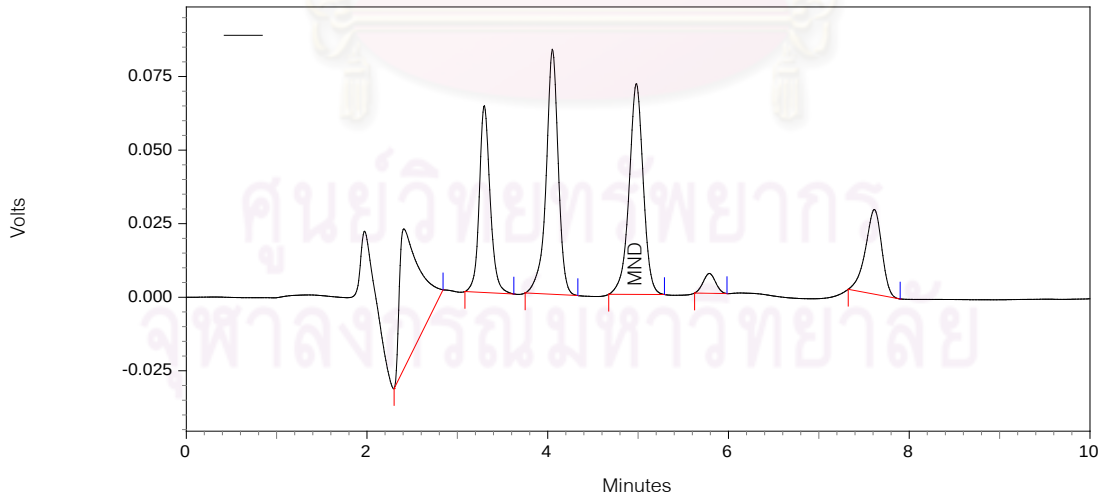

Figure 4.7 Effect of buffer pH on alkaline stress MND standard solution under condition of 25 mM ammonium formate pH 3.1 : ACN (45:55), detection wavelength of UV 230-nm and column temperature at 30°C

At pH 3.3 (Figure 4.8), MND peak was detected at retention time of 4.1 min and just three degradant peaks were found at retention times of 3.6, 5.5 and 7.5 min, respectively. At pH 3.3 of 25 mM ammonium formate buffer, the first degradant peak that usually found when using the buffer at pH range of 2.8-3.1 was coelute with the solvent peak at about 2.6 min. MND was acceptable resolved from an adjacent degradant peak of 3.6 min.

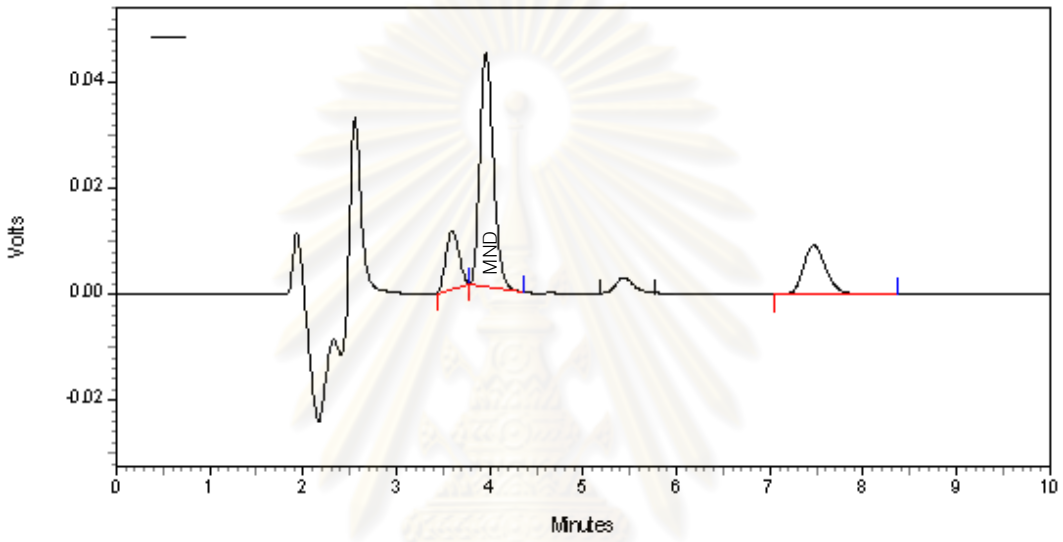

Figure 4.8 Effect of buffer on alkaline stress MND standard solution under condition of 25 mM ammonium formate pH 3.3 : ACN (45:55), detection wavelength of UV 230-nm and column temperature at 30°C

At pH 3.4 (Figure 4.9), MND was eluted faster at retention time of 3.9 min and just two degradant peaks were found after MND peak at retention times of 5.2 and 7.4 min, respectively. Two degradant peaks found before MND peak at lower pH (2.8-3.1) were collapse and coeluted with the solvent front. MND was completely resolved from degradant peaks.

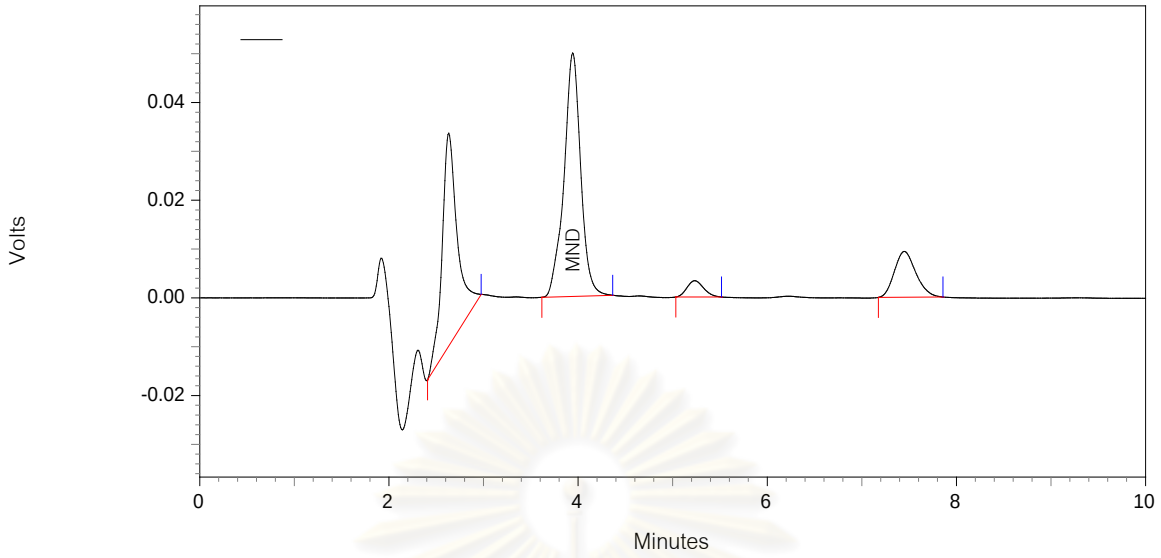

Figure 4.9 Effect of buffer on alkaline stress MND standard solution under condition of 25 mM ammonium formate pH 3.4 : ACN (45:55), detection wavelength of UV 230-nm and column temperature at 30°C

MND which has a pKa of 9.4 would be essentially protonated throughout the pH range used in this study. As the pH is increased, carboxylic functional groups in the pyridine ring of MND are probably partially deprotonated, leading to an enhancement of the stability of the labile diastereomeric complexes formed between carboxylic and pyridine.

The effect of pH of ammonium formate buffer on retention time of alkaline stress standard solution of MND hydrochloride was summarized and displayed in Table 4.2 and Figure 4.10, respectively.

Table 4.2 Retention times of MND and degradants at various pH of ammonium formate buffer (* degradant coelute with the solvent peak)

Compound	Retention time (min)				
	pH 2.8	pH 3.0	pH 3.1	pH 3.3	pH 3.4
MND	4.2	4.9	4.9	4.1	3.9
Degradant 1	2.9	3.3	3.3	2.6*	2.6*
Degradant 2	3.8	3.9	4.1	3.6	2.6*
Degradant 3	5.6	5.5	5.8	5.5	5.2
Degradant 4	7.2	7.3	7.7	7.5	7.4

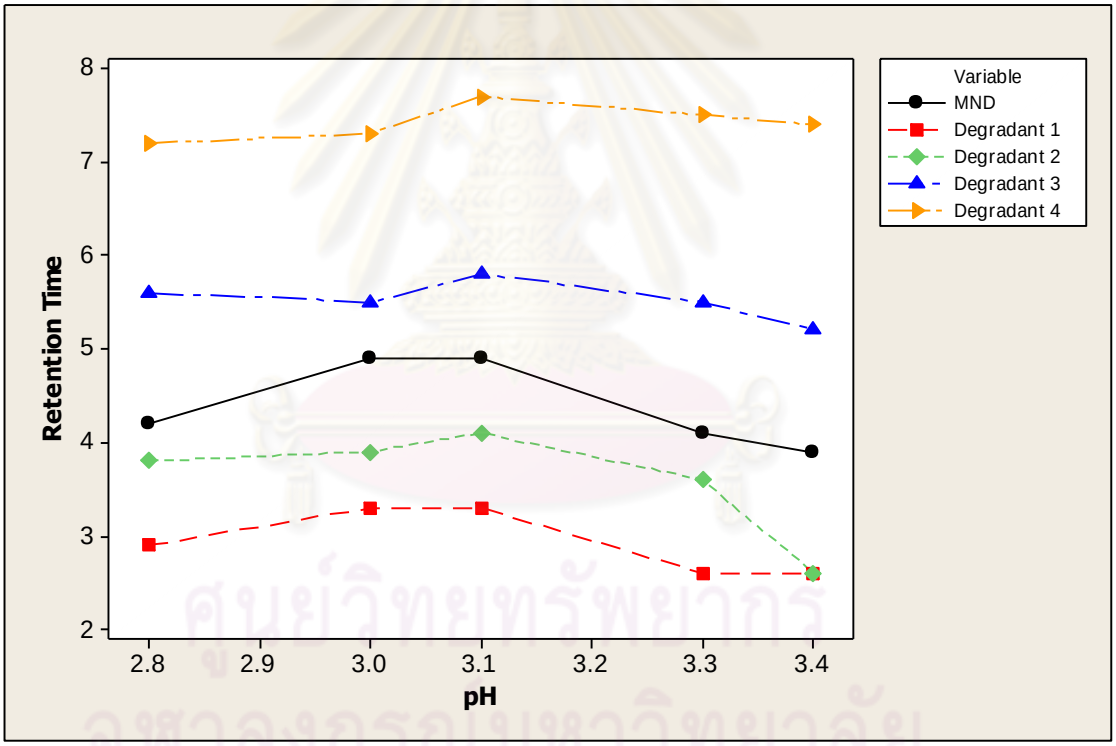

Figure 4.10 Effect of pH of ammonium formate buffer on retention time of alkaline stress standard MND hydrochloride solution

From Figure 4.10, it was obvious that the mobile phase with ammonium formate buffer of pH 3.1 gave a chromatogram of optimal reasonable retention time.

Therefore, the pH value of 3.1 was chosen as the aqueous phase of HPLC mobile phase for this study.

2.2 Effect of buffer concentration

The effect of concentration of ammonium formate buffer, pH 3.1, in mobile phase was also investigated at concentration of 20 mM, 25 mM, and 30 mM. Figure 4.11 represented chromatograms of changing concentration of buffer from 20 to 30 mM. It was observed that at concentration of buffer 20 mM, the main peak of MND at retention time of 4.2 min was not well completely resolve from its degradant peak as shown in Figure a. The concentration of buffer 25 mM, the main peak of MND at retention time of 4.9 min was well completely resolve from its degradant peak as shown in Figure b. However, the concentration of buffer 30 mM, the main peak of MND at retention time of 4.8 min was not completely resolve from its degradant peak as shown in Figure c.

Due to the concentration of buffer in the mobile phase effect to the separation of MND hydrochloride and its degradant, so when increase the buffer concentration to 30 mM, the mobile phase was more polarization which induces to the structure of MND effected to its separation was not completely resolve from degradants. Nevertheless, when the buffer concentration was decreased to 20 mM, the mobile phase was less polarized and the MND peak was not well separated from its degradant peaks.

Therefore, the concentration of 25 mM of ammonium formate buffer was chosen, in order to achieve better resolution of MND.

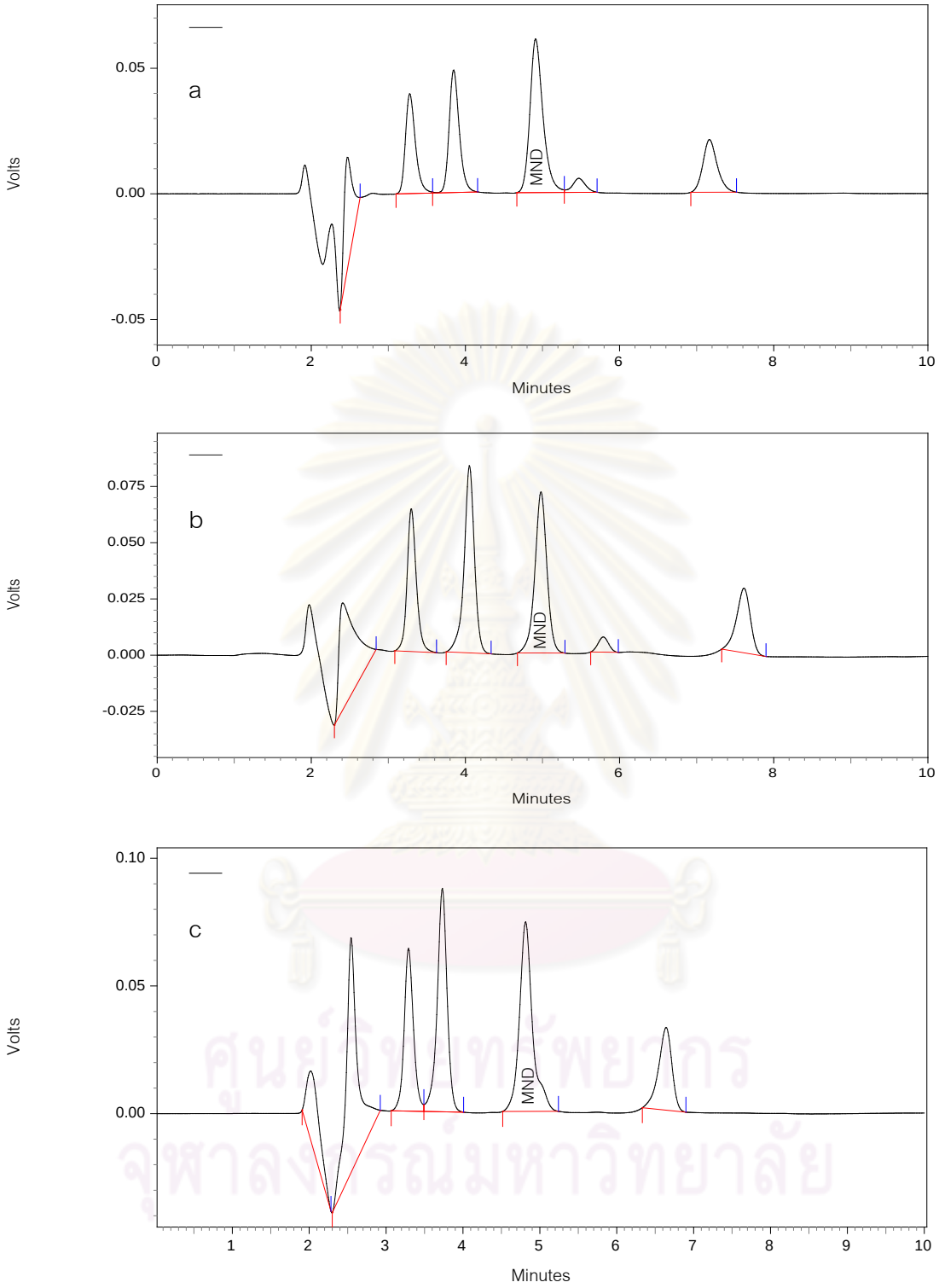

Figure 4.11 Effect of buffer concentration on alkaline stressed MND standard solution under conditions of a:25, b:25 and c:30 mM ammonium formate pH 3.1 : ACN (45:55), detection wavelength at UV 230-nm and column temperature at 30°C

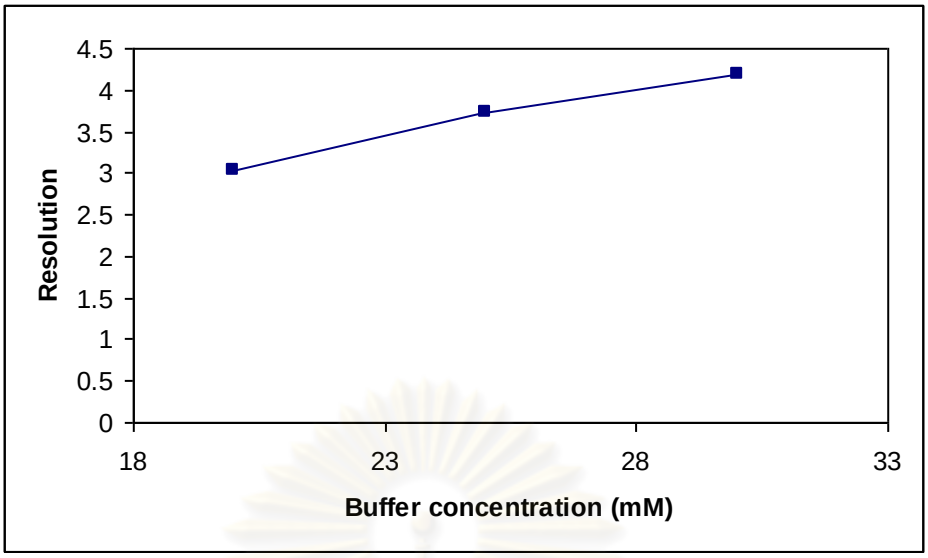

Figure 4.12 Effect of buffer concentration on the resolution of alkaline stress MND standard solution

2.3 Effect of mobile phase composition

From Figure 4.13, the significant increase in resolution was obvious in the low composition of mobile phase range (54 – 55% of acetonitrile). A similar result was observed in the high composition of mobile phase range (55 – 56 % of ACN).

At mobile phase composition of 54% ACN (Figure a), the main peak of MND detect at retention time of 4.2 min was not well separated from its degradant peaks at 2.9, 3.5, 4.7 and 6.1 min, respectively. Also, at mobile phase composition of 55% ACN (Figure b), MND was completely separation at retention time of 4.9 min and 3.3, 3.9, 5.6 and 7.4 min of degradant peak, respectively.

At mobile phase composition of 56% ACN (Figure c), the peak detect was similar result as percentage composition of 55% ACN but longer retention time detection and MND was not fully separated from its degradant peak throughout this

percentage of ACN. MND was detect at the retention time of 5.2 min and 3.4, 3.9, 5.7 and 7.5 min of degradants peak, respectively.

From the experiment, the exact ratio of organic modifier is critical for the resolution of the analyte. The percentage composition of ACN in the mobile phase is effected to its separation, when increase the ACN to 56% the mobile phase was more polarization result to the separation of MND and its degradants was not completely separated. And also when decrease the ACN to 54%, the mobile phase was less polarized and the MND was not well separated from its degradant. Thus the optimized mobile phase composition of buffer selected was 55% of ACN since it provided better retention time and resolution.

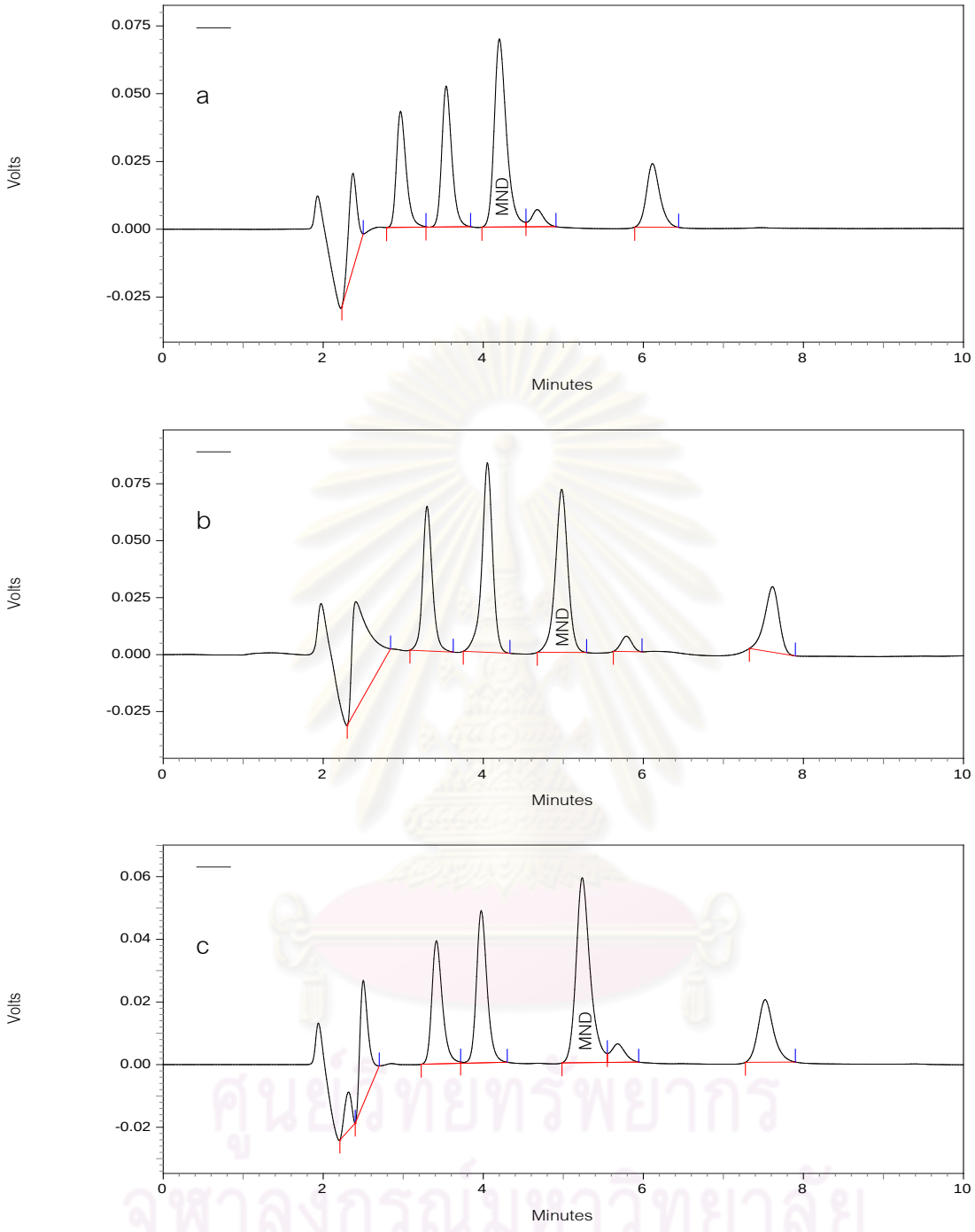

Figure 4.13 Effect of mobile phase composition on alkaline stressed MND standard solution under condition of 25 mM ammonium formate, pH 3.1 : ACN (a=46:54, b=45:55 and c=44:56), detection wavelength of UV 230-nm and column temperature at 30°C

2.4 Effect of column temperature

As expected, the retention time decreased by increasing the temperature of the analytical column due to a decrease in the viscosity of the running buffer. In this study, the satisfactory decrease in retention time was observed in the low temperature range (27 – 30°C) which led to an effect on the composite response (Figure 4.14).

The retention time of MND from three different column temperatures were not different, the main peak of MND from alkaline stressed MND standard solution at the column temperature of 27°C was 5.0 min and the degradant peak detect at retention time of 3.3, 4.1, 5.8 and 7.8 min, respectively as shown in Figure a . Also, in Figure b at column temperature at 30°C MND peak was detected at retention time of 4.9 min and the degradant peaks detected at retention times of 3.2, 3.9, 5.6 and 7.4 min. At column temperature of 33°C (Figure c), the chromatogram showed the same result as Figure b; which MND detected at retention time of 4.9 min and the degradant peaks detected at retention times of 3.3, 3.9, 5.5 and 7.3 min.

In this experiment, the temperature at 30°C was chosen since it provided shorter retention time and better resolution.

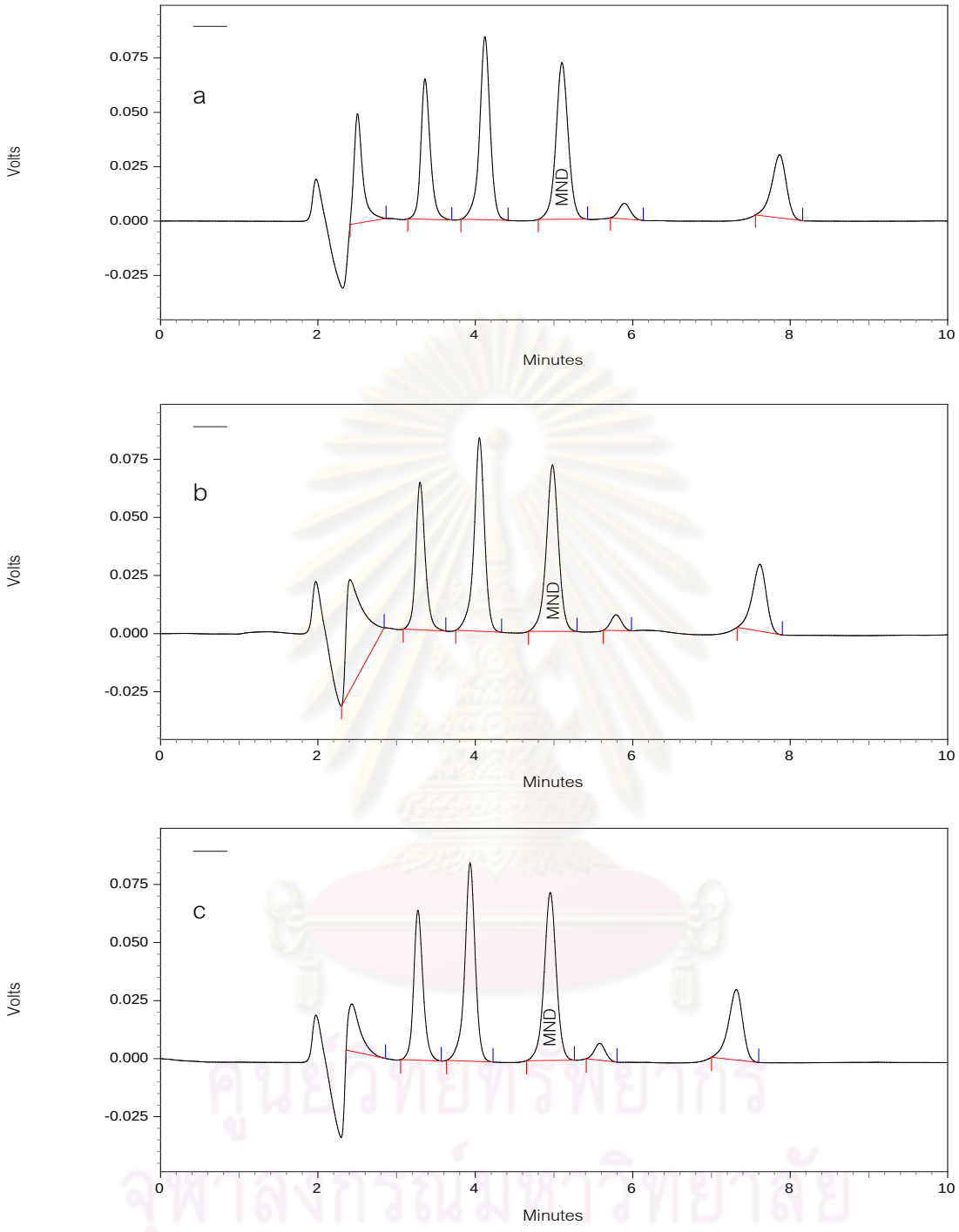

Figure 4.14 Effect of column temperature on alkaline stressed MND standard solution under condition of 25 mM ammonium formate pH 3.1 : ACN (45:55), detection wavelength of UV 230-nm and column temperature at a: 27°C, b: 30°C and c: 33°C

2.5 Effect of detection wavelength

Effect of detection wavelength at 225, 230, and 235-nm were investigated and chromatograms obtained were presented in Figure 4.15.

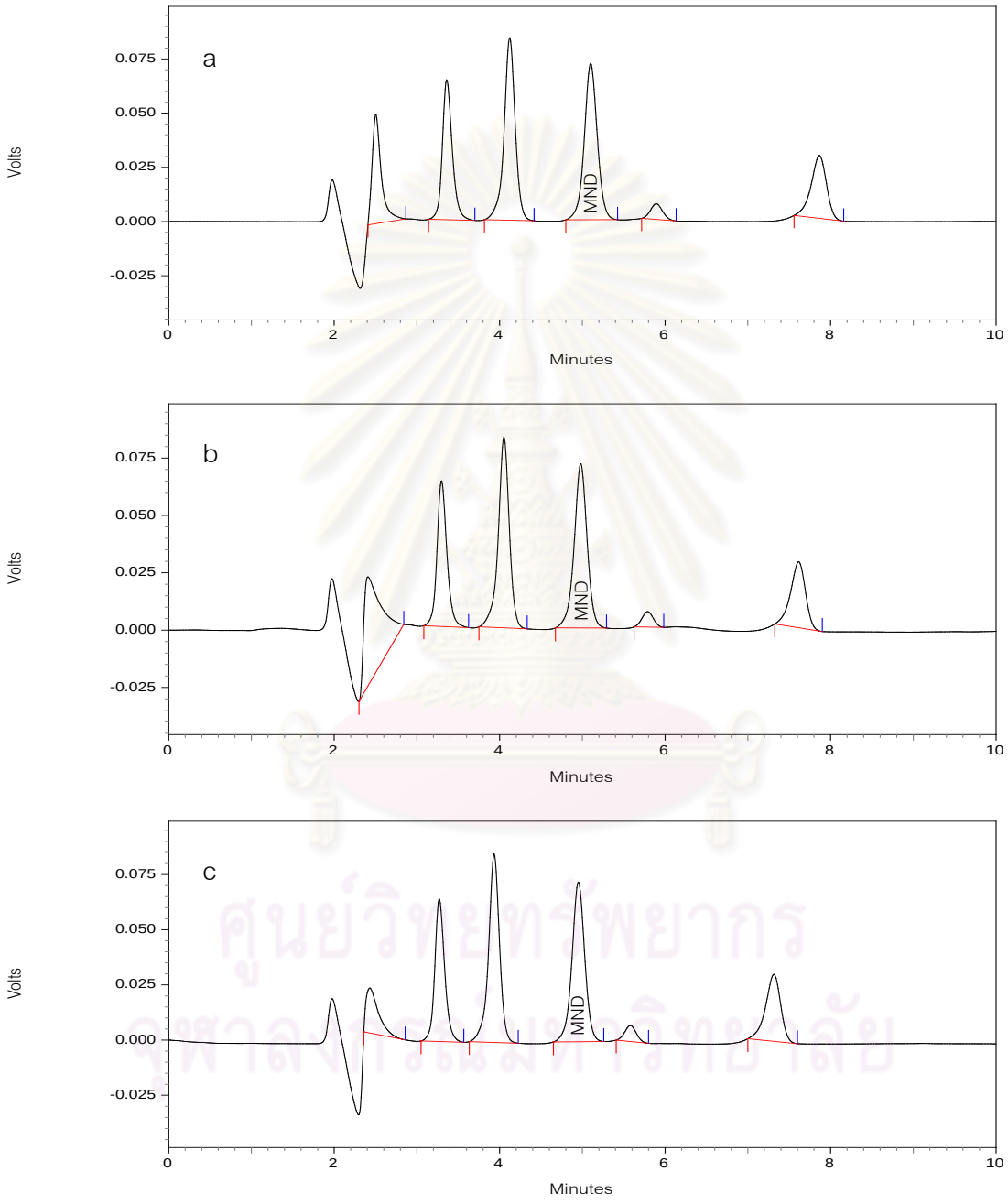

Figure 4.15 Chromatograms of alkaline stress MND hydrochloride standard solution at different detection wavelengths, a: 225-nm, b: 230-nm, and c: 235-nm.

The retention time of MND from three different detection wavelengths were not different, the main peak of MND from alkaline stressed MND standard solution at the detector wavelength of 225-nm was 4.9 min and the degradant peak detect at retention time of 3.3, 4.1, 5.8 and 7.8 min, respectively as shown in Figure a . Also, in Figure b at the detector wavelength of 230-nm MND peak was detected at retention time of 4.9 min and the degradant peaks detected at retention times of 3.3, 3.9, 5.6 and 7.5 min. At the detector wavelength of 235-nm (Figure c), the chromatogram showed the same result as Figure b; which MND detected at retention time of 4.9 min and the degradant peaks detected at retention times of 3.3, 3.9, 5.6 and 7.5 min.

Results obtained from optimization of analytical method parameters indicated that the optimal HPLC condition for analysis of MND hydrochloride was a filtered and degassed mixture of 25 mM ammonium formate buffer, pH 3.1 and acetonitrile (45 : 55) as a mobile phase, a 230-nm detector, and 4.6-mm x 15-cm Symmetry column C18, 3.5 μm which was maintained at a temperature of 30°C. The flow rate was about 0.7 ml/min.

2.6 Effect of hydrolysis degradation on MND standard and tablet sample solution

MND degraded in 1N NaOH at 60°C after 3 hr and four degradation peaks were found in both standard solution and sample solution. In MND standard solution, the main peak of MND was detected at retention time of 4.9 min and four degraded peaks were observed at 3.2, 3.8, 5.4 and 7.1 min, respectively (Figure 4.16). In MND tablet sample solution, after exposure for 3 hr in 1N NaOH at 60°C; the main peak of MND was detected at retention time of 4.9 min and four degraded peaks were at 2.9, 3.2, 3.8 and 7.1 min, respectively (Figure 4.17). The four degradant peaks may be nitrophenylpyridine derivative, nitrzophenylpyridine derivative, 2,6-dimethyl-5-methoxycarbonyl-4-(3-nitrozophenyl)-1,4-dihydropyridine-3-carboxylic acid and 1,4-

dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic acid 2-(1-piperazinyl) ethyl methyl ester, respectively from Mass Spectrophotometry studied.

In 1N HCl at 60°C after 3 hr, only MND peak was observed in the chromatograms of acid stress samples of MND standard solution and MND tablet sample solution, with retention time of 4.3 min. No degradant peak was detected. (Figure 4.18 – 4.19)

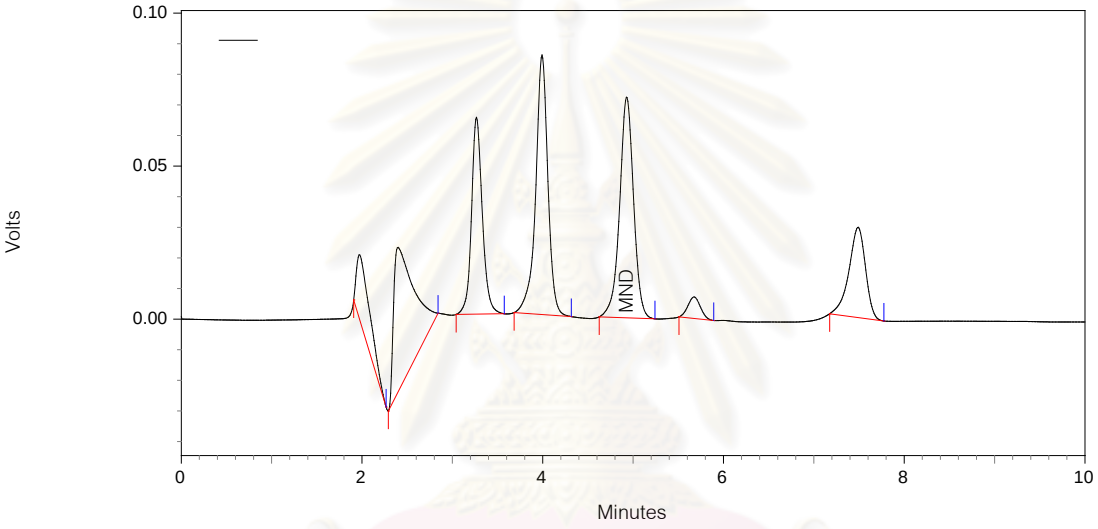

Figure 4.16 Chromatogram of alkaline stress MND standard solution

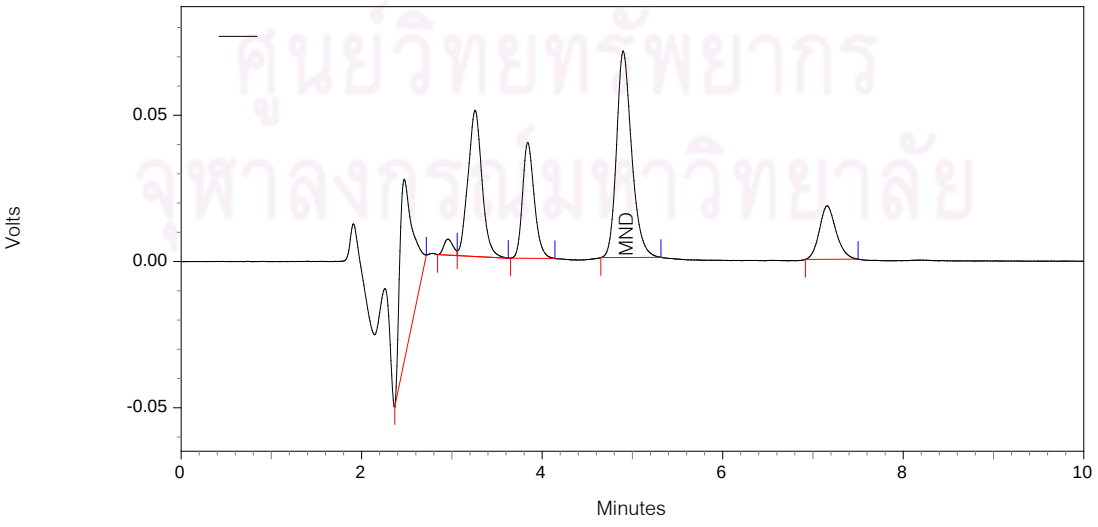

Figure 4.17 Chromatogram of alkaline stress MND tablet sample solution

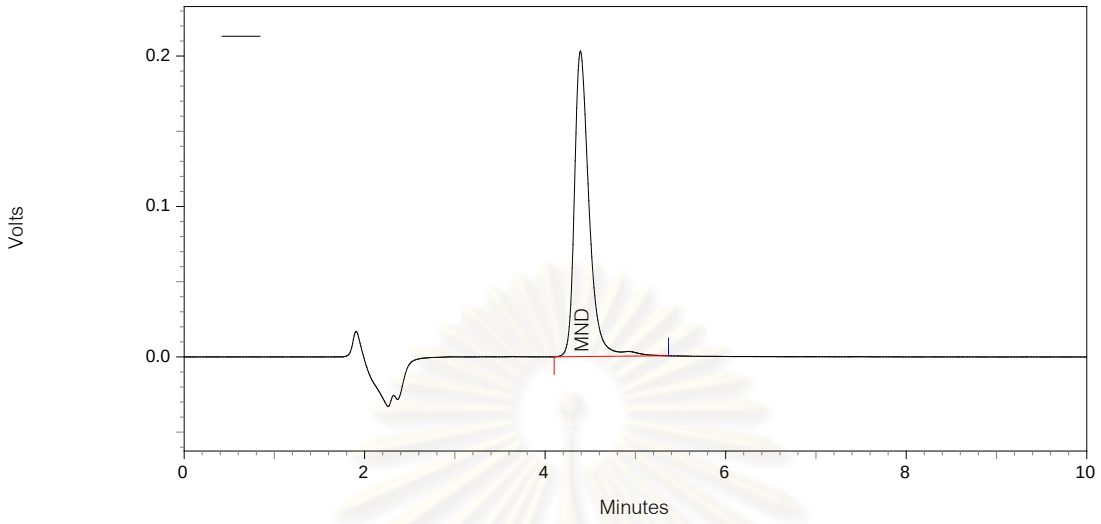

Figure 4.18 Chromatogram of acid stressed MND standard solution

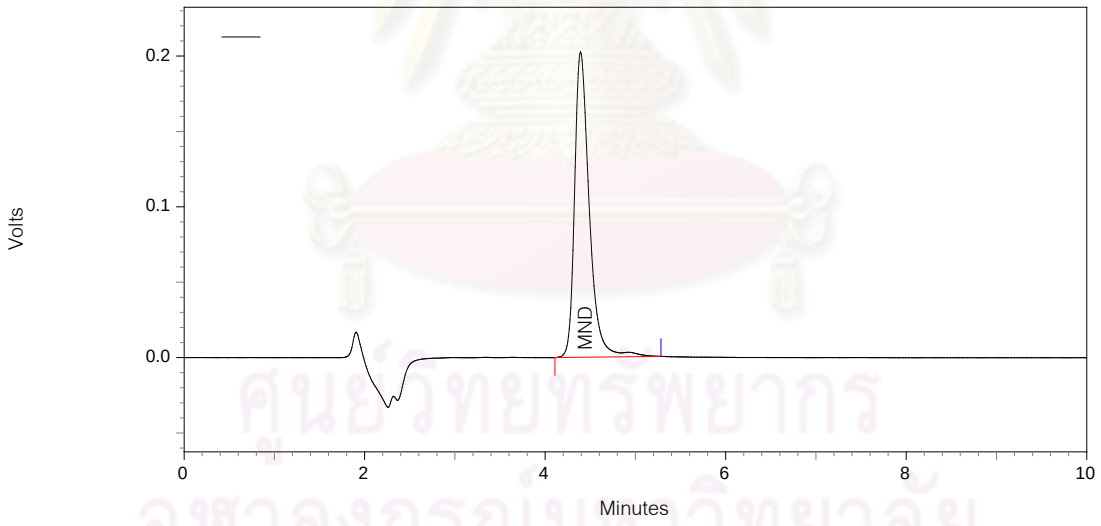

Figure 4.19 Chromatogram of acid stressed MND tablet sample solution

2.7 Effect of oxidation degradation on MND standard and tablet sample solution

In stressed MND standard and MND tablet sample solution, the main peak of MND was detected at retention time of 4.95 min and 4.94 min, respectively. With not well separated of both degraded peaks at 5.40 min and a good separation of both degraded peak at 2.2 min (Figure 4.20 – 4.21).

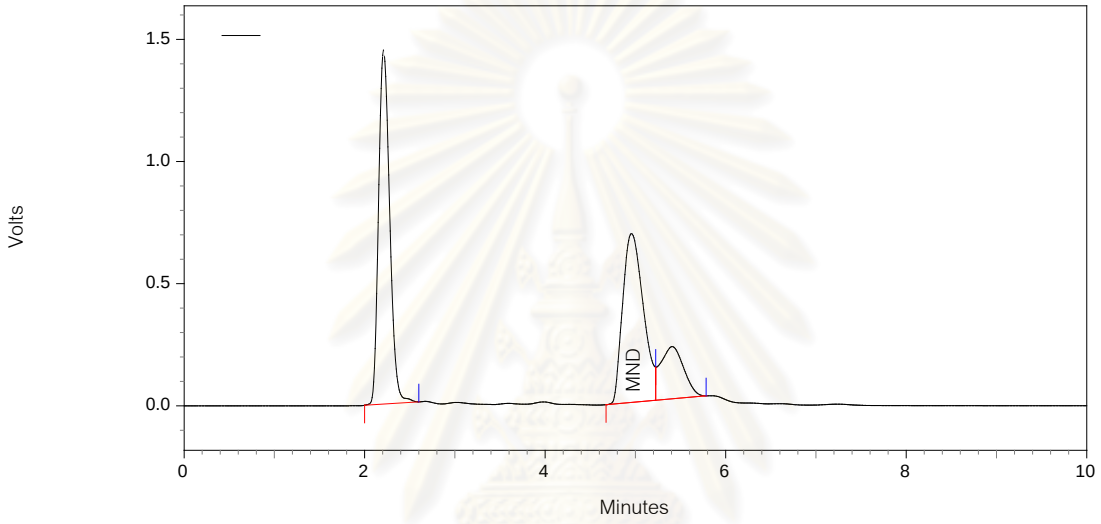

Figure 4.20 Chromatogram of oxidation stressed MND standard solution

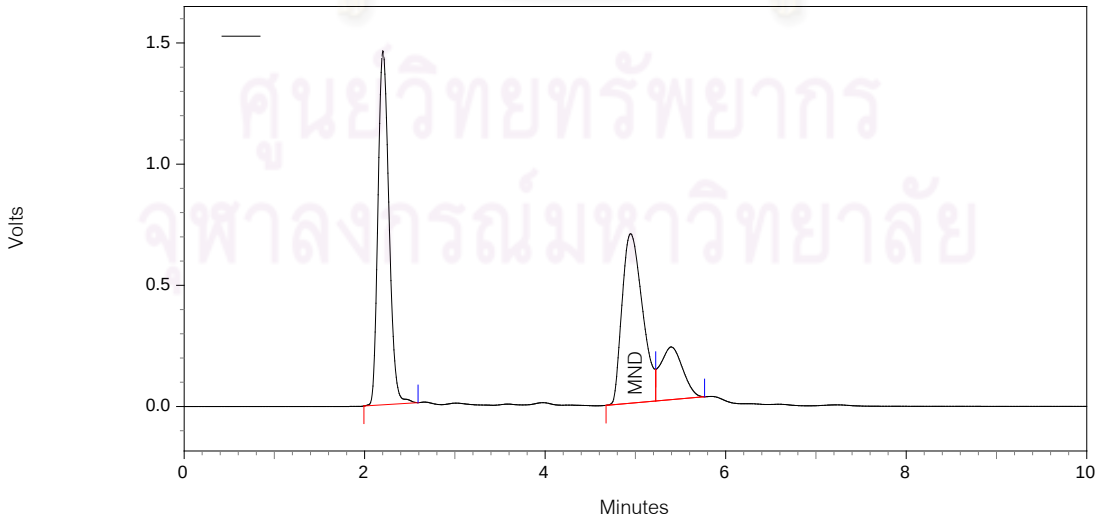

Figure 4.21 Chromatogram of oxidation stressed MND tablet sample solution

2.8 Effect of photolysis degradation on MND standard and tablet sample solution

In stressed MND standard and tablet sample solution, the main peak of MND was detected at retention time of 4.8 min and 4.7 min, respectively. The degradant peaks detect at retention time 5.6 min and 5.5 min, respectively. The degradation peak from the main peak of MND might be nitrophenylpyridine derivative from Mass Spectrophotometry studied (Figure 4.22 – 4.23).

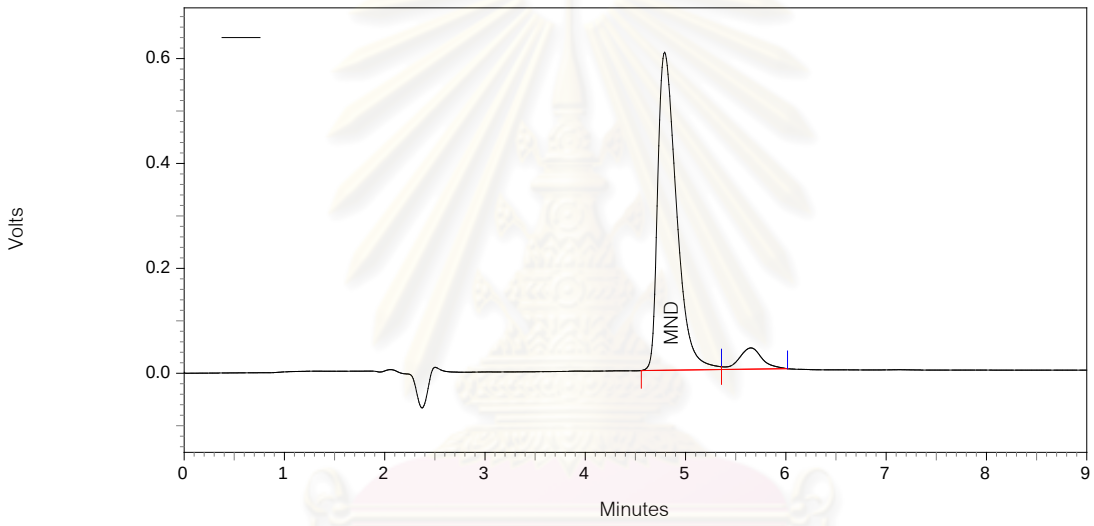

Figure 4.22 Chromatogram of photolysis stressed MND standard solution

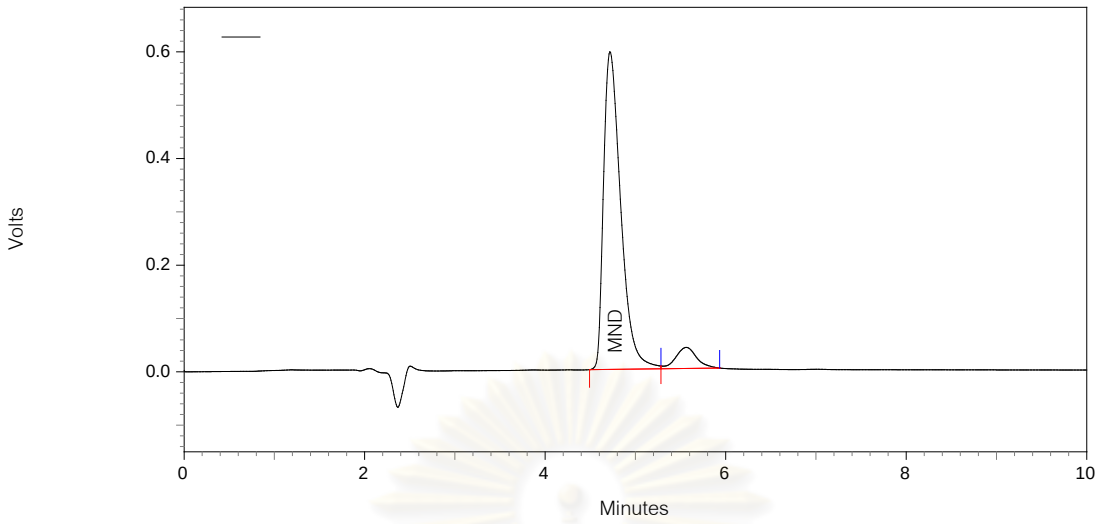

Figure 4.23 Chromatogram of photolysis stressed MND tablet sample solution

2.9 Effect of thermal degradation on standard solution and pharmaceutical preparation

In stressed MND standard and tablet sample solution, the main peaks of MND were detected at retention time of 4.93 min and 4.94 min, respectively. The degradant peaks were detected at retention time of 5.9 min and 5.8 min, respectively. The degradation peaks from the main peak of MND might be nitrophenylpyridine derivatives from Mass Spectrophotometry study (Figure 4.24 – 4.25).

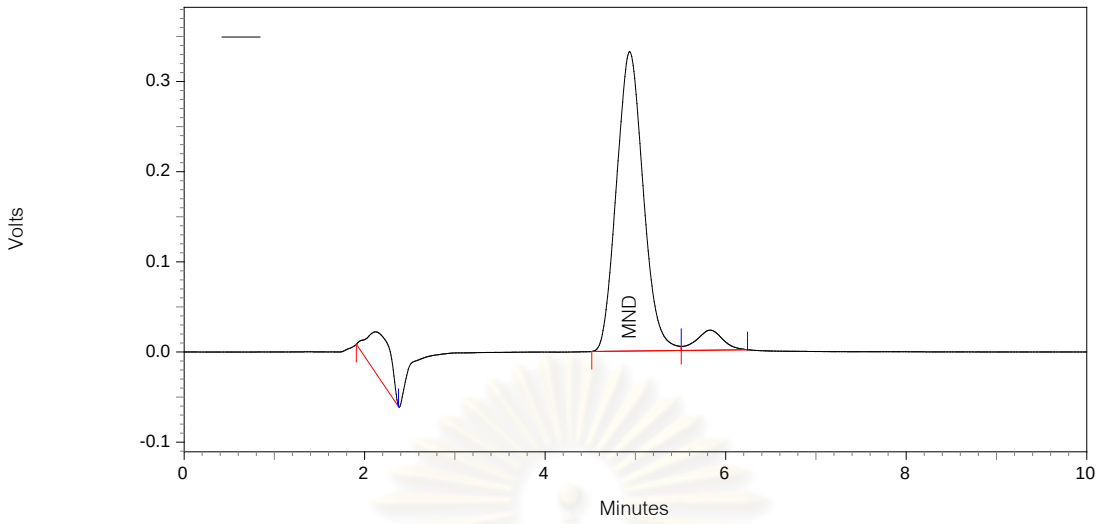

Figure 4.24 Chromatogram of thermal stressed MND standard solution

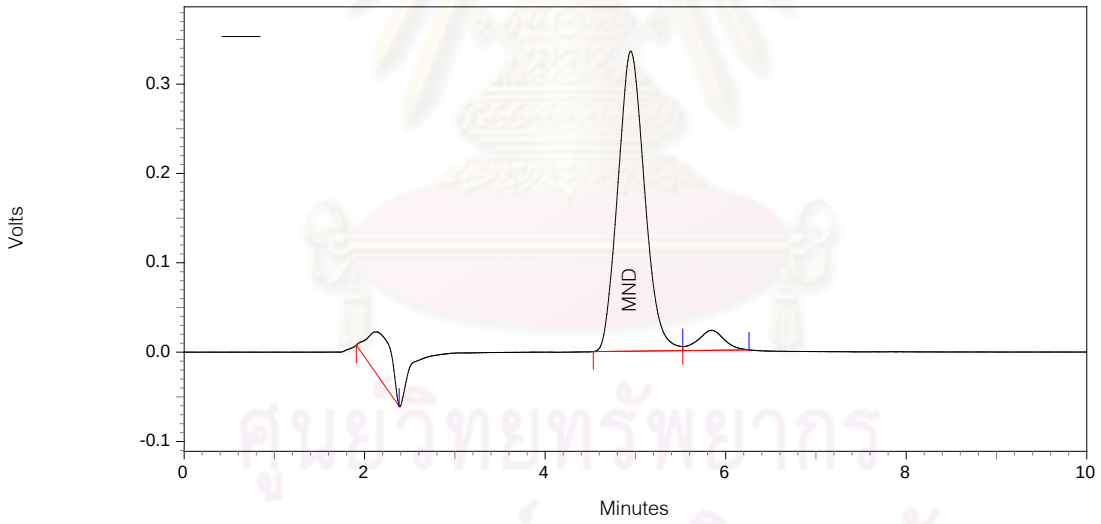

Figure 4.25 Chromatogram of thermal stressed MND tablet sample solution

The Mass spectra of stressed degradation study were recorded in the range 100 – 1000 [M-zH$^+$], using the Electron Spray Ionization (ESI) method. The molecular ions corresponding to the compounds separated by HPLC in base-catalysis (1N NaOH) were found at Rt=4.9 min, [M-zH$^+$] of 611 for MND, Rt=5.6 min, [M-zH$^+$] of 609 for nitrophenylpyridine derivative, Rt=3.9 min, [M-zH$^+$] of 597 for nitrozophenylpyridine derivative, Rt=3.3 min, [M-zH$^+$] of 297 for 2,6-dimethyl-5-methoxycarbonyl-4-(3-nitrozophenyl)-1,4-dihydropyridine-3-carboxylic acid and the Rt=7.4 min, [M-zH$^+$] of 453 for 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic acid 2-(1-piperazinyl)ethyl methyl ester, respectively (Figure 4.26). Stressed with photolysis (UV 254nm), at Rt=4.8 min, [M-zH$^+$] of 611 for MND, Rt=5.6 min, [M-zH$^+$] of 609 for nitrophenylpyridine derivative (Figure 4.27) and stressed with thermal, at Rt=4.9 min, [M-zH$^+$] of 611 for MND, Rt=5.8 min, [M-zH$^+$] of 609 for nitrophenylpyridine derivative (Figure 4.28)

The LC-MS Mass spectra (Figure 4.26-4.28), reveal a seires of peaks corresponding to the charged, protonated apparent molecular ions [M-zH$^+$]. In the range of the highest [M-zH$^+$] values, a peak corresponding to the protonated molecular ion was identified, which permitted a fast and accurate determination of the molecular mass of the compound. Further analysis of mass fragmentation of the compounds formed indicated that the main degradation product is the nitrophenylpyridine derivative formed as a result of aromatization of the dihydropyridine ring.

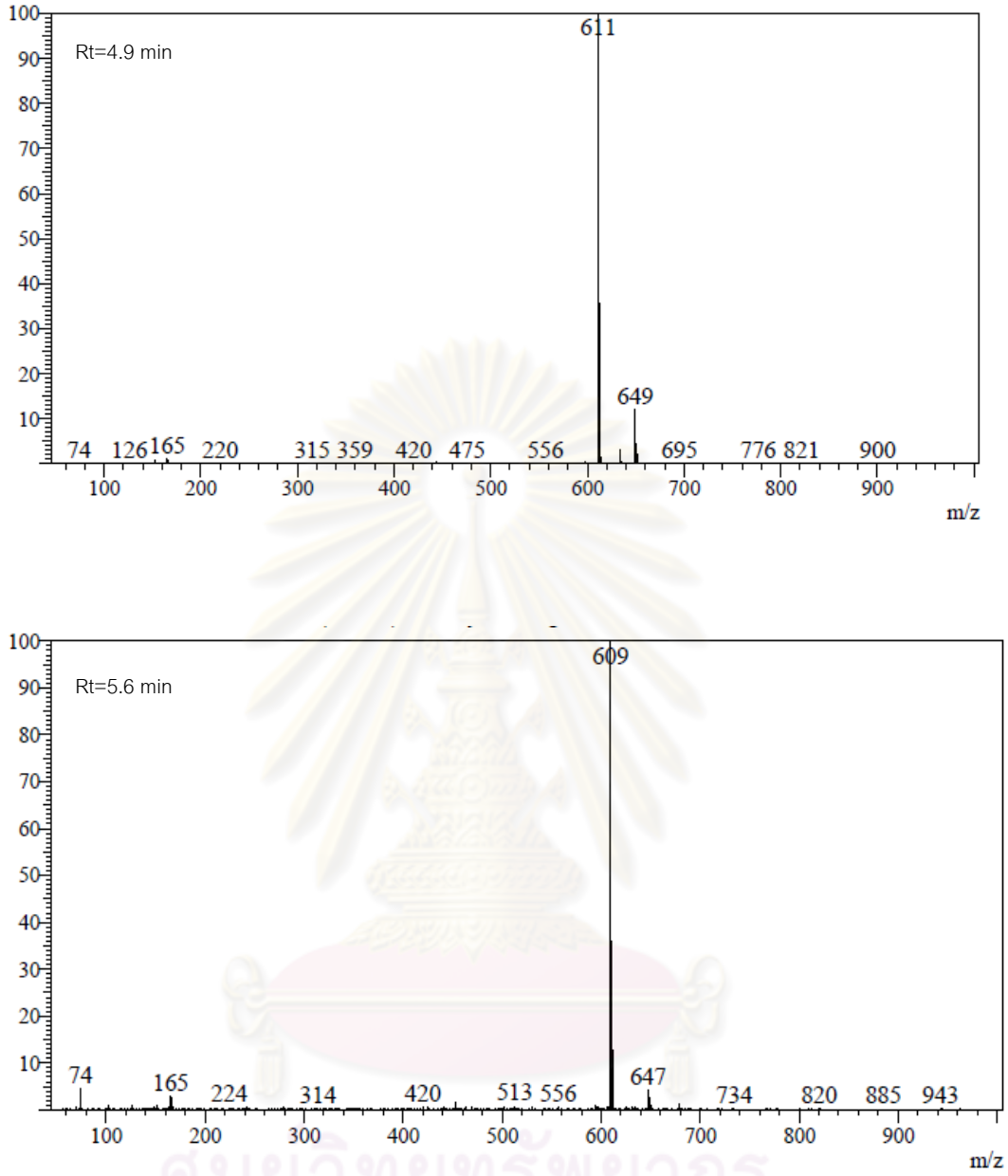

Figure 4.26 Mass spectra (LC-ESI-MS) of alkaline stressed MND standard solution and its degradant

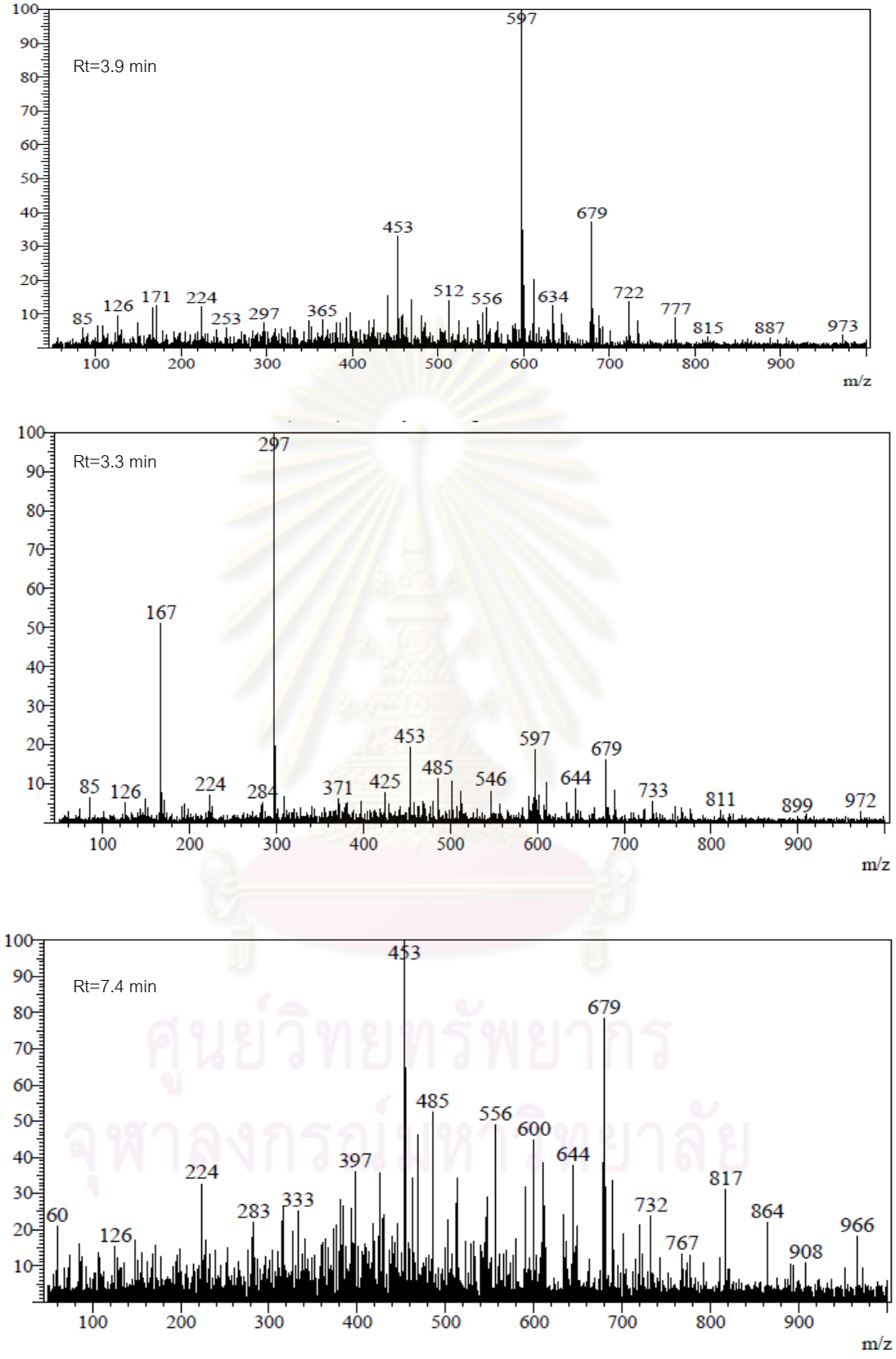

Figure 4.26 Mass spectra (LC-ESI-MS) of alkaline stressed MND standard solution and its degradant (Continued)

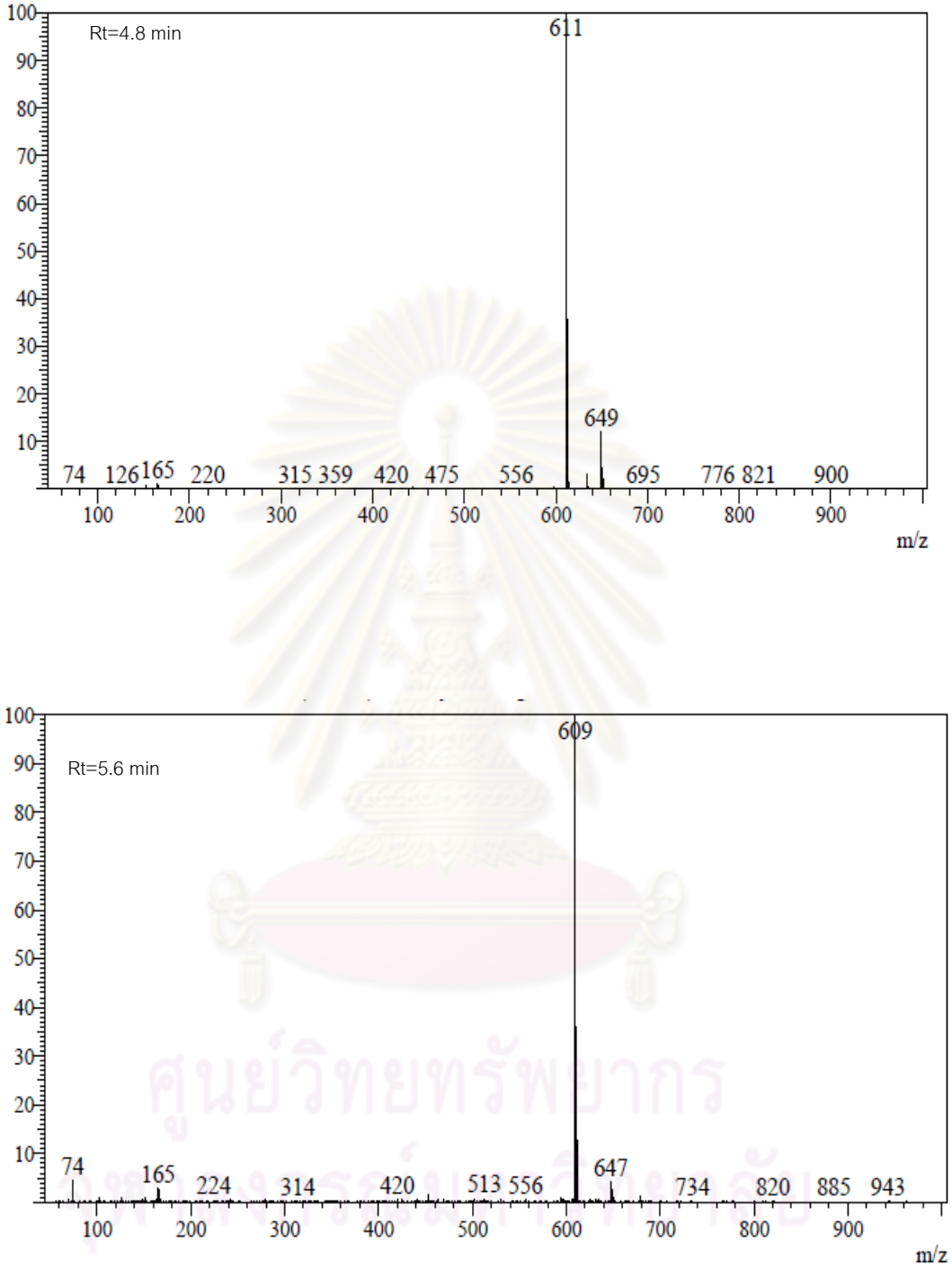

Figure 4.27 Mass spectra (LC-ESI-MS) of photolysis stressed MND standard solution and its degradant

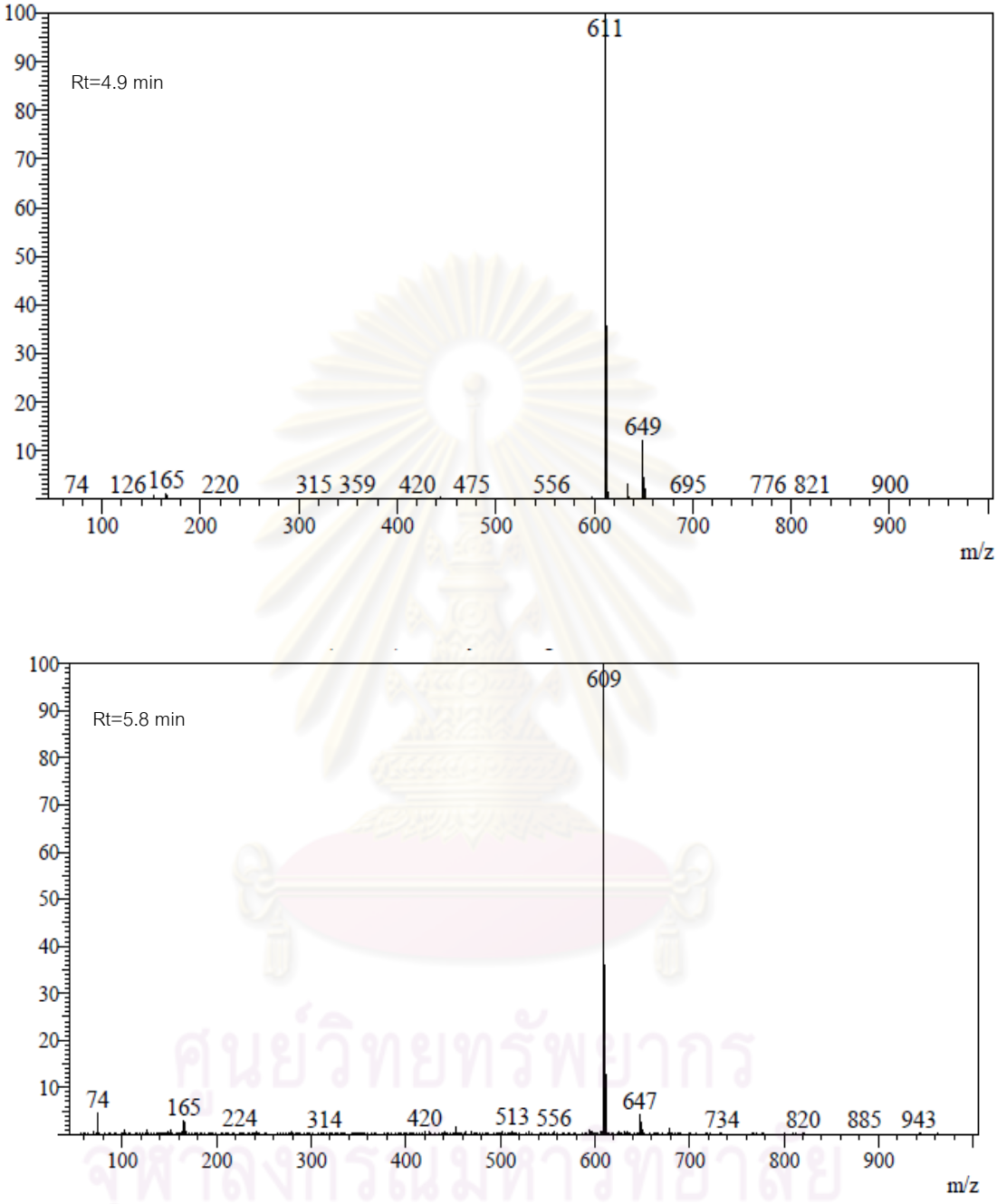

Figure 4.28 Mass spectra (LC-ESI-MS) of thermal stressed MND standard solution and its degradant

3. Analytical Method Validation

Analytical method validation includes all of procedures recommended to demonstrate that a developed method for the quantitative analysis of MND hydrochloride (MND) in drug substance and tablet dosage form is reliable and reproducible. The validation was performed according to a procedure recommended by the International Conference on Harmonization (ICH, 1996). The following measures of method performance were assessed: specificity, linearity and range, precision, accuracy, determination of limit of detection (LOD), and quantification (LOQ), stability of standard solution and system suitability.

3.1 Specificity

Chromatograms of non-stress MND hydrochloride standard solution (100 μg/ml) and non-stress solution of MND hydrochloride sample tablet were compared and presented in Figure 4.29 retention time of MND peak of sample solution was about 4.9 min and corresponded to that of the standard solution. Similar results were obtained when comparing chromatograms of MND hydrochloride standard solutions with those of solutions of MND hydrochloride sample tablet, providing that both standard solution and sample solution were alkaline stress (Figure 4.30), acid stress (Figure 4.31), oxidation stress (Figure 4.32), photolysis stress (Figure 4.33), and thermal stress (Figure 4.34).

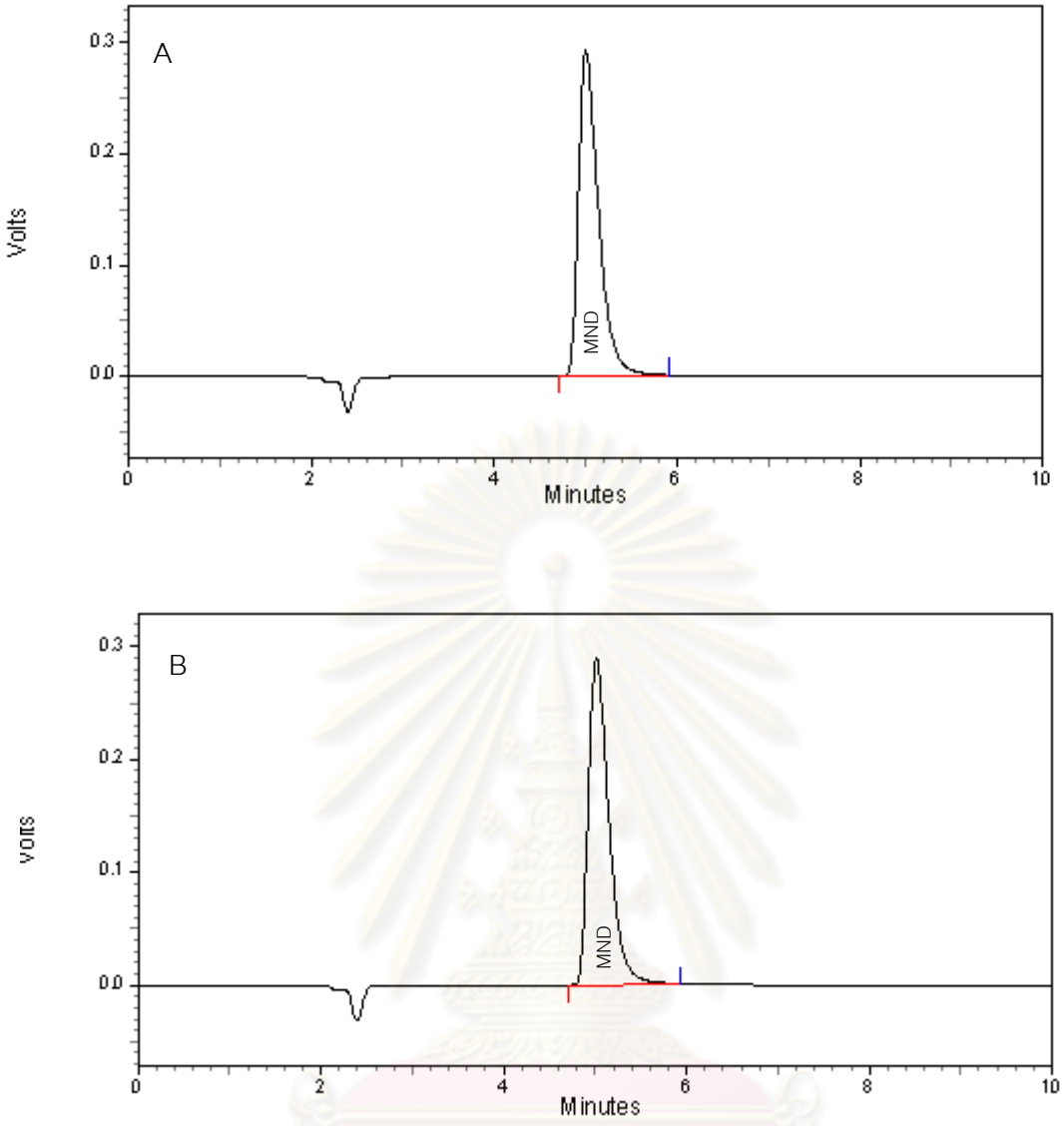

Figure 4.29 Chromatogram of MND hydrochloride standard solution (A) and MND hydrochloride tablet sample solution (B)

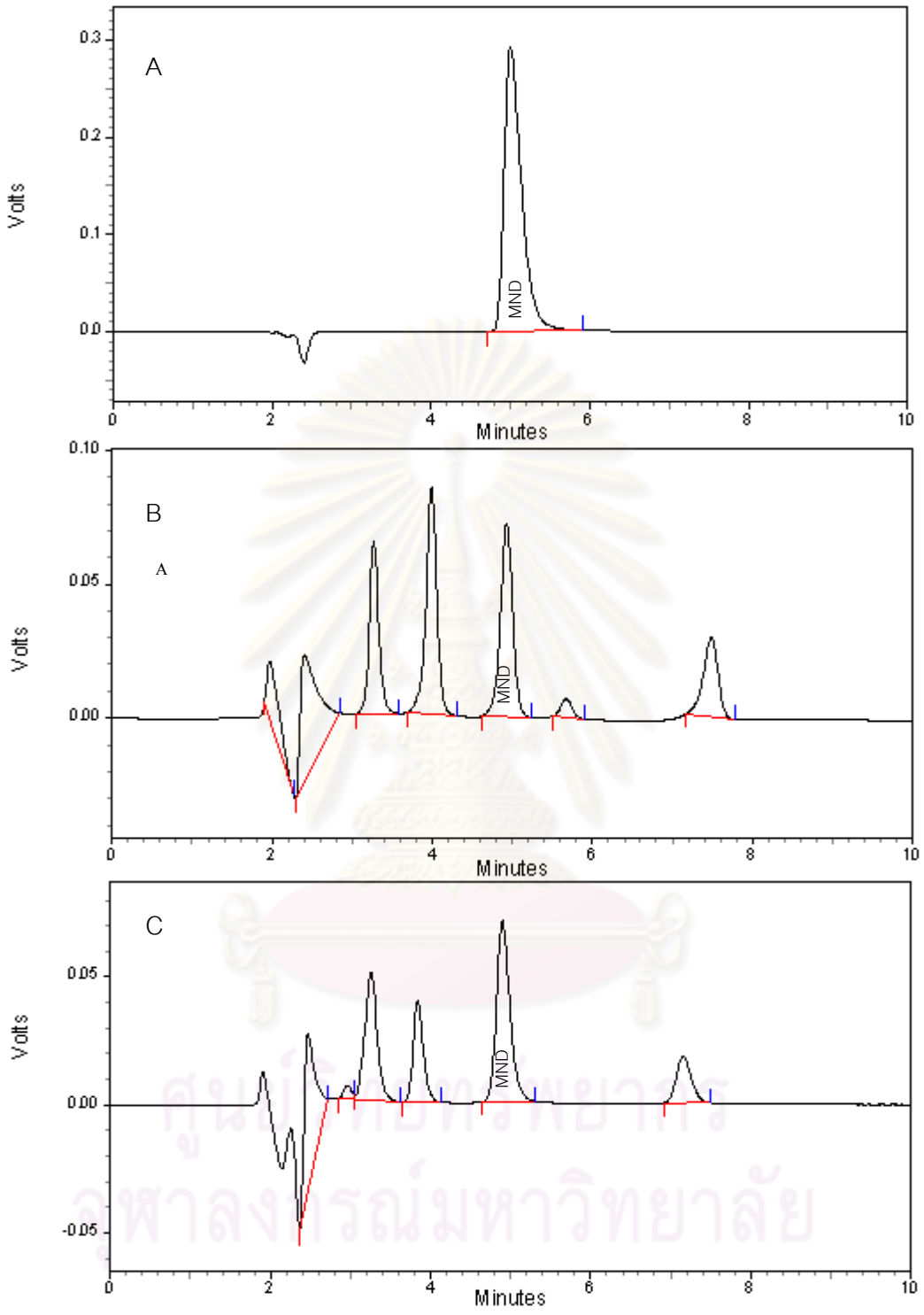

Figure 4.30 Chromatogram of MND hydrochloride standard solution (A), alkaline stress MND hydrochloride standard solution (B) and alkaline stress MND hydrochloride tablet sample solution (C)

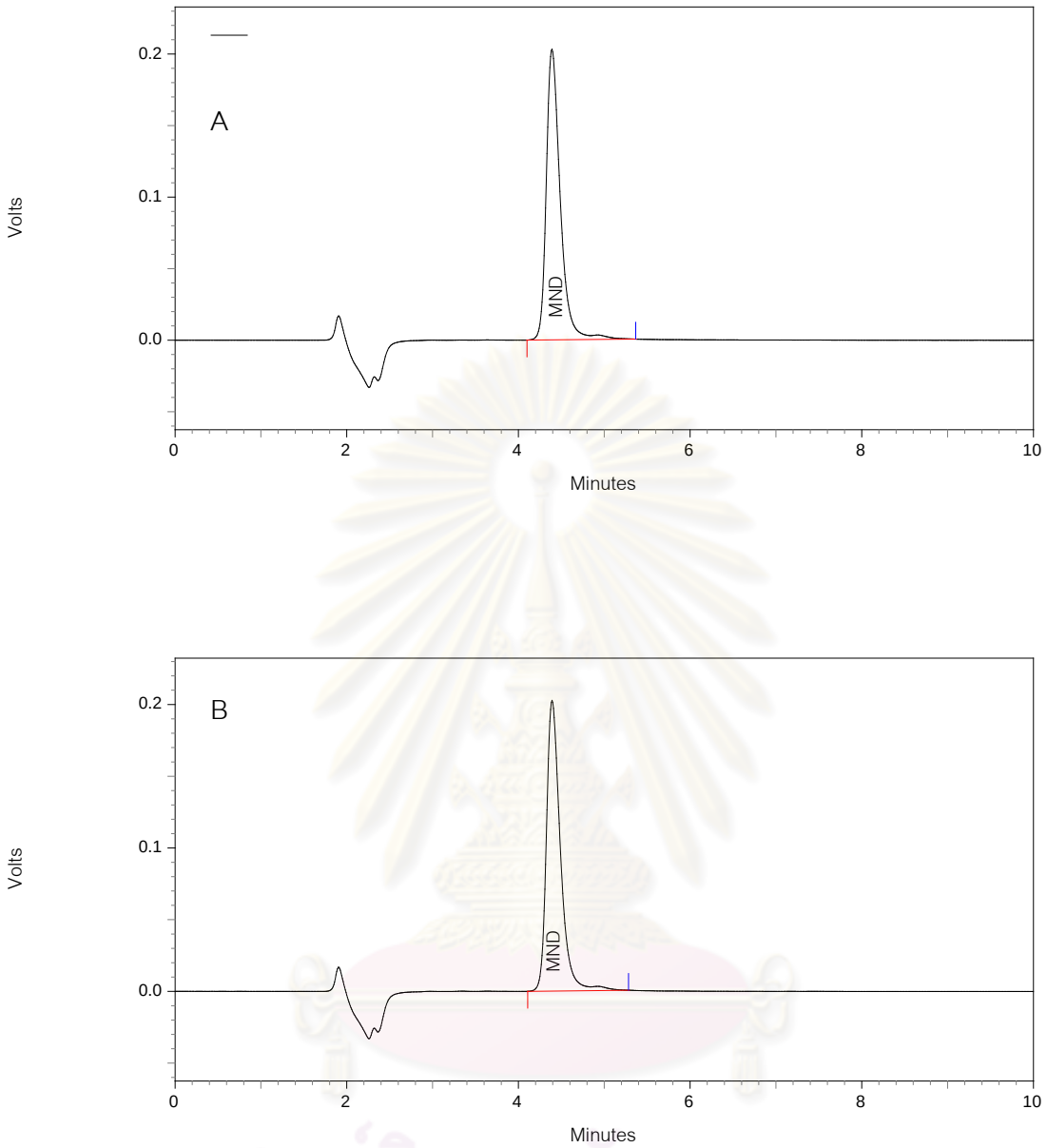

Figure 4.31 Chromatogram of acid stress MND hydrochloride standard solution (A) and acid stress MND hydrochloride tablet sample solution (B)

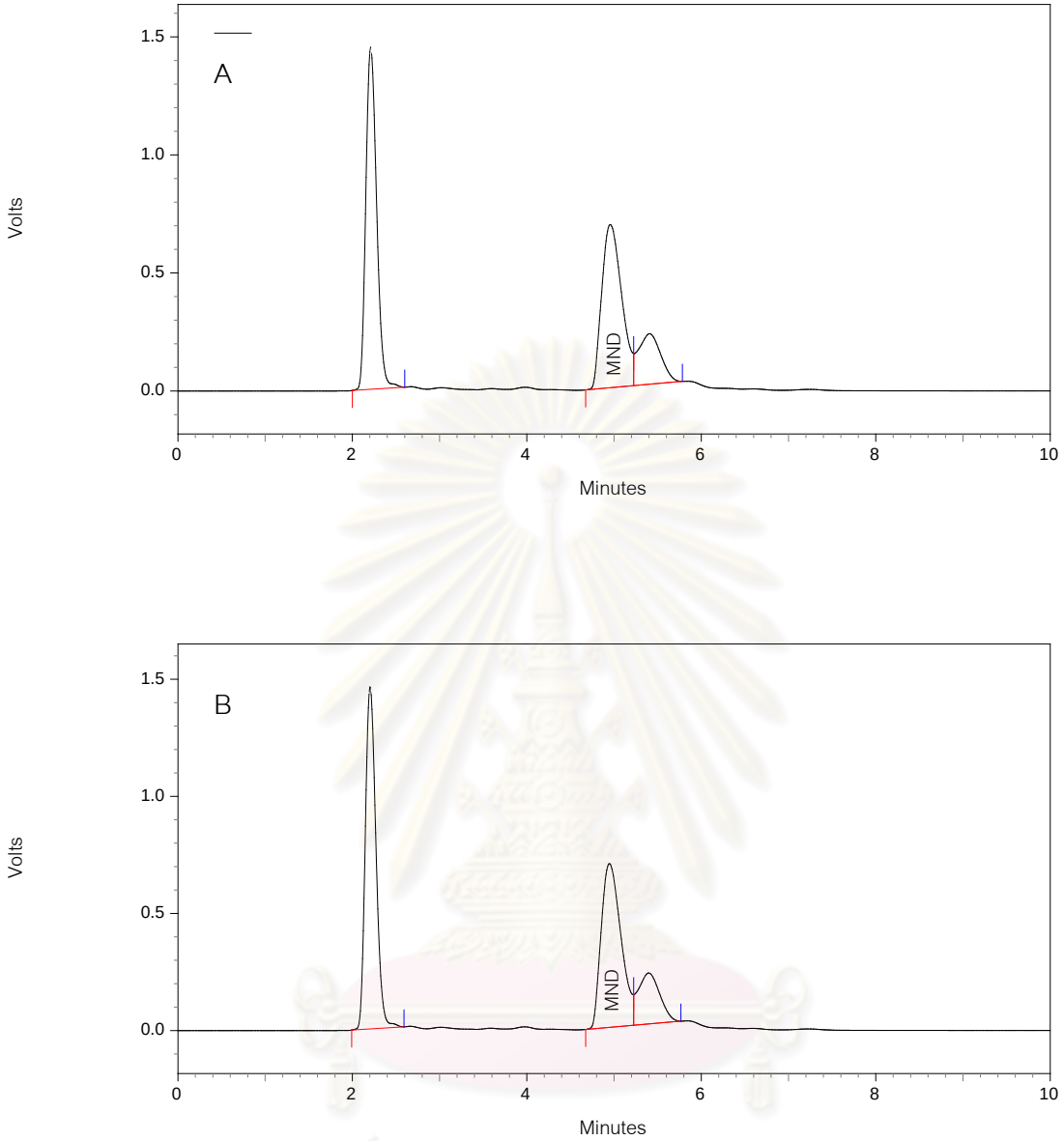

Figure 4.32 Chromatogram of oxidation stress MND hydrochloride standard solution (A) and oxidation stress MND hydrochloride tablet sample solution (B)

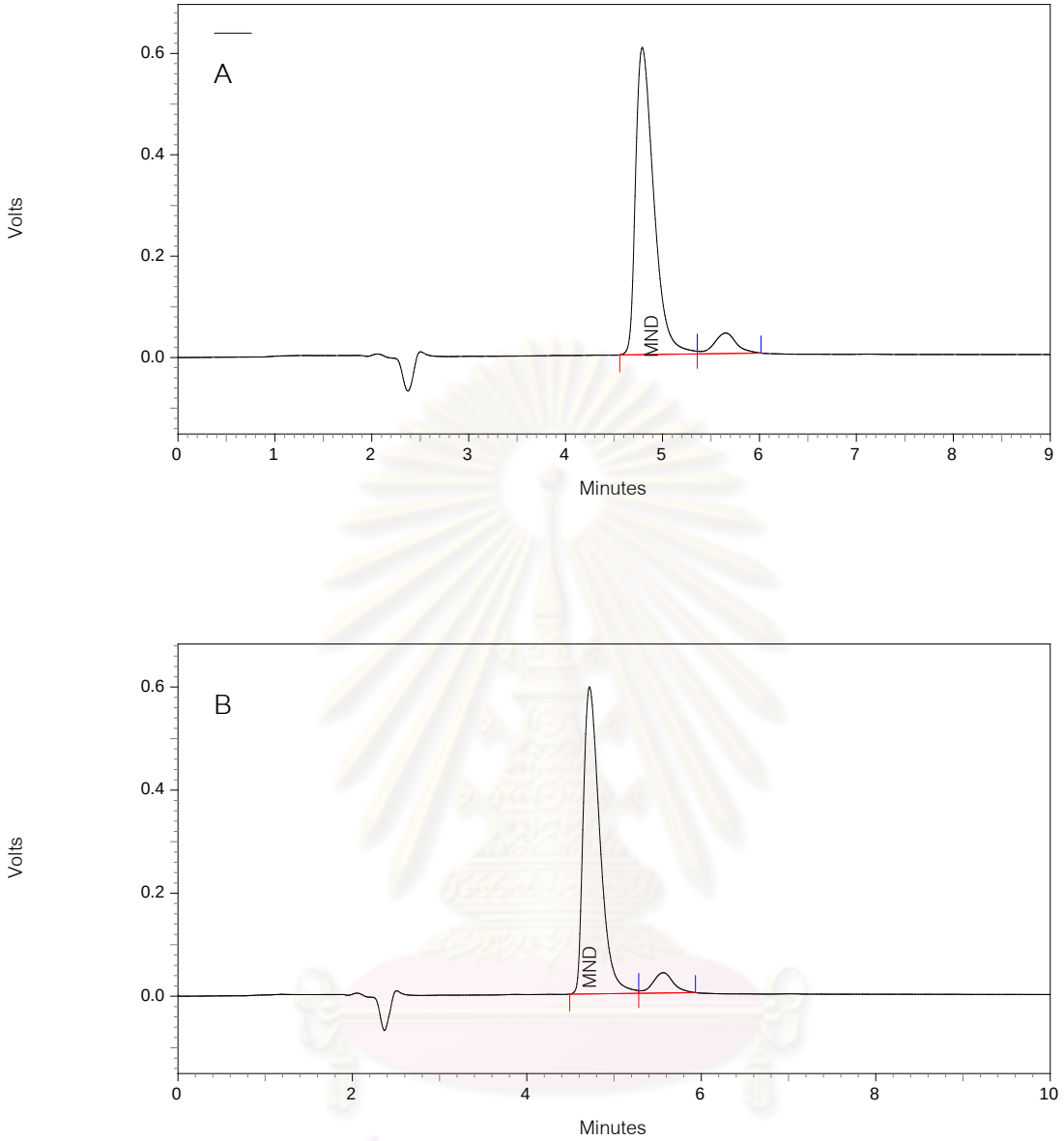

Figure 4.33 Chromatogram of photolysis stress MND hydrochloride standard solution (A) and photolysis stress MND hydrochloride tablet sample solution (B)

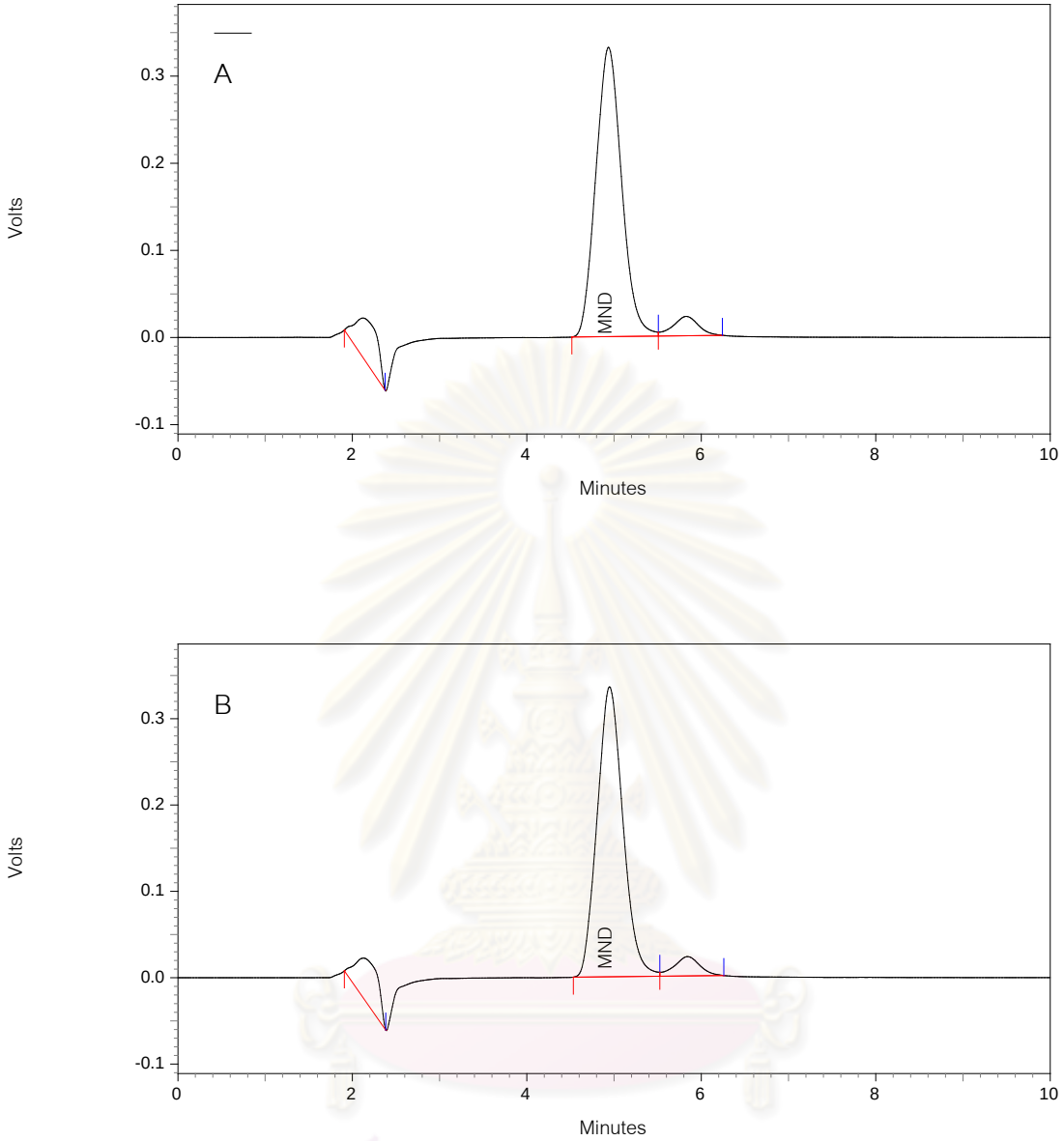

Figure 4.34 Chromatogram of thermal stress MND hydrochloride standard solution (A) and thermal stress MND hydrochloride tablet sample solution (B)

3.2 Linearity and range

The linearity and range of an analytical method is its ability to elicit test results that are directly, or by a well defined mathematical transformation, proportional to concentration of analyte in samples within a given range. Linearity can be expressed as a calibration curve which is a relationship between instrument response and known concentration of the analyte.

MND hydrochloride standard solutions of five concentrations, covering a range of 50–150 µg/ml, were prepared and analyzed. Analytical data of peak area and concentration (Table 4.3) were used to construct the calibration curve of MND hydrochloride (Figure 4.35) by using a least-squares linear regression method.

Table 4.3 Analytical data of linearity and range of MND hydrochloride standard solution

Concentration (µg/ml)	% Nominal concentration	Peak area		
		Injection 1	Injection 2	Injection 3
49.9	50.0	3878053	3898167	3894907
74.8	75.0	5910080	5914908	5939309
99.8	100.0	7766458	7752396	7776750
124.8	125.0	9912597	9843349	9921955
149.8	150.0	11574406	11602488	11674175

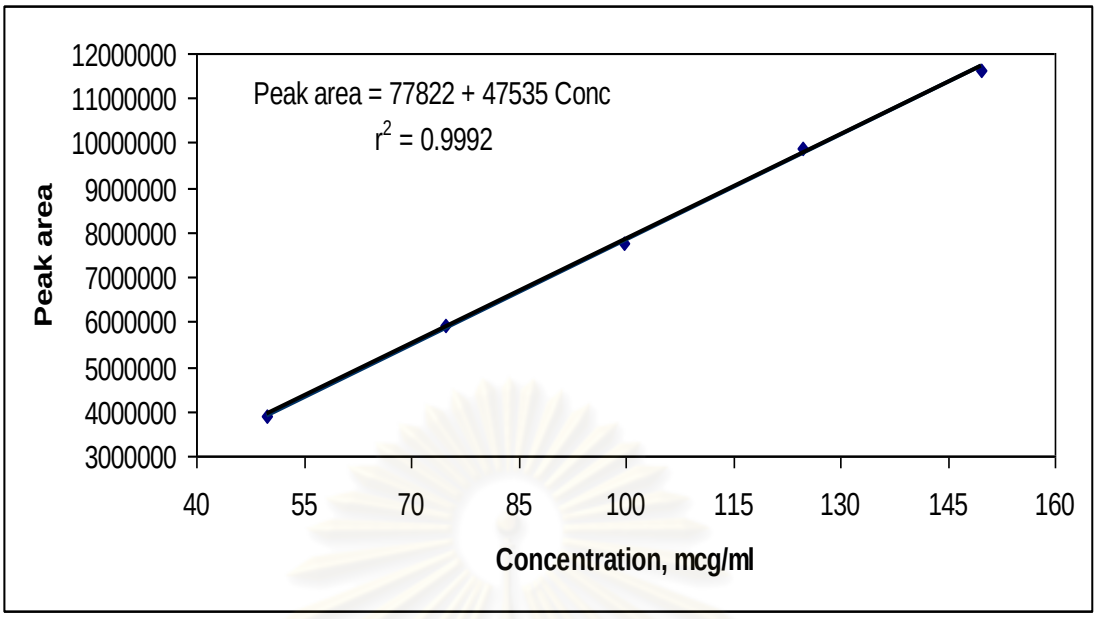

Figure 4.35 Calibration curve of MND hydrochloride standard

3.3 Precision

Precision is subdivided into repeatability (system precision and method precision) and intermediate precision. Demonstration of the system precision by checking the injection volume and retention time repeatability using a standard solution. Excellent system precision is one of the key factors for obtaining trustworthy quantitative results. The precision of an analytical method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple aliquots of a single homogeneous MND hydrochloride powdered tablets sample. The repeatability assesses precision during a single analytical run using the same analyst with the same equipment. The intermediate precision measures precision with time, as on different days, and may involve different analysts, equipments, and reagents. The precision of an analytical method is usually expressed as the relative standard deviation (RSD) or coefficient of variation (CV).

The system precision was determined by six replicate injections of standard solution with a concentration of 100 µg/ml. The method precision was determined by analyzing triplicate injections of six determinations of MND tablet sample solution with a concentration of 100 µg/ml for three days. As shown in Table 4.4 – 4.6, %RSD of system precision and method precision for MND standard and tablet sample solution were less than 2 % for all determinations.

Therefore, the precision of the proposed method was acceptable according to the criteria that the %RSD obtained at each determination should not exceed than 2 % except for the LOQ, where it should not exceed than 10 % [42].

Table 4.4 Analytical data of system precision of MND standard (n = 6)

Injection No.	Peak Area	Retention Time (Min)
1	7681827	4.87
2	7770086	4.88
3	7684620	4.87
4	7785157	4.89
5	7789583	4.89
6	7798959	4.90
Mean	7751615	4.88
SD	54006.712	0.012111
% RSD	0.70	0.25

Table 4.5 Analytical data of intra-day precision of MND tablet sample (n = 3)

Determination	Retention time (Min)	Peak Area
1	5.06	8016799
2	5.08	8120909
3	5.07	8152912
4	5.08	8143781
5	5.08	8136428
6	5.08	8092012
Mean	5.07	8110474
SD	0.008367	56013.69
%RSD	0.16	0.62

Table 4.6 Analytical data of inter-day precision of MND tablet sample (n = 3)

Determination	Day1		Day2		Day3	
	Retention time (Min)	Peak Area	Retention time (Min)	Peak Area	Retention time (Min)	Peak Area
1	5.06	8079281	5.06	8422302	5.08	8419971
2	5.08	8170484	5.07	8465603	5.09	8571455
3	5.07	8098480	5.08	8369006	5.1	8464611
4	5.08	8148090	5.06	8423122	5.09	8497514
5	5.07	8114872	5.08	8390498	5.1	8463564
6	5.08	8083504	5.08	8306599	5.09	8456328
Mean	5.07	8115785	5.07	8366188	5.09	8478907
SD	0.008165	36634.69	0.009832	80774.13	0.006206	51641.6
%RSD	0.16	0.45	0.19	0.97	0.12	0.61

3.4 Accuracy

Due to the lack of placebo accuracy of the proposed method, percent recovery was employed by the standard addition method. Three levels of solutions were made by adding three different amounts of MND hydrochloride standard to solutions of MND hydrochloride Tablets. The three added amounts corresponded to 60, 100 and 120% of the nominal analytical concentration. Each level was made in triplicate. Analytical data of accuracy of MND hydrochloride was shown in Table 4.7 and the plot of peak area vs concentration was presented in Figure 4.36.

The %LA of MND tablet sample (MND) before spiked MND standard were 98.52 - 99.53 % and the recoveries of MND tablet from spiked MND standard were with the range of 98.96 – 100.05 % (Table 4.7). The mean recovery of MND in Madiplot ® Tablet was 99.42 %. The accuracy test of this method was in the acceptance criteria (98-102 %R) so it could be used to determine the MND sample.

Table 4.7 Recoveries of MND from 10 mg Manidipine Hydrochloride Tablets

Compound	Expected concentration (%)	%LA before spike MND standard	Amount added (mg)	Amount found (mg)	Recovery (%)
MND	60	99.53± 0.51	69.98	69.99	100.05 ± 0.66
	100	98.52± 0.78	109.98	109.05	99.25 ± 0.21
	120	98.95± 0.43	129.98	127.94	98.96 ± 0.75

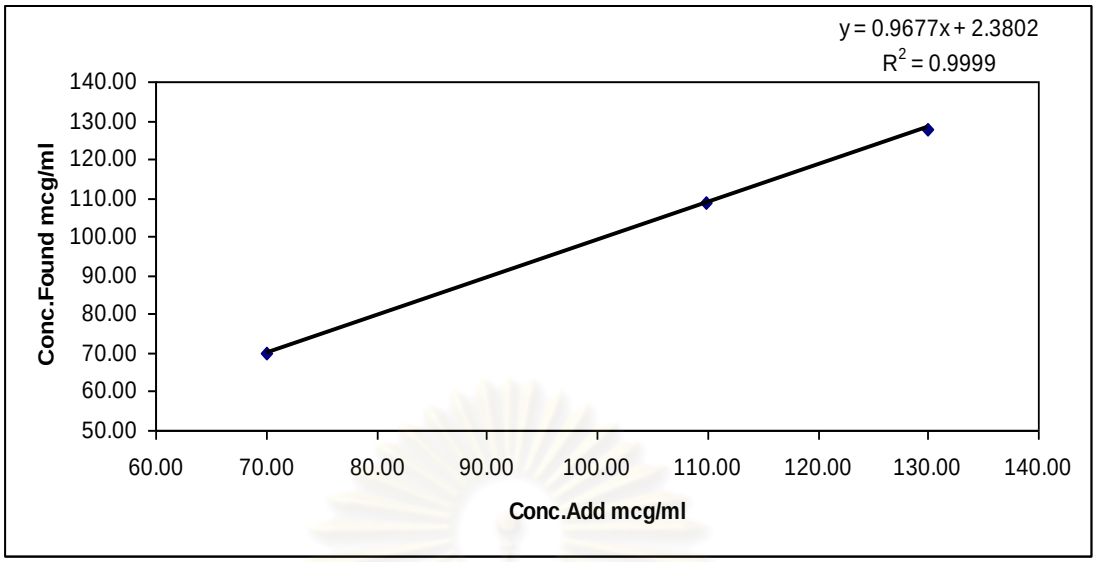

Figure 4.36 Calibration curve of recoveries of MND from Madiplot® Tablet

As presented in Figure 4.36, the R^2 of recovery of MND in Maiplot tablet and MND tablet (Madiplot) before spike with MND standard were observed that no different for all the analyte. When comparing the %LA of MND tablet sample (Madiplot) from the optimum developed method to Japanese Pharmacopoeia (JP) XV Supplement I were closely the same result which in the limit range (98 – 102 %) as shown in Table 4.8.

Table 4.8 Analysis of MND in Madiplot® tablet by the developed method compare to JP XV Supplement I

Sample No.	% LA [a] from developed method	% LA [a] from JP XV Supplement I
1	99.29 ± 0.17	99.89 ± 0.45
2	99.47 ± 0.11	100.37 ± 0.31
3	99.43 ± 0.26	99.93 ± 0.56

[a] mean ± SD of triplicate analyses

3.5 Limit of detection (LOD) and limit of qualification (LOQ)

Limit of detection (LOD) is the lowest concentration of an analyte in a sample that can be detected, but not necessary quantitated under the stated conditions of the test.

Limit of quantification (LOQ) is the lowest concentration of an analyte that can be determined with acceptable precision and accuracy under the stated conditions of the test. For this study, five levels of MND hydrochloride standard solutions, covering a concentration range of 3 - 5 µg/ml, were prepared and analyzed. Each standard solution was triplicate injected. Thereafter a calibration curve of MND hydrochloride was created by using a least-squares linear regression method. Least square linear regression analysis was performed to determine slope (S), intercept and the standard deviation of the y-intercept were shown in Table 4.9.

Table 4.9 Calibration data for calculating LOD and LOQ of MND standard

Standard No.	Conc. (µg/ml)	Peak Area # 1	Peak Area # 2	Peak Area # 3	Mean
1	3.01	207736	208172	207787	207898
2	3.49	258211	259121	259407	258913
3	4.01	313939	314694	315904	314846
4	4.52	353846	354836	353035	353906
5	5.01	402631	405634	406374	404880
Intercept	-81422				
Intercept, SD (σ) Reg. line SD	6133 4150.93				
Slope, S	97183				
LOD	0.141				
LOQ	0.427				
R^2	0.9966				

The LOD and LOQ were determined as 3.3 σ/S and 10 σ/S, respectively. The LOD of 0.141 µg/ml and LOQ of 0.427 µg/ml (%RSD < 10) were found in this study.

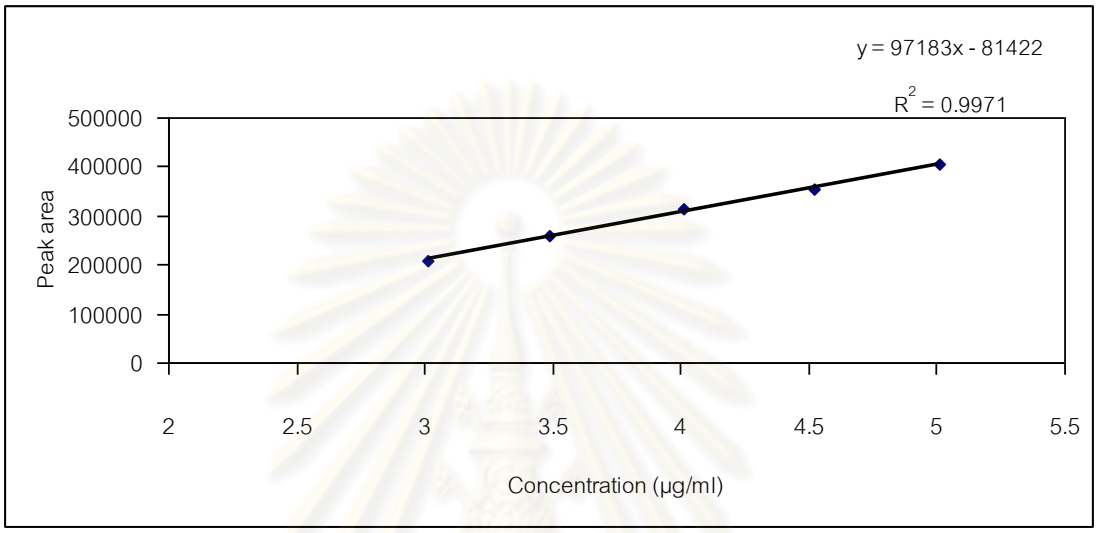

Figure 4.37 Calibration curve of MND standard on determined LOD and LOQ

3.6 Stability of MND standard solution

The stability of MND standard and tablet solution was determined at concentration of 100 µg/ml from stock standard and sample solution of MND. The concentrations (n = 3) of MND obtained from freshly prepared solutions were compared with those obtained from solutions that had been stored in the bench top for 1, 2, 3 and 4 hrs. As shown in Table 4.10 - 4.11, the concentration of MND in a standard solution was found to be in the range of 98.91 – 101.1 % of those at the freshly prepared. Therefore, standard solution of MND could be assumed stable after being in the bench top for at least 4 hrs.

Table 4.10 Stability of MND standard solution stored in the bench top.

Hour	Concentration (µg/ml)	Concentration found (µg/ml)[a]
0	99.7	99.4 %
1	100.6	100.3 %
2	99.87	100.04 %
3	99.8	98.91 %
4	100.2	101.1 %

[a] Value in the concentration found represented the percentage of the analyte at the specified time comparing to that of freshly prepared solution.

Table 4.11 Stability of MND tablet sample solution stored in the bench top.

Hour	Determination 1		Determination 2		Determination 3	
	Concentration (µg/ml)	Concentration found (µg/ml)[a]	Concentration (µg/ml)	Concentration found (µg/ml)[a]	Concentration (µg/ml)	Concentration found (µg/ml)[a]
0	99.8	99.7 %	99.8	99.8 %	99.8	100.01 %
1	100.1	100.1 %	100.1	100.01 %	100.1	100.04 %
2	99.8	100.08 %	99.8	100.2%	99.8	100.12 %
3	99.9	98.9 %	99.9	99.65 %	99.9	99.19 %
4	100.2	101.1 %	100.2	101.06 %	100.2	99.6 %

[a] Value in the concentration found represented the percentage of the analyte at the specified time comparing to that of freshly prepared solution.

3.7 System suitability

System suitability testing was evaluated by six replicate injections of a base catalysis of standard solution of MND hydrochloride at concentration of 10 µg/ml. Four parameters such as precision (%RSD), number of theoretical plate (N), tailing

factor (T) and resolution (R) were determined the system suitability [43]. Table 4.12 represented data from the system suitability test.

Percentage of relative standard deviation (%RSD) from the precision determination of six replications of standard solution was 0.29. This precision of system was passed the system suitability test because %RSD of an interested peak was less than 2.

The average of number of theoretical plates (N) of MND peak was 4153. Because of the number of theoretical plate was more than 2000, this system was passed systems suitability test.

Tailing factor (T) of an analyte peak was 1.09. Resolution (R) for MND peak was 6.89. These systems were passed the system suitability test because the tailing factor of the interested peak was less than 2 and resolution was more than 2, respectively.

Table 4.12 System suitability data of MND standard (n = 6)

Number of Injection	Parameters			
	Precision	N	T	R
1	801262	4158	1.08	6.98
2	803667	4137	1.09	6.97
3	802446	4145	1.09	6.98
4	805607	4155	1.09	7.00
5	806623	4158	1.10	6.98
6	807147	4163	1.10	7.00
Mean	804459	4153	1.09	6.89
SD	2371.77	9.73	0.0075	0.16
% RSD	0.29	0.23	0.69	0.59

4. Assay application

With the optimum HPLC condition, three commercial brands of Madiplot® tablet dosage forms were analyzed in triplicate injections. The results of analyses of manidipine hydrochloride in Madiplot® tablet was shown in Table 4.13.

The manufacturer's specification limit of manidipine hydrochloride was 90.0 - 110.0 % of the labeled amount. The percentage of labeled amount of manidipine hydrochloride was 99.39%; therefore, the results showed that all Madiplot® tablet studied met the requirements of the manufacturers.

Table 4.13 Analysis of MND in Madiplot® tablet

Sample No.	% Labeled amount [a]
1	99.29 ± 0.17
2	99.47 ± 0.11
3	99.43 ± 0.26

[a] mean ± SD of triplicate analyses

CHAPTER V

CONCLUSION

An analytical method which is rapid, sensitive and reproducible to determine manidipine hydrochloride (MND) at 100 µg/ml has been developed. The method was optimized the influences of buffer pH and concentration, composition of mobile phase, detection wavelength and column temperature were investigated. The optimum method was achieved on an analytical column RP-18, (4.6 x 150 mm, 3.5 µm i.d) using 25 mM of ammonium formate buffer at pH 3.1, applied to a mobile phase consist of acetonitrile at ratio percent of 45 : 55, detection wavelength of UV 230 nm and column temperature at 30°C.

The optimum method was validated according to the ICH guidelines to determine the suitability of the method, the linear ranges of 50 - 150 µg/ml was found for Manidipine hydrochloride. The system and method precision calculated from the relative standard deviation was less than 2%. The percentage recovery on accuracy was 99.42. The limit of detection and quantification were 0.141 and 0.427 µg/ml, respectively. The percentage of relative standard deviation (%RSD) was 0.29, the number of theoretical plates (N) was 4153, the tailing factor (T) of an analyte peak was 1.09 and the resolution (R) for MND peak was 6.89 of the system suitability test. With results confirming that the method is highly suitable for its intended purpose, The percentage of labeled amount of manidipine hydrochloride was 99.39 %

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