STABILITY INDICATING HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF CANDESARTAN CILEXETIL AND HYDROCHLOROTHIAZIDE IN PHARMACEUTICALS

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ABSTRACT

A simple and specific HPLC method was developed for simultaneous determination of candesartan cilexetil and hydrochlorothiazide in pharmaceuticals. Method development was performed by using a Zobax SB CN column (4.6×150 mm, 5µm). The optimum conditions were in a mixture of acetonitrile and 10 mM phosphate buffer (pH 7.0, 32:68, v/v) with a flow rate of 1 mL/min, the column temperature at 25^{o}C, and the UV detection at 254 nm. Hydrochlorothiazide and candesartan cilexetil were eluted at 2.65 and 8.21 min, respectively. The method was validated for linearity and range, accuracy, precision, and specificity according to ICH guidelines. Linearity was obtained in the range of 50 to 150% of target concentrations. Percent recovery was in the range of 99.1% to 101.0% for candesartan cilexetil and 99.4% to 100.2% for hydrochlorothiazide. The %RSD of intra- and inter-day precision for both drugs was less than 1.66% and 1.84%, respectively.

A stability study of candesartan cilexetil and hydrochlorothiazide was conducted using the developed and validated HPLC method. The method proved to be stability indicating as it can analyze the drugs in the presence of their degradation products with a resolution of the critical peak pair of 1.5 and peak purity values of more than 990. The drugs were stressed under acid/base/neutral hydrolysis, oxidation, photolysis, and thermal degradation. Candesartan cilexetil was stable under neutral (water) hydrolysis, photolysis, and thermal degradation, but it degraded under acid (0.1 N HCl) and base (0.1 N NaOH) hydrolysis, and oxidation (30% H_2O_2). Similarly, hydrochlorothiazide was stable under oxidation (30% H_2O_2), photolysis, and thermal degradation, but it degraded under neutral, acid (1 N HCl), and base (1 N NaOH) hydrolysis. The method was successfully applied to analyze commercial drug products with %RSD of five replication of less than 0.80%.

KEY WORDS: HPLC/ SIMULTANEOUS ANALYSIS/ CANDESARTAN CILEXETIL/ HYDROCHLOROTHIAZIDE

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LIST OF ABBREVIATIONS

%B	organic solvent concentration
%RSD	percent relative standard deviation
µg	microgram
µL	microlitre
1-N Ethyl Oxo CCX	1-[(cyclohexyloxy) carbonyl] oxy}ethyl 2-oxo-1-{[2'-(1-ethyl-1H-tetrazol-5-yl)biphenyl-4-yl] methyl}-1H-benzimidazole-7-carboxylate
2-N Ethyl CCX	1-{[(cyclohexyloxy) carbonyl]oxy} ethyl 2-ethoxy-1-{[2'-(2-ethyl-1H-tetrazol-5-yl)biphenyl-4-lyl]methyl}-1Hbenzimidazole-7-carboxylate
2-N Ethyl Oxo CCX	1-{[(cyclohexyloxy)carbonyl]oxy}ethyl 2-oxo-1-{[2'-(2-ethyl-1H-tetrazol-5-yl)biphenyl-4-yl] methyl}-1H-benzimidazole-7-carboxylate
ACE	angiotensin converting enzyme
ARBs	angiotensin receptor blockers
AT_1	angiotensin II type 1
AT_2	angiotensin II type 2
C	concentration
C18	n-octadecyl
C8	n-octyl
CDS-5	methyl-2-ethoxy-1-[[(2'-(1H-tetrazol-5-yl)biphenyl)-4-yl-] methyl] benzimidazole -7-carboxylate
CDS-6	2-ethoxy-1-[[(2'-(1H-tetrazol-5-yl)biphenyl)-4-yl-]methyl] benzimidazole -7-carboxylic acid
CDS-7	2-ethoxy-1-[[2'-(1-triphenylmethyl-1H-tetrazol-5yl) biphenyl-4-yl]methyl]benzimidazole-7-carboxylic acid
CE	capillary electrophoresis

LIST OF ABBREVIATIONS (cont.)

CTZ	clorothiazide
Des Ethyl CCX	(RS)-1-[[(cyclohexyloxy)carbonyl]oxy]ethyl-2-oxo-3-[[2'-(1H-tetrazol-5-yl)biphenylyl]methyl]-2,3-dihydro-1H-benzimidazole-4-carboxylate
DSA	4-amino-6-chloro-1,3-benzendisulfonamine
F	flow rate
GC	gas chromatography
H	hour
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HCTZ-CH_2-HCTZ	hydrochlorothiazide dimer
HPLC	high performance liquid chromatography
HPTLC	high performance thin layer chromatography
i.d.	inner diameter
ICH	International Conference on Harmonization
LC-MS/TOF	liquid chromatography-mass spectrometry/time of flight
LC-MS/MS	liquid chromatography tandem mass spectrometry
Log P	partition coefficient
lux	luminous flux per square meter
M	molarity
mg	milligram
min	minute
mL	millilitre
mM	millimolar
MS	mass spectrometry
N	normality
n	number of replications

LIST OF ABBREVIATIONS (cont.)

N Ethyl CCX	(RS)-1-[[(cyclohexyloxy)carbonyl]oxy]ethyl2-ethoxy-1-[[2'-(2-ethyl-2H-tetrazol-5-yl)biphenylyl]methyl]-1H-benzimidazole-7-carboxylate)
NaOH	sodium hydroxide
ng	nanogram
nm	nanometre
NMR	nuclear magnetic resonance
ODS	octadecylsilane
PDA	photo diode array
pKa	acid dissociation constant
psi	pounds per square inch
r^2	coefficient of determination
RP-HPLC	reverse phase high performance liquid chromatography
R_s	resolution
SD	standard deviation
SIM(s)	stability indicating method(s)
T	temperature
TLC	thin layer chromatography
UHPLC	ultra-high performance liquid chromatography
UNK	unknown
US-FDA	United States Food and Drug Administration
USP	United States Pharmacopeia
UV	ultraviolet
v/v	volume per volume
Wh/m^2	Watt- hours per square meter

CHAPTER 1
INTRODUCTION

Hypertension is a global phenomenon and is common to all population with high risks of future cardiovascular symptoms. It accounts for up to 6% of adult deaths worldwide. Hypertension is a complex disease with many factors contributing to its development and maintenance, among which is the renin–angiotensin–aldosterone system along with abnormalities in the renal tubules. Therefore, the pharmacological management of hypertension involves modulating the renin–angiotensin–aldosterone system at various molecular sites. Angiotensin receptor blockers (ARBs) work by direct antagonism of the angiotensin receptors. They inhibit angiotensin II type 1 (AT_1) receptors, which has the most profound effect on increasing blood pressure through stimulating vasoconstriction, renal sodium resorption, and further sympathetic nervous system activation. Diuretics also play an important role in the treatment of hypertension. They are effective both as sole agents and as combination agents with other antihypertensive drugs (1). The combination of candesartan cilexetil, an AT_1 receptor antagonist, and hydrochlorothiazide, a thiazide diuretic, is available under a variety of trade names. The combination has been used in the treatment for patients with hypertension as it gives synergistic effect with good tolerance (2).

Chemical stability of pharmaceutical molecules is a matter of great concern as it affects the safety and efficacy of the drug product. Stability study gives information on how the quality of a drug substance and drug product changes with time under the influence of various environmental factors, which helps in selecting suitable formulation and package as well as providing proper storage conditions and shelf life (3). Stability indicating assay is a method that is employed for the analysis of stability samples in pharmaceutical industry. The method is able to test features which are susceptible to changes during storage and are likely to influence quality, safety and/or efficacy of a drug substance and/or a drug product. The requirement of

establishment of stability indicating method (SIM) has been mentioned in many guidelines and pharmacopoeias and has become mandatory (4).

Official methods for analysis of candesartan cilexetil are offered in the United States Pharmacopoeia 36 (USP 36) for bulk drug by potentiometric technique using 0.1N perchloric acid as titrant, and for its impurities by liquid chromatography method on an octadecylsilane (ODS) column and gradient elution mode (5). In literature, developed methods for determination of candesartan cilexetil, its impurities and degradation products were available for drug products and human plasma by using ultra-high performance liquid chromatography (UHPLC) (6), reverse phase high performance liquid chromatography (RP-HPLC) (7, 8), liquid chromatography-mass spectrometry/time of flight (LC-MS/TOF) (9) and liquid chromatography-tandem mass spectrometry (LC-MSMS) (10). Methods for analysis of hydrochlorothiazide material and tablet dosage form are official in USP 36. They are liquid chromatography using an ODS column with gradient and isocratic elution mode for material and tablet form, respectively (5). In addition, literature shows many studies on the analysis of hydrochlorothiazide alone (11-13) or in its combination with other drugs either by spectroscopy (14, 15) or chromatography (16-19). Standard method for simultaneous analysis of candesartan cilexetil and hydrochlorothiazide in fixed dose combination is not available in current pharmacopoeias. There were a few researches on developing HPLC methods (20-23) and chemometric methods (24) for determination of these two agents in pharmaceutical dosage form. However, these methods were unable to analyze the drugs in the presence of potential degradation products which were formed during storage and distribution process. Therefore, the objectives of this research are as followed:

1) To develop and validate a specific stability-indicating HPLC method for routine analysis of candesartan cilexetil and hydrochlorothiazide in combined dosage form.

2) To establish stability profiles of candesartan cilexetil and hydrochlorothiazide under different conditions.

A RP- HPLC method was developed and optimized by varying pH of buffer, types and concentrations of organic solvents. Subsequently, forced degradation studies were performed on raw material of candesartan cilexetil and

hydrochlorothiazide. The drugs were stressed under acid/base/neutral hydrolysis, oxidation, photolysis and thermal degradation condition to generate sample solutions for SIM and to establish stability profiles for the drugs. Finally, the method was validated in term of linearity, accuracy, precision, specificity and range according to International Conference on Harmonization (ICH) guidelines for its stability indicating power, followed by its application to analyze four commercial pharmaceuticals products (25).

CHAPTER II
LITERATURE REVIEW

2.1 Candesartan cilexetil and hydrochlorothiazide

2.1.1 Candesartan cilexetil

2.1.1.1 Physicochemical properties

Candesartan cilexetil, 2-ethoxy-3-[21-(1H-tetrazol-5-yl)biphenyl-4ylmethyl]-3H-benzoimiadazole-4-carboxylic acid 1-cyclo-hexyloxycarbonyloxy ethyl ester, is an ester pro-drug of candesartan. It is a tetrazole derivative and highly hydrophobic. Its chemical formula is $C_{33}H_{34}N_6O_6$, the chemical structure is illustrated in Figure 2.1. Candesartan cilexetil is a racemic mixture containing one chiral center at the cyclohexyloxy-carbonyloxy- ethyl ester group (26).

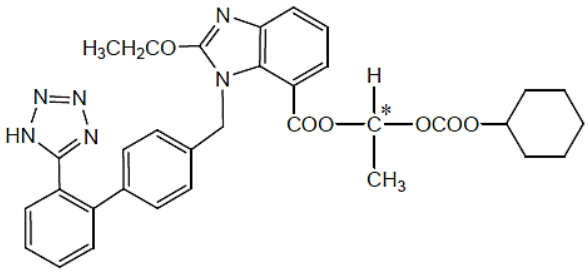
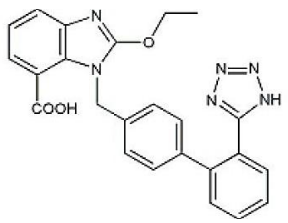
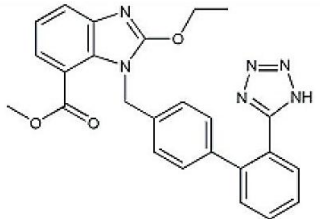


Figure 2.1 Chemical structure of candesartan cilexetil.

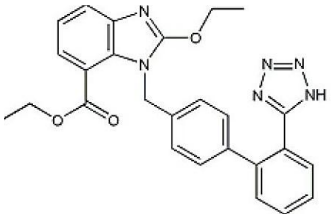
Impurities of candesartan cilexetil can be occurred by several pathways. Candesartan cilexetil undergoes base hydrolysis to give impurity CDS-6, acid hydrolysis to give impurity Des Ethyl CCX, and thermal degradation to give impurities Des Ethyl CCX, 1-N Ethyl Oxo CCX, 2-N Ethyl Oxo CCX, 2-N Ethyl CCX, N Ethyl CCX (6). The chemical structures of candesartan cilexetil impurities are shown in Figure 2.2.



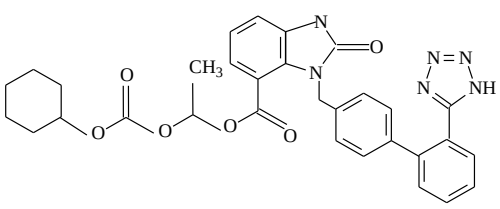
CDS-6
Impurity G (BP 2013)



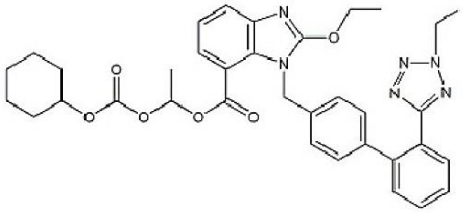
CDS-5
Impurity I (BP 2013)



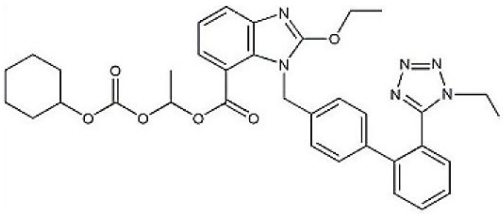
Ethyl CCX (USP 36)
Impurity A (BP 2013)



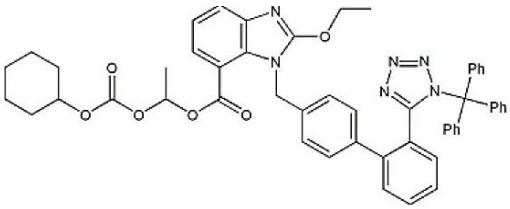
Desethyl CCX (USP 36)
Impurity B (BP 2013)



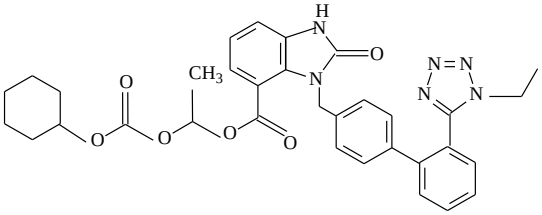
2-N Ethyl CCX (USP)
Impurity F (BP 2013)



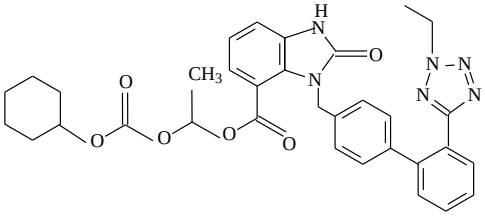
N Ethyl CCX
Impurity E (BP 2013)



CCX-1
Impurity H (BP 3013)



1-N Ethyl Oxo CCX (USP 36)
Impurity C (BP 2013)



Ethyl Oxo CCX
Impurity D (BP 2013)

Figure 2.2 Chemical structures of candesartan cilexetil impurities.

2.1.1.2 Pharmacology

Candesartan cilexetil, a pro-drug, is rapidly converted to the active drug, candesartan, during absorption from the gastrointestinal tract. Candesartan antagonizes the action of angiotensin II by blocking the angiotensin type-1 (AT_1) receptor. Angiotensin II is the primary vasoactive hormone of the renin angiotensin-aldosterone system with effects that include vasoconstriction, stimulation of aldosterone secretion, and renal reabsorption of sodium. When binding to the AT_1 receptor, candesartan prevents angiotensin II from binding to the receptor, and thereby blocking the vasoconstriction and the aldosterone secreting effects of angiotensin II (Figure 2.3). Its action is independent of the pathways for angiotensin II synthesis. Candesartan has a much greater affinity (>10,000-fold) for the AT_1 receptors than for the AT_2 receptors that are found in many tissues but play no known role in cardiovascular homeostasis to date. Candesartan does not inhibit angiotensin converting enzyme (ACE), also known as kininase II, the enzyme that converts angiotensin I to angiotensin II and degrades bradykinin, nor does it bind to or block other hormone receptors or ion channels known to be important in cardiovascular regulation (26, 27).

Candesartan is used in the management of hypertension and may also be used in heart failure in patients with impaired left ventricular systolic function. Studies revealed that candesartan cilexetil showed a significant and long lasting decrease of systolic and diastolic blood pressure. In multiple-dose studies with hypertensive patients, there were no clinically significant changes in metabolic function, including serum levels of total cholesterol, triglycerides, glucose, or uric acid. In heart failure patients, Candesartan ≥8 mg resulted in decreased systemic vascular resistance and pulmonary capillary wedge pressure (26).

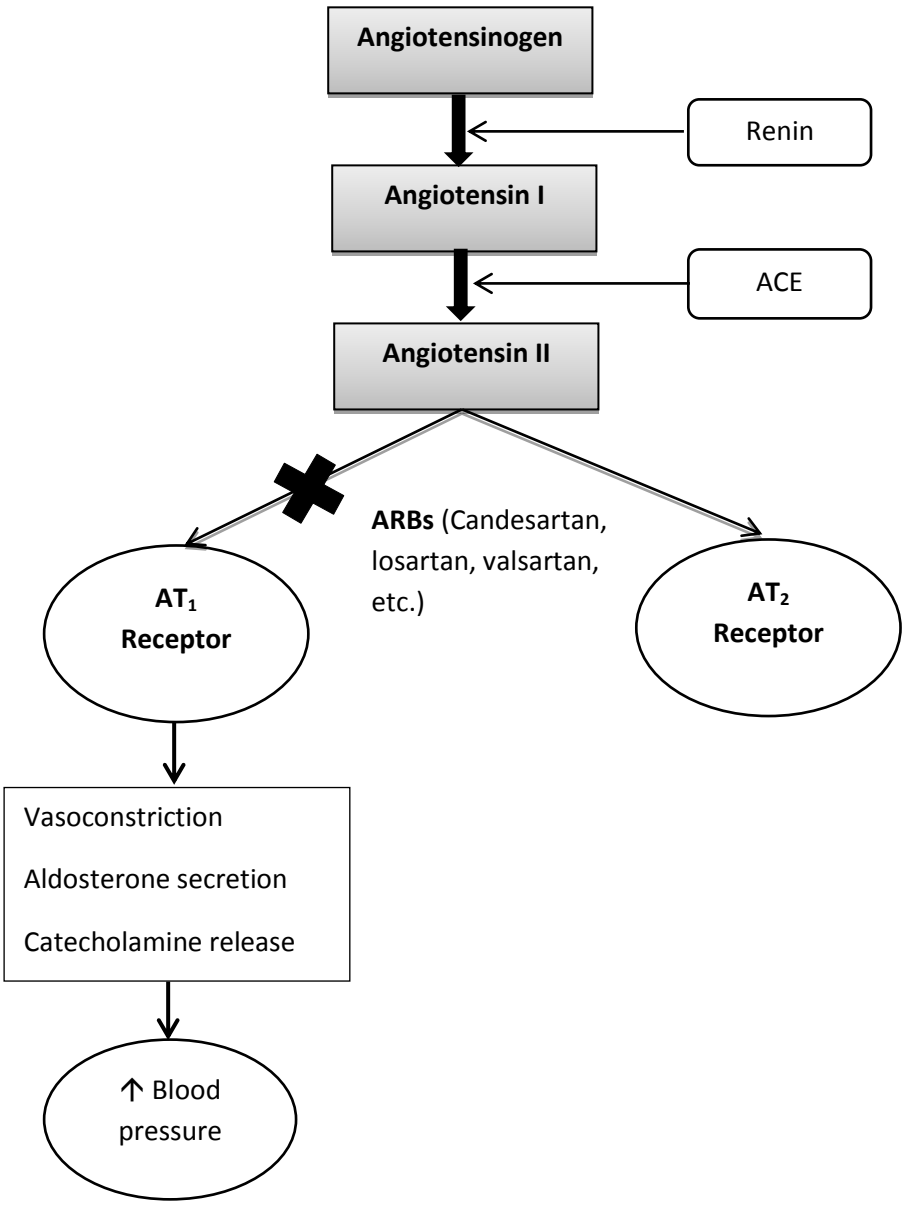


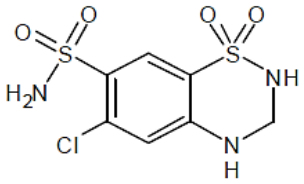
Figure 2.3 Mechanism of action of candesartan cilexetil (1).

2.1.2 Hydrochlorothiazide

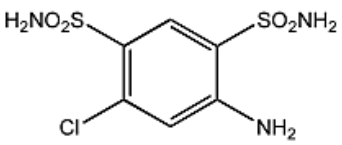
2.1.2.1 Physicochemical properties

Hydrochlorothiazide is a white or practically white, practically odorless crystalline powder. It is moderately water soluble. Chemically, hydrochlorothiazide is [2H-1,2,4-benzothiadiazine-7-sulfonamide, 6-chloro-3,4-dihydro-1,1-dioxide] with chemical formula $C_7H_8ClN_3O_4S_2$ (28).

Hydrochlorothiazide has a main primary degradation pathway which yields 4-amino-6-chloro-1,3-benzendisulfonamine (DSA) and formaldehyde by hydrolysis. Chlorothiazide (CTZ) and hydrochlorothiazide dimer (HCTZ–CH_2–HCTZ) are two major impurities present in hydrochlorothiazide (16, 29). The chemical structures of hydrochlorothiazide and its impurities are displayed in Figure 2.4 and physicochemical properties are summarized in Table 2.1.



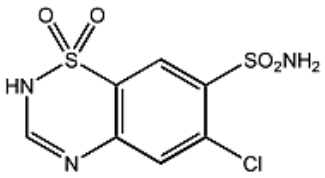
Hydrochlorothiazide



DSA

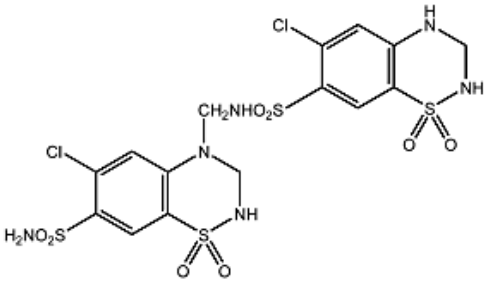
Impurity A (USP 36)

Impurity B (BP 2013)



CTZ

Impurity A (BP 2013)



HCTZ-CH_2-HCTZ

Impurity C (BP 2013)

Figure 2.4 Chemical structures of hydrochlorothiazide and its impurities.

Table 2.1 Physicochemical properties of candesartan cilexetil and hydrochlorothiazide

Properties	**Candesartan cilextil**	**Hydrochlorothiazide**
Molecular formula	$C_{33}H_{34}N_6O_6$	$C_7H_8ClN_3O_4S_2$
Molecular weight	610.67	297.7
pKa	6.0	7.9
Melting point	157-160^oC	274^oC
Solubility	in water < 8×10^{-8} M	in water 722 mg/L (25^oC)
LogP ($C_{octanol}/C_{aqueous}$)	at pH 1.1, 6.9 & 8.9 >1000	-0.07
Wavelength maxima	UV 258.14nm	UV 271nm

2.1.2.2 Pharmacology

Thiazide-type diuretics have been recognized as first-line agents for the majority of patients with hypertension (27). They mainly act within the lumen of the distal nephron, blocking the luminal transmembrane-coupled Na-Cl transport system. It has been proposed that during long-term therapy, thiazides act by reducing total peripheral resistance probably through a direct vascular effect (2). Thiazide diuretics have limited efficacy in patients whose estimated renal function is reduced, such as the elderly (27).

Hydrochlorothiazide is a synthetic thiazide diuretic compound. It inhibits the reabsorption of sodium and chloride at the distal tubule and at the ascending loops of Henlé. Hydrochlorothiazide is available as mono-therapy for the treatment of hypertension or edema (28). However, despite their wide use and proven efficacy in patients with hypertension, their mechanism of blood pressure reduction is not fully understood. Beside typical characteristics of a thiazide diuretic, hydrochlorothiazide has also been known by its ability to inhibit vascular smooth cell carbonic anhydrase, which results in a rise of intracellular pH, activation of potassium channels and vasorelaxation (2). Since the relationship between antihypertensive efficacy and metabolic/electrolyte- related side effects of thiazide diuretics is considered to be dose-related, national guidelines recommend doses that should not exceeding 50 mg/day for hydrochlorothiazide (27). Hydrochlorothiazide is more frequently used in combination therapy with other pharmaceuticals such as angiotensin

converting enzyme inhibitors, angiotensin receptor antagonists and potassium sparing diuretics.

2.1.3 Combined formulation of candesartan cilexetil and hydrochlorothiazide

2.1.3.1 Justification for combined formulation

Hypertension has remained a major problem worldwide and detailed international and national guidelines for its management are frequently updated. Most patients with hypertension are in the medium to high risk category, indicating that many patients will require antihypertensive drugs to control blood pressure. The low compliance levels of patients with hypertension remains a major problem. Fifty to 70% of patients change or discontinue their medication in the first 6 months of therapy, probably because of drug adverse events, cost of treatment and/or poor efficacy, and because of several drugs to be taken at several times in a day. Moreover, control of blood pressure is seldom achieved with monotherapy. Data from randomized, double-blind clinical trials of patients with hypertension, some of whom had type II diabetes mellitus or renal diseases, indicated that approximately 2.75 to 3.80 different anti-hypertensive drugs may be required to achieve target blood pressure levels. Therefore, fixed-dose combination products may be particularly useful in achieving target blood pressure levels and improve patients' compliance (2).

The combination between candesartan cilexetil and hydrochlorothiazide is available under a variety of trade names e.g., Atacand Plus®, Atasart-H® (in Vietnam) and Blopress Plus® (in Thailand). Data from clinical trials indicated that candesartan cilexetil/ hydrochlorothiazide combination substantially reduced blood pressure in patients with mild to severe hypertension. In a dose-finding study, patients receiving candesartan cilexetil 16 mg plus hydrochlorothiazide 12.5 mg showed the greatest diastolic blood pressure and systolic blood pressure reductions and a higher proportion of patients responding to treatment than those receiving placebo or either monotherapy (2, 26).

2.1.3.2 Advantages of combined formulations

The combination between candesartan cilexetil and hydrochlorothiazide gives synergistic effect in treating hypertension. Data from randomized, double-blind, placebo-controlled clinical trials showed that this

combination is significantly more efficacious than either agent alone. The combination is suitable in patients with hypertension who fail to respond to first-line therapy with a diuretic or patients that do not respond to candesartan cilexetil monotherapy. Also, the candesartan cilexetil/hydrochlorothiazide combination is efficacious and well tolerated during long-term treatment of patients with severe hypertension who do not respond to hydrochlorothiazide monotherapy (2, 26).

Additionally, compared to other combinations, candesartan cilexetil 16 mg/hydrochlorothiazide 12.5 mg combination was more efficacious than losartan 50 mg/hydrochlorothiazide 12.5 mg in patients with mild to severe hypertension, while candesartan cilexetil 8 mg/hydrochlorothiazide 12.5 mg showed similar antihypertensive action to lisinopril 10 mg/hydrochlorothiazide 12.5 mg in patients with mild to moderate hypertension (2).

Furthermore, the combination of these two drugs was well tolerated in patients with hypertension as most adverse events were uncommon and not serious. Therefore, this combination has been used as an alternative when mono-therapy with either agent is ineffective (2).

2.2 Assays of candesartan cilexetil and hydrochlorothiazide

2.2.1 Analytical methods for candesartan cilexetil

Method for analysis of candesartan cilexetil in bulk drug is provided in USP 36 by potentiometric technique using 0.1N perchloric acid as titrant. Standard method for analysis of its impurities is also available, which is liquid chromatography method on an ODS column and gradient elution mode using acetonitrile, glacial acetic acid and water as mobile phase. However, USP does not offer method for analysis of candesartan cilexetil in pharmaceutical dosage forms (5).

In literature, developed methods for separation of candesartan cilexetil, its impurities and degradation products were available for drug products by using UHPLC (6) and by RP-HPLC (7, 8). In addition, a validated LC-MSMS method was available for quantitation of candesartan cilexetil in human plasma (10). Stressed

behavior of this drug was also studied in a report from Mehta S et al, followed by structure elucidation of its degradation products by LC-MS/TOF (9). Summary of methods for analysis of candesartan cilexetil was shown in Table 2.2.

2.2.2 Analytical methods for hydrochlorothiazide

Standard methods for analysis of hydrochlorothiazide material and tablet dosage form are presented in USP 36. These methods are liquid chromatography using an octadecylsilane column with gradient and isocratic elution mode, respectively (5). In addition, literature shows many studies on the analysis of hydrochlorothiazide alone in pharmaceutical formulations (11-13) and its combination with other drugs either by spectroscopy (14, 15) or chromatography (16-19). Methods for analysis of hydrochlorothiazide were tabulated tin Table 2.3.

2.2.3 Analytical methods for combinations

Standard method for simultaneous analysis of candesartan cilexetil and hydrochlorothiazide in fixed dose combination is not available in current pharmacopoeias. As shown in Table 2.4, there were a few researches on developing HPLC methods (20-23) and chemometric methods (24) for determination of these two agents in pharmaceutical dosage form. However, these methods were unable to analyze the drugs in the presence of potential degradation products which were formed during storage and distribution process. Therefore, it was necessary to develop a stability-indicating method for routine analysis and quality control of the combined formulation of candesartan cilexetil and hydrochlorothiazide.

Table 2.2 Methods for analysis of candesartan cilexetil

Method	Sample matrix	Stationary phase	Mobile phase	Significant findings	Reference
UPLC-UV	Tablet	Aquity UPLC^{TM} BEH Shield RP18 column	(A) Phosphate buffer pH 3.0 (B) Milli Q water: acetonitrile (5:95, v/v)	Detection: UV 254 nm Stress studies: 5 mL reagents (0.5 N HCl, 0.5 N NaOH, water and 10% H_2O_2), room temperature.	(6)
HPLC-UV	Bulk drug	C18 column	(A) ACN: Glacial acetic acid: water (57:1:43, v/v) (B) ACN: Glacial acetic acid: water (90:1:10, v/v)	Standard methods for assay of candesartan cilexetil impurities. Detection: UV 254 nm	(5)
HPLC-UV	Tablet	Purosphere Star RP18e column	(A) Acetonitrile: ammonium acetate (pH 5.0; 20 mM) (10:90. v/v) (B) Acetonitrile: ammonium acetate (pH 5.0; 20 mM) (90:10. v/v)	Detection: UV 254 nm	(7)
HPLC-UV	Tablet and bulk drug	Zobax SB CN column	Phosphate buffer (pH 3.0, 20 mM): acetonitrile (50:50, v/v)	The stress studies: reagents (0.1 M HCl, 0.1 M NaOH, water and 0.1% H_2O_2 in 48h), light (ICH Q1)	(8)

Table 2.2 Methods for analysis of candesartan cilexetil (cont.)

Method	Sample matrix	Stationary phase	Mobile phase	Significant findings	Reference
				Linearity range: 12.5–75 µg/mL ($r^2 \geq$ 0.999) % recovery: 99.0-101.0% (bulk drug) 99.2-101.4% (tablet) Precision: %RSD = 0.3%	(8)
LC-MS/MS	Human plasma	Betasil C8 column	Methanol: ammonium tri-floroacetate buffer with formic acid (60: 40, v/v)	Linearity range: 1.2-1030 ng/mL ($r^2 \geq$ 0.9996) Extraction recovery: 101.9-110.6%	(10)
HPLC-UV	Bulk drug	Phenomenex Luna C-18 column	(A) Acetonitrile (B) Potassium dihydrogen orthophosphate buffer (pH 2.8, 0.01M)	Detection: UV 254 nm Stress studies: reagents (0.1N HCl, 0.1N NaOH and water at 80^{o}C and 30% H_2O_2 at room temperature), light (ICH Q1B) and heat (50^{o}C, 21 days)	(9)

Table 2.3 Methods for analysis of hydrochlorothiazide

Method	Sample matrix	Stationary phase	Mobile phase	Significant findings	Reference
HPLC-UV	Bulk drug	C18 column	(A) Acetonitrile: phosphate buffer pH 2.7 (3:7) (B) 0.5% anhydrous formic acid in water	Standard methods for assay of hydrochlorothiazide raw material.	(5)
HPLC-UV	Tablet	C18 column	0.1 M monosodium phosphate : acetonitrile (9:1, pH= 3.0)	Standard methods for assay of hydrochlorothiazide in tablet dosage form.	(5)
HPLC-UV	Bulk drug	Kromasil C18 column	Methanol: water (30: 70, v/v)	Linearity range: 3-120 µg/mL (r^2 = 0.9999) % recovery: 98.0–102.0% Precision: %RSD< 2% LOD= 0.9 µg/mL; LOQ= 3.0 µg/mL	(11)
HPLC-UV	Bulk drug	Thermo Hypersil C18 column	Methanol: phosphate buffer pH=3.2 (60:40 v/v)	Linearity range: 60-140 µg/mL (r^2 = 0.9990) % recovery: 100.51–101.21% Precision: %RSD< 2%	(12)

Table 2.3 Methods for analysis of hydrochlorothiazide (cont.)

Method	Sample matrix	Stationary phase	Mobile phase	Significant findings	Reference
				LOD=10 µg/mL; LOQ= 15µg/mL Stress studies: reagents (1 N HCl, 1 N NaOH, and water at 96-98^{o}C for 6h, and 30% H_2O_2, at room temperature for 6 h), sunlight for 30h and heating oven at 110^{o}C for 12h	(12)
HPLC-UV	Oral suspension	Phenomenex Luna C18 column	0.1 M sodium phosphate buffer pH 3.0: acetonitrile (70:30 v/v)	Detection: UV 254 nm Linearity range: 10-50 µg/mL (r^2 = 0.9998) Mean recovery = 100.3% Precision: %RSD <2% Stress studies: reagents (0.1 N HCl, 0.1 N NaOH, and water at 60^{o}C for 2h, and 3% H_2O_2, at ambient temperature for 24 h), photostability chamber for 72h and heating oven at 80^{o}C for 72h	(13)

Table 2.4 Methods for simultaneous analysis of candesartan cilexetil and hydrochlorothiazide

Method	Sample matrix	Stationary phase	Mobile phase	Significant findings	Reference
HPLC-UV	Tablet	Hypersil C18 column	10 mM tetra butyl ammonium hydrogen sulphate: methanol (15:85, v/v)	Linearity range: Candesartan cilexetil: 0.8-80 µg/mL (r^2>0.9990) Hydrochlorothiazide: 0.625-62.5 µg/mL (r^2>0.9990). Precision: %RSD < 2% % recovery: Candesartan cilexetil: 99.58-100.39% Hydrochlorothiazide: 99.31-100.08%	(20)
HPLC-UV	Tablet	Hypersil C8 column	Acetonitrile: triethylamine 0.02% (60:40, v/v)	Linearity range: Candesartan cilexetil: 50-150 µg/mL (r^2=0.9999) Hydrochlorothiazide: 75-225 µg/mL (r^2=0.9999). Precision: %RSD < 2% % recovery: Candesartan cilexetil: 99.88-99.95%	(21)

Table 2.4 Methods for simultaneous analysis of candesartan cilexetil and hydrochlorothiazide (cont.)

Method	Sample matrix	Stationary phase	Mobile phase	Significant findings	Reference
				Hydrochlorothiazide: 99.79-99.91%	(21)
HPLC-UV	Bulk drug Tablet	Chromosil C18 column	Methanol: tetrahydrofurane: 0.1% ortho phosphoric acid (85:5:10, v/v)	Linearity range: Candesartan cilexetil: 5-35 µg/mL (r^2=0.9996) Hydrochlorothiazide: 5-35 µg/mL (r^2=0.9999). Precision: %RSD < 2% Mean recovery: Candesartan cilexetil: 99.95% Hydrochlorothiazide: 99.46%	(22)
HPLC-UV	Tablet	Hypersil phenyl 2 column	0.02M potassium dihydrogen phosphate: methanol: triethylamine (25:75:0.2, v/v)	Linearity range: Candesartan cilexetil: 12-56 µg/mL (r^2>0.9991) Hydrochlorothiazide: 5-45 µg/mL (r^2>0.9993). Precision: %RSD < 2% % recovery:	(23)

Table 2.4 Methods for simultaneous analysis of candesartan cilexetil and hydrochlorothiazide (cont.)

Method	Sample matrix	Stationary phase	Mobile phase	Significant findings	Reference
				Candesartan cilexetil: 99.44-101.02% Hydrochlorothiazide: 98.60-100.72%	(23)

2.3 High performance liquid chromatography

2.3.1 Principle and separation mechanisms

Chromatography is an analytical technique based on the separation of molecules due to their differences in structure and/or composition. Chromatography can be defined as a separation process that is achieved by distributing the components of a mixture between two phases, a stationary phase and a mobile phase. Those components held preferentially in the stationary phase are retained longer in the system than those that are distributed selectively in the mobile phase. As a consequence, solutes are eluted from the system as local concentrations in the mobile phase in the order of their increasing distribution coefficients with respect to the stationary phase (30).

Chromatography is probably the most powerful and versatile analytical technique. In a single step process, it can separate a mixture into its individual components and simultaneously provide a quantitative estimation of each constituent. Different modes of chromatography are available for certain analytes. Gas chromatography (GC) is for volatile substances, whereas liquid chromatography (LC) is for involatile chemicals and materials of extremely high molecular weight (including biopolymers). In addition, thin layer chromatography (TLC) is inexpensive and is available for identification (30).

2.3.1.1 Principle

High performance liquid chromatography or HPLC is a type of liquid chromatography which is used to separate and quantify compounds dissolved in solution. It is the most commonly used technique for the analysis of pharmaceutical formulations.

A liquid mobile phase is pumped under high pressure through a stainless steel column that contains particles of stationary phase with a diameter of 3-10 μm. Analytes are loaded onto the head of the column via a loop valve and separation of a mixture occurs according to the relative lengths of time spent by its components in the stationary phase. Each component in a mixture spends more or less the same time in the mobile phase in order to exit the column. Monitoring the column effluent can be carried out with the use of a variety of detectors (31).

Elution techniques are methods of pumping mobile phase through a column. In the isocratic method, the composition of the mobile phase remains constant, whereas in the gradient method the composition changes during the separation process. The isocratic method is the simplest technique and should be the first choice when developing a separation. Eluent gradients are usually generated by combining the pressurized flows from two pumps and changing their individual flow rates with an electronic controller or data system while maintaining the overall flow rate constant (32).

2.3.1.2 Mechanisms

Systems used in chromatography are often categorized into one of four types based on the mechanism of action: absorption, partition, ion- exchange and size exclusion.

2.3.1.2.1 Absorption chromatography

Absorption chromatography indicates interactions between solutes and the surface of the solid stationary phase.

2.3.1.2.2 Partition chromatography

Partition chromatography involves the interaction between solutes and a liquid stationary phase. The liquid stationary phase is immiscible with the eluent and is coated on an inert support.

2.3.1.2.3 Ion- exchange chromatography

In Ion- exchange chromatography, retention is based on the attraction between solute ions and charged sites bound to the stationary phase. This technique is based on the ionization of the sample. The stronger the charge of the sample, the stronger the attraction to the stationary phase; therefore, it will take longer to elute off the column. Ions of the same charge are excluded (32, 33).

2.3.1.2.4 Size exclusion chromatography

Size exclusion chromatography, also called as gel permeation chromatography or gel filtration chromatography mainly separates particles on the basis of size. It is also useful for determining the tertiary structure and quaternary structure of proteins and amino acids. This technique is widely used for the molecular weight determination of polysaccharides (33).

Several other types of chromatographic separation have been described, including ion-pair chromatography, which is used as an alternative to ion-exchange chromatography, affinity chromatography and chiral chromatography (to separate enantiomers) (32).

2.3.1.3 Reversed phase high performance liquid chromatography

Reversed phase HPLC (RP-HPLC), which is the most commonly encountered form of HPLC, is a kind of partition chromatography. RP-HPLC has a non-polar stationary phase and an aqueous, moderately polar mobile phase. RP-HPLC operates on the principle of hydrophobic interactions, in which the separation results from the distribution or partition coefficient of analytes between the polar mobile phase and the non-polar stationary phase. In other words, analytes with high hydrophobicity will be strongly retained in non-polar stationary phase and increasing the polarity of the mobile phase will lead to longer retention times of analytes. The binding of the analyte to the stationary phase is proportional to the contact surface area around the non-polar segment of the analyte molecule (30, 32, 33).

In RP-HPLC, the most common non-polar stationary phases use an organochlorosilane for which the R group is an n-octyl (C8) or n-octadecyl (C18) hydrocarbon chain. Most reversed-phase separations are carried out using a buffered aqueous solution as a polar mobile phase. Because the silica substrate is subject to hydrolysis in basic solutions and loss of the stationary phase ligand in acidic solution, the pH of the mobile phase must be controlled in the range from 2.0 to 8.0 (32, 34).

2.3.2 Instrumentation

A typical HPLC system (Figure 2.5) consists of:

- Solvent reservoirs
- A pump capable of pumping solvent at high pressure (up to 4000 psi) and high flow rate (up to 10 mL/min)
- An injector, which is loop injector or auto-sampler, capable of injecting accurate volume of sample to the column (1-200 μL)

- A column, which is usually a stainless steel tube packed with particles of stationary phase
- A detector, which is often UV-Visible detector or Photo Diode Array (PDA) detector
- Integrator or acquisition and display system (31)

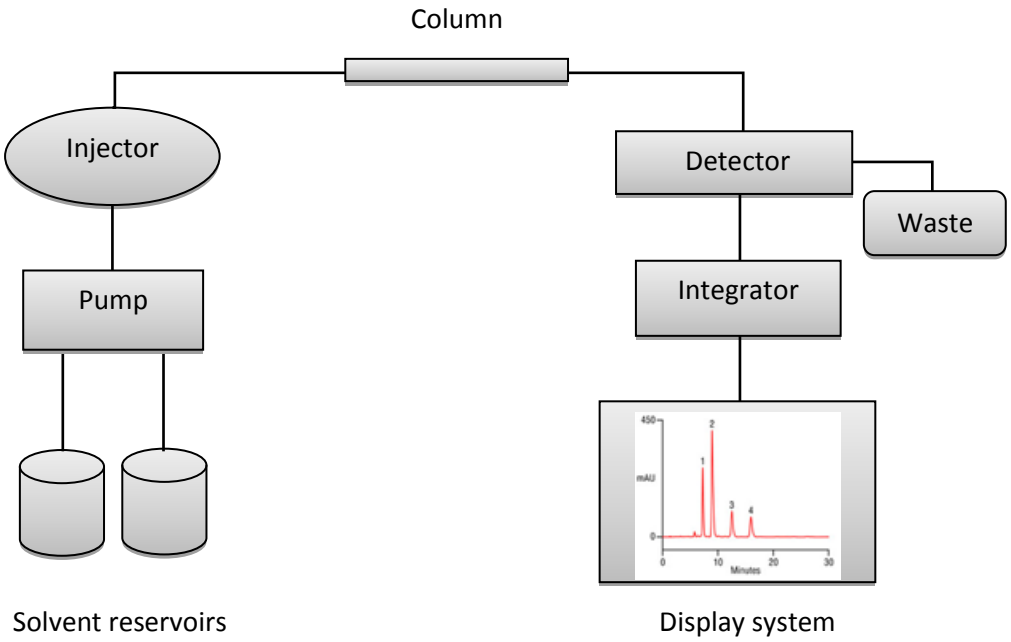


Figure 2.5 A typical HPLC system (31).

2.3.3 Quantitative analysis

The quantification methods incorporated in HPLC have basic theory involving the measurement of peak height or peak area. To determine the concentration of a compound, the peak area or height is plotted versus the concentration of the substance. For peaks that are well resolved, both peak height and area are proportional to the concentration. Three different calibration methods, each with its own benefits and limitations, can be utilized in quantitative analysis i.e., external standard, internal standard and the standard addition method.

2.3.3.1 External standard

To perform the external standard method, standard solutions of known concentrations of the analyte are prepared with one standard that is similar in

concentration to the sample. Peak area or height is then plotted versus the concentration for each compound. The plot should be linear and go through the origin. A fixed amount of sample is injected. The concentration of the analyte in the sample is then determined according to the following formula:

$$C_{sample} = \frac{\text{Peak area of sample}}{\text{Peak area of standard}} \times C_{standard}$$

Where: C_{sample} = concentration of sample

$C_{standard}$ = concentration of standard

The external standard method is the simplest of the three methods. The accuracy of this method depends on the reproducibility of the injection volume (32).

2.3.3.2 Internal standard

Internal standard is a component that is not present in the sample. It should be chemically similar to, have similar retention time and derivatize similarly to the analyte. Besides, it is important that the internal standard is stable and does not interfere with any of the sample components.

In this method, an equal amount of an internal standard is added to both the sample and standard solutions. The internal standard should be added before any preparation of the sample so that extraction efficiency can be evaluated. Quantification is achieved by using ratios of peak height or area of the component to the internal standard (32).

$$C_{sample} = \frac{\text{Peak area of internal std in standard}}{\text{Peak area of internal std in sample}} \times \frac{\text{Peak area of sample}}{\text{Peak area of standard}} \times C_{standard}$$

Where: C_{sample} = concentration of sample

$C_{standard}$ = concentration of standard

2.3.3.3 Standard addition

The standard addition method is commonly used to determine the concentration of an analyte that is in a complex matrix such as biological fluids,

soil samples, etc. The reason for using the standard addition method is that the matrix may contain other components that interfere with the analyte signal causing inaccuracy in the determination of concentration. When using standard addition method, the change in instrument response between the sample and the spiked samples is assumed to be due only to change in analyte concentration.

One of the most common standard addition procedures involves preparing a series of samples containing increasing known amounts of the external standard while the amount of samples are identical. Each solution is then diluted to a fixed volume before measurement. The instrument response is then measured for all of the diluted solutions and the data is plotted with volume standard added in the x-axis and instrument response in the y-axis. Linear regression is performed and the slope (m) and y-intercept (b) of the calibration curve are used to calculate the concentration of analyte in the sample (35).

2.4 Stability-indicating methods

2.4.1 Definition

Stability-indicating methods (SIMs) were defined by the United States-Food and Drug Administration (US-FDA) in the draft guideline of 1998 as validated quantitative analytical methods that can detect the changes with time in the chemical, physical, or microbiological properties of the drug substance and drug product, and that are specific so that the contents of active ingredient, degradation products, and other components of interest can be accurately measured without interference (36).

Stability-indicating methods can be classified into two categories specific stability-indicating and selective stability-indicating. Specific stability-indicating method is a method that is able to measure unequivocally the drug(s) in the presence of all degradation products, excipients and additives, expected to be present in the formulation. Selective stability-indicating method, on the other hand, is defined as a method that is able to measure unequivocally the drug(s) and all degradation products in the presence of excipients and additives, expected to be present in the formulation (4).

2.4.2 Forced degradation studies

Forced degradation studies or stress testing of a drug can help identifying its likely degradation products and degradation pathways. In addition, stability profile of the molecule and stability indicating power of the procedure can be established (37). General protocol for stress testing of drug substances and drug products is shown in Table 2-5 (38).

Forced degradation studies typically involve the exposure of representative samples of drug substance or drug product to the relevant stress conditions of light, heat, humidity, acid/base hydrolysis, and oxidation. Although some regulatory guidelines provide definitions and comments about forced degradation studies, details about the scope, timing, and how hydrolytic, photolytic and oxidative studies have to be actually performed is lacking (4). As a result, there are some papers that give suggestions (3, 4) and stepwise approaches (39) on conducting stress testing.

Table 2.5 General protocol for stress testing of drug substances and drug products (38)

Stressed condition	**Drug substance**		**Drug product**	
	As neat solid	**As solution or suspension**	**Solid dosage form**	**Liquid**
Hydrolysis (acid, base and thermal)		✓		✓
Oxidation		✓		✓
Photolysis	✓	✓	✓	✓
Thermal degradation	✓		✓	✓
Thermal/humidity	✓		✓	

It is found to be practical to begin with extreme conditions such as high temperatures, strong acid/base concentrations and testing at shorter (2, 5, 8, 24 h, etc.) multiple time points, so that the rate of degradation can be evaluated. The primary degradants and their secondary degradations products can be distinguished by testing at early time points. In another approach, degradation is started by doing degradation at milder conditions and testing at longer time points (1, 2, 3 days, etc.). Then stress would be increased or decreased to obtain sufficient degradation. As compared to

harsher conditions, this strategy is more time consuming, however, it is more advantageous due to the following reasons: (i) there may be a change in mechanism of reaction when a severe condition is used, and (ii) there is a practical problem in neutralizing or diluting every sample, when it contains a high concentration of reactants, e.g., acid or base, before an injection can be made on the HPLC column (3).

The sufficient level of degradation has been a matter of debates among scientists. Degradation of drug substances between 5% and 20% has been accepted as reasonable for validation of chromatographic assays. Some pharmaceutical scientists think 10% degradation is optimal for use in analytical validation for small pharmaceutical molecules. However, it is not necessary that forced degradation would result in a degradation product. The study can be terminated if no degradation is seen after drug substance or drug product has been exposed to stress conditions which are more severe than those mentioned in an accelerated stability protocol. Over-stressing a sample may lead to the formation of a secondary degradation product that would not be seen in formal shelf-life stability studies and under-stressing may not generate sufficient degradation products (3).

The concentration of drug that should be used for degradation study has not been specified in regulatory guidance. It is recommended that the studies should be initiated at a concentration of 1 mg/mL. By using drug concentration of 1 mg/mL, it is usually possible to even minor decomposition products in the range of detection (3, 4).

2.4.2.1 Hydrolytic conditions

Hydrolysis is by far the most commonly encountered drug degradation mechanism, both in solution and also in the solid state. Many drug molecules contain functional groups derived from relatively weakly bonding groups such as carboxylic acids. Hydrolysis of such derivatives is expected both in solution, and in the solid state, in the presence of water. Hydrolytic study under acidic and basic condition involves catalysis of ionizable functional groups present in the molecule (3, 40).

The hydrolytic degradation of a new drug in acidic and alkaline conditions can be studied by refluxing the drug in 0.1 N HCl/NaOH for 8 h (Figure 2.6). If reasonable degradation is seen, testing can be stopped at this point. However, in case no degradation is seen under these conditions, the drug should be refluxed in acid/alkali of higher strengths and for longer duration. Alternatively, if

total degradation is seen after subjecting the drug to initial conditions, acid/alkali strength can be decreased along with decrease in the reaction temperature (4).

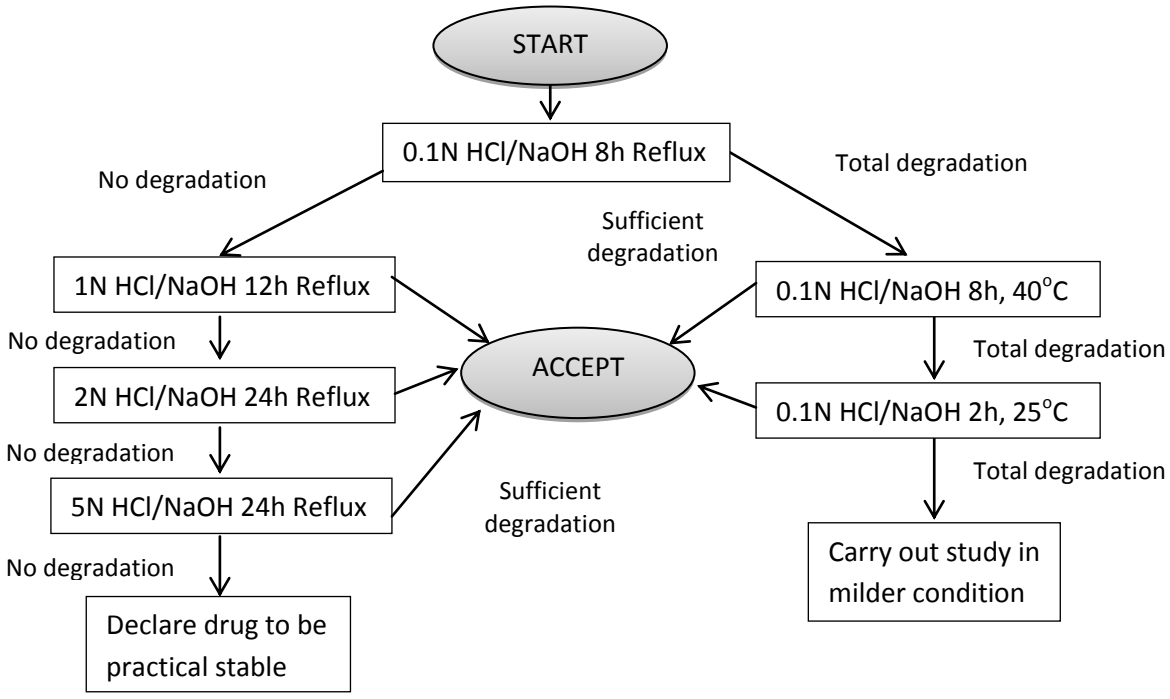


Figure 2.6 Stepwise for performing hydrolytic degradation (39).

Similarly, degradation under neutral conditions can be started by refluxing the drug in water for 12 h. Refluxing time should be increased if no degradation is seen. If the drug is found to degrade completely, both time and temperature of study can be decreased.

2.4.2.2 Oxidative conditions

Oxidation presents an important drug degradation pathway, and is second only to hydrolysis. Oxidation of organic compounds occurs primarily via three mechanisms: nucleophilic/electrophilic processes, free radical processes (autoxidation), and electron transfer reactions. Nucleophilic/electrophilic processes typically occur between peroxide and an organic reactant. The autoxidation process involves the initiation of free radicals, which propagate through reaction with oxygen and drug molecule to form oxidation products. In an electron-transfer process, an electron is transferred from a low electron affinity donor to an oxidizing species,

which may be catalyzed by transition metals (40). Amines, sulfides and phenols are susceptible to electron transfer oxidation to give N-oxides, hydroxylamine, sulfones and sulfoxide. The functional group with labile hydrogen like benzylic carbon, allylic carbon, and tertiary carbon or α-positions with respect to hetero atom is susceptible to oxidation to form hydro peroxides, hydroxide or ketone (3).

For oxidation study, it is suggested to use hydrogen peroxide (H_2O_2) in the concentration range of 3–30% (Figure 2.7) (4). Although hydrogen peroxide is widely used for oxidation of drug substances in forced degradation studies, other oxidizing agents such as metal ions, oxygen, and radical initiators (e.g., azobisisobutyronitrile, AIBN) can also be used (3).

2.4.2.3 Photolytic conditions

Photo-stability studies are performed to generate primary degradants of drug substance by exposure to UV or fluorescent conditions. Some recommended conditions for photo-stability testing are described in ICH guidelines. Exposure energy should be minimum of 1.2 million lux h fluorescent light and 200Wh/m^2 UV (Figure 2.8) (3, 4).

Light stress conditions can induce photo oxidation by free radical mechanism. Functional groups like carbonyls, nitro aromatic, N-oxide, alkenes, aryl chlorides, weak C–H and O–H bonds, sulfides and polyenes are likely to introduce drug photosensitivity.

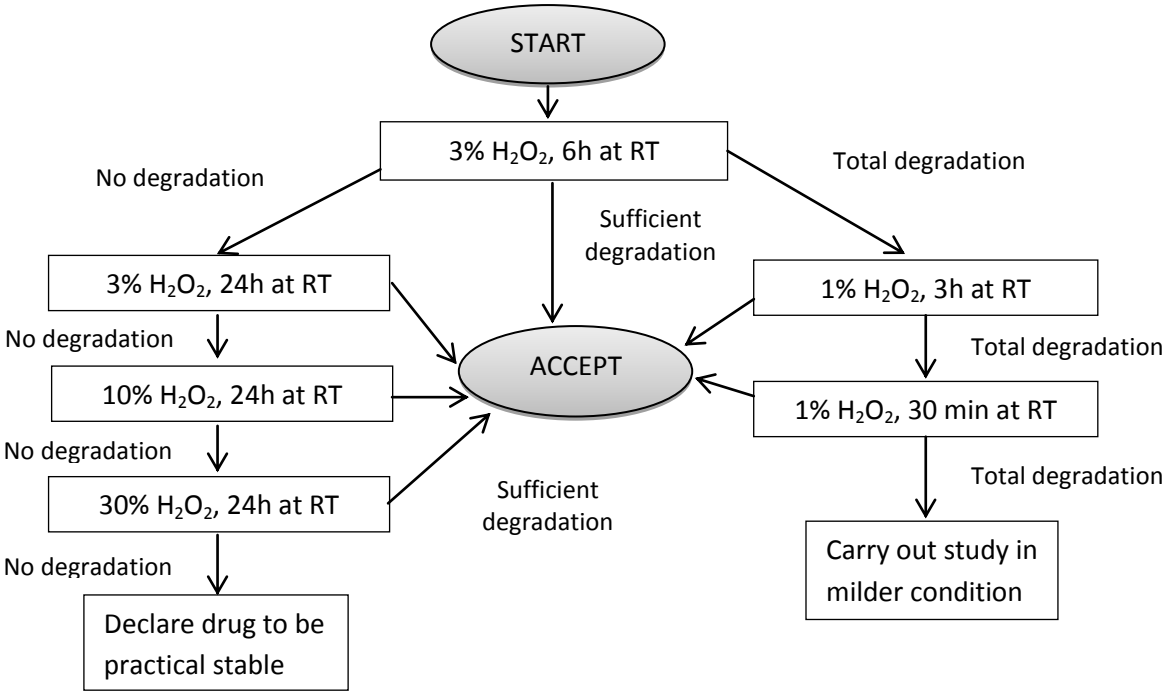


Figure 2.7 Stepwise for performing oxidative degradation (39).

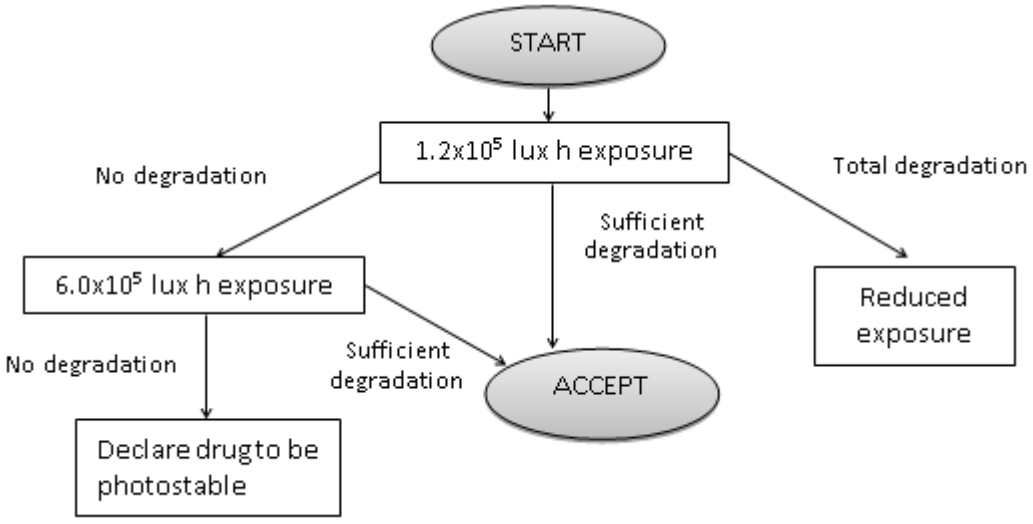


Figure 2.8 Stepwise for performing photolytic degradation (39).

2.4.2.4 Thermolytic conditions

Thermal degradation should be carried out at more strenuous conditions than recommended ICH Q1A accelerated testing conditions. Samples of

solid-state drug substances and drug products should be exposed to dry and wet heat, while liquid drug products should be exposed to dry heat. Studies may be conducted at higher temperatures for a shorter period (3).

2.4.3 Method development

Because of the requirement to separate multiple components of stability sample, chromatography has become the most prominent technique. Various chromatographic methods that have been used are TLC, HPTLC (high-performance thin-layer chromatography), GC, HPLC and CE (capillary electrophoresis). Among these, HPLC was proved to be the most employed method in literature (4).

Although SIMs are mentioned in many regulatory documents, basic steps for method development and validation are not provided in detail in any guidelines or pharmacopoeias. Therefore, some papers recommended practical approaches to developed validated SIMs (4, 38). In general, the approaches involve the following steps:

- Studying the physicochemical properties of drug molecules
- Generate appropriate samples by forced degradation studies
- Preliminary separation on stressed samples
- Method optimization
- Method validation

Before starting the method development, various physicochemical properties such as pKa value, log P, solubility and absorption maximum of the drug must be known, because they lay a foundation for HPLC method development. Log P and solubility helps select mobile phase and sample solvent while pKa value helps determine the pH of the mobile phase (3). Moreover, 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 particular stationary phase (4).

The next step in the development of a SIM is the conduct of forced degradation studies to generate degradation products of the drug. The stress samples are then subjected to preliminary analyses to study the number and types of degradation products formed under various conditions. The simplest way for preliminary separation is to start with a reversed-phase octadecyl column, preferably a

new one (4). During the method development step, the drug peak may hide an impurity or degradant peak that co-elutes with the drug. This requires peak purity analysis which determines the specificity of the method. Direct analysis can be done on line by using PDA detection. PDA provides information of the homogeneity of the spectral peak but it is not applicable for the degradants that have the similar UV spectrum to the drug. The method is then optimized for separating closely eluting peaks by changing flow rate, injection volume, column type and mobile phase ratio (3).

The developed SIM is finally validated according to USP/ICH guideline for the characteristics of linearity, accuracy, precision, specificity, quantitation limit, detection limit, ruggedness and robustness of the method (3).

In the case when the structures of degradation products are unknown, there will be a step of identification and characterization of these compounds. This step can be done conventionally by spectroscopic techniques after isolation such as mass spectrometry (MS) or nuclear magnetic resonance (NMR). However, the conventional way is often time consuming, which leads to the increasing use of hyphenated techniques (HPLC-MS, HPLC-MS-MS, HPLC-NMR, ect.) (4).

2.4.4 Method validation

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. According to ICH guideline Q2(R1), the validation of analytical procedures is mostly directed to the four most common types of analytical procedures:

- Identification tests;
- Quantitative tests for impurities' content;
- Limit tests for the control of impurities;
- Quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product.

Identification tests are intended to ensure the identity of an analyte in a sample, which is normally achieved by comparison of a property of the sample (e.g., spectrum, chromatographic behavior, chemical reactivity, etc.) to that of a reference standard. Testing for impurities can be either a quantitative test or a limit test for the

impurity in a sample. Assay procedures are intended to measure the content of analyte present in a given sample. The sample may be drug substance of drug product (25).

Typical validation characteristics which should be considered are accuracy, precision, specificity, limit of detection, limit of quantitation, linearity and range (25).

2.4.4.1 Accuracy: 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.

2.4.4.2 Precision: 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.

Repeatability: Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

Intermediate Precision: Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

2.4.4.3 Specificity: The ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

2.4.4.4 Detection Limit: The lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

2.4.4.5 Quantitation Limit: the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

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

2.4.4.7 Range: 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.

A summary of the characteristics applicable to identification, control of impurities and assay procedures was given in Table 2.6 (25).

Table 2.6 Characteristics for common types of analytical procedures (25)

Type of analytical procedure characteristics	**Identification**	**Testing for impurities**		**Assay**
		quantitative	**limit**	**- dissolution - content/potency**
Accuracy	-	+	-	+
Precision				
- Repeatability	-	+	-	+
- Interm.Precision	-	+[2]	-	+
Specificity [1]	+	+	-	+
Detection Limit	-	- [3]	+	-
Quantitation	-	+	+	-
Limit	-	+	-	+
Linearity	-	+	-	+
Range	-	+	-	+

- signifies that this characteristic is not normally evaluated

+ signifies that this characteristic is normally evaluated

[1] lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s)
[2] in cases where reproducibility has been performed, intermediate precision is not needed
[3] may be needed in some cases

CHAPTER III
MATERIALS AND METHODS

This chapter covers chemicals and instruments which are used for the study. Procedures and techniques as well as calculations are also presented.

3.1 Materials and instrumentation

3.1.1 Chemicals

Table 3.1 Lists of chemicals and reagents

Name	Grade	Source/Supplier
Acetonitrile	HPLC	Honeywell Burdick & Jackson, USA
Candesartan cilexetil	Working standard	GPO, Thailand
Hydrochloric acid	Analytical reagent	RCI Labscan Limited, Thailand
Hydrochlorothiazide	Working standard	GPO, Thailand
Hydrogen peroxide	Analytical reagent	Univar, Australia
Methanol	HPLC	Honeywell Burdick & Jackson, USA
Ortho- phosphoric acid	Analytical reagent	Carlo Erba, France
Potassium dihydrogen phosphate	Analytical reagent	Fluka, Switzerland
Sodium hydroxide	Analytical reagent	Univar, Australia
Sterile water	---	Thai Nakorn Patana Co., Ltd, Thailand

3.1.2. Equipment and Instruments

Table 3.2 List of equipment and instruments

Name	Source/Supplier
Electronic analytical balance	Mettler Toledo, Kentucky, USA
Heating oven	---
HPLC column (Zobax SB CN, 150 x 4.6 mm i.d., 5 µm)	Agilent Technologies, Kansas, USA
HPLC instrument (Dionex Ultimate 3000 UHPLC)	Thermo Scientific, USA
Micropipettes	TreffLab, Degersheim, Switzerland
Nylon filter membrane (0.45 µm)	National Scientific, Tennessee, USA
Nylon syringe filter (0.22 µm)	National Scientific, Tennessee, USA
pH meter	Denver Instrument, Colorado, USA
Disposable syringe (1 mL, 5 mL)	Nipro, Thailand
Ultrasonic bath	D.S.C Group, Nonthaburi, Thailand
Vacuum filtration set	Santorius, Gottingen, Germany
Water bath	---

3.2 HPLC method

3.2.1 Instrumentation

HPLC method development was performed on Dionex Ultimate 3000 UHPLC system (Thermo Scientific, USA) with an auto-sampler and PDA detector. The separation was carried out using a Zobax SB CN column 4.6×150mm i.d., 5µm (Agilent Technologies, USA). Detection of the desired drugs was carried out at UV 254 nm. Data was analyzed by Chromeleon Chromatography Data System software.

3.2.2 Preparation of standard and sample solutions

3.2.2.1 Preparation of standard solutions

Standard stock solutions of candesartan cilexetil (320 μg/mL) and hydrochlorothiazide (500 μg/mL) were separately prepared by dissolving accurately weighed amount of drugs in a mixture of acetonitrile and water (80:20, v/v).

Standard mixture solution was prepared from the stock solution. An accurate volume of each stock solution was pipetted out and transferred into a volumetric flask. Further dilution was performed by using mobile phase to obtain the final concentration of 32 μg/mL and 50 μg/mL for candesartan cilexetil and hydrochlorothiazide, respectively.

3.2.2.2 Preparation of calibration curve

Standard mixtures of candesartan cilexetil (16, 24, 32, 40, 48 μg/mL) and hydrochlorothiazide (25, 37.5, 50, 62.5, 75 μg/mL) were prepared in 10 mL volumetric flask by diluting stock solutions of the two drugs with mobile phase.

3.2.1.3 Preparation of sample solution

Twenty tablets of each pharmaceutical product (C1, C2, S1 or S2) shown in Table 3.3 were weighed and ground. The powder equivalent to one tablet was weighed, transferred into a 50 mL volumetric flask and dissolved in 30 mL of 80% acetonitrile in water. The mixture solution was sonicated for 15 minutes and adjusted to volume with 80% acetonitrile in water. The solution was filtered through 0.45 μm membrane. The filtrate was diluted five times (C1 and S1) and ten times (C2 and S2) with mobile phase and filtered through 0.22 μm nylon syringe membrane before injected to HPLC system.

Table 3.3 List of pharmaceutical products and their composition

Sample name	Type	Composition
C1	Combined formulation	Candesartancilexetil: 8 mg/tablet Hydrochlorothiazide: 12.5 mg/tablet
C2	Combined formulation	Candesartancilexetil: 16 mg/tablet Hydrochlorothiazide: 12.5 mg/tablet
S1	Single drug	Candesartan cilexetil: 8 mg/tablet
S2	Single drug	Hydrochlorothiazide: 25 mg/tablet

3.2.3 Method development

A HPLC method for simultaneous determination of candesartan cilexetil and hydrochlorothiazide was optimized. Optimization of mobile phase was performed by varying pH, types and concentrations of organic modifiers. Standard mixture containing 32 µg/mL of candesartan cilexetil and 50 µg/mL of hydrochlorothiazide was used for all investigations.

3.2.3.1 pH of buffer

Effects of pH were studied in the mobile phase consisting of methanol and 10 mM phosphate buffer (50:50, v/v). The pH of phosphate buffer was varied from 3.0 to 8.0.

3.2.3.2 Types of organic solvents

Effects of types of organic solvents were studied by varying commonly used solvents, acetonitrile and methanol, as organic modifier in mobile phase.

3.2.3.3 Concentrations of organic solvents

Ratios of organic solvent and 10 mM phosphate buffer were adjusted to obtain optimum separation and short run time.

3.3 Method validation

3.3.1 Stability of analytes in the solutions

Stability of candesartan cilexetil and hydrochlorothiazide in solutions was studied by storing standard stock solutions and standard mixture solution of the drugs for 48 hours at room temperature, followed by the analysis of the solutions. The results were compared with those of freshly prepared solutions. Acceptable stability criteria for the assay method are not more than 2.0% change in peak areas and retention time (41).

3.3.2 Linearity

Linearity was performed by analyzing six concentrations of standard mixture in the concentration range from 25.0µg/mL to 75.0 µg/mL for hydrochlorothiazide and from 16 µg/mL to 48 µg/mL for candesartan cilexetil (n = 3). Calibration curves were plotted (responses versus concentrations). The coefficients of determination, y-intercept and slope of the regression lines of each drug were calculated using Microsoft® Excel 2010 program. Correlation coefficient (r) of ≥ 0.99 is acceptable criterion for linearity.

Calibration curve equation is presented as follow:

$$y = ax + b$$

Where: y = peak area of the analyte
a = slope of the regression line
x = concentration of the analyte
b = intercept of the regression line

3.3.3 Accuracy

Accuracy of the method was assessed by standard addition technique. Because there was no placebo available, the sample that was used for accuracy study was C1. Different known amounts of standard solutions of candesartan cilexetil and hydrochlorothiazide, which were in the concentration range of linearity, were added to the same concentration of samples (8 µg/mL of candesartan cilexetil and 12.5 µg/mL of hydrochlorothiazide) (Table 3.4). The experiments were triplicately performed at three concentration levels.

Accuracy was expressed as percent recovery of known added amounts of standard to the sample:

$$\% \text{ Recovery} = \frac{\text{Amount found}}{\text{Amount added}} \times 100$$

Where:

Amount found = total concentration of spiked sample – concentration of sample

Table 3.4 Accuracy protocol

Standard addition	Candesartan cikexetil standard solution added (mL)	Hydrochlorothiazide standard solution added (mL)	Sample solution (160 µg/mL candesartan cilexetil + 250 µg/mL hydrochlorothiazide) (mL)	Adjust volume (mL)
0%	0.00	0.00	0.50	10
50%	0.25	0.25	0.50	10
100%	0.75	0.75	0.50	10
150%	1.25	1.25	0.50	10

3.3.4 Precision

3.3.4.1 Intra-day precision

Three concentration levels of standard mixtures (8, 24, 40 µg/mL for candesartan cilexetil and 12.5, 37.5, 62.5 µg/mL for hydrochlorothiazide) were analyzed. Three determinations were performed for each concentration level on the same day. Each concentration was injected three times.

3.3.4.2 Inter-day precision

For inter-day precision, the same three concentrations as for intra-day were studied on six different days. Each concentration was injected three times.

Precision was expressed as relative standard deviation (%RSD):

$$\%RSD = \frac{SD}{Mean} \times 100$$

Where: SD = standard deviation

Mean = average value of determinations

Standard deviation was calculated as follow:

$$SD = \sqrt{\frac{(x - X)^2}{N - 1}}$$

Where: x = each value of the samples

X = average value of the samples

N = number of samples

3.3.5 Specificity

Specificity was performed to exclude the possibility of interference (e.g. excipients, impurities, degradation products and mobile phase composition) in the region of elution of hydrochlorothiazide and candesartan cilexetil.

Specificity was done by comparing the chromatograms of standard solutions, sample solution, degraded drug solutions and blank. Standard mixture contained 32 µg/mL of candesartan cilexetil and 50 µg/mL of hydrochlorothiazide. Sample solution was obtained from drug tablets also contained 32 µg/mL of candesartan cilexetil and 50 µg/mL of hydrochlorothiazide. Degraded solutions of the two drugs in forced degradation studies were mixed together, diluted and injected to HPLC. Blank was prepared in the same way as standard mixture without adding the drugs.

3.3.6 Range

Range was set up by the evaluation of linearity, accuracy and precision

3.3.7 Robustness

The robustness of the method was evaluated by varying four analytical parameters: Buffer pH, flow rate (F), column temperature (T) and organic solvent concentration (%B). Each parameter was varied in a specified range. Buffer pH was from 6.8 to 7.2 (nominal pH ± 0.2), flow rate from 0.9 mL/min to 1.1 mL/min (nominal flow rate ± 0.1 mL/min), temperature from 23^{o}C to 27^{o}C (nominal temperature ± 2^{o}C) and organic solvent concentration from 31% to 33% (nominal concentration ± 1%). All of the parameters were studied by varying one factor at a time. The influence of the variables on the resolution of candesartan cilexetil and hydrochlorothiazide was determined.

3.4 Forced degradation studies

3.4.1 Preparation of sample solutions

Sample solutions of candesartan cilexetil (1 mg/mL) and hydrochlorothiazide (1 mg/mL) were separately prepared by dissolving accurately weighed amount of drug raw materials in a mixture of acetonitrile and water (80:20, v/v).

Solutions of the two drugs were subjected to acid/base/neutral hydrolysis, oxidation, thermal degradation and photolysis stressed tests as shown in Tables 3.4 and 3.6. The remained drugs were determined by the developed HPLC method and their stability profiles were established.

3.4.2 Forced degradation condition

For hydrolysis and oxidation, an amount of 15 mL of candesartan cilexetil or hydrochlorothiazide sample solution (1 mg/mL) was transferred into a 50 mL volumetric flask and treated with 5 mL of HCl solution, NaOH solution, water or 30% H_2O_2 (Table 3.7 and 3.8). Each mixture was heated on a water bath, withdrawn at different time intervals and adjusted to volume with 80% acetonitrile in water. The solution was diluted 10 times with mobile phase before injected to the HPLC system.

For thermal degradation and photolysis, 50 mg of candesartan cilexetil or hydrochlorothiazide raw material was spread on a petri-dish and the petri-dish was kept in an oven at 80^oC or under the sunlight for 2 weeks. The drug was collected and dissolved in 80% acetonitrile in water to the volume of 50 mL. Portions of the solution (15 mL) was transferred to a 50 mL volumetric flask and adjusted to volume with 80% acetonitrile in water, followed by ten- time- dilution with mobile phase before injected to HPLC.

Table 3.5 Stability study protocol of candesartan cilexetil

Stress test	**Condition**	**Duration**
Hydrolysis		
Base (0.1 N NaOH)	Heated at 40^{o}C	60 min
Acid (0.1 N HCl)	Heated at 40^{o}C, 80^{o}C	4 h
Neutral (water)	Heated at 80^{o}C	6 h
Oxidation		
30% H_2O_2	Room temperature	24 h
Thermal degradation	Heated at 80^{o}C	2 weeks
Photolysis	Sunlight	2 weeks

Table 3.6 Stability protocol of hydrochlorothiazide

Stress test	**Condition**	**Duration**
Hydrolysis		
Base (1 N NaOH)	Heated at 80^{o}C	6 h
Acid (1 N HCl)	Heated at 80^{o}C	6 h
Neutral (water)	Heated at 80^{o}C	6 h
Oxidation		
30% H_2O_2	Room temperature	24 h
Thermal degradation	Heated at 80^{o}C	2 weeks
Photolysis	Sunlight	2 weeks

Table 3.7 Preparation of forced degradation solutions of candesartan cilexetil

Stress test	**Sample solution (mL)**	**0.1 N NaOH (mL)**	**0.1 N HCl (mL)**	**Water (mL)**	**30% H_2O_2 (mL)**	**Volume (mL)**
Initial	15					50
Base	15	5				50
Acid	15		5			50
Neutral	15			5		50
Oxidation	15				5	50

Table 3.8 Preparation of forced degradation solutions of hydrochlorothiazide

Stress test	**Sample solution (mL)**	**1 N NaOH (mL)**	**1 N HCl (mL)**	**Water (mL)**	**30% H_2O_2 (mL)**	**Volume (mL)**
Initial	15					50
Base	15	5				50
Acid	15		5			50
Neutral	15			5		50
Oxidation	15				5	50

3.5 Analysis of pharmaceutical products

The validated method was applied to analyze four pharmaceutical products of candesartan cilexetil and hydrochlorothiazide, two fixed dose combinations and two single drug products. Products of the same batch for each dosage form were purchased from local drug stores. Five replications were carried out for each product. Percent RSD of determinations were calculated, which should not exceed 2%.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Method development

4.1.1 Effects of pH of buffer

Effects of pH on the separation and retention time of candesartan cilexetil and hydrochlorothiazide were studied. Phosphate buffer at different pH varying from 3.0 to 8.0 was investigated. The study was performed in the mobile phase consisting of methanol and 10 mM phosphate buffer (50:50, v/v).

The results showed that hydrochlorothiazide had a sharp peak at every pH and its retention time did not show significant changes with the changes of pH (Figure 4.1), the retention time was between 2.0 and 2.2. This might be due to the fact that hydrochlorothiazide is a weak acid with pKa = 7.9, thus it was mostly in neutral form in the pHs between 3.0 and 7.0. However, candesartan cilexetil is an acidic compound with pKa = 6.0, so it was strongly affected by pHs from 5.0 to 8.0, decreasing retention time as pH increased (Figure 4.2) since it changed from neutral form to ionized form. Candesartan cilexetil had high retention at pHs 3.0 and 4.0 (retention time > 20min). From pHs 5.0 to 8.0, the retention time decreased from 19.90 min to 6.89 min. Due to the limited tolerance of HPLC column, mobile phase at pH 7.0 was chosen.

4.1.2 Effects of types of organic solvents

Different organic solvents have different effects on the separation. Methanol and acetonitrile, the most commonly used organic solvents in HPLC, were studied. Results revealed that acetonitrile at concentration of 35% gave shorter retention time and better peak shape for candesartan cilexetil in comparison to methanol at concentration of 50% (Figure 4.3). When switching from methanol to acetonitrile, the number of theoretical plates of candesartan cilexetil increased from

1487 to 6203 and asymmetry factor decreased from 1.35 to 1.16. Consequently, acetonitrile was chosen as the optimal organic modifier.

The results can be explained in term of eluent strength and solvation ability of organic solvents. In RP-HPLC, acetonitrile has higher eluent strength compared to that of methanol, which means that a higher amount of methanol (roughly a 10% increase in methanol content) compared to acetonitrile is necessary in order for an analyte to obtain similar retention. Besides, candesartan cilexetil was mostly in ionic form at pH 7.0, so it was more hydrophilic and tended to be more solvated with protic solvent. Methanol is a protic solvent and it can participate in the analyte solvation, which may lead to significant distortion of the peak shape (42). Therefore, when using acetonitrile even at lower concentration than methanol we still got a shaper and more symmetric peak of candesartan cilexetil with shorter retention time.

4.1.3 Effects of concentrations of organic solvent

Ratios of acetonitrile were varied from 30% to 35% in order to obtain optimum separation and short run time between candesartan cilexetil and hydrochlorothiazide. As the concentration of the organic modifier increases, elution strength increases. As a result, the retention of the analytes decreases. Table 4.1 shows the effects of acetonitrile concentration on the retention of candesartan cilexetil and hydrochlorothiazide. Mobile phase that consists of acetonitrile and 10 mM phosphate buffer (35: 65, v/v) offered the best separation with a reasonable short run time. Therefore, this mobile phase was originally selected to apply to the following stability study of candesartan cilexetil and hydrochlorothiazide. However, the mobile phase did not offer acceptable resolution between critical peak pair of hydrochlorothiazide and its degradation product. To obtain a stability indicating method, mobile phase consisting of acetonitrile and 10 mM phosphate buffer (32: 68, v/v), which gave reasonable resolution of the critical peak pair (R_s = 1.5), was used in the final condition.

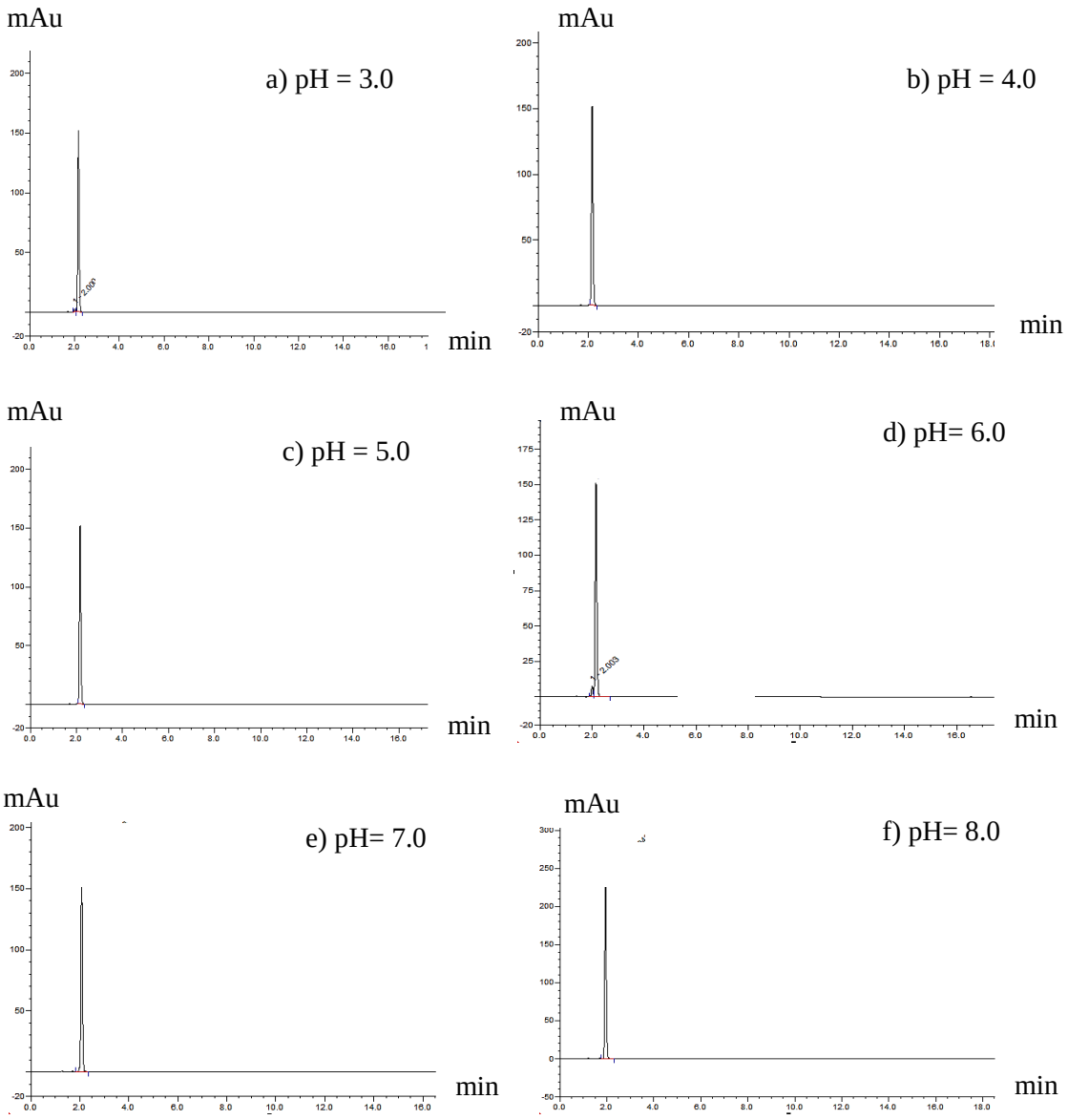


Figure 4.1 Effects of pH buffer on the retention time of hydrochlorothiazide (50 µg/mL). Conditions: column: SB CN column 150×4.6 mm i.d., 5µm; mobile phase: methanol and 10 mM phosphate buffer (50:50, v/v) a) pH 3.0, b) pH 4.0, c) pH 5.0, d) pH 6.0, e) pH 7.0 and f) pH 8.0; flow rate 1 mL/min; injection volume 20 µL; detection at 254 nm; run time 20 min; ambient temperature.

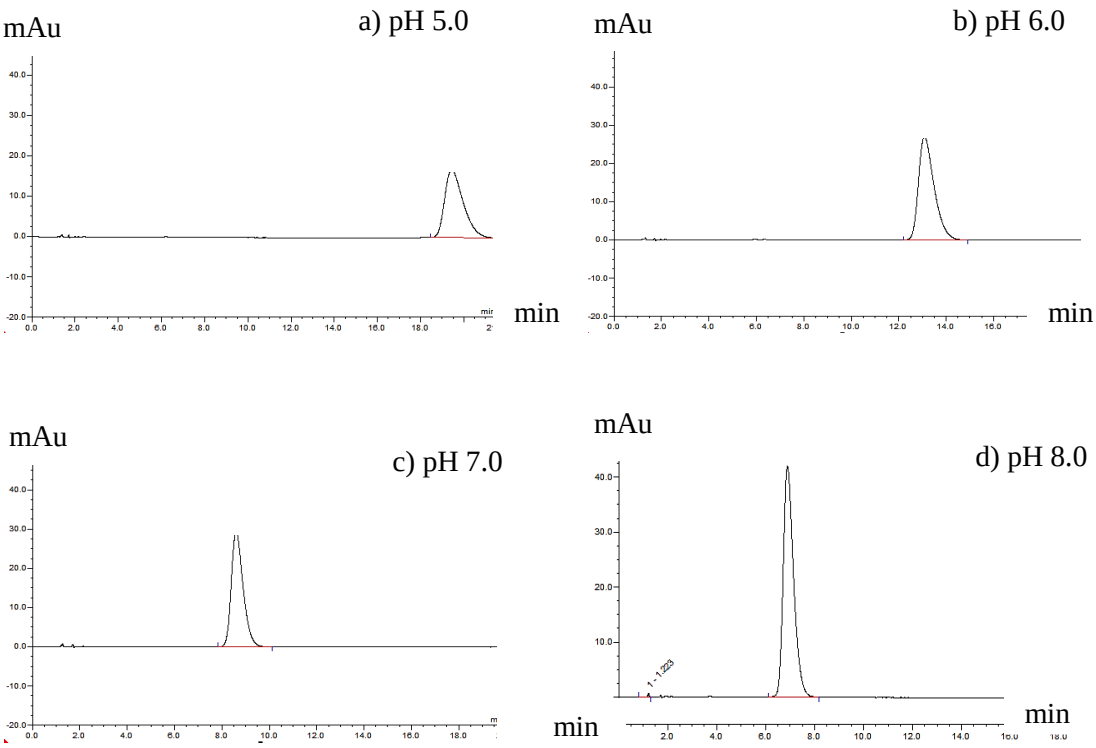


Figure 4.2 Effects of pH buffer on the retention time of candesartan cilexetil (32 µg/mL). Conditions: column: SB CN column 150×4.6 mm i.d., 5µm; mobile phase: methanol and 10 mM phosphate buffer (50:50, v/v) a) pH 5.0, b) pH 6.0, c) pH 7.0 and d) pH 8.0; flow rate 1 mL/min; injection volume 20 µL; detection at 254 nm; run time 20 min; ambient temperature.

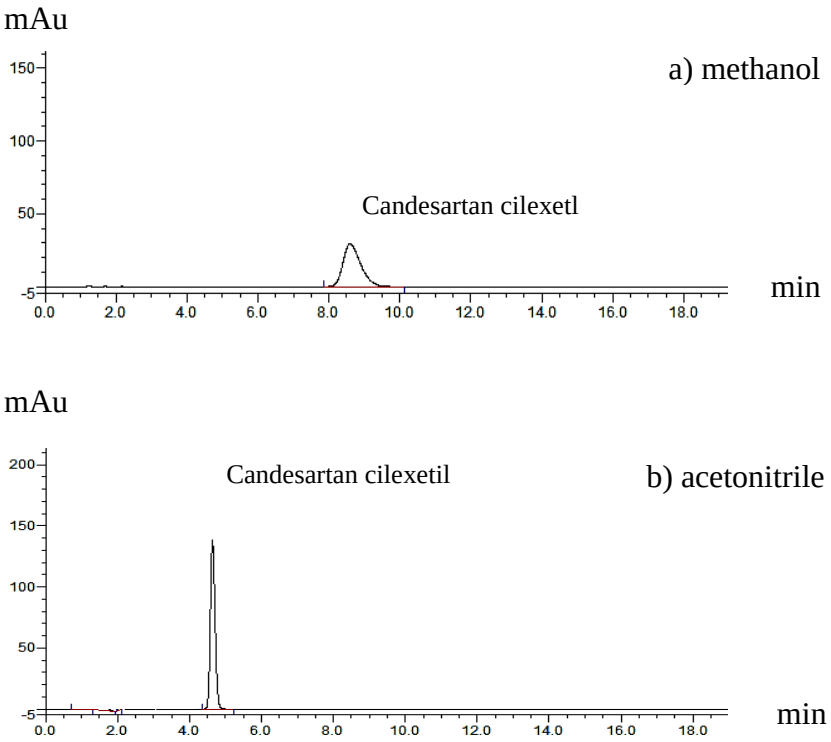


Figure 4.3 Effects of types of organic solvent on the retention time of candesartan cilexetil (32 µg/mL). Condition: column: SB CN column 150×4.6 mm i.d., 5µm; mobile phase a) methanol: 10 mM phosphate buffer pH 7.0 (50:50, v/v) and b) acetonitrile: 10 mM phosphate buffer pH 7.0 (35:65, v/v); flow rate 1 mL/min; injection volume 20 µL; detection at 254 nm; run time 20 min; ambient temperature.

Table 4.1 Effects of ratios of acetonitrile and buffer

Compound	Acetonitrile:Buffer (v/v)	Retention time	Asymmetry factor	Number of theoretical plates
Hydrochlorothiazide	35:65	2.52	1.08	8,000
	32:68	2.68	1.05	7,600
	30:70	2.81	1.06	7,500
Candesartan cilexetil	35:65	4.64	1.16	6,200
	32:68	7.81	1.17	5,700
	30:70	11.74	1.23	6,100

Finally, the optimum condition for the separation of candesartan cilexetil and hydrochlorothiazide was on a Zobax SB CN column (150 × 4.6 mm i.d., 5 µm) using the mixture of acetonitrile and 10 mM phosphate buffer pH 7.0 (32:68, v/v) as mobile phase. Flow rate was set at 1 mL/min and column temperature was controlled at 25°C. The UV detection was at 254 nm. Figure 4.4 shows the chromatogram of standard mixture in which the concentration ratio of candesartan cilexetil and hydrochlorothiazide was the same as that in combined drug. Summary of chromatographic characteristics were tabulated in Table 4.2.

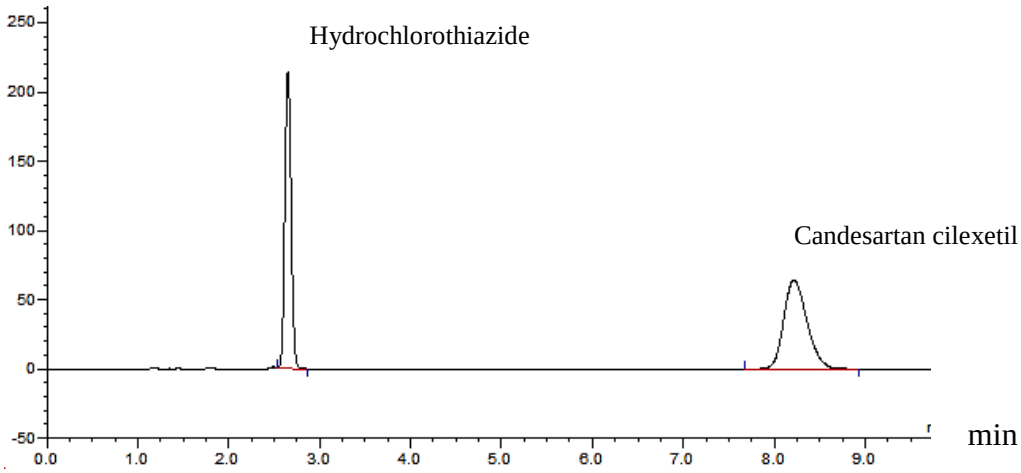


Figure 4.4 Typical chromatogram of standard mixture of candesartan cilexetil (32 µg/mL) and hydrochlorothiazide (50 µg/mL). Condition: column: SB CN column 150×4.6 mm i.d., 5µm; mobile phase: acetonitrile: 10 mM phosphate buffer pH 7.0 (32:68, v/v); flow rate 1 mL/min; injection volume 20 µL; detection at 254 nm; run time 10 min; temperature 25°C.

Table 4.2 Summary of chromatographic characteristics

Analyte	Retention time (t_R) (min)	Capacity factor (k')	Asymmetry factor	Plate number (N)	Resolution (R_s)
Hydrochlorothiazide	2.65	0.51	1.10	7,000	-
Candesartan cilexetil	8.21	3.66	1.22	5,000	18.9

4.2 Method validation

4.2.1 Stability of analytes in the solutions

Tables 4.3 and 4.4 show the stability data of the analytes in standard stock solutions and in standard mixture solution. As can be seen in the Tables, the stock solutions were stable for 48h with less than 2.00% change in peak area and retention time. The %RSD of percent peak area was 1.06 for candesartan cilexetil and 0.79 for hydrochlorothiazide. In addition, the %RSD of retention time was less than 0.29 and 0.07 for candesartan cilexetil and hydrochlorothiazide, respectively. However, standard mixture was stable for only 24 h, because after 48 h the change in peak area of hydrochlorothiazide was more than 2.00% (percent peak area = 97.47%).

4.2.2 Linearity and range

Linearity was performed by analyzing six concentrations of standard mixture in the concentration range from 50-150% target concentrations which was from 12.5 μg/mL to 62.5 μg/mL for hydrochlorothiazide and from 16 μg/mL to 48 μg/mL for candesartan cilexetil (n = 3). Calibration curves were plot and correlation coefficients were more than 0.999. Data were tabulated in Table 4.5 and 4.6. Results of linearity and range are shown in Figure 4.5.

Table 4.3 Stability data of candesartan cilexetil and hydrochlorothiazide in stock solution

Time	Candesartan cilexetil		Hydrochlorothiazide	
(h)	%peak area	t_R	%peak area	t_R
0	100.00	8.18	100.00	2.66
24	101.81	8.15	99.76	2.66
48	101.90	8.14	98.54	2.66
Average	101.24	8.16	99.43	2.66
SD	1.07	0.02	0.78	0.00
%RSD	1.06	0.29	0.79	0.07

Table 4.4 Stability data of candesartan cilexetil and hydrochlorothiazide in standard mixture solution

Time (h)	Candesartan cilexetil		Hydrochlorothiazide	
	% peak area	t_R	% peak area	t_R
0	100.00	8.19	100.00	2.66
24	100.79	8.17	98.42	2.66
48	99.33	8.15	97.47	2.66
Average	100.04	8.17	98.63	2.66
SD	0.73	0.02	1.28	0.00
%RSD	0.73	0.25	1.30	0.06

Table 4.5 Linearity data of candesartan cilexetil

Concentration of solutions (µg/mL)	Peak area	Average	SD	%RSD
16.0	9.207 9.404 9.266	9.292	0.10	1.09
24.0	13.880 13.933 13.909	13.907	0.03	0.19
28.8	16.633 16.699 16.687	16.673	0.04	0.21
32.0	18.638 18.676 18.848	18.720	0.11	0.60
40.0	23.336 23.310 23.420	23.355	0.06	0.25
48.0	28.123 28.016 28.100	28.080	0.06	0.20

Table 4.6 Linearity data of hydrochlorothiazide

Concentration of solutions (µg/mL)	**Peak area**	**Average**	**SD**	**%RSD**
25.0	8.493 8.432 8.532	8.485	0.05	0.60
37.5	12.694 12.678 12.795	12.722	0.06	0.50
45.0	15.215 15.035 15.383	15.211	0.17	1.14
50.0	16.881 16.919 16.745	16.848	0.09	0.54
62.5	21.021 21.289 20.954	21.088	0.18	0.84
75.0	25.088 25.068 24.890	25.015	0.11	0.44

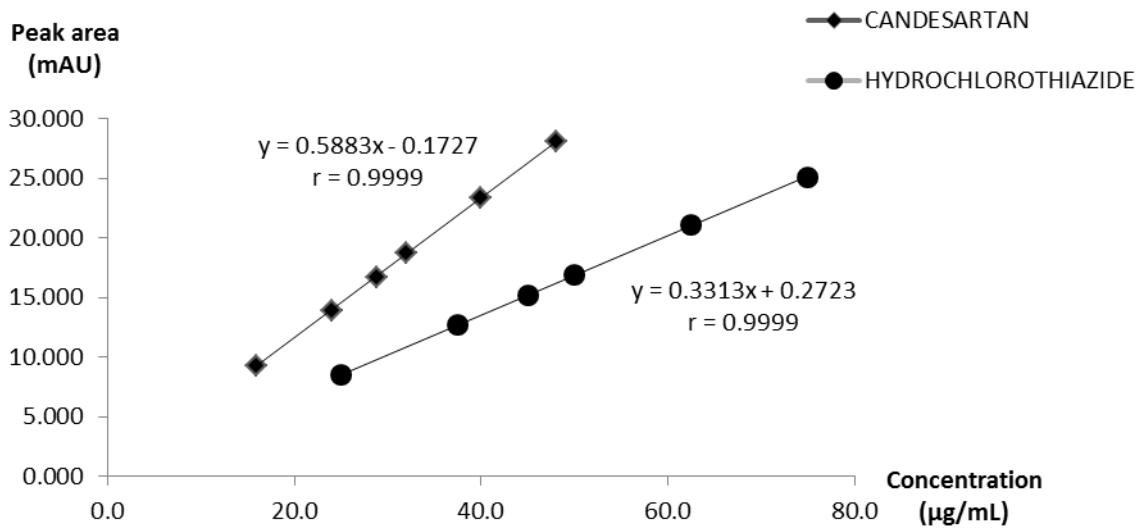


Figure 4.5 Linearity of candesartan cilexetil and hydrochlorothiazide

4.2.3 Accuracy

Accuracy of the method was evaluated by adding different known amounts of standard mixture to the same concentration of samples and the spiked samples were analyzed by the developed method. Percent recovery was in the range of 99.1% to 101.0% for candesartan cilexetil and 99.4% to 100.2% for hydrochlorothiazide (Table 4.7).

4.2.4 Precision

Precision data of the method were presented as %RSD of percent recovery of standard added. Table 4.7 shows the intra-day precision data of the method, the %RSD was less than 1.66. Table 4.8 shows the intermediate precision data of the method, the %RSD was less than 1.84.

Table 4.7 Accuracy and intra-day precision data of candesartan cilexetil and hydrochlorothiazide

Analyte	Concentration added (µg/mL)	Conncentration found (µg/mL)			Average (µg/mL)	% Recovery	%RSD
		1	2	3			
Candesartan cilexetil	8.0	7.90	8.07	7.81	7.93	99.1	1.66
	24.0	23.98	24.33	24.39	24.23	101.0	0.91
	40.0	40.53	40.27	39.89	40.23	100.6	0.81
Hydrochlorothiazide	12.5	12.59	12.47	12.46	12.51	100.1	0.60
	37.5	37.44	37.90	37.36	37.57	100.2	0.78
	62.5	62.17	61.44	62.84	62.15	99.4	1.13

4.2.5. Specificity

Figure 4.6 shows the specificity of the method for candesartan cilexetil and hydrochlorothiazide. The peaks of the two drugs were separated from each other with high resolution. Moreover, the peaks of candesartan cilexetil and hydrochlorothiazide were not interfered by peaks of degradation products with resolution of critical peak pair of 1.5. Additionally, peak purity values of candesartan cilexetil and hydrochlorothiazide were more than 990 by analysis using PDA detection, which indicated that there was no co-eluting peak.

Table 4.8 Inter-day precision data of candesartan cilexetil and hydrochlorothiazide

Analyte	Concentration added (µg/mL)	% recovery						Average (%)	SD	%RSD
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6			
Candesartan cilexetil	8.0	99.1	101.1	100.6	100.7	102.9	102.4	101.1	1.36	1.34
	24.0	101.0	101.2	100.3	100.0	100.0	100.1	100.4	0.53	0.53
	40.0	100.6	100.5	99.7	100.0	99.0	99.4	99.9	0.61	0.61
Hydrochlorothiazide	12.5	99.3	102.7	99.9	99.2	100.1	97.1	99.7	1.83	1.84
	37.5	99.6	100.3	100.3	100.2	101.1	99.0	100.1	0.71	0.71
	62.5	99.2	100.1	99.1	100.5	99.4	98.6	99.5	0.69	0.69

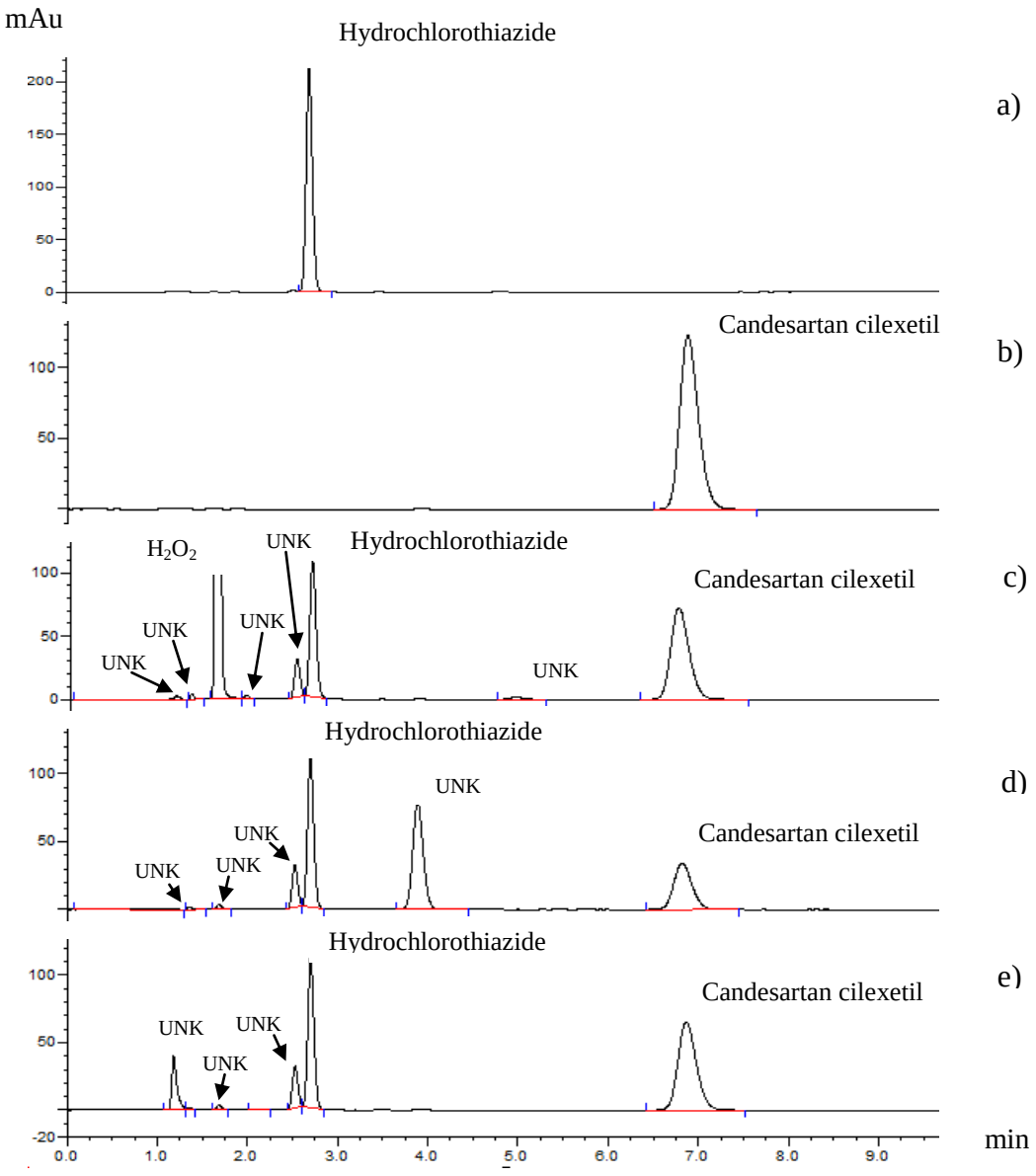


Figure 4.6 Specificity of the method for candesartan cilexetil (32 µg/mL) and hydrochlorothiazide (50 µg/mL). Chromatograms of a) standard hydrochlorothiazide, b) standard candesartan cilexetil, mixture of base hydrolysis solution of hydrochlorothiazide and c) oxidation solution of candesartan cilexetil or d) acid hydrolysis solution of candesartan cilexetil or e) base hydrolysis solution of candesartan cilexetil. Condition: column: SB CN column 150×4.6 mm i.d., 5µm; mobile phase: acetonitrile: 10 mM phosphate buffer pH 7.0 (32:68, v/v); flow rate 1 mL/min; injection volume 20 µL; detection at 254 nm; run time 20 min; temperature 25°C; UNK: Unknown.

4.2.6 Robustness

For robustness study, buffer pH, flow rate (F), column temperature (T) and organic solvent concentration (%B) were varied in specified ranges. Peak area and retention time were characteristics that showed significant changes with the changes of the parameters. Other characteristics i.e., asymmetry factor, number of theoretical plates and resolution also encountered changes with the variation of the parameters. However, the changes were not significant and the values were within acceptable limits (Table 4.9).

Table 4.9 Robustness data on asymmetry factor, plate number and resolution

Condition	Candesartan cilexetil		Hydrochlorothiazide		Resolution
	Asymmetry factor	Plate number	Asymmetry factor	Plate number	
*Nomal	1.23	5,000	1.10	6,800	18.2
%B = 31%	1.19	5,700	1.09	6,700	21.1
%B = 33%	1.26	5,200	1.10	6,900	15.1
F = 1.1 mL/min	1.23	5,200	1.11	7,200	18.9
F = 0.9 mL/min	1.21	4,600	1.08	6,600	17.8
T = 23^{o}C	1.25	4,800	1.09	6,900	18.5
T = 27^{o}C	1.23	4,900	1.10	6,900	18.3
pH = 6.8	1.25	5,200	1.10	7,000	19.8
pH = 7.2	1.19	4,900	1.08	7,000	17.0

*Normal condition: %B = 32%, F = 1.0 mL/min, T = 25^{o}C, pH = 7.0

4.3 Forced degradation studies

4.3.1 Forced degradation of candesartan cilexetil

Candesartan cilexetil was stressed under various conditions. Percent of remained drug in each condition was calculated and four unknown degradation products were observed using the developed HPLC method. Data were tabulated in

Tables 4.10 to 4.13. Stability profiles on stability of candesartan cilexetil were illustrated in Figures 4.7 to 4.10.

For hydrolysis, oxidation, thermal degradation and photolysis conditions, candesartan cilexetil showed different stability profiles. The drug was highly susceptible to base hydrolysis as it quickly degraded from 100% to 8.4% after 60 min at 40°C. It was also unstable in acid hydrolysis, which it decreased from 100% to 90.2% at 40°C and degraded completely after 4 h at 80°C. However, candesartan cilexetil was stable in neutral hydrolysis as the percent assay remained 98.0% after 6 h at 80°C. Under chemical oxidation by 30% H_2O_2, candesartan cilexetil slowly declined to 79.4% after 24 h at room temperature. The drug was found to be stable under thermal degradation and photolysis where the percent assay was 98.3% and 99.5%, respectively after 2 weeks of exposure.

Table 4.10 Degradation data of candesartan cilexetil in 0.1 N NaOH, heated at 40°C*

Time (min)	**% Candesartan cilexetil**	**Unknown 1 (RRT = 0.16)**	**Unknown 2 (RRT = 0.25)**
0	100.0	×	×
10	65.2	✓	✓
20	43.4	✓	✓
40	21.7	✓	✓
60	8.4	✓	✓

*×: Not observed, ✓: Observed

Table 4.11 Degradation data of candesartan cilexetil in 0.1 N HCl heated at 40°C, 80°C*

Time (h)	**% Candesartan cilexetil at 40°C**	**% candesartan cilexetil at 80°C**	**Unknown 3 (RRT = 0.55)**
0	100.0	100.0	×
1	96.7	14.3	✓
2	95.4	1.8	✓
3	91.2	0.7	✓
4	90.2	0.0	✓

*×: Not observed, ✓: Observed

Table 4.12 Degradation data of candesartan cilexetil in water, heated at 80°C*

Time (h)	% Candesartan cilexetil	Unknown 3 (RRT = 0.57)
0	100.0	×
1	99.4	✓
2	98.7	✓
4	98.2	✓
6	98.0	✓

*×: Not observed, ✓: Observed

Table 4.13 Degradation data of candesartan cilexetil in 30% H_2O_2 at room temperature*

Time (h)	**% Candesartan cilexetil**	**Unknown 1 (RRT = 0.14)**	**Unknown 4 (RRT = 0.70)**
0	100.0	×	×
1	89.9	✓	✓
6	86.3	✓	✓
15	83.8	✓	✓
24	79.4	✓	✓

*×: Not observed, ✓: Observed

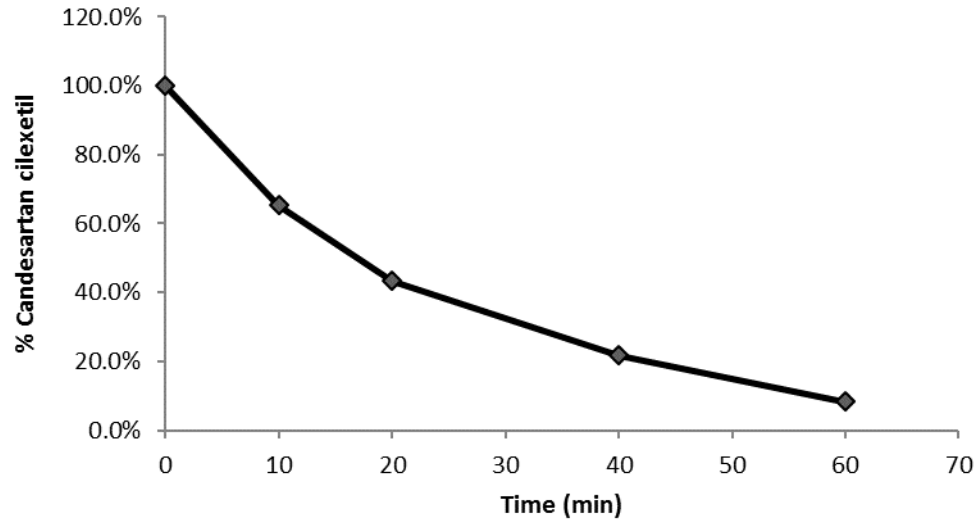


Figure 4.7 Stability profile for the degradation of candesartan cilexetil in 0.1 N NaOH heated at 40°C.

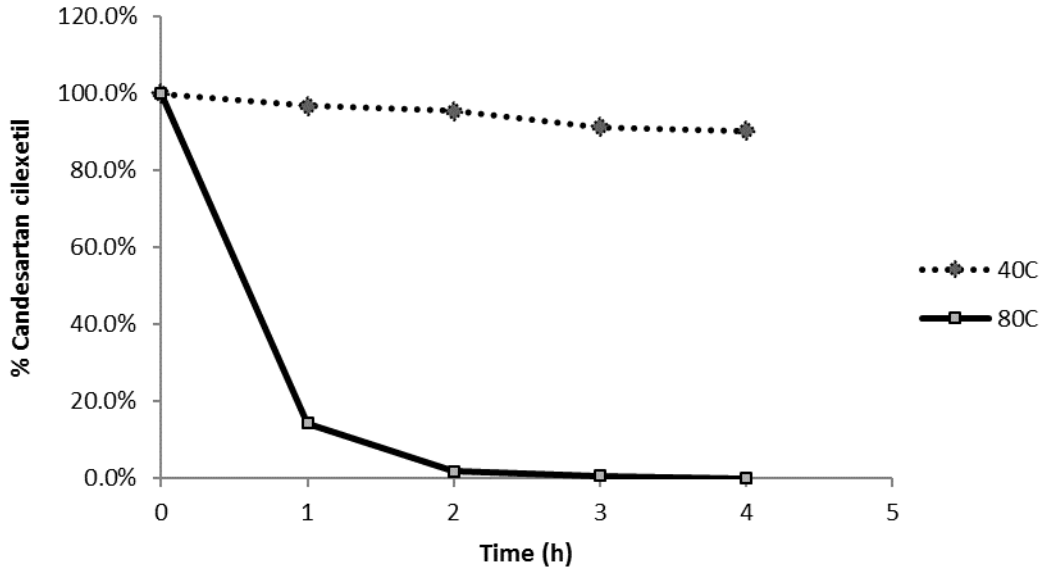


Figure 4.8 Stability profile for the degradation of candesartan cilexetil in 0.1 N HCl heated at 40°C and 80°C.

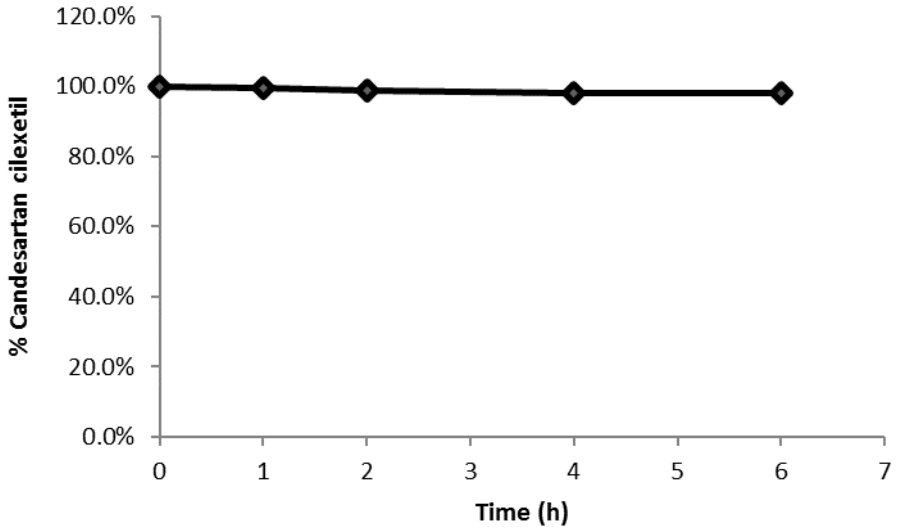


Figure 4.9 Stability profile for the degradation of candesartan cilexetil in water heated at 80^{o}C.

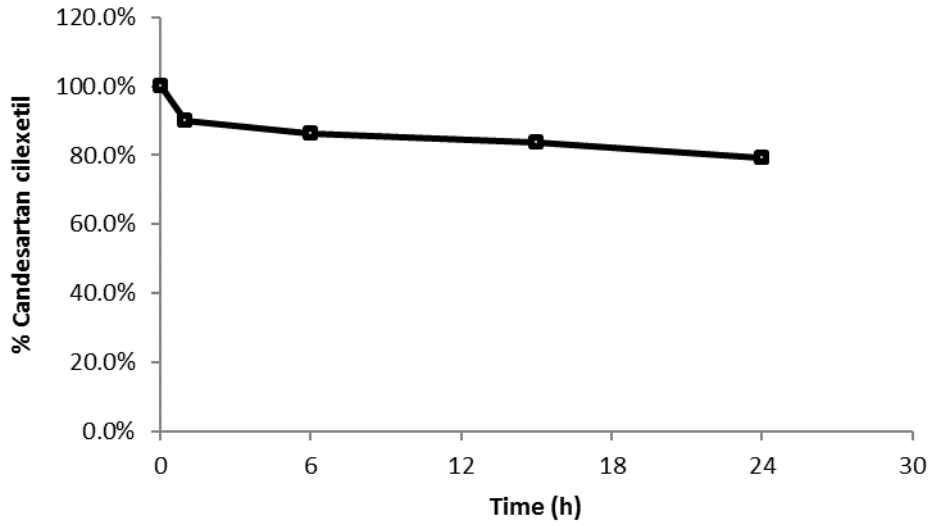


Figure 4.10 Stability profile for the degradation of candesartan cilexetil in 30% H_2O_2 at room temperature.

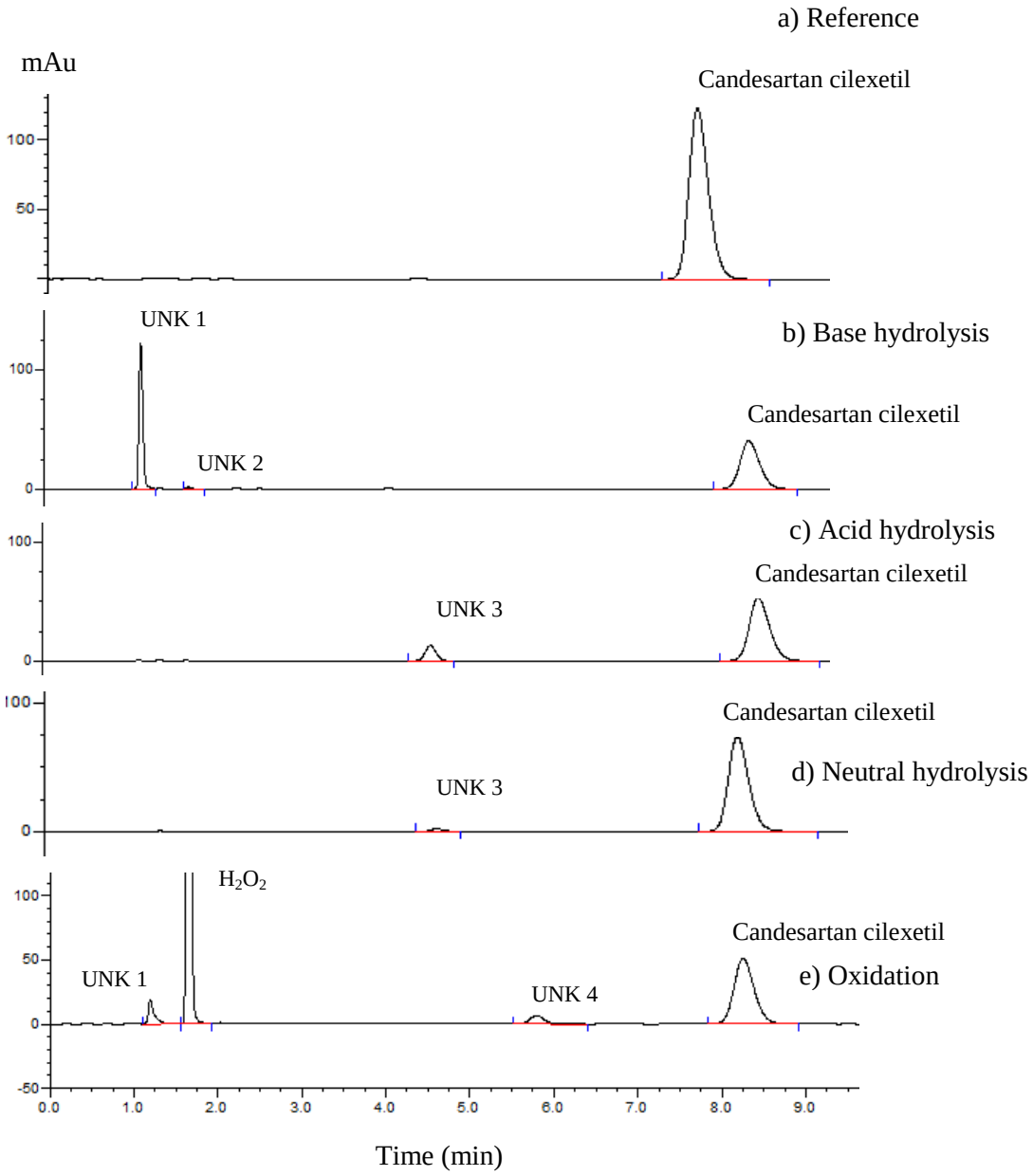
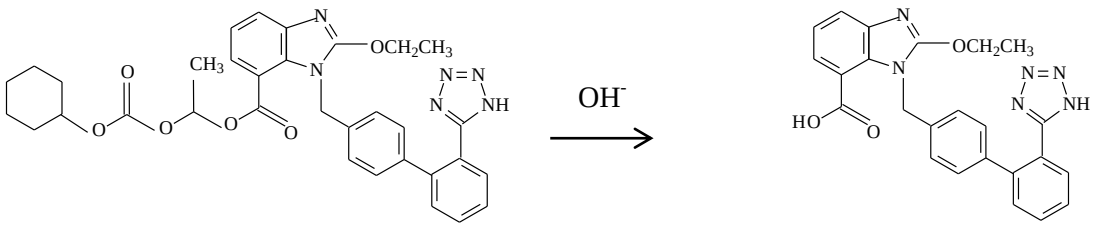


Figure 4.11 Chromatograms of forced degradation solutions of candesartan cilexetil (32 µg/mL). Condition: column: SB CN column 150×4.6 mm i.d., 5µm; flow rate 1 mL/min; ; mobile phase: acetonitrile: 10 mM phosphate buffer pH 7.0 (32:68, v/v); flow rate 1 mL/min; injection volume 20 µL; detection at 254 nm; run time 20 min; temperature 25^{o}C; UNK: Unknown.

There were four unknown degradation products, named from 1 to 4, which were observed from stressed solutions. Unknown 1 was found in base hydrolysis and oxidation. Unknown 2 and 4 appeared in base hydrolysis and oxidation respectively. Unknown 3 was observed in both acid and neutral hydrolysis. Representative chromatograms of hydrolysis and oxidation solutions were showed in Figure 4.11. As can be seen from the chromatograms, the retention time of candesartan cilexetil in stressed solutions was slightly changed comparing to that of the reference solution because of the pH modification in the degraded solution.

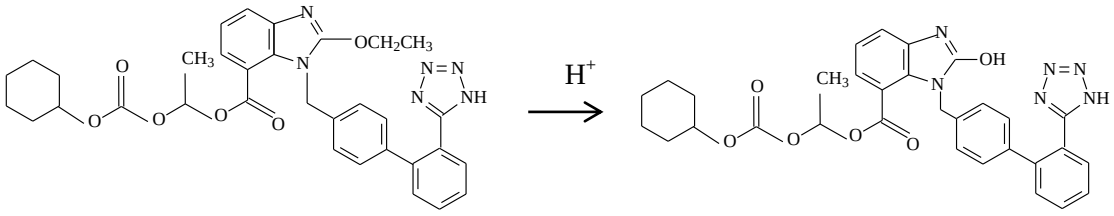
Because base-catalyzed hydrolysis of esters is fast with the powerful attacking –OH nucleophile, candesartan cilexetil was hydrolyzed in base to release candesartan active drug and cilexetil moiety. As a result, unknown 1, which was the major degradation product found in base hydrolysis, might be candesartan (Figure 4.12) (43). However, full investigation should be done for confirmation.



Candesartan cilexetil Candesartan

Figure 4.12 Base hydrolysis of candesartan cilexetil (43).

Acid-catalyzed ester hydrolysis is slower, so the main degradation pathway in acid hydrolysis was to form des ethyl candesartan cilexetil (Figure 4.13) (6). Unknown 3 might be des ethyl candesartan cilexetil.



Candesartan cilexetil Des ethyl candesartan cilexetil

Figure 4.13 Acid hydrolysis of candesartan cilexetil (6).

Candesartan cilexetil degradation occurred in acid and base hydrolysis, and oxidation. Therefore, kinetics of these reactions was investigated. The results showed that degradation process of candesartan cilexetil in base and acid hydrolysis followed first-order kinetics (Figure 4.14 and 4.15) whereas the kinetics of oxidation degradation could not be specified.

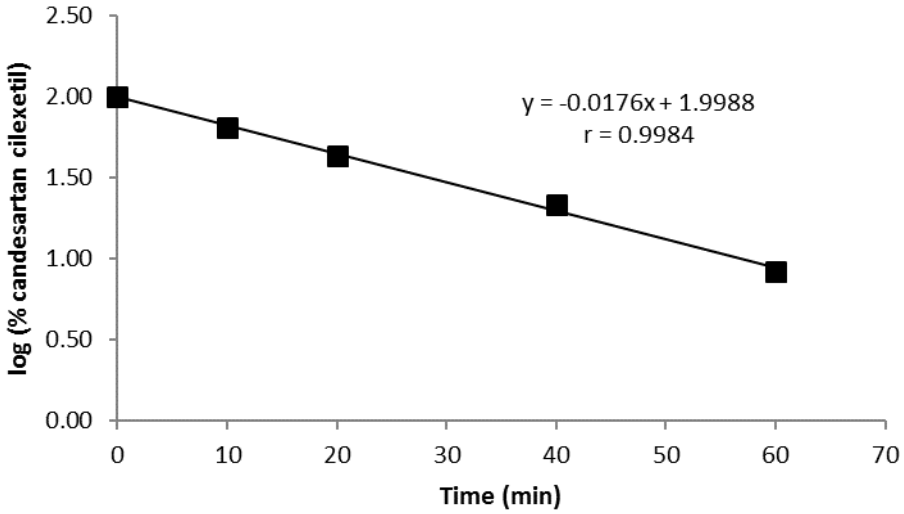


Figure 4.14 Kinetic study of the base-induced degradation of candesartan cilexetil at 80°C.

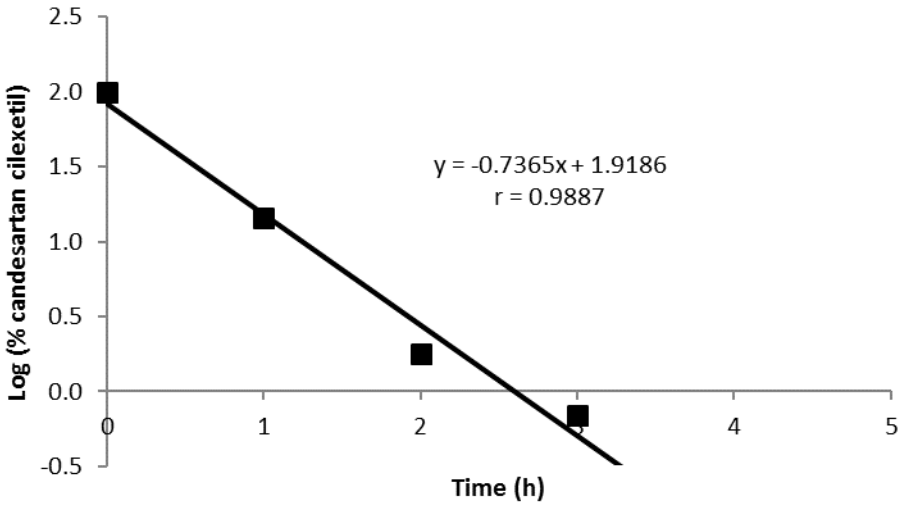


Figure 4.15 Kinetic study of the acid-induced degradation of candesartan cilexetil at 80°C.

For the first-order reaction: Drug → Product

$$\frac{-d[D]}{dt} = k[D]$$

$$\log[D] = \log[D]_0 - \frac{kt}{2.303} \quad (*)$$

$$t_{1/2} = \frac{0.693}{k}$$

$$t_{90} = \frac{0.105}{k}$$

Where D= concentration of drug solution at time t

D_0= initial concentration of drug solution

k= rate constant

Equation (*) has the form of an equation for straight line y = ax + b. Therefore plot of log[D] as a function of time gives straight line with slope of $\frac{-k}{2.303}$,

which helps to calculate half-life and shelf-life of the drug solution under the stressed conditions (Table 4.14).

Table 4.14 Kinetic parameters of candesartan cilexetil under base and acid hydrolysis

	Correlation coefficient (r)	**Rate constant k (s^{-1})**	**Half-life $t_{1/2}$ (s)**	**Shelf-life t_{90} (s)**
Base hydrolysis	0.9984	6.76×10^{-4}	1.03×10^{3}	0.16×10^{3}
Acid hydrolysis	0.9887	4.71×10^{-4}	1.47×10^{3}	0.22×10^{3}

4.3.2 Forced degradation of hydrochlorothiazide

Similar to candesartan cilexetil, hydrochlorothiazide was stressed under various conditions and percent of remained drug in each condition was calculated. The forced degradation studies were carried out following the stepwise approaches reported by Bakshi M. and Singh S.(4) and hydrochlorothiazide did not show significant degradation in 0.1 N NaOH and 0.1 N HCl. Therefore, the concentration of base and acid used for the hydrolysis of hydrochlorothiazide was increased to 1 N, which was much higher than that for candesartan cilexetil. For hydrochlorothiazide three unknown degradation products were observed in the chromatograms of the stressed solutions. Data were tabulated in Tables 4.15 to 4.18. Stability profiles on stability of hydrochlorothiazide were illustrated in Figures 4.16 and 4.17.

The results showed that hydrochlorothiazide is unstable under acid/base/neutral hydrolysis. It is more sensitive to base hydrolysis compared to acid and neutral hydrolysis with percent assay of 48.5%, 80.56% and 77.4%, respectively, after 6 h at 80^{o}C. However, hydrochlorothiazide is stable under oxidative condition at room temperature as percent assay was 97.8% after 24 h. Hydrochlorothiazide is stable under photolysis and thermal degradation with percent assay of 97.0% and 95.8% respectively.

Table 4.15 Degradation data of hydrochlorothiazide in 1 N NaOH and heated at 80°C*

Time (h)	% Hydrochlorothiazide	Unknown 1 (RRT = 0.61)	Unknown 2 (RRT = 0.93)
0	100.0	×	✓
1	88.1	✓	✓
2	84.9	✓	✓
4	62.6	✓	✓
6	48.5	✓	✓

*×: Not observed, ✓: Observed

Table 4.16 Degradation data of hydrochlorothiazide in 1 N HCl and heated at 80°C*

Time (h)	% Hydrochlorothiazide	Unknown 2 (RRT = 0.94)
0	100.0	✓
1	82.5	✓
2	82.1	✓
4	80.7	✓
6	80.6	✓

*×: Not observed, ✓: Observed

Table 4.17 Degradation data of hydrochlorothiazide in water and heated at 80°C*

Time (h)	% Hydrochlorothiazide	Unknown 2 (RRT = 0.93)
0	100.0	✓
1	91.4	✓
2	84.2	✓
4	77.9	✓
6	77.4	✓

*×: Not observed, ✓: Observed

Table 4.18 Degradation data of hydrochlorothiazide in 30% H_2O_2 at room temperature*

Time (h)	% Hydrochlorothiazide	Unknown 2 (RRT = 0.93)	Unknown 3 (RTT = 0.46)
0	100.0	✓	×
1	99.0	✓	✓
6	99.4	✓	✓
15	99.0	✓	✓
24	97.8	✓	✓

*×: Not observed, ✓: Observed

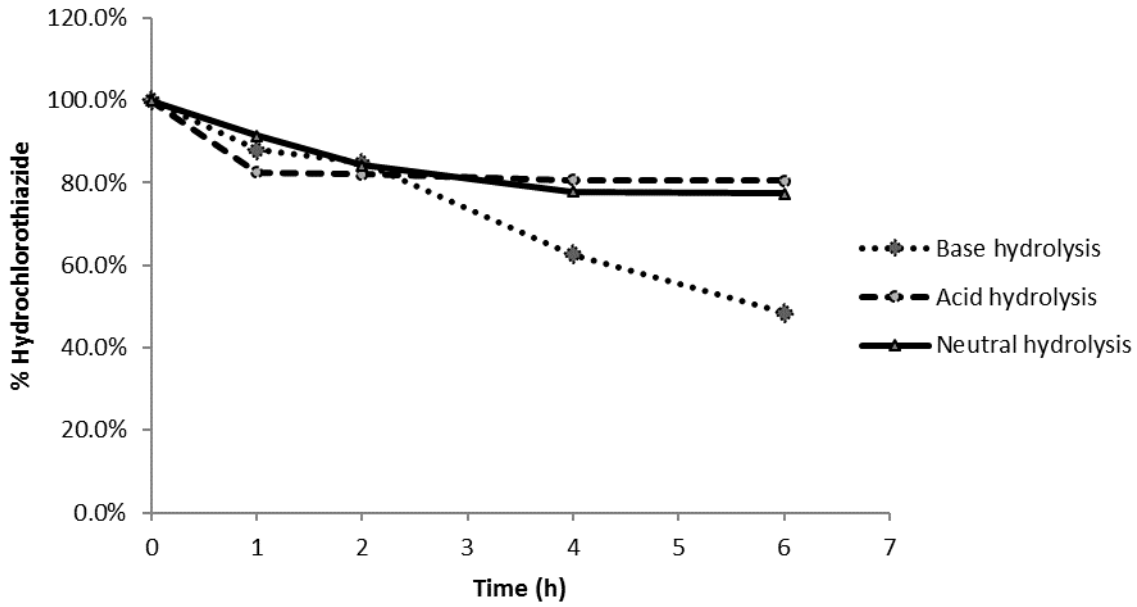


Figure 4.16 Stability profile for the degradation of hydrochlorothiazide in 0.1 N NaOH, 0.1 N HCl and water, heated at 80°C.

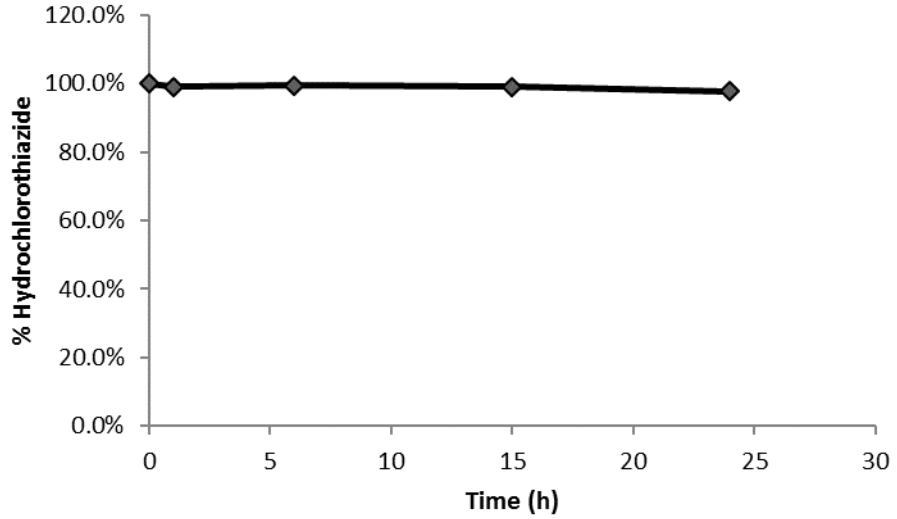


Figure 4.17 Stability profile for the degradation of hydrochlorothiazide in 30% H_2O_2 at room temperature.

Hydrochlorothiazide is synthesized from the reaction between 4-amino-6-chloro-1, 3-benzenedisulfonamide with formaldehyde. However, formaldehyde can cause undesirable dimerization of hydrochlorothiazide during synthesis. Additionally, hydrochlorothiazide is known to decompose via retro-synthetic pathway to generate formaldedyde and the starting material as disulfonamide in its synthesis. Therefore, hydrochlorothiazide dimer and disulfonamide are the two possible degradation products of hydrochlorothiazide (44). The degradation mechanism was illustrated in Figure 4.18.

There were three unknown compounds, named from 1 to 3, in the chromatograms of stressed solutions of hydrochlorothiazide. Unknown 2 appeared in all stressed solutions even in solutions at time 0, which indicated that unknown 2 was initially formed in the drug raw material. As the time increased, the amount of unknown 2 increased in hydrolysis solutions. Therefore, unknown 2 was a product of hydrolysis of hydrochlorothiazide. Unknown 1 and 3 were observerd in small amount in base hydrolysis and oxidation respectively. Representative chromatograms of hydrolysis and oxidation solutions were showed in Figure 4.19.

Unknown 2 was the main degradation products of hydrochlorothiazide. Its retention time was close to and prior to that of hydrochlorothiazide. Therefore,

unknown 2 might be 4-amino-6-chloro-1, 3-benzenedisulfonamide whose chemical structure was similar to hydrochlorothiazide. However, further investigation should be done for confirmation.

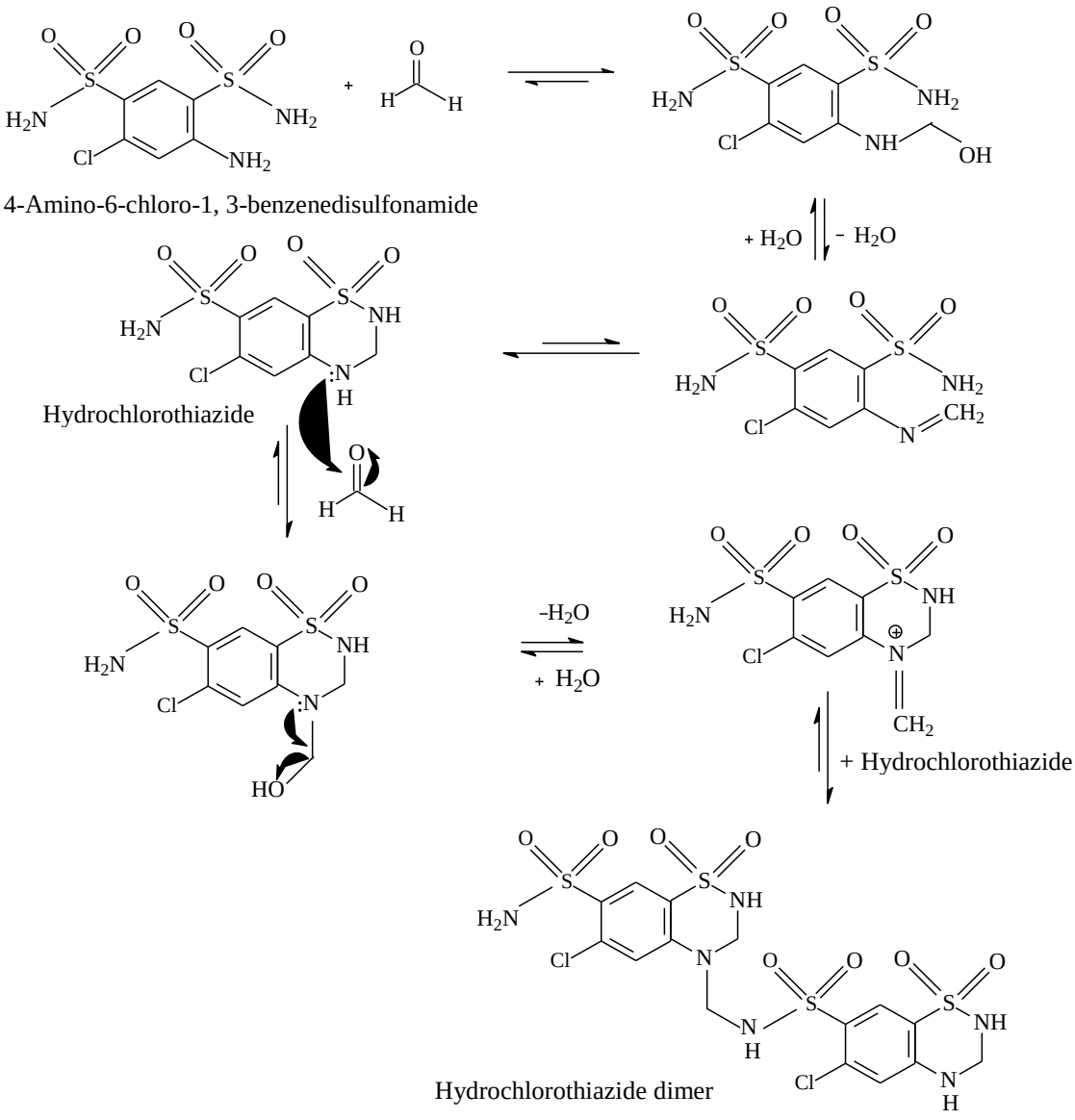


Figure 4.18 Degradation mechanism of hydrochlorothiazide to form hydrochlorothiazide dimer (44).

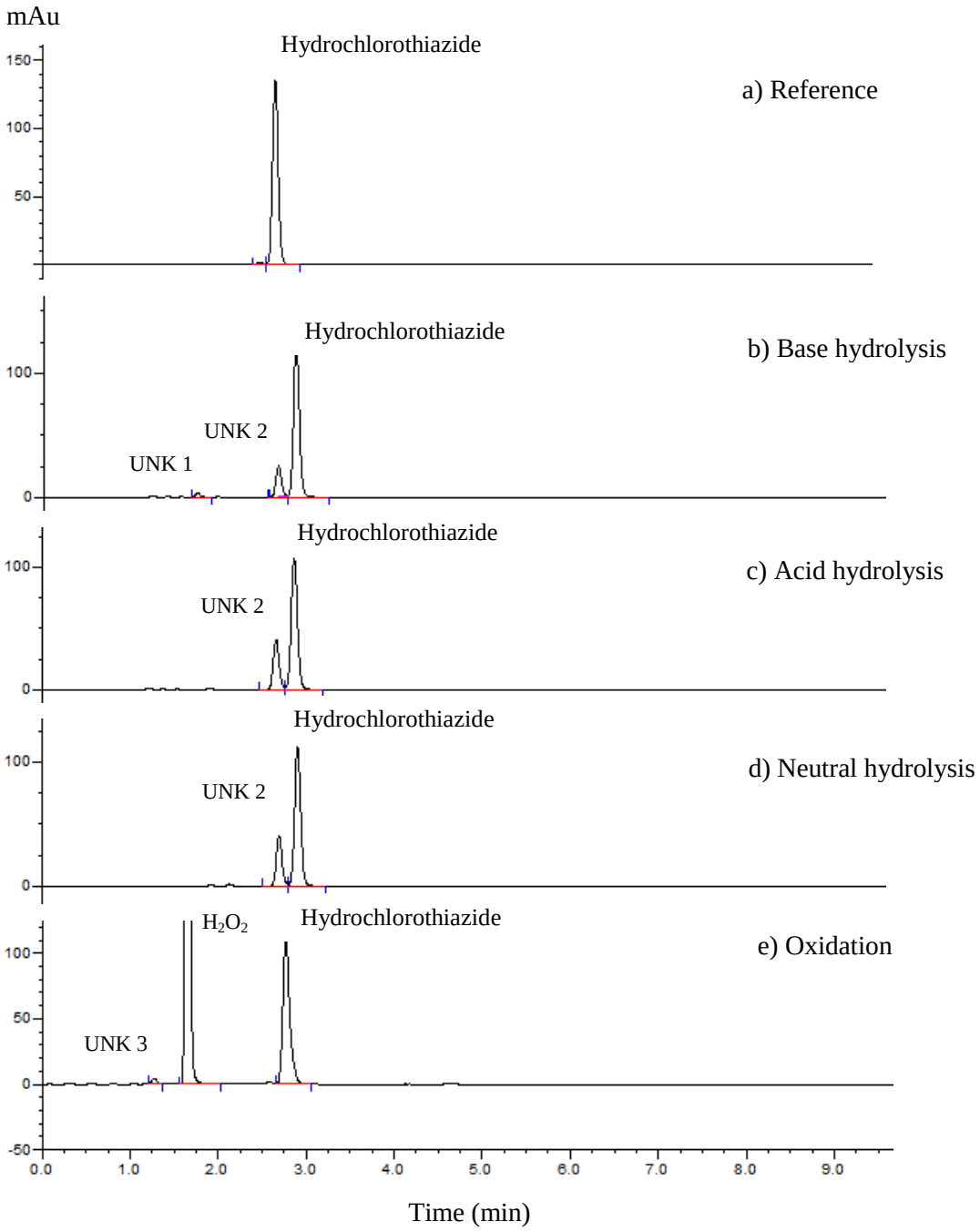


Figure 4.19 Chromatograms of forced degradation solutions of hydrochlorothiazide (50 µg/mL). Condition: column: SB CN column 150×4.6 mm i.d., 5µm; mobile phase: acetonitrile: 10 mM phosphate buffer pH 7.0 (32:68, v/v); flow rate 1 mL/min; injection volume 20 µL; detection at 254 nm; run time 20 min; temperature 25^{o}C; UNK: Unknown.

Hydrochlorothiazide degraded in acid, base and neutral hydrolysis. Therefore, kinetics of these reactions was investigated. The results showed that degradation kinetics of hydrochlorothiazide in base hydrolysis followed first-order law (Figure 4.20). Kinetic parameters of hydrochlorothiazide were given in Table 4.19. Kinetics of the other reactions could not be specified.

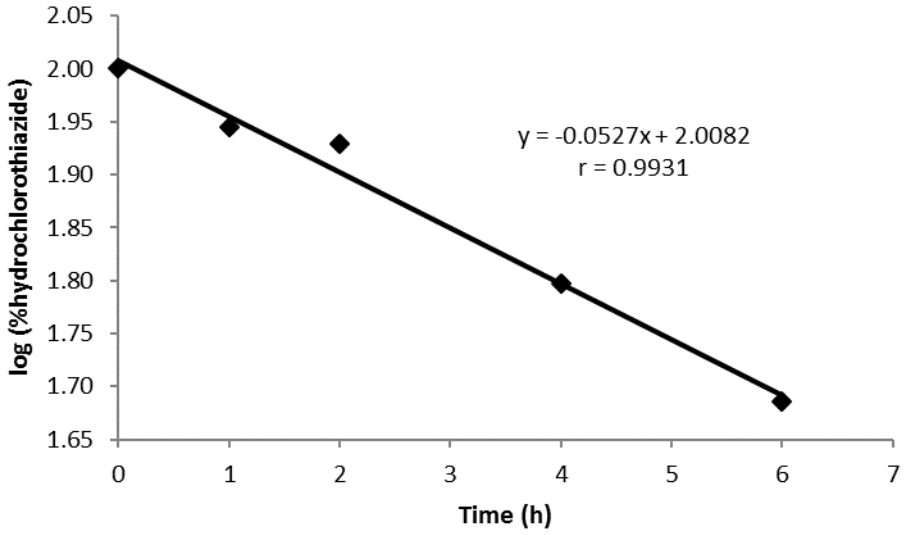


Figure 4.20 Kinetic study of the base-induced degradation of hydrochlorothiazide at 80^{o}C.

Table 4.19 Kinetic parameters of hydrochlorothiazide under base hydrolysis

	Correlation coefficient (r)	**Rate constant k (s^{-1})**	**Half-life $t_{1/2}$ (s)**	**Shelf-life t_{90} (s)**
Base hydrolysis	0.9931	0.34×10^{-4}	2.06×10^{4}	0.31×10^{4}

4.4 Analysis of pharmaceutical products

The developed and validated method was applied to analyze four samples of pharmaceutical products including two single drugs (S1 and S2) and two combined formulations (C1 and C2). The %RSD of five replications was less than 0.80 for every product (Table 4.20).

Percent labeled amounts were between 99.1% and 100.5% for candesartan cilexetil and between 124.8% and 126.9% for hydrochlorothiazide single drug. For combined formulation, percent labeled amounts were from 95.2% to 98.8% for candesartan cilexetil and from 96.2% to 100.3% for hydrochlorothiazide.

Official methods for analysis of combined formulation and for candesartan cilexetil single drug were not available in recent pharmacopoeias. For hydrochlorothiazide tablet, percent labeled amount should be in the range of 90.0% to 110.0% according to USP 36. Thus, the percent labeled amount of the hydrochlorothiazide tablets used in this study was out of the USP limit. To confirm whether the cause was from the product itself or from the method, comparing the results with those obtained by using standard method (USP 36) is recommended. Besides, standard addition method can also be applied in this situation for confirmation.

Table 4.20 Assay of samples

Product	Composition	% Labeled amount					Average (%)	SD	%RSD
		1	2	3	4	5			
C1	Candesartan cilexetil	98.8	97.8	98.1	98.1	97.9	98.1	0.40	0.41
	Hydrochlorothiazide	101.1	99.9	100.3	99.8	99.8	100.2	0.57	0.57
C2	Candesartan cilexetil	95.3	95.9	94.6	94.8	95.2	95.2	0.51	0.54
	Hydrochlorothiazide	97.1	97.9	96.2	96.6	96.8	96.9	0.62	0.64
S1	Candesartan cilexetil	100.5	99.1	99.8	99.4	99.6	99.7	0.53	0.54
S2	Hydrochlorothiazide	126.9	127.2	125.7	125.5	124.8	126.0	1.01	0.80

CHAPTER V
CONCLUSION

5.1 Method development

A RP-HPLC method for simultaneous determination of candesartan cilexetil and hydrochlorothiazide was achieved. The separation was performed on a Zobax SB CN column (4.6×150mm, 5µm). The optimum condition was in the mixture of acetonitrile and 10 mM phosphate buffer (pH 7.0, 32:68, v/v) with a flow rate of 1 mL/min and column temperature at 25^{o}C. The detection wavelength was at 254 nm. Hydrochlorothiazide and candesartan cilexetil were eluted at 2.65 and 8.21 min, respectively.

5.2 Method validation

The developed HPLC method was validated according to ICH guidelines on linearity, accuracy, precision, specificity and range. The data of validation was summarized in Table 5.1.

Table 5.1 Summary of validation data of candesartan cilexetil and hydrochlorothiazide

	Candesartan cilexetil	**Hydrochlorothiazide**
Regression	y = 0.5883x – 0.1727 (r^2 = 0.9999)	y = 0.3313x + 0.2723 (r^2 = 0.9998)
Accuracy (%Recovery)	99.08 - 100.97	99.44 – 100.19
Precision (%RSD)	0.83	1.08
Range	16 – 48 µg/mL	12.5 – 62.5 µg/mL

5.3 Forced degradation studies

Candesartan cilexetil and hydrochlorothiazide raw material were stressed under hydrolysis (acid, base and neutral), oxidation, photolysis and thermal degradation condition. Experiments were performed in solution (hydrolysis and oxidation) and solid form (photolysis and thermal degradation). Stability of both drugs was summarized in Table 5.2 and Table 5.3.

Table 5.2 Summary on stability of candesartan cilexetil

Condition	**Candesartan cilexetil**
0.1 N NaOH, 40^{o}C	Degrade
0.1N HCl, 40^{o}C and 80^{o}C	Degrade
Water, 80^{o}C	Stable
30% H_2O_2, room temperature	Degrade
Exposed to sunlight	Stable
Dry heat, 80^{o}C	Stable

Table 5.3 Summary on stability of hydrochlorothiazide

Condition	**Hydrochlorothiazide**
1 N NaOH, 80^{o}C	Degrade
1N HCl, 80^{o}C	Degrade
Water, 80^{o}C	Degrade
30% H_2O_2, room temperature	Stable
Exposed to sunlight	Stable
Dry heat, 80^{o}C	Stable

5.4 Analysis of pharmaceutical products

The developed and validated method was applied to determine candesartan cilexetil and hydrochlorothiazide in pharmaceutical formulations. Two combined formulations and two single drugs were analyzed and the %RSD of five replications was less than 0.80 for every product.

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APPENDICES

APPENDIX A
PEAK PURITY VALUES OF CANDESARTAN CILEXETIL, HYDROCHLOROTHIAZIDE AND THEIR DEGRADATION PRODUCTS

Table A1 Peak purity values of candesartan cilexetil, hydrochlorothiazide and their degradation products in forced degradation studies

Analyte	Condition	Peak	Peak purity
Candesartan cilexetil	Base hydrolysis	Candesartan cilexetil	1000
		UNK 1	992
		UNK 2	967
	Acid hydrolysis	Candesartan cilexetil	1000
		UNK 3	1000
	Neutral hydrolysis	Candesartan cilexetil	1000
		UNK 3	1000
	Oxidation	Candesartan cilexetil	1000
		UNK 1	940
		UNK 4	1000
Hydrochlorothiazide	Base hydrolysis	Hydrochlorothiazide	999
		UNK 1	882
		UNK 2	1000
	Acid hydrolysis	Hydrochlorothiazide	999
		UNK 2	1000
	Neutral hydrolysis	Hydrochlorothiazide	1000
		UNK 2	1000
	Oxidation	Hydrochlorothiazide	999
		UNK 2	1000
		UNK 3	988

Table A2 Peak purity values of candesartan cilexetil and hydrochlorothiazide in mixture of degraded drug solutions

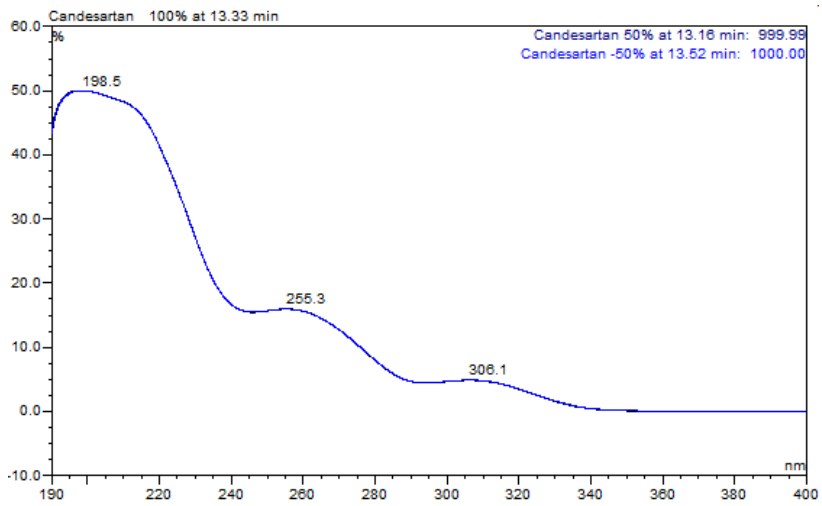
Mixture	**Peak purity value**	
	Candesartan cilexetil	**Hydrochlorothiazide**
Base hydrolysis hydrochlorothiazide + oxidation candesartan cilexetil	1000	1000
Base hydrolysis hydrochlorothiazide + acid hydrolysis candesartan cilexetil	1000	1000
Base hydrolysis hydrochlorothiazide + base hydrolysis of candesartan cilexetil	1000	1000

Table A3 Peak purity values of candesartan cilexetil and hydrochlorothiazide in the analysis of pharmaceutical dosage form

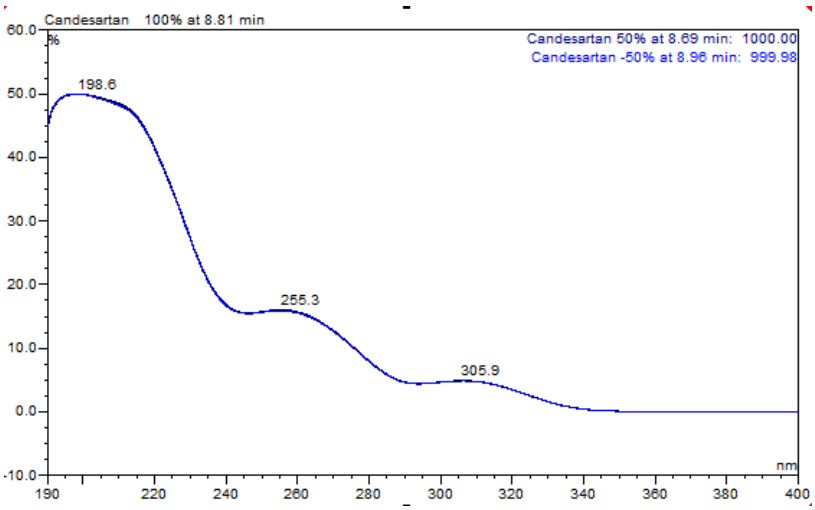
Products	**Composition**	**Peak purity value**
C1	Hydrochlorothiazide	1000
	Candesartan cilexetil	1000
C2	Hydrochlorothiazide	1000
	Candesartan cilexetil	998
S1	Candesartan cilexetil	1000
S2	Hydrochlorothiazide	1000

APPENDIX B
UV SPECTRA OF CANDESARTAN CILEXETIL IN FORCED DEGRADATION CONDITIONS

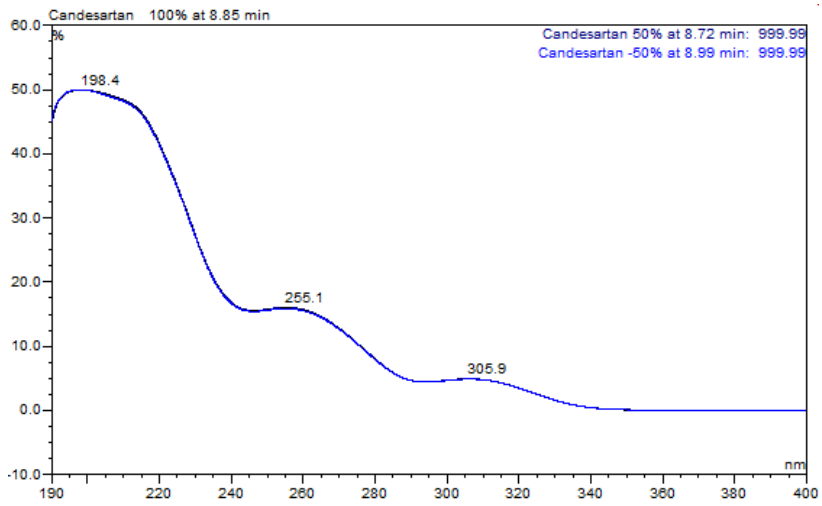
a) Standard



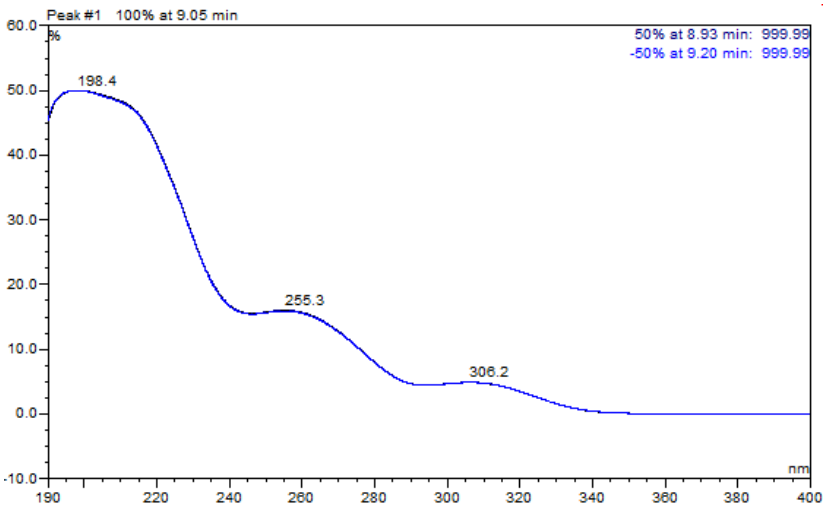
b) Base hydrolysis



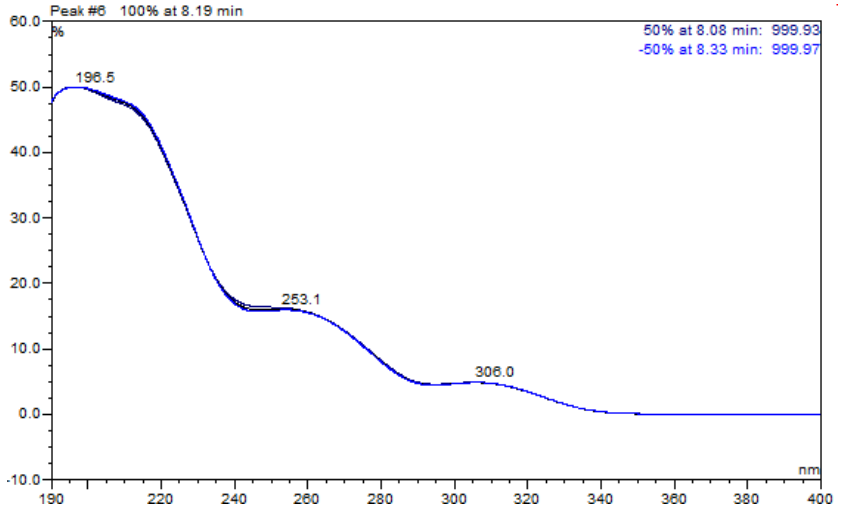
c) Acid hydrolysis



d) Neutral hydrolysis



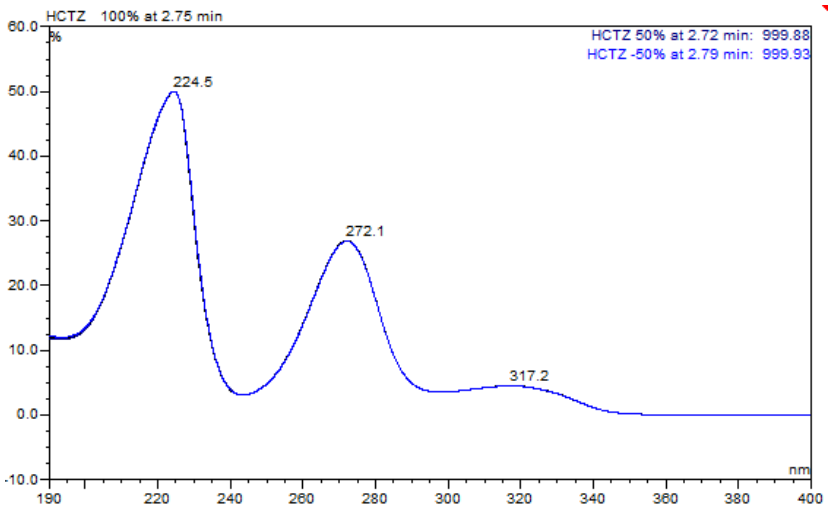
e) Oxidation


Peak #6 100% at 8.19 min
%
50% at 8.08 min: 999.93
-50% at 8.33 min: 999.97
196.5
253.1
306.0
nm
60.0
50.0
40.0
30.0
20.0
10.0
0.0
-10.0
190
220
240
260
280
300
320
340
360
380
400

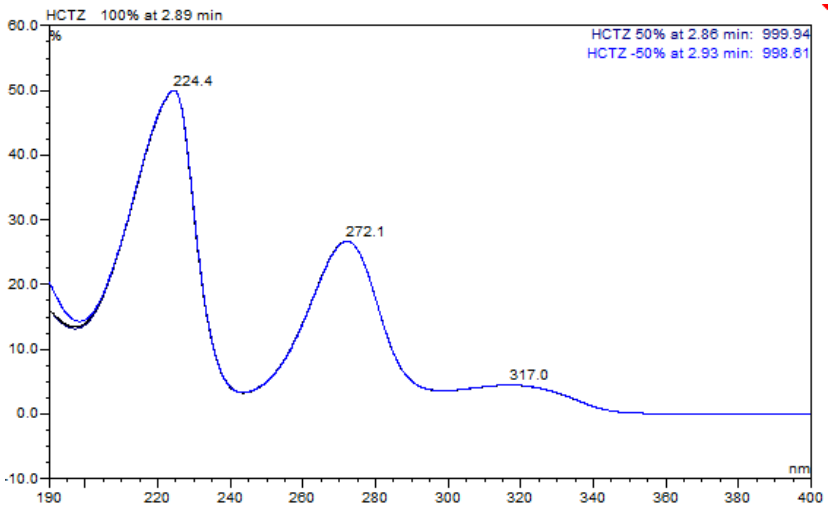
APPENDIX C

UV SPECTRA OF HYDROCHLOROTHIAZIDE IN FORCED DEGRADATION CONDITIONS

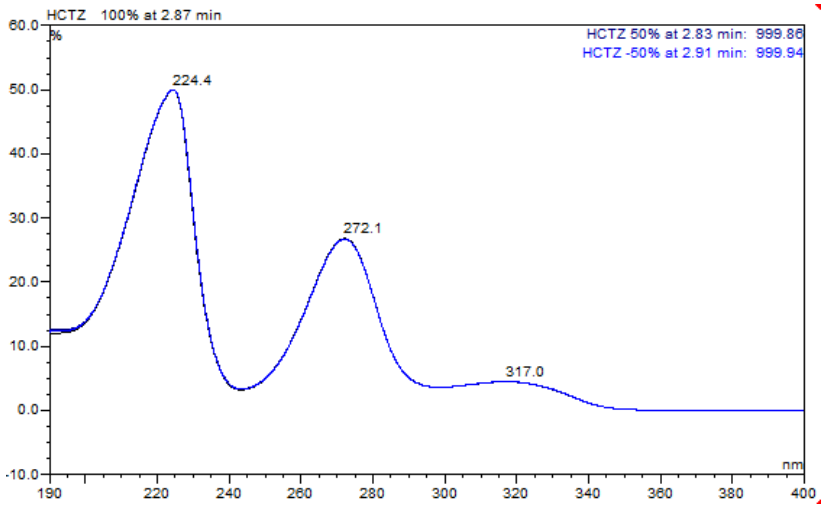
a) Standard



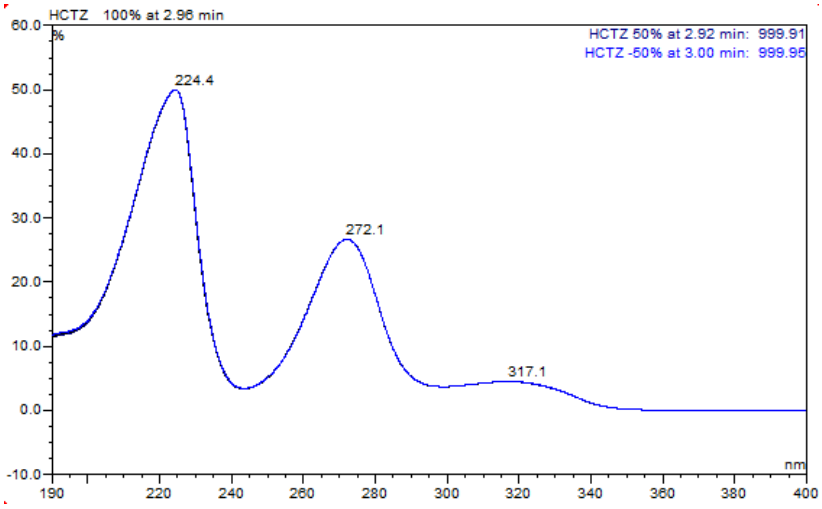
b) Base hydrolysis



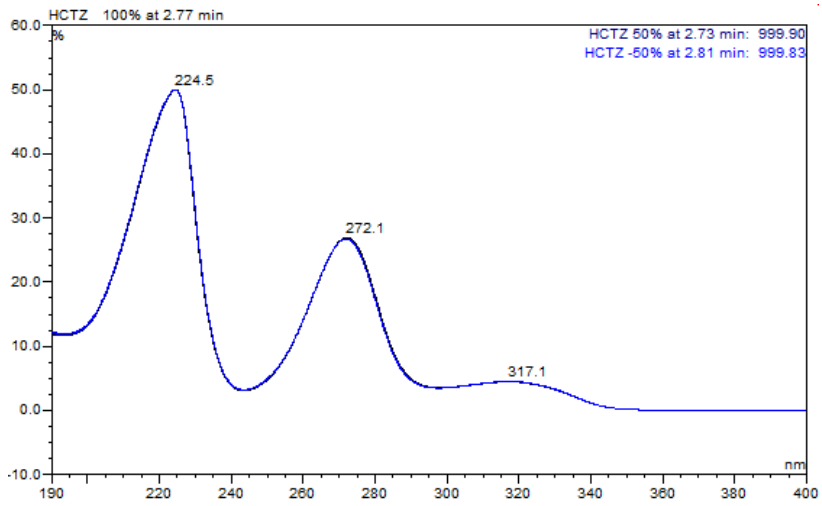
c) Acid hydrolysis



d) Neutral hydrolysis

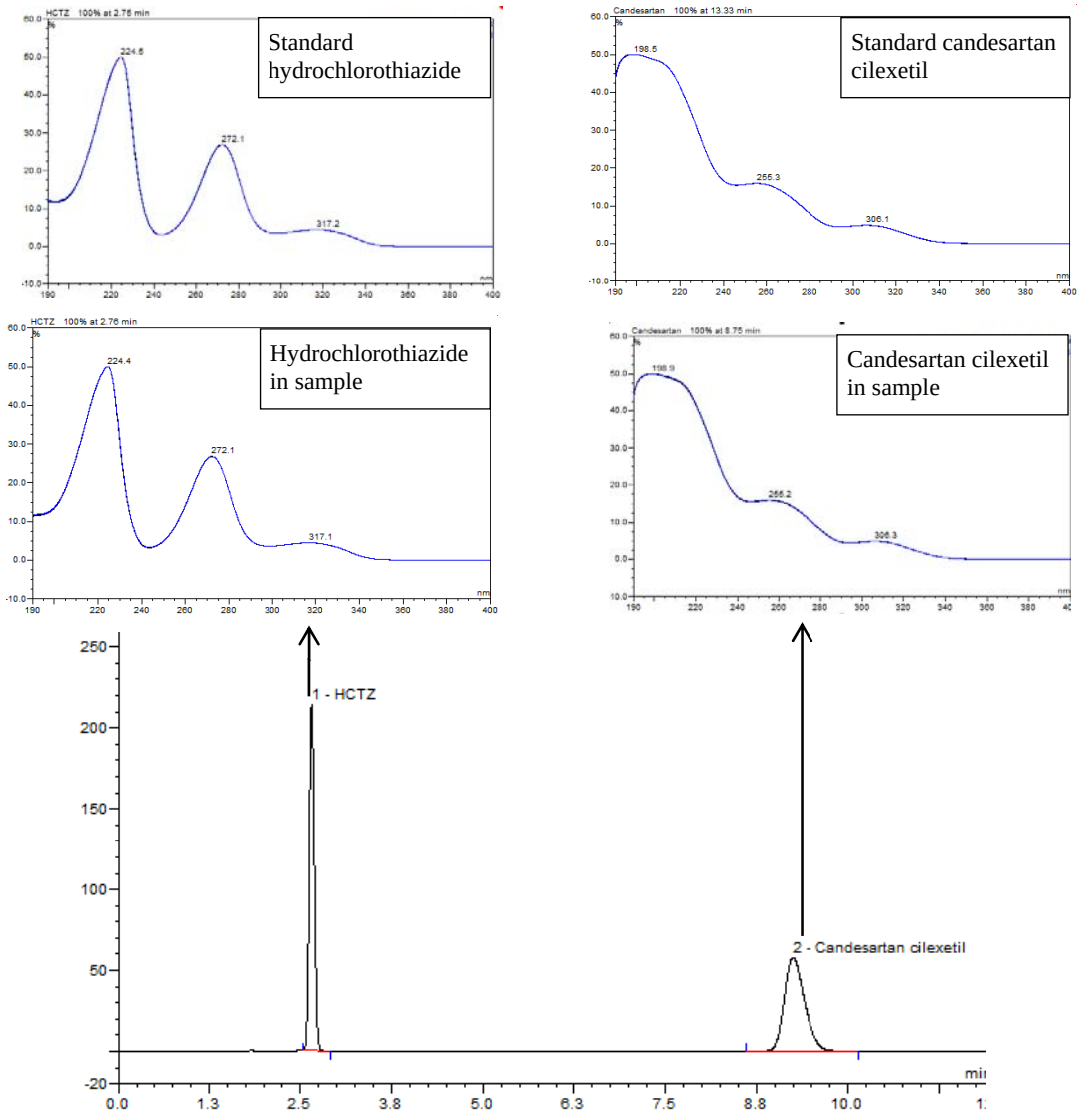


e) Oxidation

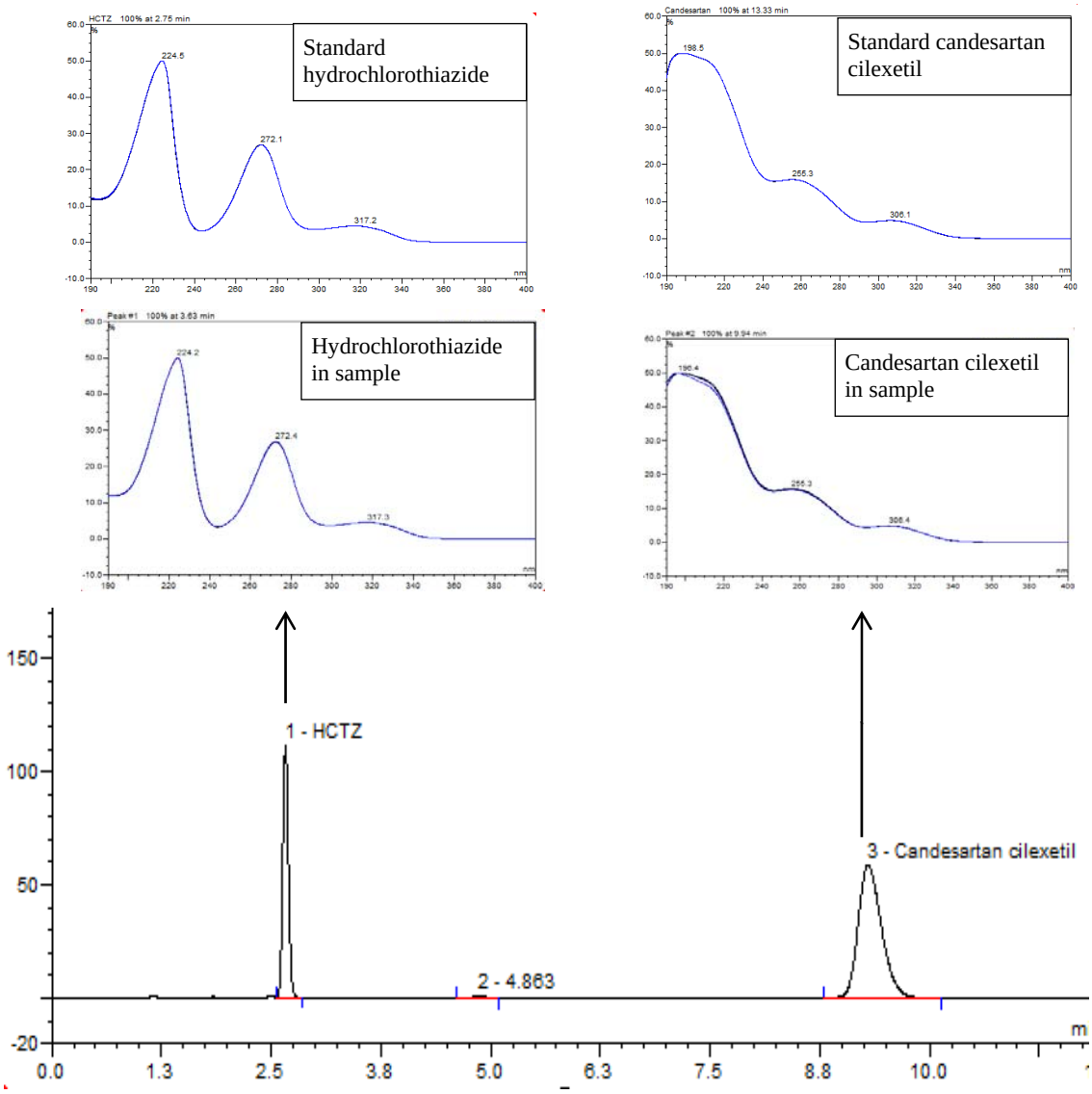

HCTZ 100% at 2.77 min
%
HCTZ 50% at 2.73 min: 999.90
HCTZ -50% at 2.81 min: 999.83
224.5
272.1
317.1
nm
60.0
50.0
40.0
30.0
20.0
10.0
0.0
-10.0
190
220
240
260
280
300
320
340
360
380
400

APPENDIX D
UV SPECTRA OF CANDESARTAN CILEXETIL AND HYDROCHLOROTHIAZIDE IN PHARMACEUTICAL PRODUCTS

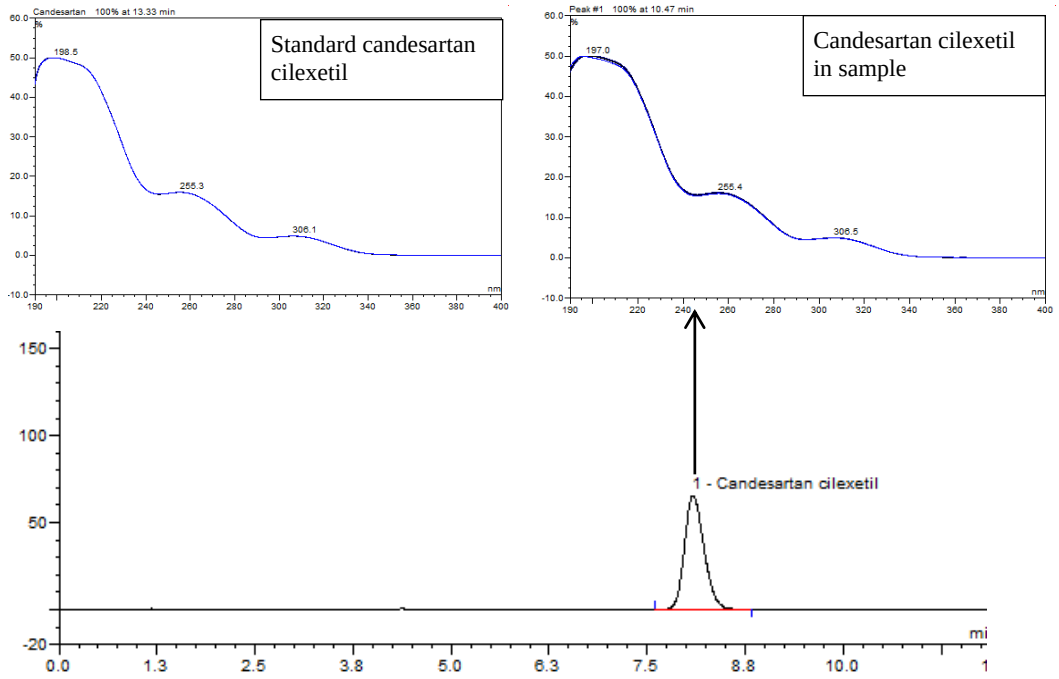
a) C1



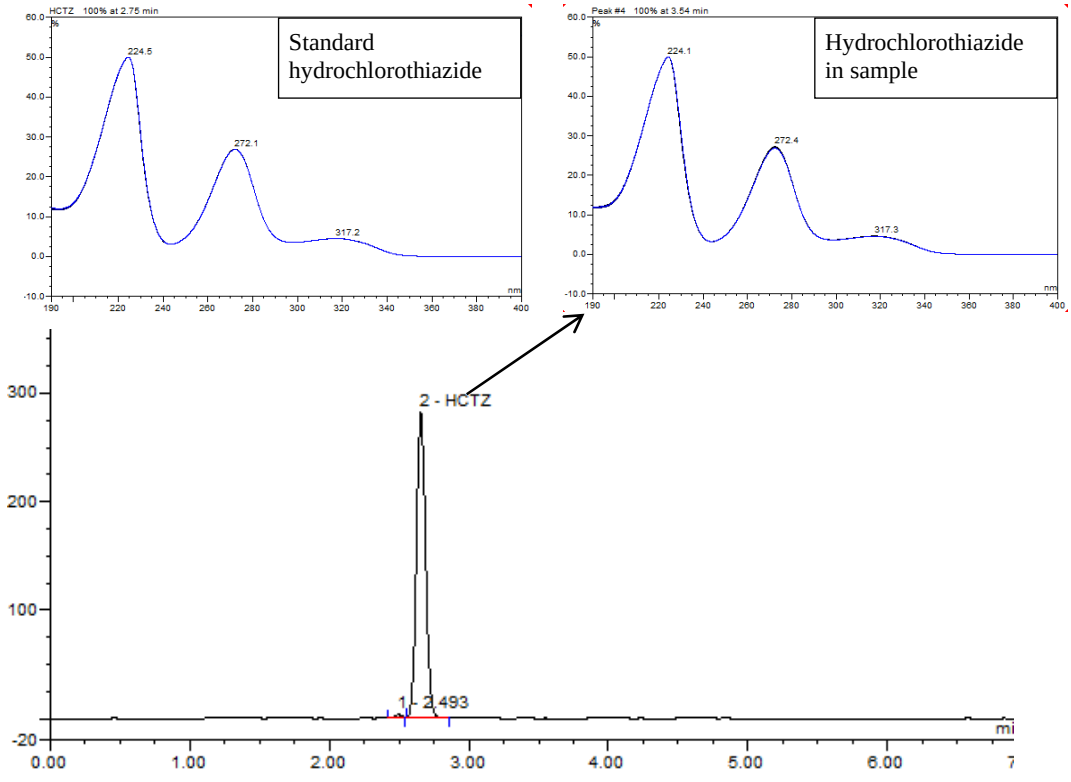
b) C2


Standard hydrochlorothiazide
Standard candesartan cilexetil
Hydrochlorothiazide in sample
Candesartan cilexetil in sample
1 - HCTZ
2 - 4.863
3 - Candesartan cilexetil

c) S1


Standard candesartan cilexetil
Candesartan cilexetil in sample
1 - Candesartan cilexetil

d) S2


Standard hydrochlorothiazide
Hydrochlorothiazide in sample
2 - HCTZ

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