

**STRESS DECOMPOSITION STUDIES AND DEVELOPMENT
OF VALIDATED STABILITY INDICATING HPLC ASSAY
METHOD FOR LAMIVUDINE**

BY

BERHANUE MUCHE (B.PHARM)

**A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE
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SCHOOL OF GRADUATE STUDIES

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Acronyms

3TC	Lamivudine
ABC	Abacavir
AIDS	Acquired immunodeficiency syndrome
API	Active pharmaceutical ingredient
CSF	Cerebral spinal fluid
CPMP	Committee for Proprietary Medicinal products
d	Day
d4T	Stavudine
ddC	Zalcitabine
ddI	Didanosine
FDA	Food and Drug Administration
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
H-	Human
ICH	International Conference on Harmonization
MS	Mass spectrometry
MS-MS	Tandem mass spectrometry (tandem quadrupole mass spectrometry)
NRTI	Nucleoside reverse transcriptase inhibitor
NRTI-TP	Triphosphorylated nucleosides reverse transcriptase inhibitors
NNRTIs	Non nucleoside Reverse Transcriptase inhibitors
MOH	Ministry of Health
PDA	Photodiode array detector
PIs	Protease inhibitors
PP	Protein precipitation
RIA	Radioimmunoassay

RSD	Relative Standard Deviation
R _T	Retention time
RTIs	Reverse transcriptase inhibitors
SPE	Solid-phase extraction
SD	Standard deviation
SIAM	Stability-Indicating Assay Method
3TC-TP	Triphosphorylated lamivudine
UF	Ultra filtration
USP	United State Phamacopeia
UV	Ultraviolet (also ultraviolet absorption)
WHO	World Health Organization
ZDV	Zidovudine.

ABSTRACT

Lamivudine belongs to the class of nucleoside reverse transcriptase inhibitors, and is potent *in vitro* and *in vivo* inhibitors of human immunodeficiency virus (HIV), which is the causative agent of the acquired immunodeficiency syndrome. Various analytical methods have been reported for the determination of lamivudine in the biological fluid such as plasma, CSF, saliva and urine. They have been used to support pharmacokinetic study. However, there are no reported stability-indicating HPLC assay methods for this drug. Though reported methods are highly specific and sensitive for this drug they may not yield precise and accurate result for determination of the API in the presences of its degradation product. Stability requirements for the world wide registrations of pharmaceutical products have undergone a dramatic change in the past few years with the advent of ICH guidelines. ICH has introduced a standardized approach for the development of stability data for registration through various guidelines. Therefore, it is necessary to develop a stability indicating method for these drugs to ensure the safety, quality and efficacy of these drugs.

The aim of the present study is thus to establish the inherent stability of lamivudine, a nucleoside reverse transcriptase inhibitor, through stress studies under a variety of ICH recommended test conditions and develop validated stability indicating assay method.

In this study all solution was prepared and used according to USP 24 and BP 1999 and forced decomposition studies were conduct to generate degradation products of the API following the condition recommended by the ICH guideline Q1A. The stressed samples obtained were subjected to preliminary analyses by employing HPLC to study the number and types of degradation products formed under various conditions.

The result of this study showed that the drug was liable to degradation in all stressed condition though the extent of degradation varied. The API was found to be more liable to decompositions in alkaline solution than in acidic solution and neutral condition. Degradation of lamivudine in neutral solution was observed relatively after long hour of refluxing indicating that the drug is relatively stable in neutral condition. Relative rate of degradation of the drug in hydrolytic and oxidative condition shows that the rate of hydrolytic degradation in

acidic solution (0.1N HCl) was less than the rate of oxidative degradation in hydrogen peroxide (3.0%). Separation of the drug and the degradation products under various conditions was successfully achieved on C-8 column by using a mobile phase composed of 0.1M ammonium acetate: methanol: 1 % (v/v) acetic acid in the ratio of 91.9:8:0.1. The method was validated with respect to linearity, precision, accuracy, selectivity, specificity and ruggedness. The response was linear ($r=0.9998$) in the drug concentration range of 5-500 μgml^{-1} . The mean values ($\pm\text{RSD}$) of slope and intercept were 46376 (± 0.006975) and 200049 (± 0.4009) respectively. The RSD values for intra- and inter-day precision studies were $<0.292\%$ and $<1.781\%$ respectively. The recovery of the drug ranged between 98.3 - 101.16% from the mixture of degradation products. The method was specific to the drug and also selective to degradation products.

The developed method is simple accurate, precise, specific and selective and rugged and thus it can be used for analysis of the drug and its degradation products.

Key words. Lamivudine, HPLC, SIAMs.

1. INTRODUCTION

1.1 Human immunodeficiency virus /Acquired immunodeficiency syndrome (HIV/AIDS)

HIV/AIDS is one of the major killer diseases in developing countries. Ethiopia, as most of other sub-Saharan African countries, is severely affected by the HIV-1/AIDS epidemic (Tegabru *et al.*, 2004). Ethiopia, with just 1% of the world's population, contributes 9% of worldwide cases of HIV/AIDS. By the end of 1997, UNAIDS estimated that in urban areas, one person in six was infected with HIV. By the end of 1999, 1.1 million persons had died due to AIDS. By the end of 2002, a total of 1.7 million Ethiopians had died from AIDS-related causes. More than 700,000 children have been orphaned by AIDS. Ethiopia is now among the most heavily affected countries, with the sixth highest number of HIV/AIDS infections in the world. The current national HIV-prevalence estimate is 6.6 percent; thus, 3.0 million people in Ethiopia are presently living with HIV. The Ministry of Health predicts that the number of people living with HIV/AIDS will increase to 3.2 million by 2006. AIDS deaths will increase from about 350,000 in 1998 to six million by 2014. Average life expectancy will drop to 47 years by 2025. 90,000 new cases of tuberculosis are reported each year. Between 40 and 50 percent of hospitalized patients with TB in Addis Ababa are thought to be infected with HIV (HIV/AIDS in Ethiopia, 2003). As of the fifth report of AIDS in Ethiopia HIV/AIDS, has a visible effect and detrimental impact on Ethiopia's society and economy. Findings from studies conducted at schools, workplaces, and among orphans also indicate similar effects (MOH, 2004).

1.2 Drugs as an instrument of public health

National drug expenditure as a proportion of total health expenditure currently ranges from 7% to 66% worldwide. The proportion is higher in developing countries (24%-66%) than in developed countries (7%-30%). In the former, at the individual and household level, drugs represent a major out-of-pocket health care cost (WHO, Health Economics and Drugs, 2000). People and governments willingly spend money on drugs because of the role they can play in saving lives, restoring health, preventing diseases and stopping epidemics. But, in order to do so, drugs must be safe, effective and of good quality, and used appropriately. This means, in turn, that their development, production, importation, exportation and subsequent distribution must be regulated to ensure that they meet prescribed standards (Ratanawijitrasin and Wondemagegnehu, 2002)

Since the mid-1930s, many new pharmaceutical products have flourished and trade in the pharmaceutical industry has taken on international dimensions. At the same time, however,

the circulation of toxic, substandard and counterfeit drugs on the national and international market has increased. This is mainly due to ineffective regulation of production and trade in pharmaceutical products in both exporting and importing countries. The use of toxic, substandard and counterfeit drugs is not only a waste of money, but may also threaten the health and lives of those who take them. Examples include the sulphanilamide incident that led to the deaths of 107 children in the United States of America in 1937 (Geiling and Cannon, 1938) and the thalidomide disaster of the 1960s, which caused birth defects in children (Dukes, 1985). More recently, diethylene glycol contamination in drug preparations, such as paracetamol, have led to multiple tragedies in Haiti and India (O'Brien, 1998; Singh *et al.*, 2001).

In Niger, fake meningitis vaccines, administered during an epidemic in which more than 26,700 people had contracted the disease, led to the deaths of 2,500 people (Ratanawijitrasin and Wondemagegnehu, 2002). Substandard and counterfeit products are not only a problem in developing countries, but in developed countries as well (Howells, 1997; Csillag, 1998; Aiken, 2000). Problems relating to drug safety and efficacy are generally due to the use of drugs containing toxic substances or impurities, drugs whose claims have not been verified or which have unknown severe adverse reactions, substandard preparations or counterfeits. All of these problems can be tackled effectively only by establishing an effective drug regulatory system. Drug regulation is a public policy that restricts private-sector activities in order to attain social goals set by the State. Drug regulation is the totality of all measures: legal, administrative and technical: which governments take to ensure the safety, efficacy and quality of drugs, as well as the relevance and accuracy of product information (Ratanawijitrasin and Wondemagegnehu, 2002).

1.3. Antiretroviral drugs

Since the identification of a virus as the cause of AIDS in 1983–84, treatment has been improved stepwise, first by using zidovudine (D'Alessandro *et al*,2000; Xiaolei *et al*,2000), later by combination therapy using two nucleoside analogues, and recently, by adding to the nucleoside combination, drugs that use the HIV-encoded protease as their target. The nucleoside analogues have been developed from naturally occurring compounds upon which the virus depends for its replication, and which therefore represent a structure optimization strategy in drug development. Conversely, the protease inhibitors were developed by molecular modeling following elucidation of the crystal structure of the HIV protease and the

shape of the target site on the enzyme. As a consequence, the protease inhibitors are larger molecules and they are more lipophilic. Hence, they tend to be eliminated from the body mainly by metabolism (Svensson *et al.*, 2000).

Recent advances in the treatment of HIV-1 infection involving co-administration of reverse transcriptase and protease inhibitors to achieve near-complete suppression of HIV-RNA concentrations have led to considerable improvements in life expectancy of infected individuals. Highly active antiretroviral therapy (HAART), i.e., the use of aggressive combination antiretroviral regimens consisting of reverse transcriptase inhibitors (RTIs) and protease inhibitors (PIs), has become the standard of care. Response to therapy is monitored by quantifying HIV-RNA copies (viral load) and CD-4 T-lymphocyte count. The objective of therapy is to reduce viral load to undetectable level by the assay used and to increase CD-4 T-cell count; both are important indicators of therapeutic success (Moyer *et al.*, 1999).

Although the benefit of HAART has been clearly demonstrated, it presents several problems for the patient. The large number of pills ($n > 20$) to be taken per day, the associated toxicities, the varying dosage regimens, and drug-drug and drug-food interactions may lead to confusion and nonadherence on the part of the patient. Nonadherence to antiretroviral therapy is particularly critical because it allows continued viral replication and the development of resistance to drugs. Other possible causes of antiretroviral therapy failure are drug-drug and drug-food interactions and individual patient variability in metabolism and clearance, and taking substandard/counterfeited drugs. These may produce suboptimal drug concentrations in some patients with subsequent incomplete viral suppression and the development of resistance. On the other hand, increased blood concentrations may relate to toxicity (Moyer *et al.*, 1999).

Currently generic and brand name antiretroviral drugs are becoming increasingly available in developing countries. In the developing world, at least 6 million of the 42 million HIV-infected patients are in urgent need of antiretroviral medications, yet because of the high costs, <300,000 are receiving treatment. However, generic antiretroviral medications, along with discounted brand name products, are quickly increasing the availability of these drugs. Although generic medications offer affordable treatment for many HIV-infected patients, little information is available regarding the integrity of these medications. Because of the huge demand for and high cost of antiretroviral medications in developing countries, brand name

drugs are a likely target for counterfeiters. In an isolated report, an HIV-infected man living in Zimbabwe purchased zidovudine tablets that, upon analysis, were found to contain no zidovudine. High cost, coupled with significant demand, suggests that antiretroviral medications may be of particular interest to counterfeiters. Therefore, in addition to generic antiretroviral medications, studies to determine drug content among branded products from developing nations are also necessary to prevent the dissemination of counterfeit drug products in the developing world (Scott *et al.*, 2004). To do these a method capable of determining the active ingredients specifically/selectively from its possible degradation products and process related impurities must be developed. These methods should be proved for its capabilities for determining time related harmful event such as: a decrease in therapeutic activity of the preparations to below some arbitrary labeled content and the appearance of a toxic substance formed as a degradation product upon storage of the formulation (Linter, 1980).

1.3.1. Nucleoside reverse transcriptase inhibitors (NRTIs).

NRTIs comprise the first class of compounds developed to treat the acquired immunodeficiency syndrome (AIDS) caused by the human immunodeficiency virus (HIV-1). NRTIs are synthetic 2', 3'-dideoxynucleoside analogs of naturally occurring nucleosides. The six NRTIs, approved for use against HIV-1, infection are: zidovudine (ZDV), didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC), and abacavir (ABC). A typical therapeutic regimen contains one or more NRTI in combination with an HIV-1PIs, non-nucleoside inhibitor of HIV-1 reverse transcriptase, or both. NRTIs are inactive prodrugs, which must be phosphorylated by intracellular enzymes to their respective dideoxynucleoside-5'-triphosphate for activity. NRTIs have two modes of action. First, triphosphorylated NRTIs (NRTI-TP) competitively inhibit viral reverse transcriptase. Second, because NRTIs lack the 3'-hydroxyl needed for 5'→3' phosphodiester DNA propagation, their incorporation into viral DNA prevents further viral replication and blocks the viral life cycle (Pereira and Tidwell , 2001).

1.3.2. Non nucleoside Reverse Transcriptase inhibitors (NNRTIs)

NNRTIs are a new class of antiretroviral drugs, representing several structurally distinct classes. Nevirapine, efavirenze and delaviridine are a representative of this class of antiretroviral drugs. Unlike the nucleoside antimetabolites, the NNRTIs do not require bioactivation by kinase to yield phosphate esters. They are not incorporated into growing

DNA chain. Instead, they bind to an allosteric site that is distinct from the substrate (nucleoside triphosphate)-binding site of reverse transcriptase (Martine, 1998)

1.3.3. Protease inhibitors (PIs)

Human immunodeficiency virus (HIV) protease inhibitors are new and potent antiretroviral drugs that have changed the treatment of infection with HIV dramatically. Inhibition of HIV protease activity by representatives of this class of antiretroviral drugs leads to production of non-infectious virions, which have the morphological features of immature particles (Van Heeswijk *et al.*, 1998). A breakthrough in the treatments of HIV-positive patients was the introductions of the HIV- protease inhibitors in 1996, to be used in combination with HIV-reverse transcriptase inhibitors. The first protease inhibitors approved by the FDA under its accelerated approval regulations were indinavir, saquinavir, zalcitabine, and zalcitabine, (Hugen *et al.*, 1999) and more recently amprenavir and lopinavir (Aarnoutse *et al.*, 2001)

1.4. Physico-chemical properties of antiretroviral drugs.

Physico-chemical properties of antiretroviral drugs are valuable clues for the choice of various feasible and plausible conditionalities for HPLC separation. Antiretroviral drugs are compounds of medium polarity with weak basic or weak acidic, and some with amphoteric properties due to ionizable substituent groups (Kashuba *et al.*, 1999; Gallicano and Kashuba, 2000; Aarnoutse *et al.*, 2001), Table: 1.1 summarizes the physicochemical parameters of some of the antiretroviral drugs: solubility data, UV-absorption maxima, pK_a value and protein binding. Lamivudine, zalcitabine, abacavir, indinavir, saquinavir and ritonavir are weak bases. The pK_a of 2.8 for ritonavir refers to loss of hydrogen from a protonated thiazole group because thiazole itself is a weak base; delavirdine is amphoteric, with weakly acidic sulfonamide hydrogen and a number of basic nitrogen atoms. The pK_{BH} (Removal of a proton from a neutral organic acid) of sulfonamides like sulfabenz is generally about 11. The reported pK_a of 4.6 is probably for deprotonation from one of the nitrogen atoms, consistent with delavirdine's ability to form a mesylate salt. Likewise, nevirapine is amphoteric. A pK_a of 2.8 suggests a moderately strong acid, but nevirapine has only a weakly acidic amide hydrogen. There are three weakly basic nitrogen atoms available for protonation, and the pK_a likely reflects deprotonation at one of these sites, consistent with nevirapine's higher aqueous solubility at a pH of <3. The pK_a values of 9 to 11 for zidovudine, didanosine, efavirenz, and

nelfinavir indicate that these compounds are weak acids. Zidovudine contains acidic hydrogen on the thymine moiety. Didanosine is amphoteric and contains acidic hydrogen on the hypoxanthine ring. Nelfinavir is amphoteric, and the pK_a of 11.1 reflects the acidic phenolic group (Kashuba *et al.*, 1999; Gallicano and Kashuba, 2000).

Complete UV spectra for most of the antiretroviral drugs are depicted in Fig 1.1. These spectra show that all PIs, abacavir, didanosine, efavirenz, nevirapine have high absorbances in the lower wavelength range (200–220 nm). For abacavir, efavirenz, indinavir, lopinavir, nelfinavir, nevirapine, and ritonavir absorbances in this range are significantly higher than their respective peak absorbances at higher wavelengths. For amprenavir and saquinavir, absorptivities at their maxima approximate those at lower wavelengths. The aqueous solubilities of indinavir and nelfinavir are strongly dependent on pH. At pH values above 3.5 these drugs show a sharp decline in solubility.

Table 1.1: physicochemical parameters of antiretroviral drugs.

Drug	Lipophilicity/hydrophilicity (partition coefficient)	Solubility in water	pKa value	UVmax (nm)	Protein binding(%)	Referenc
NRTIs						
Zidovudine	Slightly lipophilic (1.15)	20.1 mg/mL	9.7	266	20–38	Kashuba <i>et al.</i> ,1999, Aymard <i>etal.</i> , 2000
Lamivudine	Hydrophilic (NA)	70 mg/mL	4.3	270	10–50	Hoetelmans <i>etal.</i> ,1998 ;;Kashuba <i>et al.</i> ,1999
Stavudine	Hydrophilic (NA)	83 mg/ml	10.0	266	Negligible	Kashuba <i>et al.</i> ,1999 Aymard <i>etal.</i> , 2000 María <i>et al.</i> , 2000
Abacavir	Lipophilic (NA) Slightly lipophilic (NA)	77 mg/mL (sulfate)	0.4, 5.1	217,260,285	50	Kashuba <i>et al.</i> ,1999; Veldkamp <i>et al.</i> ,1999; Aymard <i>etal.</i> , 2000
Didanosine	Hydrophilic (0.04)	27.3mg/ml	9.1	252	<5	Michael,1997; Kashuba <i>et al.</i> , 1999
Zalcitabine		76.4mg/ml	4.4		<4	Kashuba <i>et al.</i> , 1999
NNRTIs						
Nevirapine	Slightly lipophilic (1.8)	0.1mg/ml	2.8	282	60	Van Heeswijk <i>etal.</i> ,1998;Kashuba <i>et al.</i> ,1999, Aymard <i>etal.</i> , 2000
Delavirdine	Slightly lipophilic (2.98) Lipophilic (NA)	0.8µg/ml Less than 10 µg/ml	4.3–4.6 10.2	225 246	98 99.5	Kashuba <i>et al.</i> ,1999 ; Aymard <i>etal.</i> ,2000
Efavirenz						Kashuba <i>et al.</i> ,1999; Veldkamp <i>et al.</i> , 1999 ;Aymard <i>etal.</i> , 2000
PIs						
Amprenavir	Lipophilic (NA)	NA	1.9	265	95	Kashuba <i>etal.</i> ,1999; Aymard <i>etal.</i> , 2000; Poirier <i>et al.</i> ,
Indinavir	Hydrophilic (NA)	100 mg/ml (sulfate) 60 mg/ml (pH 3.5)	3.7 na	260	60	2000;Aarnoutse <i>et al.</i> , 2001 Kashuba <i>et al.</i> , 1999; Poirier <i>et al.</i> , 2000; Aarnoutse <i>et al.</i> ,
Lopinavir	NA	0.3 mg/ml (pH 4.8)	6.00,11.06	259	NA	2001
Nelfinavir	Lipophilic (NA)	Practically insoluble	2.8	252	99	Kashuba <i>etal.</i> ,1999; Aymard <i>etal.</i> , 2000; Poirier <i>et al.</i> ,
Ritonavir	Lipophilic (4)	4.5 mg/ml(mesylyate) Practically insoluble	7.01	239	98	2000;Aarnoutse <i>et al.</i> , 2001 Kashuba <i>etal.</i> ,1999; Aymard <i>etal.</i> , 2000; Poirier <i>et al.</i> ,
Saquinavir	Lipophilic (NA)	2.2 mg/ml(mesylyate)		239	98	2000;Aarnoutse <i>et al.</i> , 2001 Kashuba <i>etal.</i> ,1999; Aymard <i>etal.</i> , 2000; Poirier <i>et al.</i> ,

NA=Notreported/Notavailable

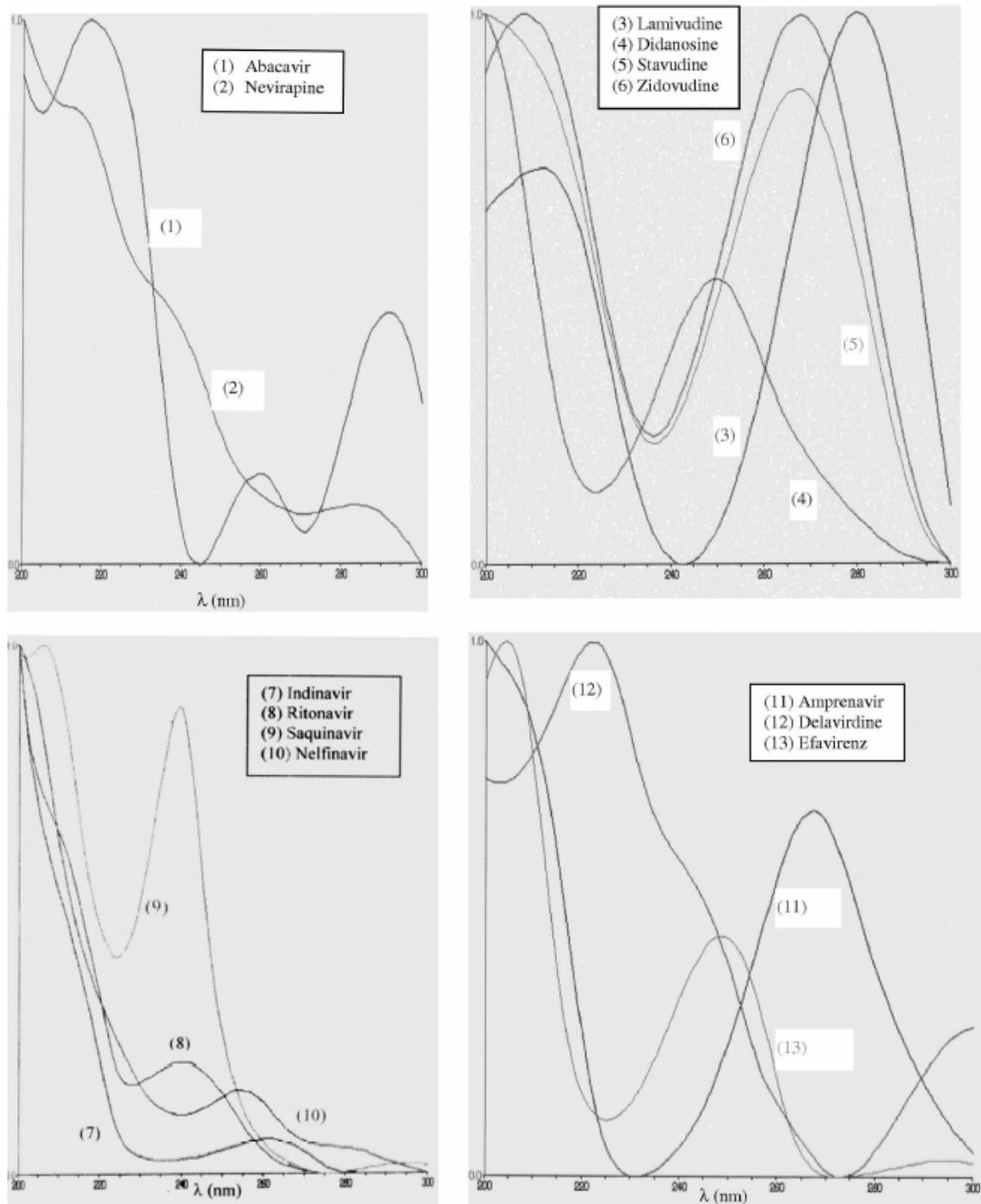


Figure 1.1: Molecular spectra of the molecules obtained under the chromatographic conditions using a UV-Vis diode-array detector. [Taken from *G. Aymard et al. J. Chromatogr. B* 744 (2000) 230.]

1.5. Lamivudine

Lamivudine is chemically (-)-2'-deoxy-3'-thiacytidine (de Clercq, 2001). It is a pyrimidine nucleoside originally developed as an antiretroviral drug (Fig.1.2). It is white or almost white powder, having molecular formula $C_8H_{11}N_3O_3S$ and molecular weight 229.26 (U.S departments of Health and Human service, 2005). It is soluble in water and sparingly soluble in methanol and melts between 172° and 178° (Indian P, 2002). It is a cytidine analogue that is metabolized intracellularly to lamivudine triphosphate, which inhibits hepatitis B DNA polymerase as well as HIV reverse transcriptase. Lamivudine is effective as monotherapy for the treatment of chronic hepatitis B and in combination with other antiretroviral drugs for the treatment of HIV-1 infection. Lamivudine has high oral bioavailability and a relatively long plasma half-life (five to seven hours), which makes once daily dosing feasible for patients with chronic hepatitis B (Balfour *et al.*, 1999)

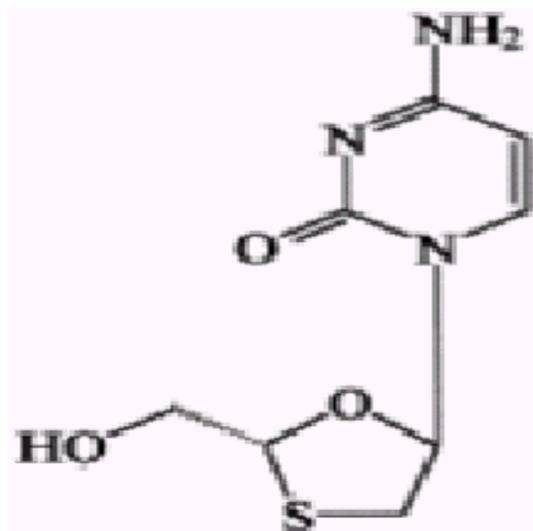


Fig. 1.2: Molecular structure of lamivudine.

1.5.1 Reported analytical methods for lamivudine

Various analytical methods have been reported for determination of the drug in biological fluid such as plasma, CSF, saliva and urine. They include a RIA method for the quantitation of intracellular 3TC-TP (Robbins *et al.*, 1998), SPE-HPLC-UV for determination of 3TC in H-Serum (Harker *et al.*, 1994), HPLC-Column Switching-UV for determination of 3TC in H-Urine (Morris and Selinger, 1994), PP-HPLC-UV for quantifying lamivudine in H-Serum (Zhou and Sommadossi, 1997), SPE-HPLC-UV for determination of 3TC in H-Plasma, H-Saliva, H-CSF, and H-Urine (Hoetelmans *et al.*, 1998), UF-HPLC-MS-MS in the determination of lamivudine in human serum with Zidovudine (Kenney *et al.*, 2000), UF-HPLC-MS-MS for determinations of Zidovudine and lamivudine in H-semen (Pereira *et al.*, 2000), SAX SPE-XAD-HPLC-MS-MS for determination of ZDV-TP and 3TC-TP in H-Whole blood (Rodriguez *et al.*, 2000), SPE-HPLC-UV for simultaneous determination of zidovudine and lamivudine from rat plasma, amniotic fluid and tissues (Alnouti *et al.*, 2004), SPE-HPLC-UV for simultaneous determination of the HIV nucleoside analogue reverse transcriptase inhibitors lamivudine, didanosine, stavudine, zidovudine and abacavir in human plasma by reversed phase high performance liquid chromatography (Verweij-vanWissen *et al.*, 2005), HPLC-MS-MS for the simultaneous determination of d4T, 3TC and ddI intracellular phosphorylated anabolites in human peripheral-blood mononuclear cells (Becher *et al.*, 2002). However, there is no report on stability-indicating HPLC assay methods for this drug.

1.6. Method development

Method of analysis is developed usually based on prior art or existing literature, using the same or quite similar instrumentations. It is rare today that an HPLC-based method is developed that does not in some way relate or compare to existing, literature-based approaches. The development of any new or improved method usually tailors existing approaches and instrumentations to the current analyte, as well as to the final needs or requirements of the method. Method development usually requires selecting the method requirements and deciding on what type of instrumentation to utilize and why. In the development stage decisions regarding choice of column, mobile phase, detector(s), and method of quantification must be addressed (Swartz and Krull, 1997).

There are several valid reasons for developing new method of analysis:

- There may not be a suitable method for a particular analyte in the specific sample matrix;
- Existing methods may be too erroneous, artifact, and/or contamination prone, or they may be unreliable (have poor accuracy or precision);
- Existing methods may be too expensive, time consuming, or energy intensive, or they may not be easily automated;
- Existing methods may not provide adequate sensitivity or analyte selectivity in samples of interest;
- Newer instrumentation and techniques may have evolved that provide opportunities for improved method, including improved analyte identification or detection limits, greater accuracy or precisions or better return on investment and
- There may be a need for an alternative method to confirm, for legal or scientific reasons, analytical data originally obtained by existing methods.

1. 7. Stability testing and the need for stability testing.

1.7.1. Stability testing: An essential quality attributes for drug products

“There never was anything by the wit of man so well devised or so sure established which hath not in the continuance of time become corrupted...”

Thomas Cranmer

Everything made by human hands-from the sublime Parthenon to the trivial milkshake-is subject to decay. Pharmaceuticals are no exception to this general statement. If there is any functionally relevant quality attributes of a drug product that changes with time, evaluation of this change fall with the purview of the pharmaceutical scientists and regulators who quantify drug product stability and shelf life (Rhodes, 2000).

The rate at which drug products degrade varies dramatically. Some radiopharmaceuticals must be used within a day or so. Other products may, if properly stored and packed, retain integrity for a decade or more, although in many jurisdictions the maximum shelf life that a regulatory agency will approve for a drug product is five years. Since the evaluation of the stability a drug is highly specialized and esoteric in nature, reliance on the patient’s suck-it-and-see organoleptic evaluation is of distinctly limited value. The governments in many parts of the world-most importantly in Western Europe, North America, and Japan-have, because of concerns about drug product safety, efficacy, and quality, found it appropriate to require some

form of stability testing for drug products. Therefore the main objective of stability testing is to provide supporting evidence on how quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity, and light (USP 24). Stability behavior in broad terms refers to overall quality of drug ingredient or drug product in terms of strength, purity, identity, safety, apparent degradation, physical or biological change, and their effect on biological performance of the drug product. It enables to establish the recommended storage conditions, retest periods, and shelf lives (Rhodes, 2000).

Potential adverse effects of instability in pharmaceutical products are (Rhodes, 2000):

- Increase in concentration of active ingredients
- Alteration in bioavailability
- Loss of active ingredient
- Loss of content uniformity
- Decline of microbiological status
- Loss of pharmaceutical elegance and patient acceptability
- Formation of toxic degradation products
- Loss of package integrity
- Reduction of label quality

1.7.2 Reasons for stability testing

Major reasons for stability testing are briefly described below:

Concern for the well being of patients

In the early 1980s a packaging stability problem with nitroglycerin tablets unfortunately resulted in some nitroglycerin tablets being available in the Midwest with potency values of less than 10% of label claim. Since nitroglycerin is used for the emergency treatments of a most cardiac condition, angina, there is unfortunately strong cause for concern that some patients may have died as a result of this stability problem. Even if death is not likely because of stability problem with a particular drug product, the inconvenience, discomfort, and cost associated with the use of product that is subpotent or exhibits an unacceptably wide range of potencies may be a serious problem needing radical remedial response.

Requirement of regulatory agencies

In many parts of the world, there are legal requirements that certain types of stability test, as required by regulatory agencies, must be performed. These requirements are also listed in

World Health Organization (WHO), European Committee for Proprietary Medicinal Products and Canadian Therapeutic Products Directorate's guidelines on stability testing of well established or existing drug substances and products (WHO, 1996; CPMP, 1998; TPD, 1997). The United States Pharmacopoeia (USP 24) has also requirements listed under 'Stability Studies in Manufacturing', which states that samples of the products should be assayed for potency by the use of a stability-indicating assay (USP 24). The requirement in such explicit manner is, however, absent in other pharmacopoeias. Current ICH guideline on Good Manufacturing Practices for Active Pharmaceutical Ingredients (Q7A), which is under adoption by WHO, also clearly mentions that the test procedures used in stability testing should be validated and be stability- indicating (ICH, 2000).

To provide a database that may be of value in the formulation of other products

Data obtained in the stability evaluation of product X today may prove to be of value to develop another product in the future.

To protect the reputation of the producer

The most important reason for manufacturer for conducting a stability-testing program is to assure that products will indeed retain fitness for use with respect to all functionally relevant attributes for as long as they are on the market.

1.8. Stress testing

For the development of a sound scientific protocol for the stability studies, an understanding of the conditions under which a drug degrades as well as the mechanism of the breakdown is needed. This is established through a series of stress studies designed to elucidate the intrinsic stability of the new molecule by establishing its degradation pathway (Rhodes, 2000). A stress stability study is often referred to as a 'preformulation study' or 'characterization study'. The result of these studies are the basis for developing appropriate dosage forms, formulations and manufacturing processes, selecting appropriate packaging and storage conditions and the analytical methods to be used in stability studies. The information derived from stress testing can be used to establish the methodology employed, the parameters followed and specifications for long-term testing under accelerated and normal storage condition conditions (Matthews, 2000].

Stress studies are different from accelerated studies because the former is carried under more sever conditions. Stress testing is testing under extreme conditions, used to characterize only the drug substance. Accelerated testing applies both to the drug substance and drug product

and involves testing under conditions more severe than normal, which serve to generate data useful in predicting what might happen during storage under normal conditions (Matthews, 2000).

1.8.1. The ICH guidance requirement in Stress testing

The International Conference on Harmonization (ICH) guideline, 'Stability Testing of New Drug Substances and Products' (Q1A), requires that stress testing be conducted to elucidate the inherent stability characteristics of the active substance (ICH, 2000). It suggests that the degradation products formed under a variety of conditions should be identified and degradation pathways established. It is stated that the testing should study the effect of temperature, humidity (where appropriate), oxidation, photolysis and susceptibility to hydrolysis across a wide range of pH values. In the guideline, it is suggested that the effect of temperature is studied in 10 °C increments above the accelerated temperature test condition (for example, 50 °C, 60 °C) and humidity tests at 75% or greater. The requirement of stress testing is also covered in other International guidelines, for instance, the ICH guidelines have been incorporated as law in the EU, Japan and in US, but in reality, other countries are also using them. The 'Note for Guidance on Stability testing: Stability Testing of Existing Active Substances and Related Finished Products' (CPMP/QWP/122/02) issued by European Committee for Proprietary Medicinal products (CPMP) states that 'when a drug substance is described in an official Pharmacopoeial monograph (European Pharmacopoeia or a Pharmacopoeia of a European Union member state), no data are required on the degradation products if they are named under the headings "purity test" and/or "impurities"; in this case no stress testing is required (CPMP, 2002]. That means no forced decomposition studies are required for drug covered under the pharmacopoeial monographs. On the other hand, stress testing is to be done when is to be done when no data are available in the scientific literature or the official pharmacopoeias. The route of stress testing for determining intrinsic stability of drugs is also mentioned as a requirement in Canadian Therapeutic products Directorate's (TPD) draft guideline entitled 'Stability testing of existing Drug Substances and Products' (TPD, 1997)

1.8.2. Practical conduct of stress testing

Various guidelines (ICH, EMEA, and FDA) provide information about the reporting and documentation of the stress studies conducted for the final dossier. The ICH guideline Q1A

suggests the following conditions to be employed: (i) 10 °C increments above the accelerated temperatures (e.g. 50 °C, 60 °C, etc.), (ii) humidity where appropriate (e.g. 75% or greater), (iii) hydrolysis across a wide range of pH values, (iv) oxidation and (v) photolysis. However, the guideline provides no details on how hydrolytic, photolytic and oxidative studies have to be actually performed. In other words, the practical aspects concerning the conduct of stress testing are addressed neither by the regulatory guidelines nor by any other document, leaving the performance of these studies to the prudence of the applicant. On the other hand, the information is available in literature but in staggered way, with suggested approaches differing a lot from one another. Few approaches towards the practical conduct of stress studies are reported in literature (Hong, 2000). A comprehensive document providing guidance on the practical conduct and issues related to stress testing under variety of ICH prescribed conditions has been published (Singh and Bakshi, 2000). This report from the authors proposes a classification scheme and offers decision trees to help in the selection of the right type of stress condition in a minimum number of attempts. This guidance document on the conduct of stress tests to determine inherent stability of drugs will be followed in the current study.

From the guidance on the conduct of stress tests to determine inherent stability of drugs, the following observations were made clearly: the condition used to study decomposition in acid revealed that 0.1N hydrochloric acid was most commonly used. A few reports indicate the use of 1N HCl and even higher strength and the use of sulfuric acid in varying strengths. Large variations were also seen in the reaction (temperature) conditions and periods of study. The temperatures varied between 40 °C and 110 °C. The reaction time varied, for example, drugs being kept at 100 °C or at boiling conditions, for periods ranging from a few minutes to as long as 2 months. The extent of decompositions also varied. For example, a 35% loss of retinoic acid was observed on refluxing in 0.1N HCl for just 5min (Caviglioli, 1994], whereas no drug decomposition was reported after refluxing nabilone in 0.1N acid for a week (Floery, 1979].

It is observed that NaOH is most often used for the hydrolysis of drugs in alkaline conditions, at strengths of 0.1N and 1N, with the occasional use of potassium hydroxide. As with acidic degradation, great variation is observed in the time and temperature of alkali exposure. Depending on their inherent stability, some drugs (for example, nabilone) show no

degradation even after refluxing in 0.1N NaOH for a week, whereas, others (such as trifluoperazine) undergo complete degradation in 0.1N alkali for 24 h at 30 °C (Floery, 1979)

At neutral pH, no significant degradation was obtained when the temperature was 37 °C for celiprolol, and refluxing conditions were used for sertraline. The testing is generally done in water. The slow rate of decomposition in neutral conditions is understandable because reactions at neutral pH are non-catalytic and hence long periods at exaggerated temperatures may be required to obtain sufficient quantities of degradation products. The most commonly used oxidizing agent, hydrogen peroxide, is used in varying strengths between 1% and 30%. Some drugs (ranitidine HCl and cimetidine HCl) degrade when exposed to 3 %H₂O₂ for short periods at room temperature (RT) (Floery, 1979). In other cases, exposure to high concentration of H₂O₂, even at extreme conditions, does not cause any significant degradation (for example sertraline HCl). This could happen since non-oxidizable drugs are not expected to show any change-even in the presence of high concentration of oxidizing agents. Photolytic studies are done on drugs in either solid form or solution, in water or in acidic and alkaline solutions and also on drugs dissolved in either methanol or acetonitrile. Mostly drugs are exposed to short /long wavelength UV or fluorescent light of varying illumination (approximately 4300-17000lux). The Various degradation conditions described for the stressed testing are shown in Table 1.2.

Table1.2: Various degradation conditions described for the stress testing.

Decomposition condition	Reagent used most frequently	Temperature	Reaction time	Extent of degradation
Acidic	HCl (0.1N/1N), H ₂ SO ₄	40°C-110 °C	Few min-2 months	Negligible-complete
Alkaline	NaOH (0.1N/1N)	30 °C/Reflux	5min-21 days	Insignificant-Extensive
Neutral	Water	35 °C/Reflux	Verylong periods	No significant degradation
Oxidation	H ₂ O ₂ (1%-3.0%)	RT- refluxing	Few hour-Week	Insignificant-Extensive
Photolytic	UV/fluorescent light(4300 to17000lux)	R.T	Few hour-months	Nil-complete

RT=room temperature

Flow charts or decision trees (Figures 1.3-1.6) for investigating the different types of stress conditions for new drug substances are shown which assume that the new drug is labile in nature to the stress conditions. Depending on the results, the strength of the reaction condition may be increased or decreased. The change, if required, is done stepwise and stress conditions are accepted when sufficient decomposition is obtained. The term ‘sufficient decomposition’ is taken in the broadest sense, meaning 80%-100% decomposition if the objective is isolation of the degradation products, or between 20-80% decompositions when the objective is to establish degradation pathways.

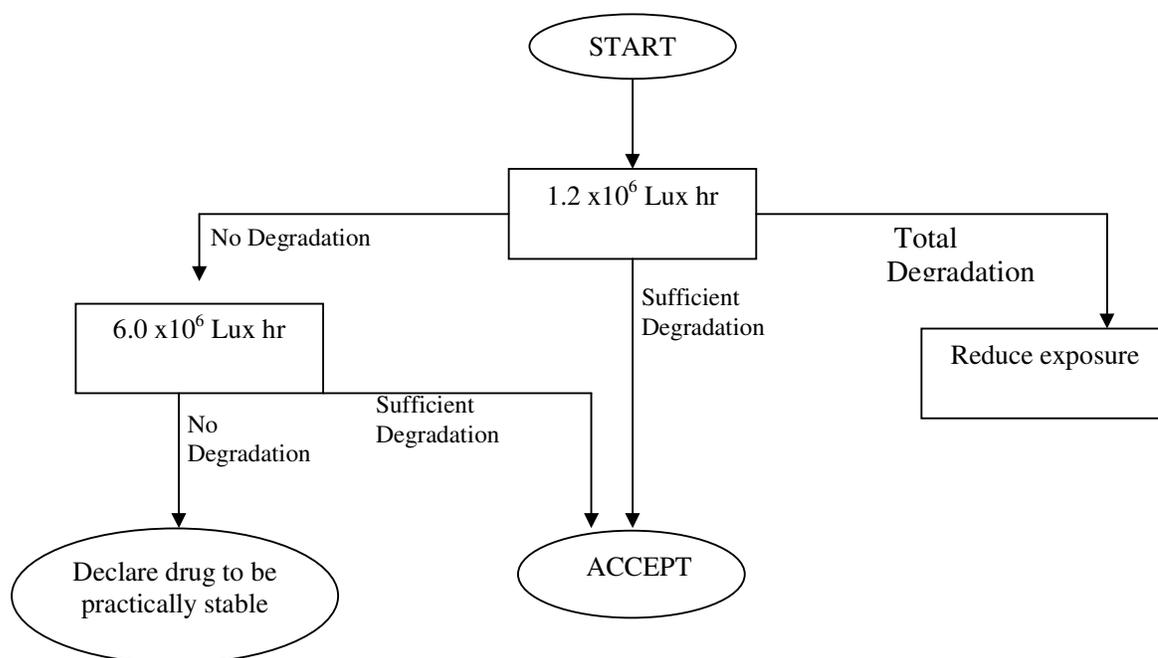


Figure1.3: Flow chart for performing stress studies for photolytic degradation

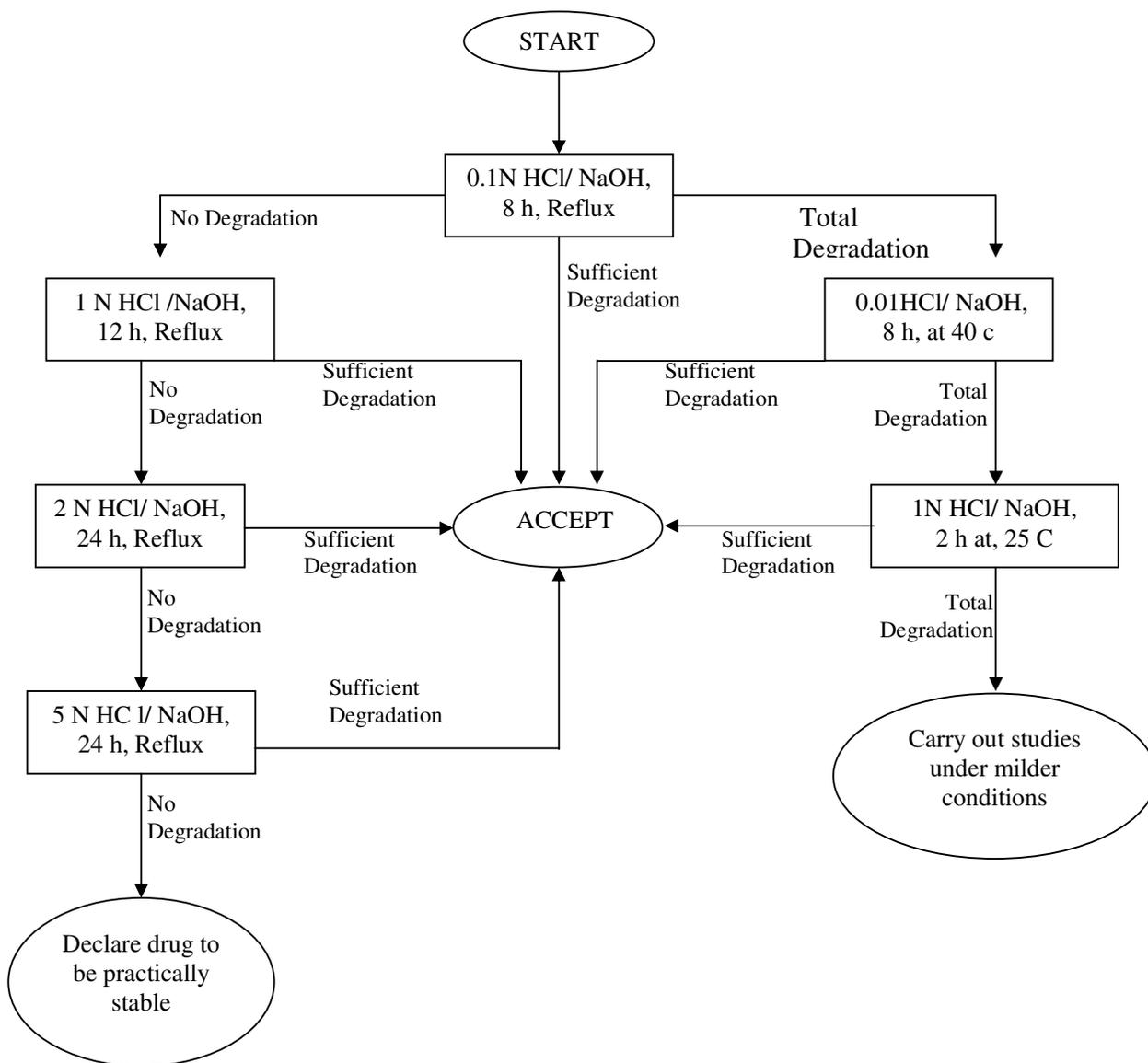


Figure 1.4: Flow chart for performing stress studies for hydrolytic degradation under acid and alkali conditions.

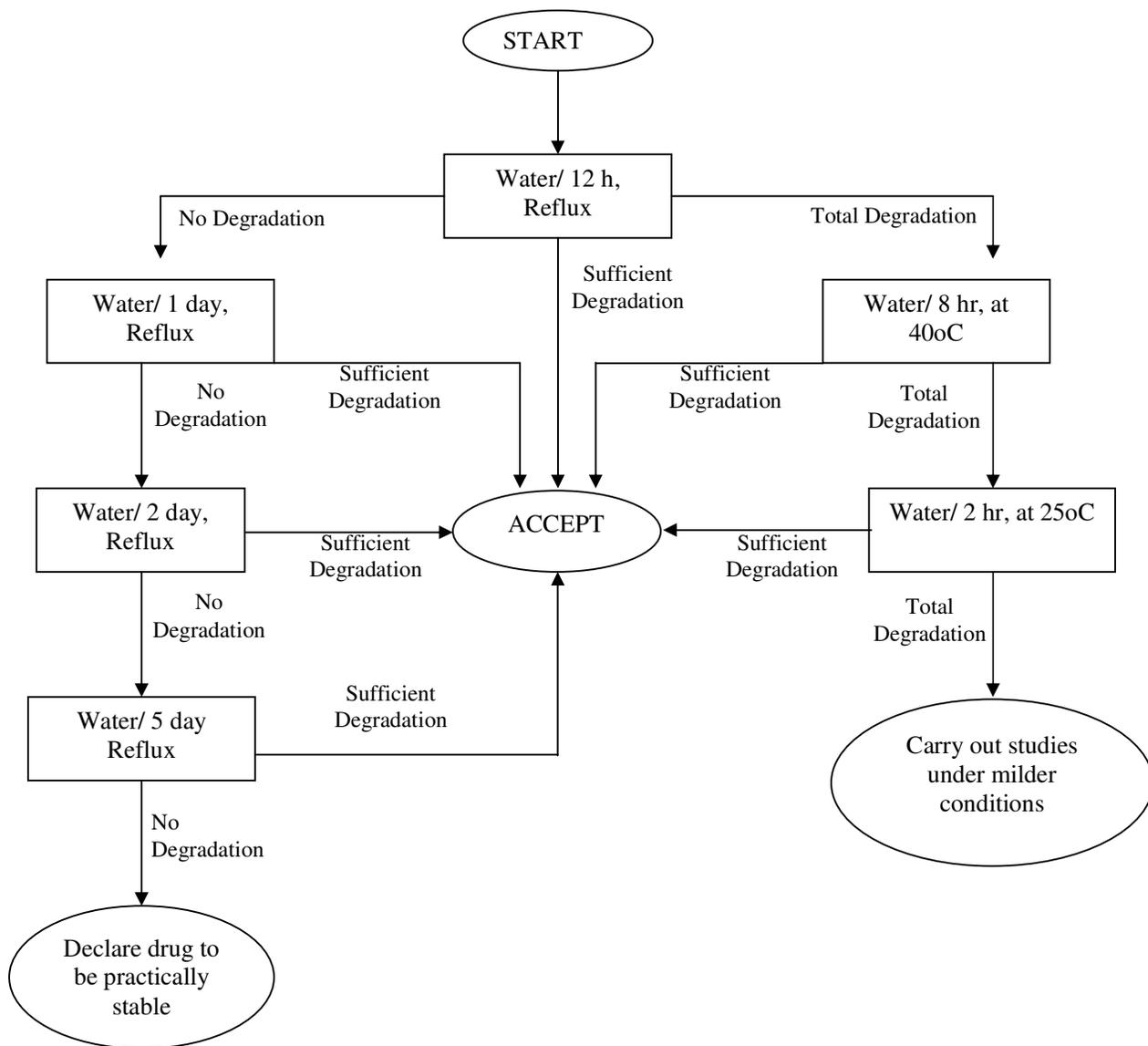


Figure 1.5: Flow chart for performing stress studies for hydrolytic degradation under neutral condition (in water).

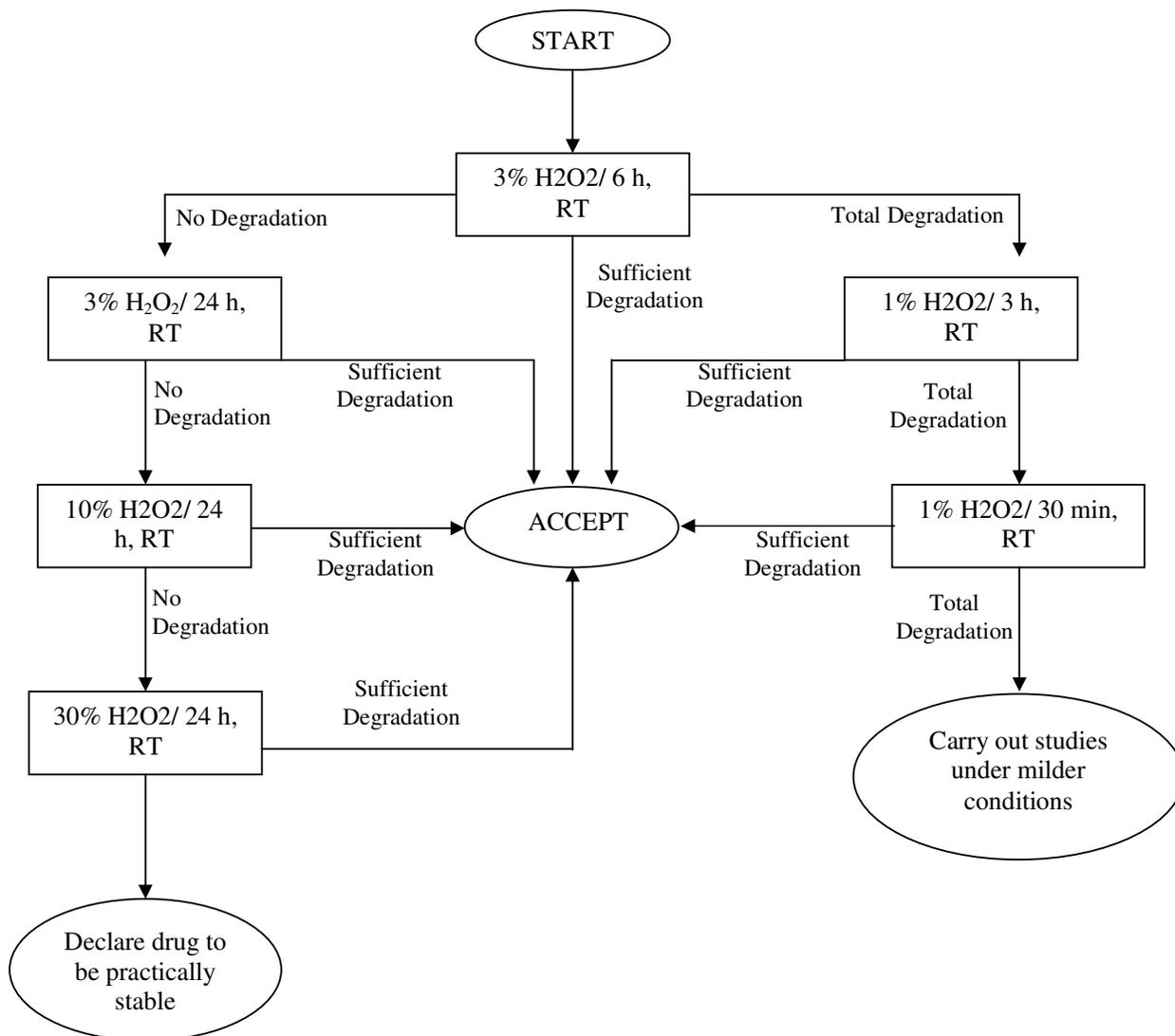


Figure 1.6: Flow chart for performing stress studies for degradation under oxidative conditions.

1.9. Stability-indicating assay methods (SIAMs)

The stability-indicating assay is a method that is employed for the analysis of stability samples in pharmaceutical industry (Bakshi and Singh, 2002). It is an analytical method procedure that is capable of discriminating between the major active (intact) pharmaceutical ingredients (API) from any degradation (decomposition) product(s) formed under defined storage conditions during the stability evaluation period. In addition, it must also be sufficiently sensitive to detect and quantify one or more degradation products (Hong and shah, 2000). With the advent of ICH guidelines, the requirement of establishment of SIAM has become more clearly mandated. The guidelines explicitly require conduct of forced decomposition studies. Under a variety of conditions, like pH, light, oxidation, dry heat, etc. and separation of drug from degradation products. The method is expected to allow analysis of individual degradation products. Unfortunately, none of the ICH guidelines provides an exact definition of a stability-indicating method. Elaborate definitions of stability-indicating methodology are, however, provided in the United States-Food and Drug Administration (US-FDA) stability guideline of 1987 (FDA, 1987) and the draft guideline of 1998 (FDA, 1997). Stability-indicating methods according to 1987 guideline were defined as the *'quantitative analytical methods that are based on the characteristic structural, chemical or biological properties of each active ingredient of a drug product and that will distinguish each active ingredient from its degradation products so that the active ingredient content can be accurately measured.'* This definition in the draft guideline of 1998 reads 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.'* The major changes brought in the new guideline are with respect to (i) introduction of the requirement of validation, and (ii) the requirement of analysis of degradation products and other components, apart from the active ingredients(s).

The ICH guidelines have been incorporated as law in the EU, Japan and in the US, but in reality, besides these other countries are also using them. As these guidelines reflect the current inspectional tendencies, they carry the *de facto* force of regulation. The ICH guideline Q1A on Stability Testing of New Drug Substances and Products (ICH, 1993) emphasizes that the testing of those features which are susceptible to change during storage and are likely to influence quality, safety and/or efficacy must be done by validated stability-indicating testing

methods. It is also mentioned that forced decomposition studies (stress testing) at temperatures in 10 °C increments above the accelerated temperatures, extremes of pH and under oxidative and photolytic conditions should be carried out on the drug substance so as to establish the inherent stability characteristics and degradation pathways to support the suitability of the proposed analytical procedures. The ICH guideline Q3B entitled 'Impurities in New Drug Products' emphasizes on providing documented evidence that analytical procedures are validated and suitable for the detection and quantitation of degradation products (ICH, 1996). It is also required that analytical methods should be validated to demonstrate that impurities unique to the new drug substance do not interfere with or are separated from specified and unspecified degradation products in the drug product. The ICH guideline Q6A, which provides note for guidance on specifications (ICH, 1997), also mentions the requirement of stability-indicating assays under Universal Tests/Criteria for both drug substances and drug products. The same is also a requirement in the guideline Q5C on Stability Testing of Biotechnological/ Biological Products (ICH, 1995). Since there is no single assay or parameter that profiles the stability characteristics of such products, the onus has been put on the manufacturer to propose a stability-indicating profile that provides assurance on detection of changes in identity, purity and potency of the product. The USP has a requirement listed under 'Stability Studies in Manufacturing', which states that samples of the products should be assayed for potency by the use of a stability-indicating assay (USP 24). The requirement in such explicit manner is, however, absent in other pharmacopoeias.

Current ICH guideline on Good Manufacturing Practices for Active Pharmaceutical Ingredients (Q7A), which is under adoption by WHO, also clearly mentions that the test procedures used in stability testing should be validated and be stability-indicating (ICH, 2000). Hong and Shah described methods that measure quantitatively the component of interest in the sample matrix without separation as 'Stability-Specific' while the ones where separation is done of the drug as well as all other degradation products as being the combination of 'stability-indicating' and 'stability-specific'(Hong and shah,2000). The term *stability* indicating has been invariably used in the vast number of publications in the literature to describe even the so-called 'stability-specific' methods (Bakshi and Singh, 2002). Bakshi and Singh used the term '*Specific stability-indicating*' and '*Selective stability indicating*' for defining, respectively, the two types of assays. Thus 'Specific stability-indicating assay method (Specific SIAM)' can be defined as '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.’ The ‘Selective stability indicating assay method (SelectiveSIAM)’ on the other hand can be 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.’ By this definition, it means that a ‘Selective SIAM’ is a procedure that is selective to the drug as well its degradation products (separates all of them qualitatively) and is also specific to all the components (measures them quantitatively) (Bakshi and Singh, 2002).

Pharmacopoeial methods historically were not ‘Selective SIAMs’ and perhaps, there are several, which are still not. However, with the advent of technology allowing resolution of a particle into its components and introduction of ICH guideline Q1A, in which there is a clear mandate for simultaneous analysis of degradation products, the situation has changed over the period. The ICH guideline was printed in USP 23 and made official, though it has been removed in USP 24 due to restraint on printed pages. The USP contains a large number (there were >2000 in USP 23) of assays and tests based on HPLC and several of them supposedly are ‘selective’ by nature. USP also defines Category II analytical methods that are meant for determination of impurities in bulk drug substances or degradation compounds in finished pharmaceutical products and provides data elements required for validation of these. Thus USP fully recognizes the necessity of compendial methods to be ‘Selective’.

Interestingly, the ‘Monograph Development: Guidance to Manufacturers’ in British Pharmacopoeia, prescribes that ‘For bulk drug substances, it has been BP policy generally to use a robust and precise method of assay (such as titration) rather than a specific, but sometimes less precise, stability-indicating method (such as liquid chromatography). Wherever possible, control of potential impurities is provided separately by means of specific impurity tests. It is appreciated, however, that a manufacturer may use, and therefore propose, a chromatographic method for both related substances and assay. In such circumstances, each case is judged on its merits on the basis of the data provided, which must relate to validate methods. Separately it is stated that ‘the method of assay will not necessarily be that used for the bulk drug substance. For formulations a specific, stability-indicating method is preferred’. Evidently, there is a slightly different approach in BP as compared to USP, although there is an endeavor for shifting to ‘Selective’ methods (Bakshi and Singh, 2002).

1.10. Reaction kinetics

Many drugs are susceptible to some form of chemical decomposition when formulated in either liquid or solid dosage forms. Such degradation not only leads to a loss of potency of the drug but also may, in some cases, cause changes in the physical appearance of the dosage forms, such as discoloration following the photochemical decomposition of the drug (Florence and Attwood, 1998). Stability is not synonymous with chemical kinetics, yet most of the rate-limiting phenomena are either associated with chemical reactions or are describable by some equation system that bears a resemblance to those encountered in chemical kinetics.

The stability guidelines make certain requirements on basic stability that are best elucidated through solution kinetics. First of all, it is necessary to develop a stability-indicating assay, that means, the assay must be capable of detecting quantitatively the amount of parent drug present, and identify, and to some degree quantitate, the decomposition products. The 1993 ICH guideline states that analytical test procedures should be fully validated and the assay should be stability-indicating and that of the 1987 stability guideline states that “the stability-indicating methodology should be validated, i.e, the accuracy and precision should be established in developing it. In developing stability-indicating assay methodology it is customary, to deliberately decompose the drug in solutions, so as to challenge the assay and to insure its capability of separating the parent drug from decomposition products. It is also desired to establish the kinetic order of the decomposition.

1.10.1. Rate and order of reaction

The rate of reaction is the velocity with which a reactant, or reactants, undergoes a chemical change. The rate of breakdown of the drug can be monitored either by its decrease in concentration with time or alternatively from the rate of appearance of one of the breakdown products. In a typical reaction;



The rate of reaction is expressed as:

$$\text{Rate} = \frac{-1}{n} \frac{d[A]}{dt} = \frac{-1}{m} \frac{d[B]}{dt} = K [A]^n [B]^m \dots$$

Where [A] and [B] are the concentration of A and B, respectively, and K is the reaction constant, then the reaction is said to be of the order (n+m+...) .The term reaction order refers

to the way in which the concentration of a reactant, or reactants, influences the rate of a chemical reaction. Therefore it is important to establish the order of reaction. The 1993 ICH guidelines specifically states that the nature of any degradation relationship will determine the need for transformation of the data for linear regression analysis. Usually the relationships can be represented by a linear, quadratic or cubic function on an arithmetic or logarithmic scale. Statistical methods should be employed to test the goodness of fit of the data on all batches and combined batches (where appropriate) to the assumed degradation line or curve. Classification of reactions according to the reaction order has been a generally applied principle.

The different orders of the reactions are:

Zero-order reactions

First-order reactions

Second-order reactions

Third-order reactions

Complex reactions.

Zero-order reactions

In zero order reaction, the rate of decomposition is independent of the concentration of the reactants. This type of degradation is typical of hydrolysis of drugs in suspensions or tablets where the drug is initially in the solid state and gradually dissolves at more or less the same rate as the drug in solution is degraded i.e. the equilibrium concentration in free solution remains constant (Florence and Attwood, 1998). Photochemical reactions-in that the rate determining factor is the light intensity, rather than the concentration of reactant-are examples of reactions that may be found to be zero order. The equation for zero-order reactions is:

$$\frac{dc}{dt} = -k_0C$$

Where C is concentration, t is the time, and k_0 is the zero order rates constant

It can be also expressed as:

$$C = C_0 - K_0t$$

C is the concentration at time t, C_0 the concentration at $t = 0$; initial concentration.

There is a linear relationship between the concentration and time in zero-order reactions. The reaction can also be characterized by the half-life ($t_{1/2}$), which is the time required for one half of the starting material to disappear. There are not many truly zero-order reactions in the pharmaceutical field. There are many examples of reactions that appear to be zero order, i.e are pseudo-zero order. When an only small amount of decompositions occurs it is difficult to

distinguish between zero- and first-order reactions, then such reaction is called a pseudo-order reaction.

First order reaction

When the rate of a reaction is proportional to the first power of the concentration of a reactant, then the reaction is said to be first-order with respect to reactant. The rate of first order reactions is determined by one concentration term and may be written as:

$-d[A]/dt = k[A]$, Where A is the concentration of the drug which will change as degradation proceeds. This expression can be written as:

$$\frac{dx}{dt} = k(a-x) \quad \text{or } t = 1/k \left[\ln \left(\frac{a}{a-x} \right) \right]$$

Where x is the amount of degraded product and a is the starting concentration of the drug.

The rate constant k and the half-life for the first order reaction do not depend on the initial concentration of the reactant (A₀).

First order degradation would be typical of the hydrolysis of a drug in solution. Such reactions are pseudo first order since the concentration of water is usually in such large excess that it is regarded as constant even though it does participate in the reaction.

Complex reaction

Many reactions cannot be expressed by simple zero-, first-, and second, or higher order equations. Often they consist of a combination of two or more reactions; some times the overall reaction can be characterized as one of these orders, but the rate of equation may be a complicated function involving first-,second-,or third-order intermediate steps. Experiments may indicate a reaction order which is nonintegral, or fractional, when reactions are complex. Nevertheless, concentration of the various reactants usually can be controlled to permit determinations of the order of reaction with respect to each reactant, and hence integral reaction order with respect to each component can be established. Among complicating factors that may be involved in the kinetic study of a complex reaction are simultaneous reactions, consecutive reactions, and opposing reactions (Kostenbauder, 1980).

a) Reversible reactions

Treatments of the kinetics of a reversible reaction involves two rate constants; one, k_f , to describe the rate of the forward reaction and the other, k_r , to describe the rate of the reverse reaction. For the simplest example in which both of these reactions are first order, that is



The rate of decomposition of a reactant is

$$\frac{-d[A]}{dt} = k_f[A] - k_r[B]$$

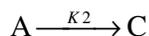
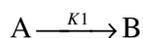
the integrated form of the rate equation is

$$t = 2.303 / (k_f + k_r) \log [(A_0 - A_{eq}) / (A - A_{eq})]$$

Where A_0 , A , and A_{eq} represents the initial concentration, the concentration at time t and the equilibrium concentration of reactant A , respectively

b) Simultaneous Reactions

Consider a substance, A which is simultaneously converted into B and C , each at characteristic rates:



$C_A = C_{A0} e^{-(k_1 + k_2)t}$, Where C_{A0} is the initial concentration of A at $t=0$ and C_A is the concentration at time t

c) Consecutive Reactions

If two first order reaction occur consecutively, thus



If C_{A0} , C_{B0} , and C_{C0} is the concentration of A , B , C at $t=0$, then C_A , C_B , C_C at time t can be obtained

$$C_A = C_{A0} e^{-k_1 t}$$

$$C_B = (k_1 C_{A0}) [e^{-k_1 t} - e^{-k_2 t}] / (k_2 - k_1)$$

$$C_C = C_{A0} [1 + (k_2 e^{-k_1 t} - k_1 e^{-k_2 t}) / (k_1 - k_2)]$$

As discussed in the above knowledge of the order of reaction is of great importance in stability determination of drug substance, in particular in solution. In this study the order of reaction of lamivudine in 0.1N HCl, 0.1N NaOH and 3.0% H_2O_2 will be determined.

2. OBJECTIVE

Nucleoside analogues occupy a prominent position among inhibitors of HIV. Being recent drugs, not much has been done on their stability aspects. In order to ensure the purity, quality and safety of the drugs in the pure and formulation form a specific, sensitive method must be available or developed. Several methods have been developed for these drug determinations in biological fluids with HPLC. These methods are biological in nature. They have been used to support pharmacokinetic study, determination of the drugs and its metabolite in various physiological fluids such as saliva, plasma/serum, and urine. These methods though highly specific and sensitive for these drugs, may not yield precise and accurate results during *in vitro* analysis of the drugs dosage forms, and so far no method has been developed for these drugs in the dosage forms except for few of them. However, there is no report on stability indicating assay methods for most of these drugs. Therefore, it is necessary to develop a stability indicating assay method for these drugs to ensure the safety, efficacy and quality of these drugs. Hence, it is mandatory to establish inherent stability of these drugs through a variety of ICH recommended stress conditions and develop validated stability indicating assay methods and compare their rate of degradation under the various stress conditions.

The aim of the present study is thus to establish the inherent stability of lamivudine, a nucleoside reverse transcriptase inhibitor, through stress studies under a variety of ICH recommended test conditions and develop validated stability indicating assay method.

2.1 General objective:

- ❖ To develop optimized and valid stability-indicating HPLC-based method for the assay of lamivudine.

2.2 Specific objective:

- ❖ Undertake stability studies of lamivudine under stress conditions
- ❖ Conduct separation studies on stressed samples using HPLC
- ❖ Develop and optimized the various HPLC conditions that provide best separation
- ❖ Validate the developed and optimized method
- ❖ Determine the rate of degradation of the drug

3. EXPERIMENTAL

3.1 Materials

Lamivudine were received free of charge from Addis Pharmaceutical Factory Sh.Co. (Adigrat, Tigray) and GlaxcoSmithkline and was used without further purification. Sodium hydroxide (Lot K17645418, BDH Laboratory supplies Poole, BH151TD, England), Hydrochloric acid GRG (Avondale Laboratory Banbury, oxon, England), Glacial acetic acid AR (May and Baker LTD, Dagenham, England), Hydrogen peroxide (Lot ZU39033679 BDH Laboratory supplies Poole, BH151TD, England), Ammonium acetate (E.Merk, 64271Darmstadt Germany and Pharmacos LTD, Southend-on-sea essex england) were all used as received. Ultra pure water was obtained from Aquatorm double dirstiller and NaNoPure (Barnstead) water purification unit.

3.2. Instruments

Dri-Bath (model DB-16520, Themolyne, Iowa, USA) was used for oxidative degradation of lamivudine in H₂O₂. Bistabil flask condenser in *Gerhardt* heating plate was used for degradation of lamivudine in all three conditions viz. acid, basic and neutral. Thermal stability studies were performed in dry air oven (Memmert 854 Schabach, W-Germany)

Photostability studies were done in a stability chamber (PSC062, SANYO GallenkampPLC, Pharma-safe, UK) capable of controlling tolerances in temperature (± 1 °C) and humidity ($\pm 3\%$ RH). The chamber was set at 40 °C and 75%RH and was equipped with illumination bank made of light sources defined under option 2 in the ICH guideline Q1B. The light bank consisted of six black light Pharma-Light UV lamps and six Pharma-lights VIS cool white fluorescent lamps. The Pharma-light near UV fluorescent lamps have a spectral distribution from 320 nm to 400 nm with a maximum energy emission between 350 nm and 370 nm. A significant proportion of UV is in the 320 nm to 360 nm and 360 nm to 400nm bands with maximum out put at 350nm. The output of cool white fluorescent lamps was similar to that specified in ISO10977 (1993). The chamber is equipped with high quality amplified Lux light and UVA light sensor. The samples were placed at a distance of 9 inches from light bank. The overall illumination at the point of placement was 6million lux. for visible light and 1000watt UVlight.

The HPLC system consisted of a LC-10ATVP pump, SPD-10AVVP UV-Vis dual wavelength detector, a SIL-10ADVP autoinjector, and a DGU-14A degasser module; data were acquired and processed using CLASS-VP software (all equipment from Shimadzu, Kyoto, Japan).

The chromatographic separations were carried out on 5 μ m Teknokroma nucleosil C-18 column (250 X4.6mm i.d) and 10 μ m Teknokroma Kromacil C-8 column (250 X4.0mm i.d). Ruggedness testing of methods was done on another HPLC system, equipped with Perkin Elmer series 200 pump, autosampler, UV/Vis detector, vacuum degasser, NCI 900 Network chromatography interface; data were acquired and processed using PerkinElmer total chrom Navigator-HPLC 600.

Mobile phase was degassed using a sonicator (Branson Ultrasonic, Smith Kline Company). The pH of the mobile phase was checked using microcomputer pH/ion analyzer (HI 8424, HANNA, Portugal). The mobile phase was filtered using whatman filtration apparatus and cellulose nitrate filter paper (pore size 0.45 micrometer).

3.3. Methods

3.3.1. Preparation of solutions

0.1N NaOH, 0.1 N HCl, 0.03%, 0.09%, 0.9% and 3.0% hydrogen peroxide solution was prepared following the procedure outlined in BP 1999 and USP 24.

3.3.2. Degradation studies (Bakshi and Singh, 2002)

All degradation studies were done at a drug concentration of 1mg/ml. A minimum of four samples were generated for every stress condition, viz. the blank subjected to stress in the same manner as the drug solution, zero time sample containing the drug, which was stored under normal condition and the drug solution subjected to stress treatment. The specific stress conditions were as follows:

Neutral studies

For study in neutral conditions, drug at strength of 1 mg/ml was prepared in water and the solution was heated at 60 °C and at refluxing temperature for different time periods.

Alkaline degradation studies

The drug was dissolved in 0.1N sodium hydroxide to get 1 mg/ml and the study was conducted at refluxing temperature and at 60 °C for different time periods.

Acid degradation studies

Acid decomposition studies were carried out at drug strength of 1 mg/ml in 0.1 N HCl at 60 °C and refluxing temperature for different time periods.

Oxidative studies

The oxidative stress studies were done at drug strength of 1 mg/ml in 0.03%, 0.9%, and 3% H₂O₂ for different times. The drug was kept at room temperature.

Photolytic studies

Photolytic studies were done in 0.1 M HCl, water and 0.1 M NaOH and the solution was exposed in a photostability chamber. Control samples were kept in dark for the same period.

Thermal studies

Thermal decomposition studies were conducted by exposing the solid sample to dry heat at 60 °C for two month and sampling were done every 15 days.

3.3.3. Preparation of samples for HPLC analysis

50 microlitres were taken from the samples withdrawn at various time intervals from the specified degradation studies and were diluted to 5 ml with the mobile phase.

3.3.4. Separation studies

The stressed samples so obtained were subjected to preliminary analyses to study the number and types of degradation products formed under various conditions. For this, a new reversed phase octadecyl (C-18) and octyl (C-8) column and different concentration of methanol with water at initial stage and finally 0.1 M ammonium acetate: methanol: 1%V/V acetic acid at a ratio of 91.9:8:0.1 was used as a mobile phase. Studies were carried out first on individual drugs, second on all reaction solutions individually, and then on a mixture of those solution in which decomposition was observed.

3.3.5. Kinetic studies

The solutions of lamivudine prepared in 0.1 N NaOH, water and 0.1 N HCl refluxed in refluxing apparatus and Lamivudine solution in 3% H₂O₂ was kept in dry bath at 25 °C. Samples were withdrawn at intervals such that approximately 4-6 data points could be depicted on the kinetics plots.

3.3.6. Development of SIAMs

For development of stability-indicating methods, stressed samples of the drug in which sufficient decomposition was observed were mixed and analyzed using HPLC method in order to see that the drug and the degradation products were separated well.

3.3.7. Validation Studies

Linearity and range

A stock solution of the drug (1 mg/ml) was prepared. This stock solution was diluted to prepare solutions containing 5-500 µg/ml of the drug. Each 20 µl of solutions were injected six times in to the HPLC column, using 0.1M-ammonium acetate-methanol-1%V/V acetic acid (91.9: 8:0.1) as the mobile phase.

Precision

Intra-day precision were performed by hexaplicate injections of the drug eight different dilutions (10, 25, 50, 100, 200, 300, 400, and 500 μgml^{-1}) on the same day and the values of relative standard deviations were calculated. The studies were also repeated on different days to determine inter-day precision.

Accuracy

Accuracy was evaluated by fortifying a mixture of decomposed reaction solutions with four known concentrations of the drug. The recovery of the added drug was determined.

Specificity and selectivity

The specificity of the method towards the drug was established through study of resolution factor of the drug peak from the nearest resolving peak. Overall selectivity was established through determination of purity for each degradation product peak using PDA detector.

Ruggedness

The ruggedness was established through separation studies on the mixture reaction solutions by different analysts on the same chromatographic system. A study was conducted on a different chromatographic system on a different day. Seven different dilutions (10, 50, 100, 200, 300, 400, 500 $\mu\text{g/ml}$) were prepared on different day by different analyst (person) and four injections from each dilutions were performed, and the values of relative standard deviation were calculated to determine intra-analyst and inter-analyst difference. Separation studies on the same chromatographic system with different brands of C-8 columns were also conducted. Inter and intra-column variations (C-8 kromacil, C-8 Nucleosil, C-8 supelco) were studied on seven different dilutions and three injections from each dilutions were made to the columns under the study.

4. RESULT

4.1. Development and optimization of the stability-indicting method

The HPLC procedure was optimized with a view to develop a stability indicating method so as to resolve the degraded products from the drug. Initially separation studies were started using a new reversed-phase octadecyl column (Teknokroma, nucleosil 100 C-18, TR 011349, N29789) and 50:50, water-methanol, as mobile phase so as to obtain well-separated and good quality peaks for the drug of interest since such result at the outset provide better confidence because of the unknown nature of the products formed during stressing. However, using this mobile phase, the retention time of the drug obtained was very short which is not acceptable for studying the degraded product from the drug, as degradation products from drugs are generally polar in nature and elute quickly in the reverse phase HPLC. Several studies were carried out by decreasing the percentage of methanol from 50% downwards (up to 15%), till satisfactory retention time was obtained. Figure 4.1: shows the chromatograms obtained using different hydroalcoholic solutions as mobile phase.

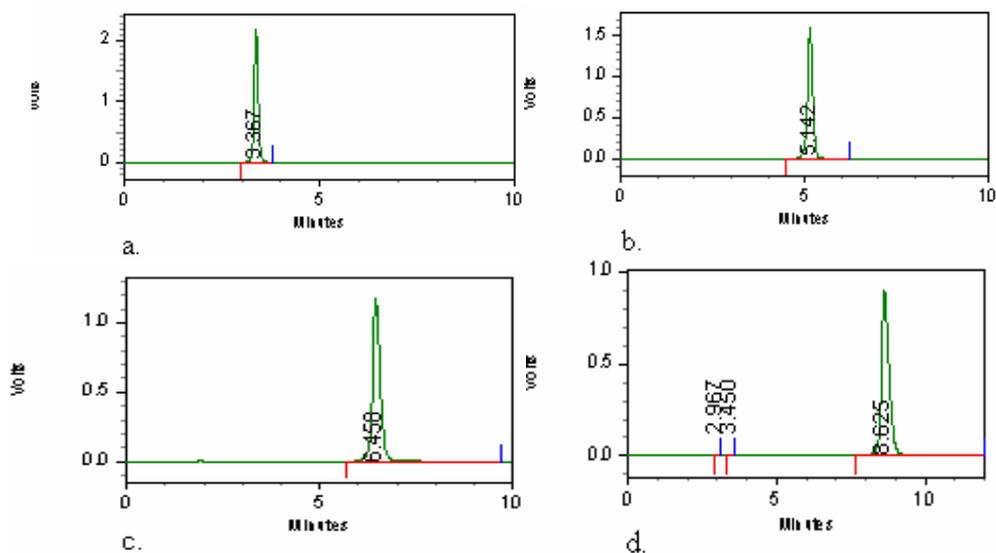


Figure 4.1: HPLC chromatograms obtained in the retention time study of lamivudine using different proportion of methanol and water a) CH₃OH:H₂O (50:50) b) CH₃OH:H₂O (25:75) c) CH₃OH:H₂O (20:80) d) CH₃OH:H₂O (15:85) as mobile phase at flow rate of 1 ml/min, C-18 column, at 30⁰C, 20µl injection volume and detection at 270 nm UV-Vis diode-array detector.

As shown in the Figure 4.2 the chromatogram obtained for the individual reaction solution. As it can be seen in the Figure good separations were not achieved when 15% methanol in water were used as mobile phase to resolve the degraded products from the drug in the individual reaction solution. Further studies were carried out to see the separation pattern of these individual reaction solutions by decreasing the methanol to 10%. However, even at such low percentage of methanol the desired separation was not achieved (Figure 4.3)

The chromatographic condition was optimized to separate the degraded product from the drug by using 0.1 M ammonium acetate: methanol: acetonitrile: 1%v/v acetic acid (90.9:8:1:0.1) as mobile phase. This solvent system was used in the determination of lamivudine from biological fluids (Harker *et al*, 1994). Though a relatively better separation was achieved by using this mobile phase it was not considered to be satisfactory. The chromatograms obtained with this chromatographic condition for individual reaction solution shown in Figure 4.4. Further variation of the percentage of methanol did not improve the resolution.

Finally, changing the stationary phase to octyl (C-8) column and the mobile phase to 0.1 M ammonium acetate-methanol-1%(v/v) acetic acid in the ratio of 91.9% : 8%:0.1% at a flow rate of 1 ml/min not only separates the degradation products from the drug but also improved the sharpness of the chromatographic peaks significantly. Chromatogram showing separation of the drug and the degradation products in individual reaction solution using different mobile phases and octyl column are depicted in Figure 4.5.

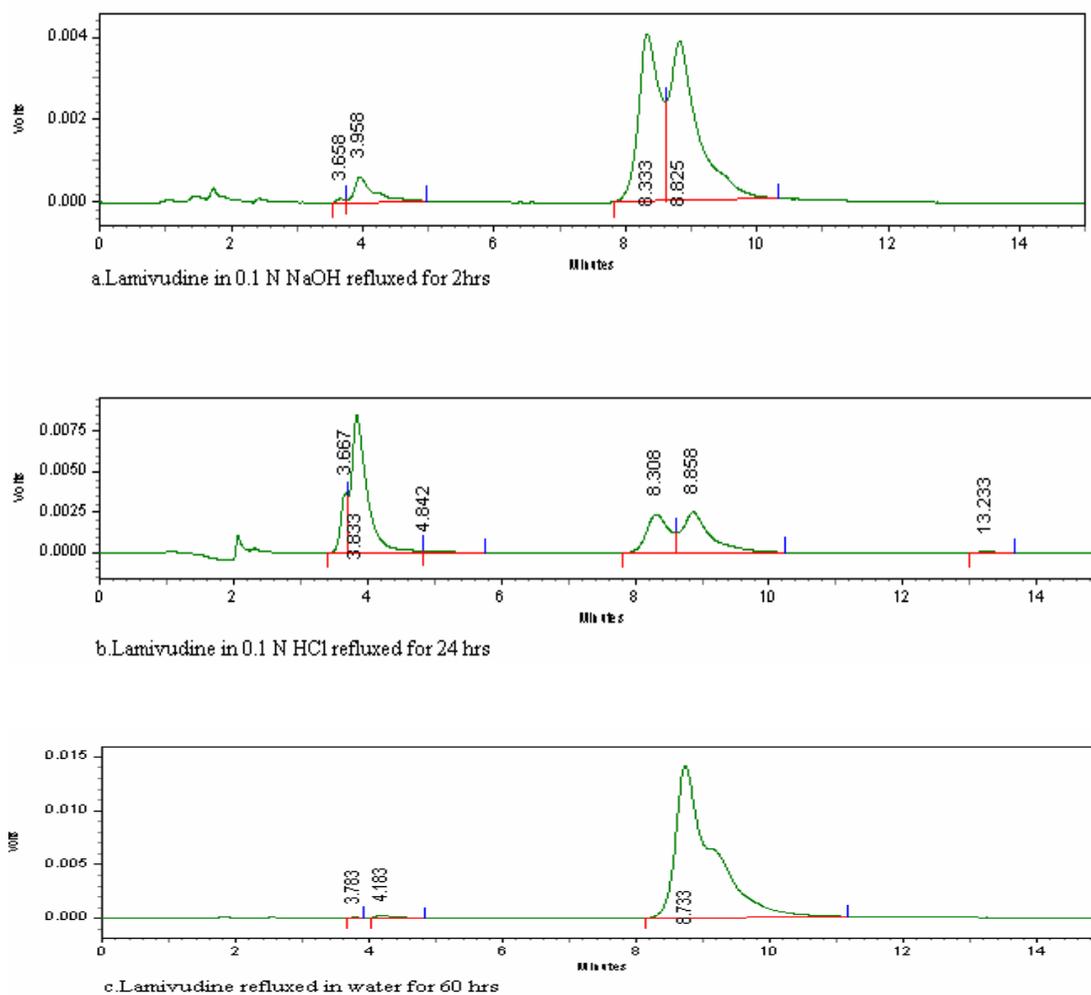
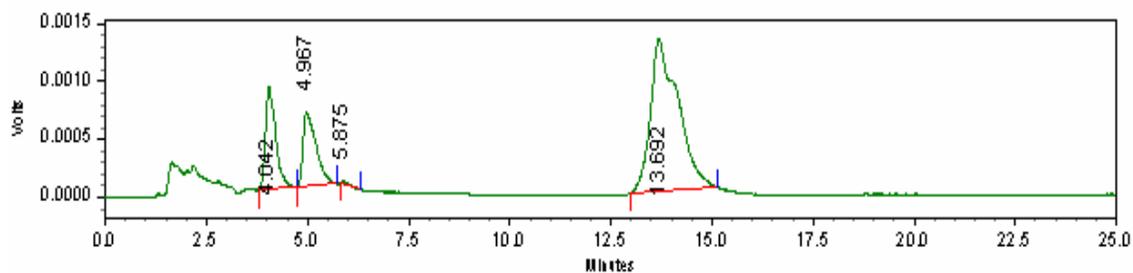
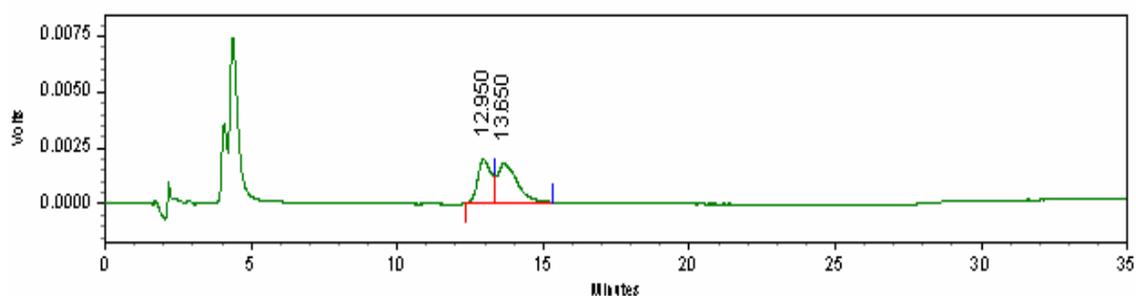


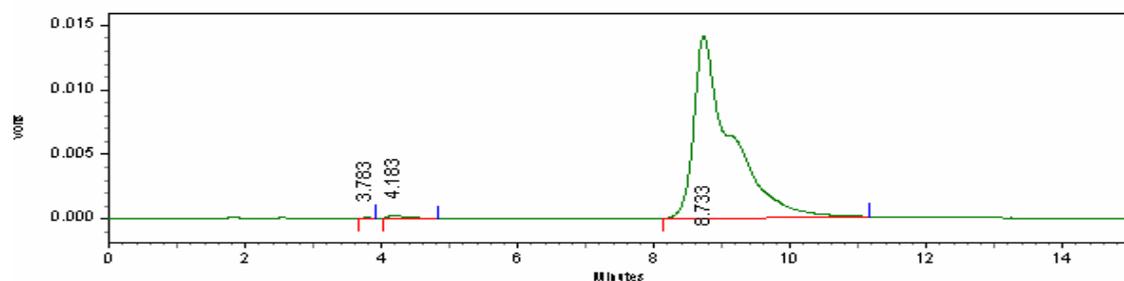
Figure 4.2: HPLC chromatograms of lamivudine and its degradation products obtained using 15% methanol in water as mobile phase at flow rate of 1ml/min, C-18 column, at 30⁰C , injection volume of 20µl and detection at 270 nm UV-Vis diode array detectors.



a. Lamivudine refluxed in 0.1N NaOH for 10 hrs.

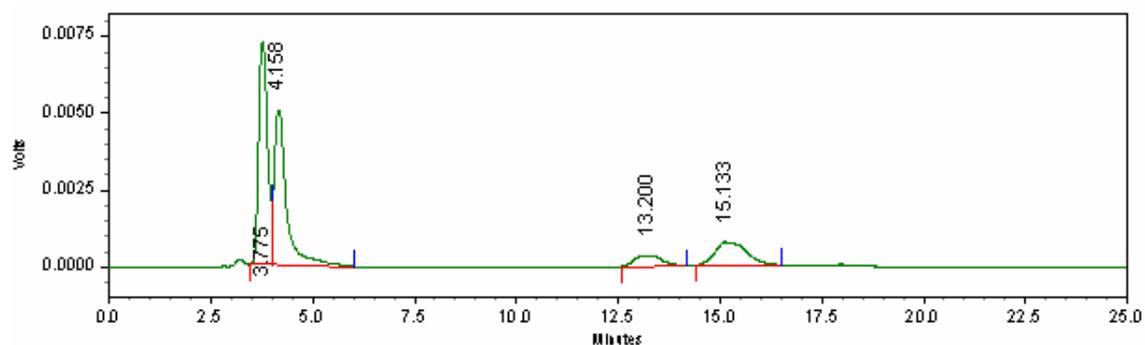


b. Lamivudine refluxed in 0.1 N HCl for 24 hrs.

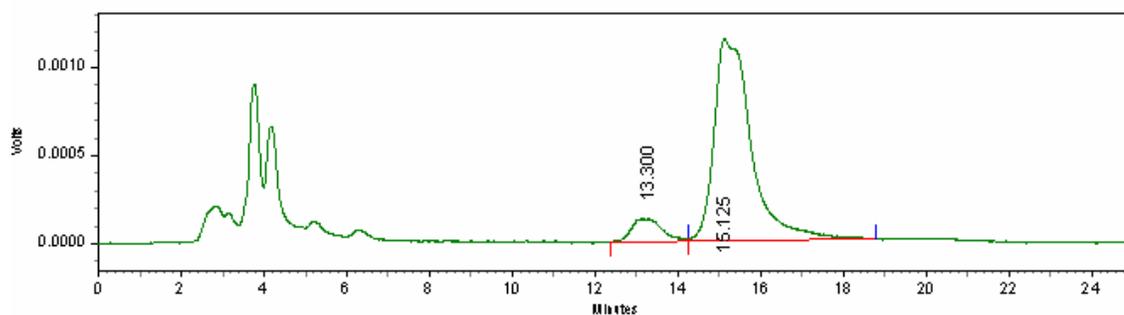


c. Lamivudine refluxed in water for 60 hrs

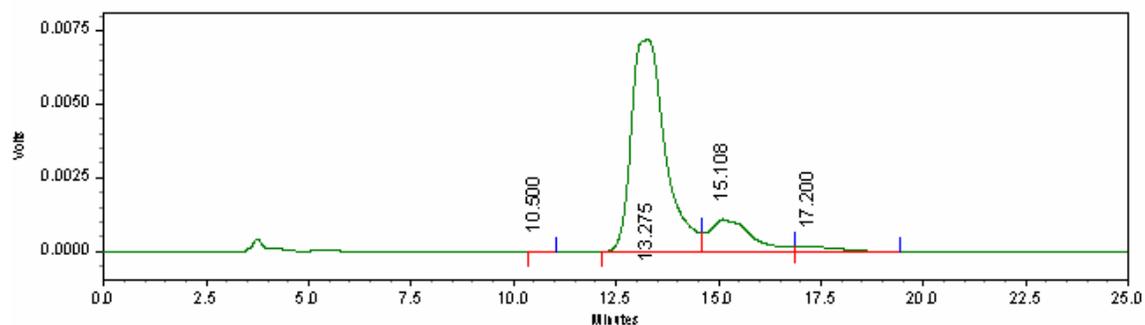
Figure 4.3: HPLC chromatograms of lamivudine and its degradation products using 10 % methanol in water as mobile phase at flow rate of 1ml/min, C-18 column, at 30 °C , injection volume of 20µl and detection at 270 nm UV-Vis diode array detectors.



a. Lamivudine refluxed in 0.1 N HCl for 36hrs.



b. Lamivudine refluxed in 0.1N NaOH for 8hrs.



c. Lamivudine refluxed in water for 72hrs.

Figure 4.4: HPLC chromatograms of lamivudine and its degradation products using 0.1 M ammonium acetate: methanol: acetonitrile: (1%) v/v acetic acid: (90.9%:8%:1%:0.1%) as mobile phase at flow rate of 1ml/min, C-18 column at 30 °C , 20µl injection volume and detection at 270nm UV-Vis diode array detector.

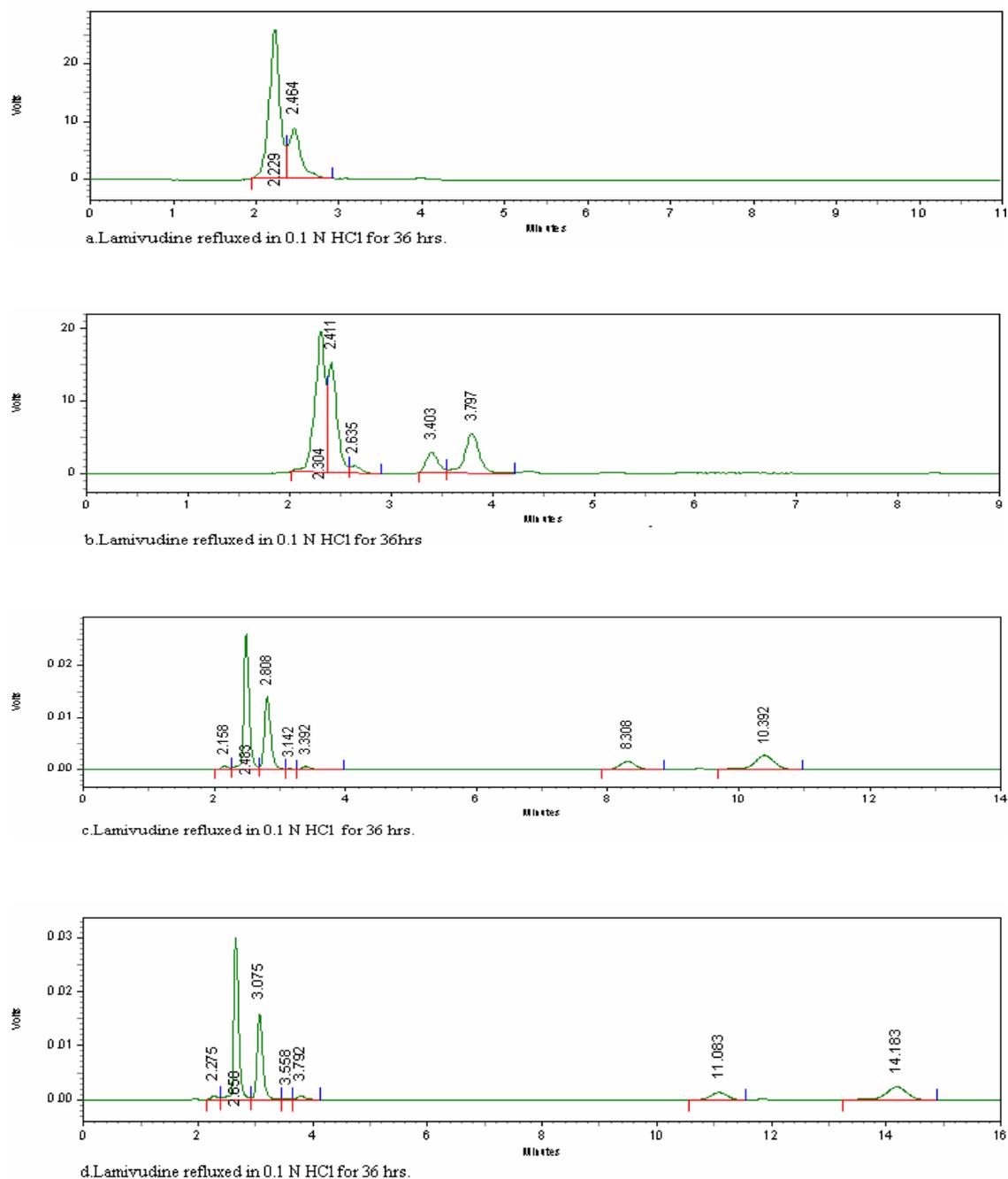


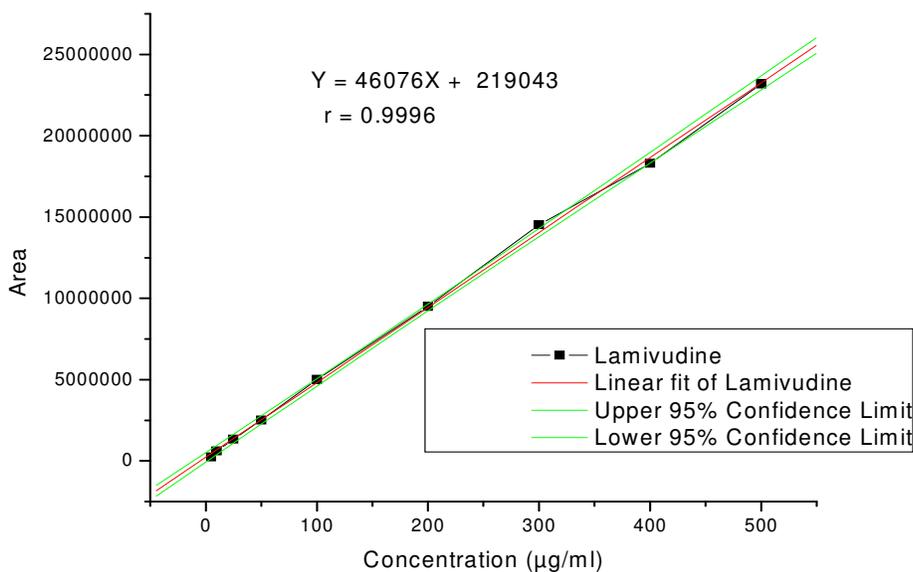
Figure 4.5: HPLC chromatograms of lamivudine and its degradation products using 0.1M ammonium acetate: Methanol: 1%v/v acetic acid at a ratio of a) 49.9:50:0.1 b) 74.9:25:0.1 c) 89.9:10:0.1 d) 91.9:8:0.1 as mobile phase at 1ml/min flow rate, C-8 column, at 30 °C , 20µl injection volume and detection at 270nm UV-Vis diode array detector.

4.2. Validation of the method

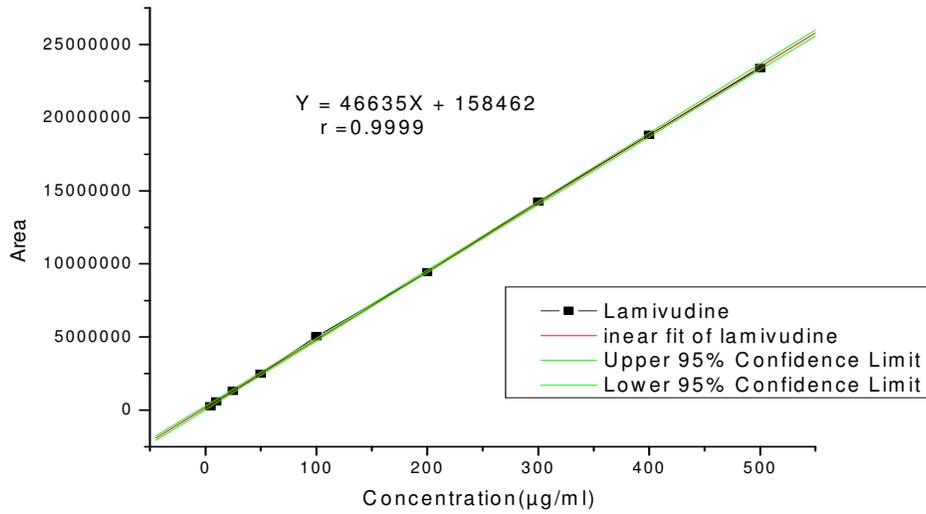
The method was validated with respect to parameters like linearity, precision, accuracy, specificity, selectivity and ruggedness.

4.2.1. Linearity

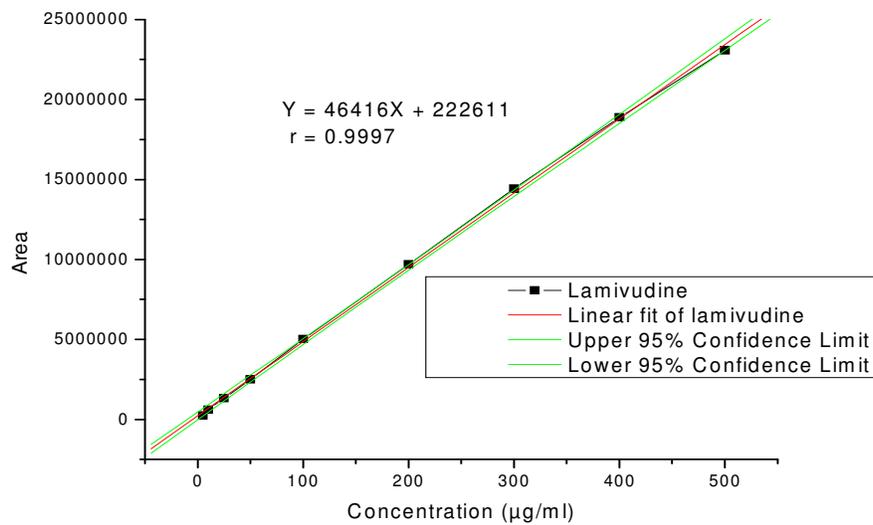
Linearity was established using 0.1M ammonium acetate: methanol: 1%V/V acetic acid (91.9:8:0.1) solvent system on three different days. The mean HPLC area response VS concentrations were plotted for all of the three days using GraphPad Instant and Origin 7 software in the concentration range of 5 -500 μ g/ml. The plot obtained in the three days (day 1, 2 and 3) were strictly linear in the investigated concentration range ($r > 0.999$). The mean (\pm RSD) values of slope and intercept were 46076(\pm 0.0106) and 219043 (\pm 0.555) for day1, 46635(\pm 0.00472) and 158462 (\pm 0.34439) for day 2, 46416(\pm 0.00896) and 222611(\pm 0.4634) for day 3 respectively. Figure 4.6 shows the linearity plot obtained on the three days.



a) Day 1



b) Day 2



c) Day 3

Figure: 4.6 Calibration plots of mean HPLC Area responses versus concentration (5-500 µg/ml) obtained on three different days using 0.1M ammonium acetate:methanol:1%v/v acetic acid (91.9:8:0.1) as mobile phase at 1ml/min flow rate, C-8 column, at 30°C, 20µl injection volume and detection at 270nm UV-Vis diode array detector.

The mean HPLC Area response of the three days was strictly linear in the investigated concentration range ($r = 0.9998$). Table 4.1 shows the average of the mean HPLC area response of the three days. The mean (\pm RSD) values of slope, intercept were $46376(\pm 0.006975)$ and $200049(\pm 0.4009)$ respectively.

Table 4.1: The mean HPLC Area responses obtained on three days in the concentration range of 5-500 μ g/ml ($n=3$)

Conc. (μ gml ⁻¹)	Mean	STD	%RSD
5	246793,9	5162.021	2.0916
10	609283.4955	7576.991	1.2435
25	1319792.47	13681.8	1.03666
50	2500912.127	16543.43	0.66149
100	5022726.654	19412.97	0.3865
200	9544916.018	134206.7	1.40605
300	14406463.04	137319.6	0.95318
400	18665711.74	332576.8	1.781753
500	23221106.36	155678.9	0.67042

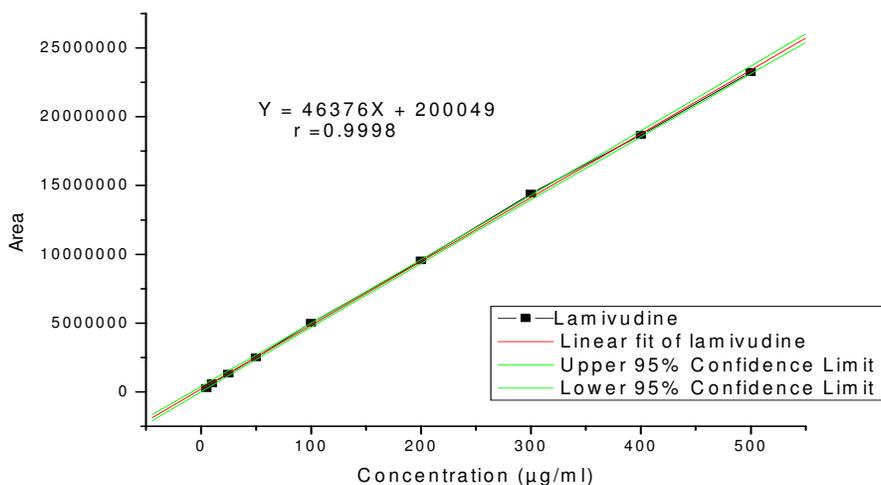


Figure: 4.7. Calibration plots of mean HPLC Area responses versus concentration (5-500 μ g/ml) obtained on the mean of the area of three days using 0.1Mammonium acetate: methanol: 1%v/v acetic acid(91.9:8:0.1) as mobile phase at 1ml/min flow rate and C-8 column, at 30 $^{\circ}$ C , 20 μ l injection volume, and detection at 270 nm UV-Vis diode array detector.

4.2.2. Precision

Data obtained from the precision experiments for six multiple injections at eight different dilutions (10, 25, 50, 100, 200, 300, 400, and 500 $\mu\text{g/ml}$) during intra-day precision studies are given in Table 4.2. The corresponding mean data for inter-day studies are given in Table 4.3. The RSD values for intra-day and inter-day precision studies are <0.292 and <1.781 respectively which confirm that the method is sufficiently precise.

Table 4.2: Peak area values for lamivudine from intra-day precision studies.

Concentration (μgml^{-1})	Area						Mean	STD	%RSD
	A1	A2	A3	A4	A5	A6			
10	602846,3	602542,8	600984,4	599385,2	602188,7	598636,2	601097,3	1751,613	0,291403
25	1303883	1304660	1306394	1303897	1304955	1304350	1304690	934,8913	0,071656
50	2484879	2480118	2484875	2485590	2480172	2483925	2483260	2470,268	0,099477
100	5044324	5041163	5042661	5045164	5045745	5037178	5042706	3188,667	0,063233
200	9435170	9435536	9431661	9433766	9413569	9430434	9430023	8300,314	0,08802
300	14257438	14247156	14260037	14259856	14262652	14258826	14257661	5424,763	0,038048
400	18811922	18806486	18814235	18819710	18835428	18822261	18818340	10077,64	0,053552
500	23388383	23392288	23371848	23378514	23388315	23407035	23387731	12094,62	0,051714

Table 4.3: Mean area values (n=3) for lamivudine from inter-day precision studies.

Days	Mean area							
	$10\mu\text{gml}^{-1}$	$25\mu\text{gml}^{-1}$	$50\mu\text{gml}^{-1}$	$100\mu\text{gml}^{-1}$	$200\mu\text{gml}^{-1}$	$300\mu\text{gml}^{-1}$	$400\mu\text{gml}^{-1}$	$500\mu\text{gml}^{-1}$
1	601097,3	1304690	2483260	5042706	9430023	14257661	18818340	23387731
2	610702,7	1323328.749	2503415.37	5021538.911	9692424.125	14433451.36	18894578.79	23079371.6
3	616050.5837	1331358.949	2516061.284	5003934.825	9512301.556	14528298.64	18284215.95	23196216.93
Mean	609283.4955	1319792,477	2500912.127	5022726.654	9544916.018	14406470.17	18665711.74	23221106.36
STD	7576.991	13681.8	16543.43	19412.97	134206.7	137321.7	332576.8	155678.9
%RSD	1.24359	1.036663	0.661496	0.386503	1.406055	0.953194	1.78	0.67042

The data for Table 4.2 and 4.3 obtained using 0.1M ammonium acetate: methanol: 1%v/v acetic acid (91.9:8:0.1) solvent system at 1ml/min. flow rate, C-8 column, at 30°C, 20 μ l injection volume and detection at 270 nm UV-Vis diode array detector.

4.2.3. Accuracy

The mixture of stressed samples was spiked with drug at four concentrations. Percentage recovery was calculated from difference between the peak areas obtained for fortified and unfortified solutions. As shown from the data in Table 4.4, excellent recoveries were made at each added concentration, despite the fact that the drug was fortified to a mixture that contained drug as well as the degradation products, formed under various reaction conditions.

Table 4.4: Recovery data for lamivudine spiked into a mixture of stressed samples (NB-Observed area of drug in mixture of stressed samples before spiking = 27811) the data collected using 0.1M ammonium acetate: methanol: 1%v/v acetic acid (91.9:8:0.1) solvent system at 1ml/min flow rate and C-8 column, at 30 °C, 20µl injection volume and detection at 270 nm UV-Vis diode array detector (n=3).

Spiked concentration (µgml ⁻¹)	Observed area of drug in mixture of stressed samples + spiked drug	Observed area of spiked drug	Calculated concentration of spiked drug (µgml ⁻¹)	±STD	%(RSD)	%(Recovery)
25	1367668.111	1339857.111	24.577	1425.575	0.10625	98.3%
60	2992011	2964200.222	59.603	1327.367	0.04436	99.33%
100	4919351	4891540	101.162	3983.781	0.080982	101.16%
300	14197508	14121300.89	300.184	43301.03	0.306034	100.06%

4.2.4. Specificity

Specificity of the method was established by verifying purity of the drug in a mixture of stressed samples by PDA detector and through the study of resolution factor of the drug peak from the nearest resolving peak. As shown in Figure 4.8 the resolution factor for the drug peak from the nearest resolving peak was greater than 2.9 indicating that the method was sufficiently specific to the drug. The method was also selective to degradation products as all peaks were pure. The purity curve and peak profile of the drug and the major degradation product in a mixture of stressed samples are shown in Figures 4.9-4.13. The peak purity index value for the drug and degradation products in a mixture of stressed samples was found to be

not less than the threshold value, indicating the absence of any co-eluting peak in the drug peak and degradation products.

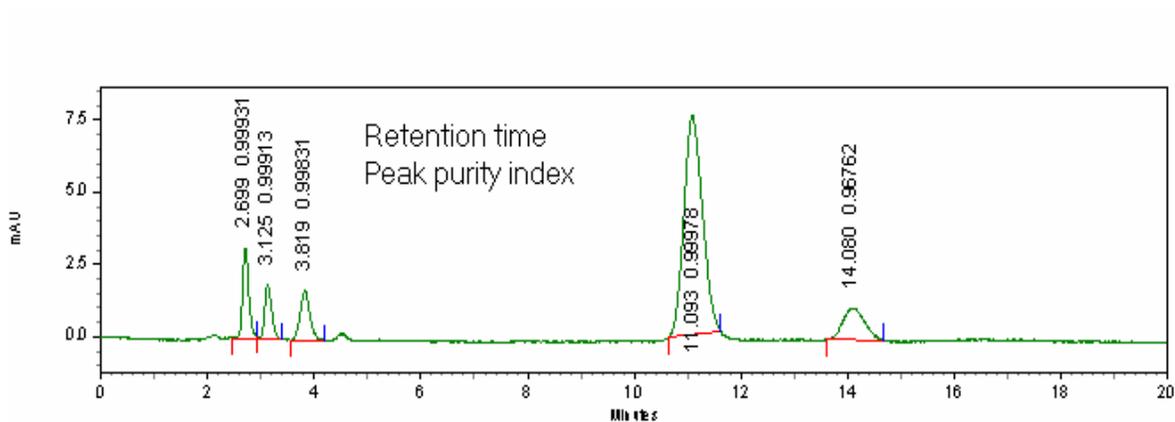


Figure4.8: Chromatogram showing the separation of different degradation products of lamivudine and its degradation products in a mixture of reaction solution. The chromatogram shows the retention time and the peak purity index. The chromatogram collected using C-8 column, at 30 °C , 20µl injection volume and 0.1M ammonium acetate: methanol: 1%v/v acetic acid (91.9:8:0.1) as solvent system at 1ml/min. flow rate and detection at 270nm UV-Vis diode array detector.

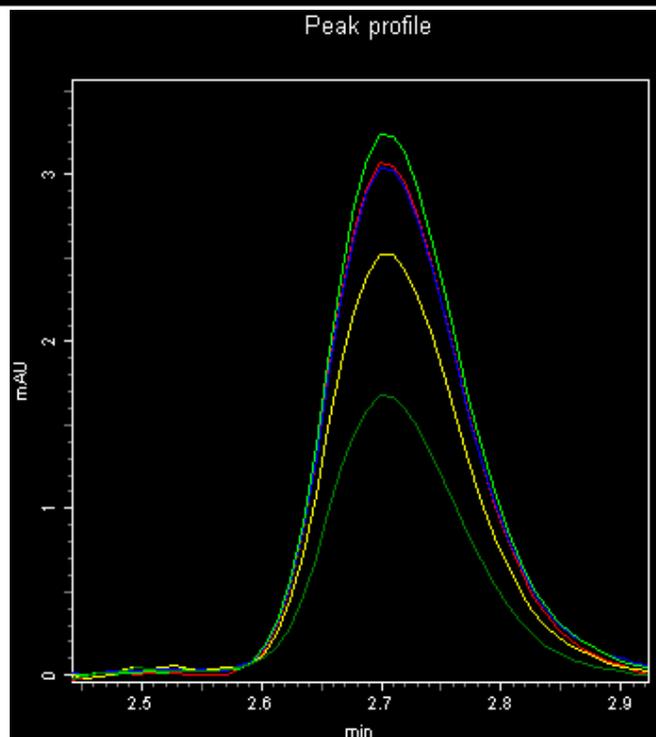


Figure 4.9: Peak purity and peak profile of the degradation product in a mixture of stressed samples ($R_T \approx 2.69$ min.) obtained using 0.1M ammonium acetate: methanol: 1%v/v acetic acid (91.9:8:0.1) as solvent system at 1ml/min flow rate, C-8 column, at 30 °C , 20 μ l injection volume ,and detection at 270nm UV-Vis diode array detector.

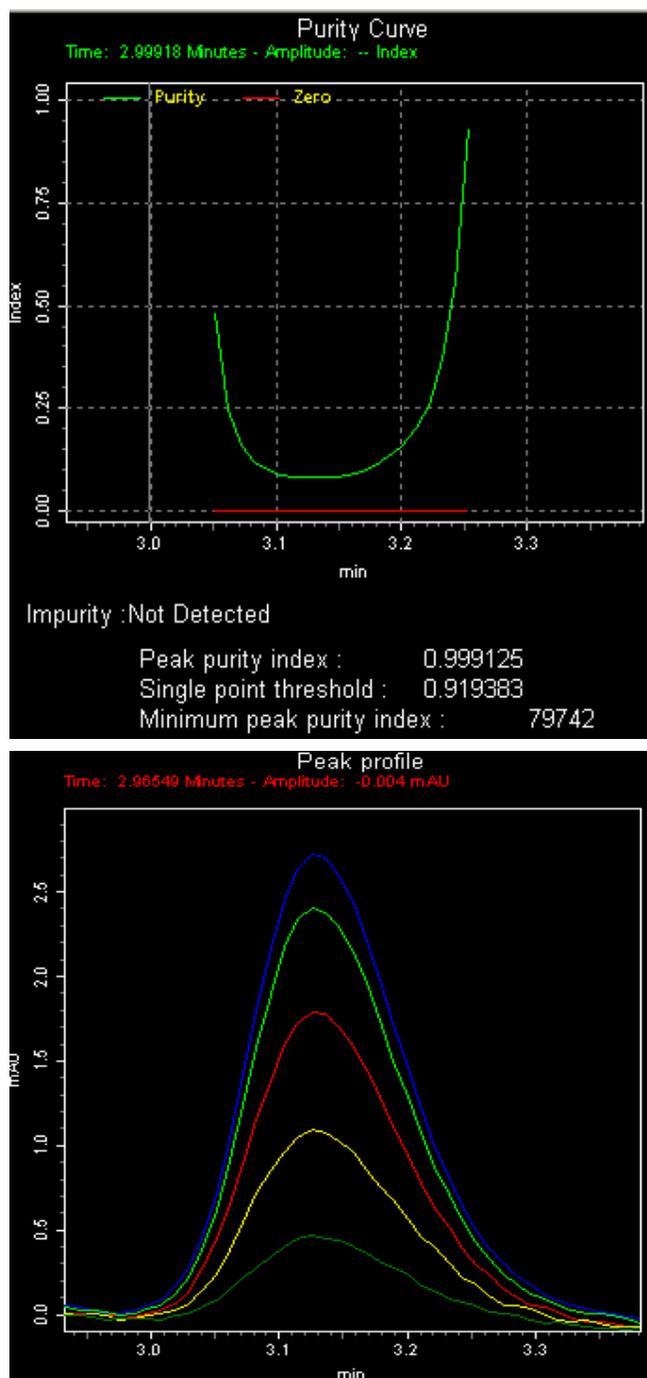


Figure 4.10: Peak purity and peak profile of the degradation product in a mixture of stressed samples ($R_T \approx 3.1$ min) obtained using 0.1M ammonium acetate: methanol: 1%v/v acetic acid (91.9:8:0.1) as solvent system at 1ml/min flow rate, C-8 column, at 30 °C , 20 μ l injection volume ,and detection at 270 nm UV-Vis diode array detector.

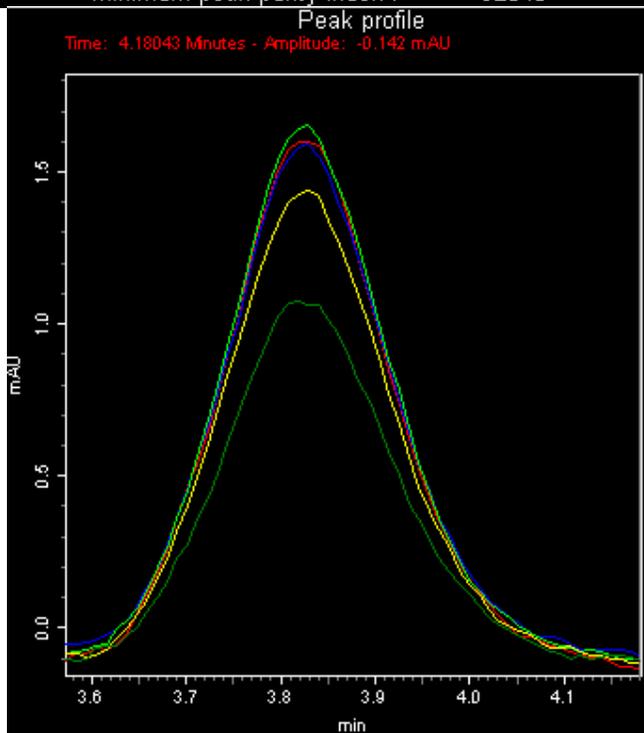
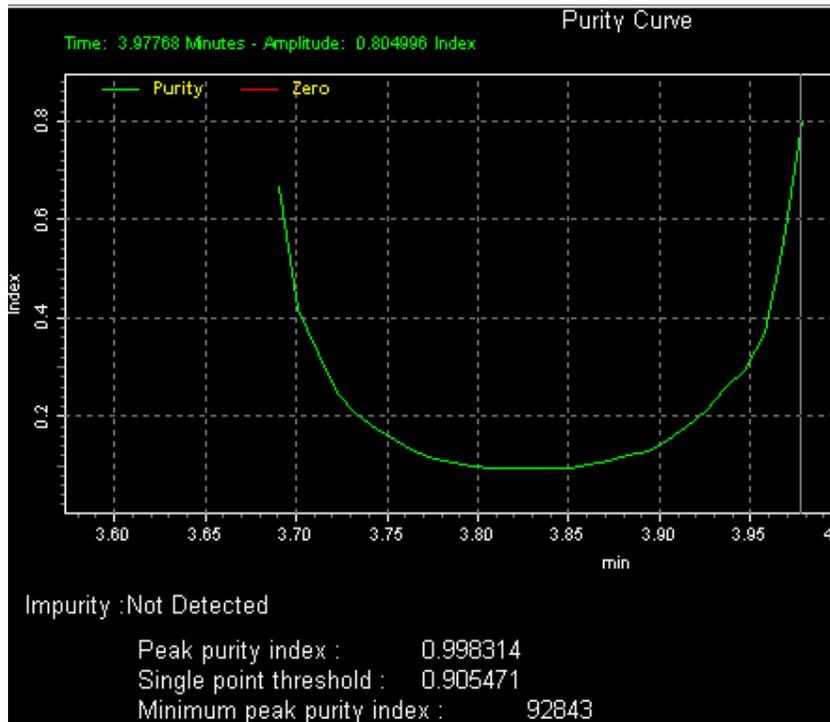


Figure 4.11: Peak purity and peak profile of the degradation product in a mixture of stressed samples ($R_T \approx 3.8$ min.) obtained using 0.1M ammonium acetate: methanol: 1%(v/v) acetic acid (91.9:8:0.1) as solvent system at 1ml/min flow rate, C-8 column, at 30 °C , 20 μ l injection volume ,and detection at 270 nm UV-Vis diode array detector.

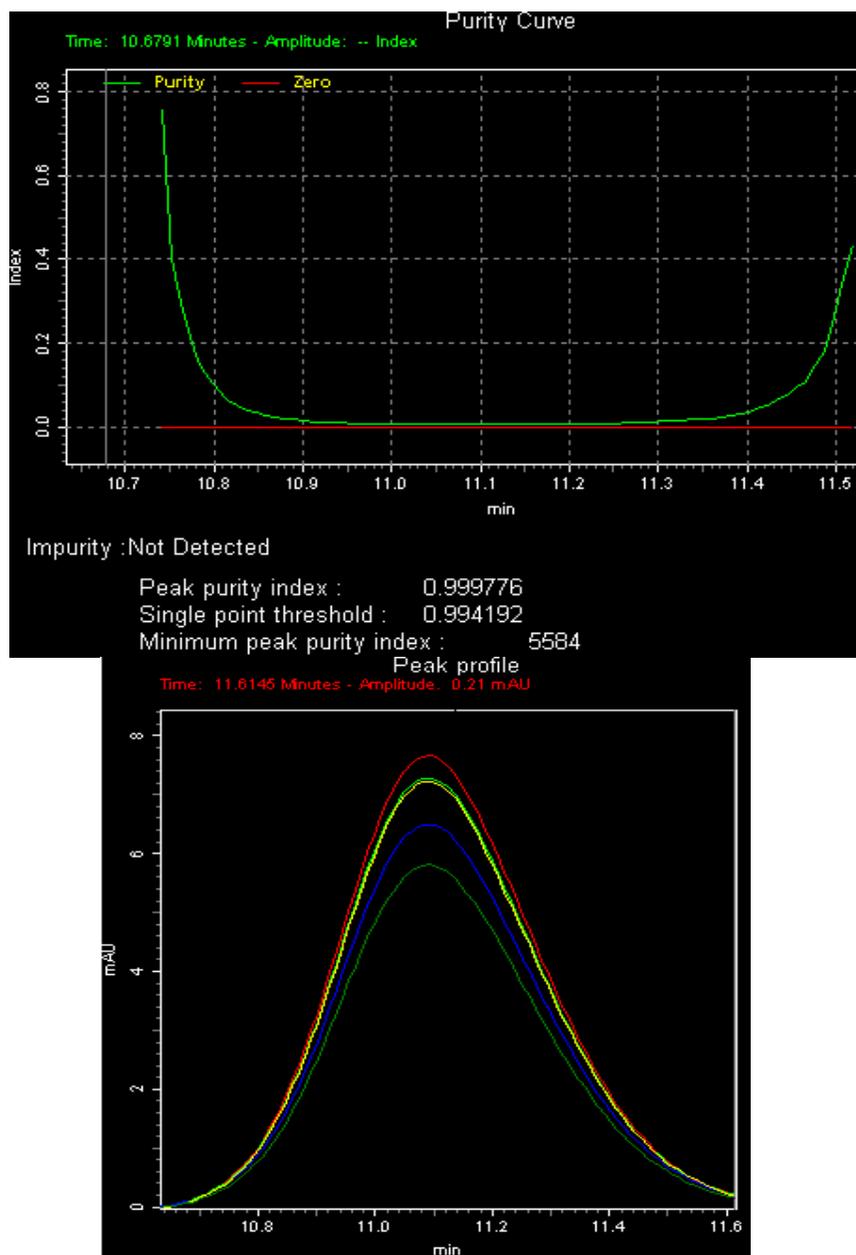


Figure 4.12: Peak purity and peak profile of lamivudine in a mixture of stressed samples ($R_T \approx 11.1$ min.) obtained using 0.1M ammonium acetate: methanol: 1%(v/v) acetic acid (91.9:8:0.1) as solvent system at 1ml/min flow rate, C-8 column, at 30 °C , 20 μ l injection volume ,and detection at 270 nm UV-Vis diode array detector.

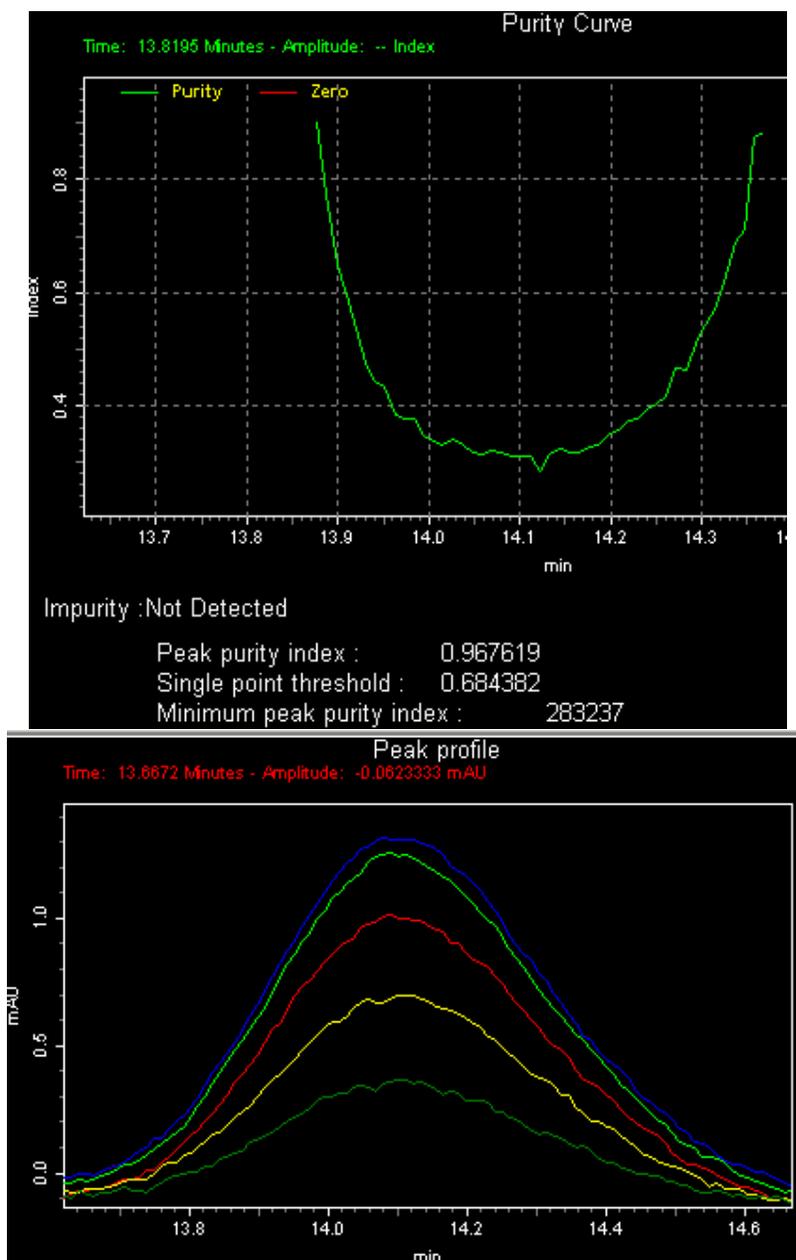


Figure 4.13: Peak purity and peak profile of the degradation product in a mixture of stressed samples ($R_T \approx 14.1$ min) obtained using 0.1M ammonium acetate: methanol: 1%(v/v) acetic acid (91.9:8:0.1) as solvent system at 1ml/min flow rate, C-8 column, at 30 °C , 20µl injection volume ,and detection at 270 nm UV-Vis diode array detector.

4.2.5. Ruggedness

Ruggedness was performed to confirm that separation was satisfactory under conditions external to the method. Good separations were always achieved with three analysts under the same chromatographic conditions and using different chromatographic system with same analysts indicating that the method remained selective for all components.

4.2.5.1 Inter and intra analyst precision studies

Data obtained from different analyst for four multiple injections at seven different concentrations (10, 50,100,200,300,400, and 500 μgml^{-1}) during intra-analyst precision studies are given in Table 4.5. The corresponding mean data for inter-analyst studies are given in Table 4.6. The RSD values for intra-analyst precision study are <0.848132 and for inter-analyst study are <3.3662, which confirm that the method is precise with differ analysts.

Table 4.5: Peak area values of lamivudine obtained by Analyst 1(Bekele Tefera,Senior expert, instrumental analyst) with 0.1 M ammonium acetate: methanol: 1%v/v acetic acid (91.9:8:0.1) solvent system at 1ml/min flow rate, C-8 column, at 30 $^{\circ}\text{C}$, 20 μl injection volume and detection at 270nm UV-Vis diode array detector.

Concentration (μgml^{-1})	Area						
	A1	A2	A3	A4	Mean	STD	%RSD
10	488564	485461	486120	484862	486251,8	1624,864	0,334161
50	2455299	2499620	2495411	2496986	2486829	21091,59	0,848132
100	4920172	4972754	4978702	4971014	4960661	27192,29	0,548159
200	9411298	9579174	9547047	9514802	9513080	72766,1	0,764906
300	13931513	13905304	13921577	13936806	13923800	13852,68	0,099489
400	18371371	18385796	18382368	18393638	18383293	9242,542	0,050277
500	22659864	22643006	22659208	22678791	22660217	14632,78	0,064575

Table 4.6: Peak area value of lamivudine obtained by analyst 2 (Lantider Kassaye, Physicochemical analyst expert I) with 0.1 M ammonium acetate: methanol: 1%v/v acetic acid (91.9:8:0.1) solvent system at 1ml-min⁻¹ flow rate, C-8 column, at 30⁰C, 20µl injection volume, and detection at 270nm UV-Vis diode array detector.

Concentration n (µg/ml)	Area				Mean	STD	%RSD
	A1	A2	A3	A4			
10	482449	482574	483208	478279	481627,5	2256,927	0,468604
50	2406047	2410484	2408973	2407798	2408326	1875,118	0,07786
100	4919711	4920721	4918291	4910385	4917277	4701,537	0,095613
200	9369590	9371406	9398588	9383666	9380813	13398,23	0,142826
300	13923325	13907038	13908115	13914334	13913203	7475,048	0,053726
400	18732818	18750206	18731556	18732376	18736739	8993,212	0,047998
500	23442560	23415236	23409290	23372472	23409890	28846,58	0,123224

Table4.7: Peak area value of lamivudine obtained by analyst 3(Berhanue Muche Physicochemical analyst expert II) with 0.1 M ammonium acetate: methanol: 1%v/v acetic acid (91.9:8:0.1) solvent system at 1 ml/min flow rate, C-8 column, at 30⁰C, 20µl injection volume and detection at 270nm UV-Vis diode array detector.

Concentration (µgml ⁻¹)	Area				Mean	STD	%RSD
	A1	A2	A3	A4			
10	512750	511959	514421	510583	512428,3	1602,053	0,312639
50	2479229	2477764	2 475 811	2481163	2478492	2265,33	0,0914
100	4704592	4698259	4692294	4698591	4698434	5022,477	0,106897
200	9626082	9633047	9611492	9614442	9621266	10067,83	0,104641
300	14185777	14168324	14165388	14170073	14172391	9131,276	0,06443
400	18773784	18747704	18745800	18728112	18748850	18818,3	0,10037
500	23478832	23479256	23476654	23444116	23469715	17103,69	0,022501

Table 4.8: Mean area values (n=3) of lamivudine obtained from inter-analyst precision studies with 0.1M ammonium acetate: methanol: 1%(v/v) acetic acid (91.9:8:0.1) solvent system at 1 ml/ml flow rate, C-8 column, at 30 °C, 20µl injection volume and detection at 270 nm UV-Vis diode array detector.

Analyst	Mean area						
	10µgml ⁻¹	50µgml ⁻¹	100µgml ⁻¹	200µgml ⁻¹	300µgml ⁻¹	400µgml ⁻¹	500µgml ⁻¹
1	486251,8	2486829	4960661	9513080	13923800	18383293	22660217
2	481627,5	2408326	4917277	9380813	13913203	18736739	23409890
3	512428,3	2478492	4698434	9621266	14172391	18748850	23469715
Mean	493435,8	2457882	4858791	9505053	14003131	18622961	23035053
STD	16609,63	43119,24	140556,7	120427,5	146678,6	207646,5	451086,3
%RSD	3,366118	1,754325	2,892833	1,266983	1,04747	1,115002	1,95826

4.2.5.2 HPLC column brand variation studies

HPLC area response of the drug obtained from different columns for three multiple injections at seven different concentrations (5, 25, 50, 100, 200, 300, and 400 µg/ml) during intra-column precision studies are given in Tables 4.9-4.11. The corresponding mean data for inter-column studies are given in Table 4.12. The RSD values for intra-column precision study are < 1.52 and for inter-column study are < 2.54, which confirm that the method is sufficiently precise even under different brands of C-8-columns. As shown in Figure 4. 14. Resolution of the drug in a mixture stressed samples with different brands of C8-column was found to be similar.

Table 4.9: Peak area value of lamivudine obtained with Teknokroma C-8 kromacil column, at 30 °C, 0.1M ammonium acetate: methanol:1%(v/v) acetic acid (91.9:8:0.1) solvent system at 1 ml/ml flow rate at, 20 µl injection volume and detection at 270nm UV-Vis diode array detector.

Concentration (µgml ⁻¹)	Area					
	A1	A2	A3	Mean	STD	%RSD
5	242628	247265	249997	246630	3725,313	1,510487
25	1263369	1261324	1257228	1260640	3127,063	0,248054
50	2375623	2384675	2382158	2380819	4672,262	0,196246
100	5086447	5091729	5090428	5089535	2751,984	0,054071
200	9999592	9998732	9995341	9997888	2247,572	0,02248
300	14261982	14257926	14259843	14259917	2029,012	0,014229
400	18872624	18878426	18886382	18879144	6907,046	0,036586

Table 4.10: Peak area value for lamivudine obtained with Supelco C-8 BDS column , at 30 °C, 20 µl injection volume, 0.1M ammonium acetate: methanol: 1% (v/v) acetic acid (91.9:8:0.1) solvent system at 1ml·min⁻¹ flow rate and detection at 270 nm UV-Vis diode array detector.

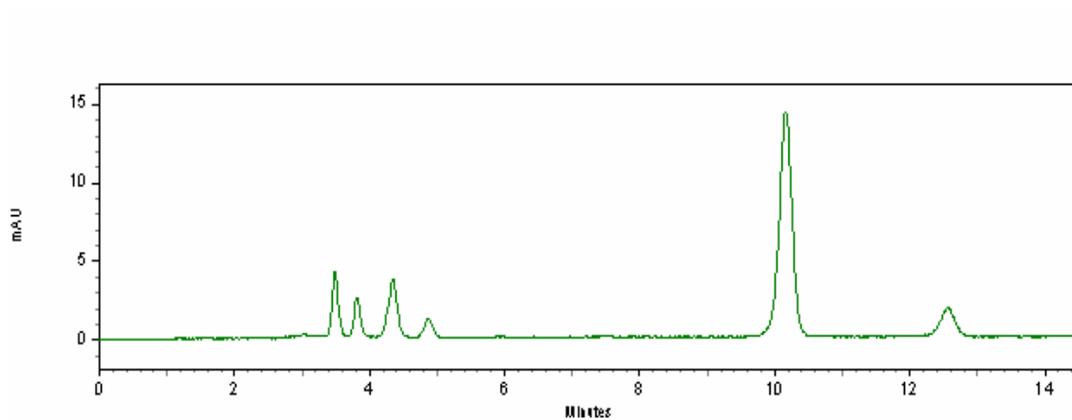
Concentration (µgml ⁻¹)	Area					
	A1	A2	A3	Mean	STD	%RSD
5	246859	246793	246405	246685,7	245,2944	0,099436
25	1283009	1283108	1282055	1282724	581,4817	0,045332
50	2417359	2420126	2416753	2418079	1798,178	0,074364
100	4885058	4884051	4879034	4882714	3226,787	0,066086
200	9798539	9779566	9787168	9788424	9548,689	0,097551
300	14374283	14372839	14357061	14368061	9553,601	0,066492
400	17988300	17973808	17949826	17970645	19431,09	0,108127

Table 4.11: Peak area value of lamivudine obtained with C-8 Nucleosil column, at 30 °C, 0.1 M ammonium acetate: methanol: 1% (v/v) acetic acid (91.9:8:0.1) solvent system at 1 ml/min. flow rate , 20 µl injection volume and detection at 270 nm UV-Vis diode array detector.

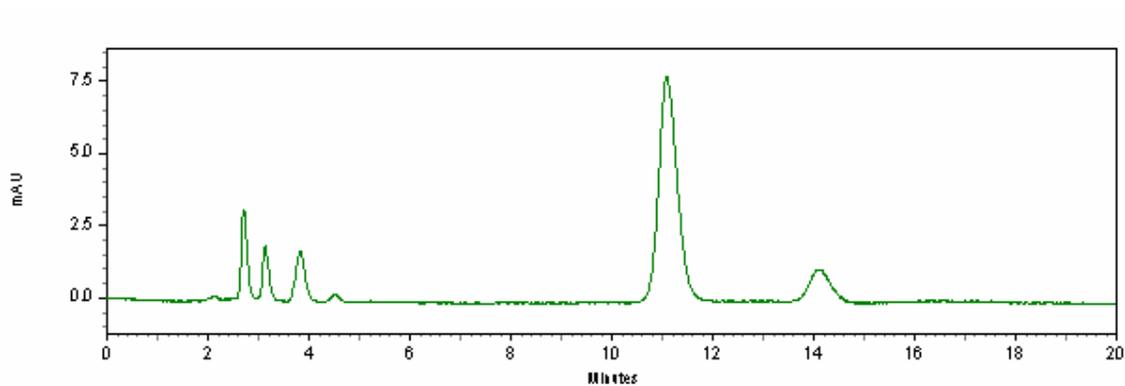
Concentration (µgml ⁻¹)	Area					
	A1	A2	A3	Mean	STD	%RSD
5	243185	244503	245844	244510,7	1329,517	0,543746
25	1274050	1268217	1272683	1271650	3050,621	0,239895
50	2404234	2407998	2408620	2406951	2373,169	0,098596
100	4866756	4861524	4860771	4863017	3259,884	0,067034
200	9829467	9828067	9835021	9830852	3677,976	0,037413
300	14627171	14643517	14641897	14637528	9006,213	0,061528
400	18494912	18495612	18517340	18502621	12751,54	0,068917

Table 4.12: Mean area value (n=3) of lamivudine obtained from inter-column precision studies with different C-8 column, at 30 °C, 0.1 M ammonium acetate: methanol: 1% (v/v) acetic acid (91.9:8:0.1) solvent system at 1 ml/ml flow rate, 20 µl injection volume and detection at 270 nm UV-Vis diode array detector.

Columns (Brand)	Area						
	5 µgml ⁻¹	25 µgml ⁻¹	50 µgml ⁻¹	100 µgml ⁻¹	200 µgml ⁻¹	300 µgml ⁻¹	400 µgml ⁻¹
C8(Kromacil)	246630	1260640	2380819	5089535	9997888	14259917	18879144
C8(Nucleosil)	244510,75	1272683	2406951	4863017	9830852	14641897	18502621
C8(supelco)	246685,7	1282724	2418079	4882714	9788424	14368061	17970645
Mean	245942,1	1272016	24019501	4945089	9872388	14423292	18450803
STD	1239,97	11057,11	19126,86	125481,3	110737,2	196888,3	456460,7
%RSD	0.504172	0.869259	0.796306	2.537493	1.121686	1.365072	2.473934



a



b.

Figure 4.14: HPLC Chromatograms obtained with 20 μ l injection volume, 0.1 M ammonium acetate: methanol: 1% (v/v) acetic acid (91.9:8:0.1) solvent system at 1 ml/min. flow rate, detection at 270 nm UV-Vis diode array detector and a) Supelco BDS C-8 column, at 30 $^{\circ}$ C, b) Teknokroma C-8 Kromacil column at 30 $^{\circ}$ C.

4.3. Degradation behavior

HPLC degradation studies of lamivudine under different stress conditions using 0.1 M ammonium acetate: methanol: 1% (v/v) acetic acid (91.9%:8%:0.1%) as the mobile phase indicate that the drug has the following behaviour.

4.3.1. Acidic conditions.

HPLC analysis of the reaction solution of lamivudine after it has been refluxed with 0.1 N HCl resulted in the formation of at least three major degradation products with R_t values of 2.7, 3.1, 14.01 (major products) 3.81 min (minor products). After 12, 24, 36, 48 and 72 hrs of refluxing around 26.3%, 53.1%, 63.9%, 73.7, 87.5% degradations were seen, respectively with a corresponding rise in the peak of the degradation product (Figure 4.15). The degradation products with retention times of 2.7, 3.1, and 14.01 min. has λ_{max} at 266 nm, 258 nm, 261 nm respectively (Figure 4.16).

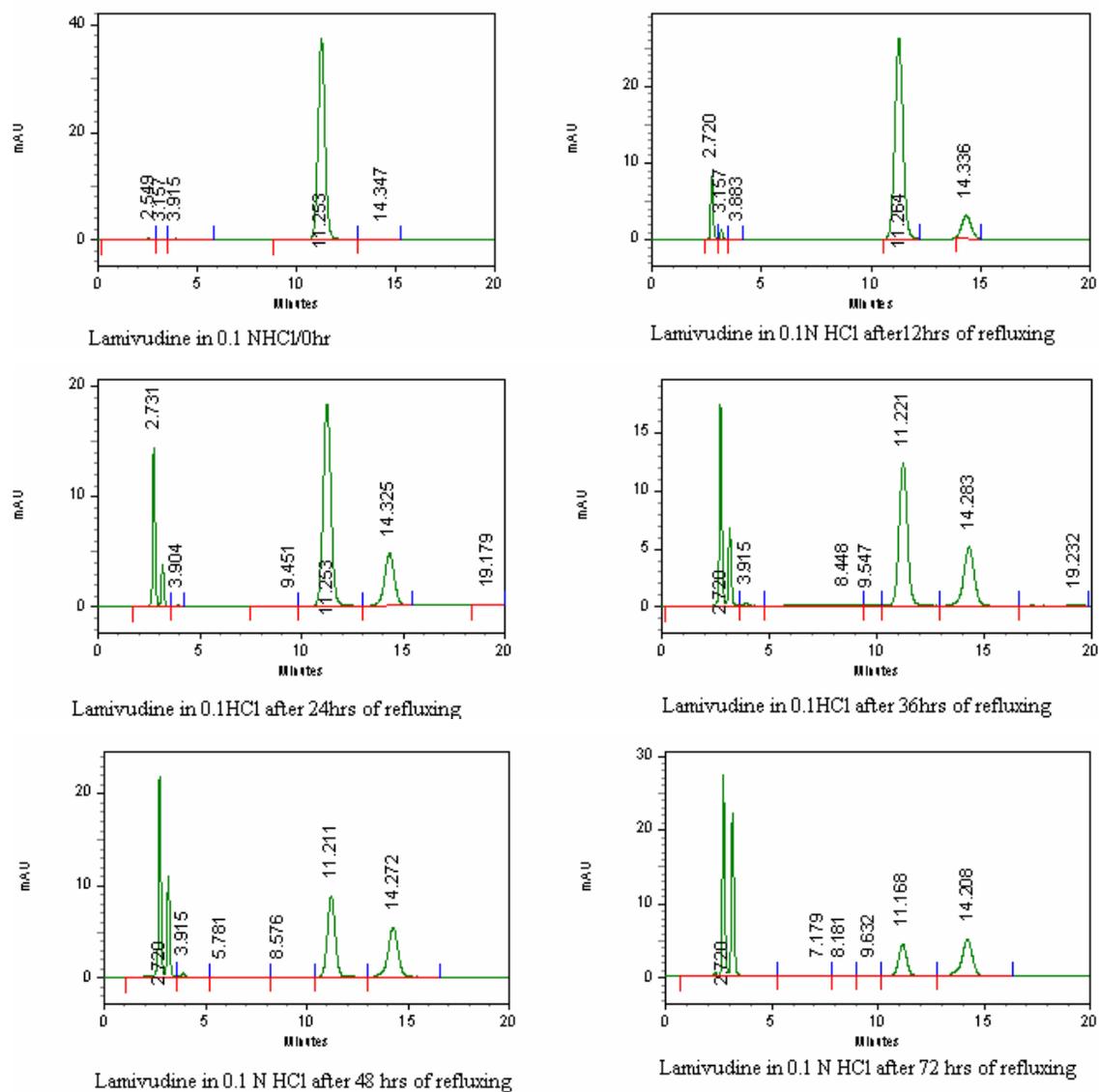


Figure 4.15: HPLC Chromatograms of lamivudine and its degradation products after treatment with 0.1 N HCl obtained with 0.1 M ammonium acetate: methanol: 1 % (v/v) acetic acid (91.9:8:0.1) solvent system at 1 ml/min. flow rate, C-8 column, at 30 °C, 20 µl injection volume and detection at 270 nm UV-Vis diode array detector

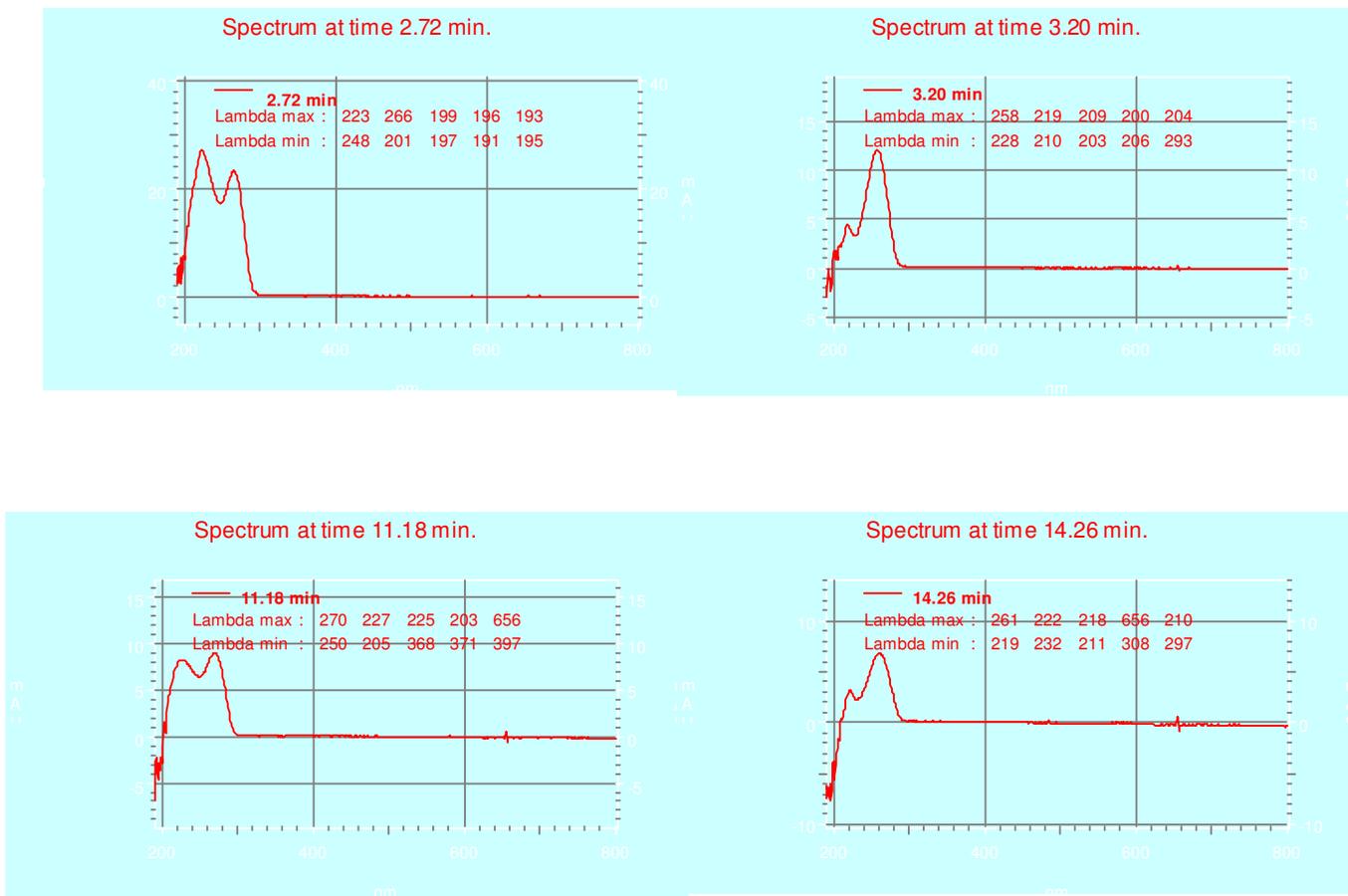


Figure 4.16: UV-spectra of the lamivudine (at 11.18 min.) and its degradation products after treatment with 0.1N HCl obtained with 0.1 M ammonium acetate: methanol: 1 % (v/v) acetic acid (91.9:8:0.1) solvent system at 1 ml/min flow rate, C-8 kromacil column, at 30 °C, 20 µl injection volume and detection at 270 nm UV-Vis diode-array detector.

When lamivudine in 0.1N HCl was heated at 60 °C for 15, 30 and 45 days around 14.5%, 28.3%, 39.3% degradation was seen respectively with a corresponding rise in degradation products peaks (Figure 4.17). The retention time of the separated major degradation products and their spectra obtained with PDA detector were similar to the one obtained from refluxed solution. It was also observed that lamivudine in 0.1N HCl kept at normal laboratory working condition for 30 and 60 days shows around 0.44 %, 0.72 % decompositions respectively indicating that the drug is relatively stable at room temperature in 0.1 N HCl (Figure 4.18).

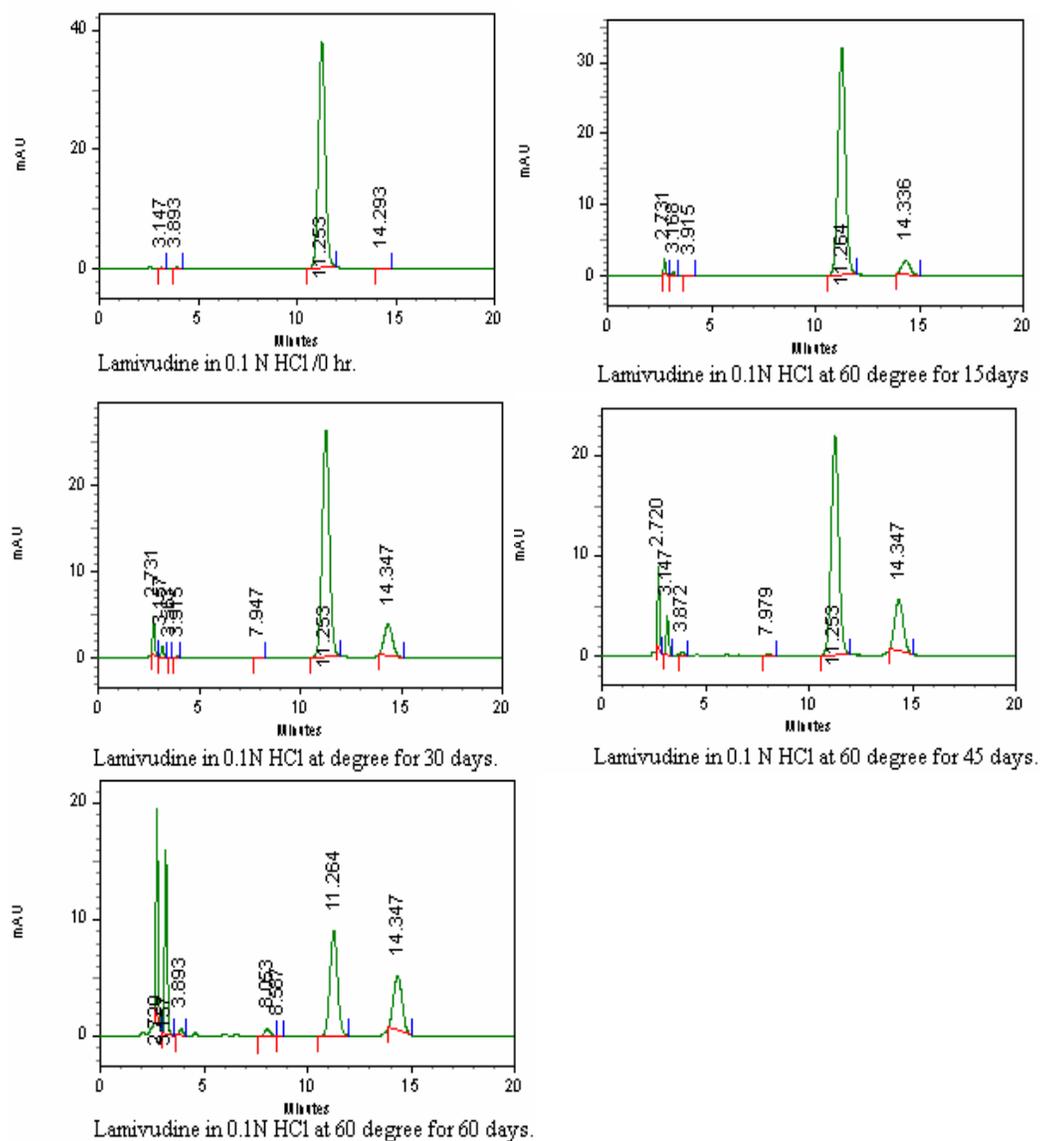


Figure 4.17: HPLC Chromatograms of lamivudine treated with 0.1N HCl at 60 °C obtained with 0.1 M ammonium acetate: methanol: 1% (v/v) acetic acid (91.9:8:0.1) solvent system at 1 ml/min flow rate, C-8 column, at 30 °C, 20 µl injection volume and detection at 270 nm UV-Vis diode array detector.

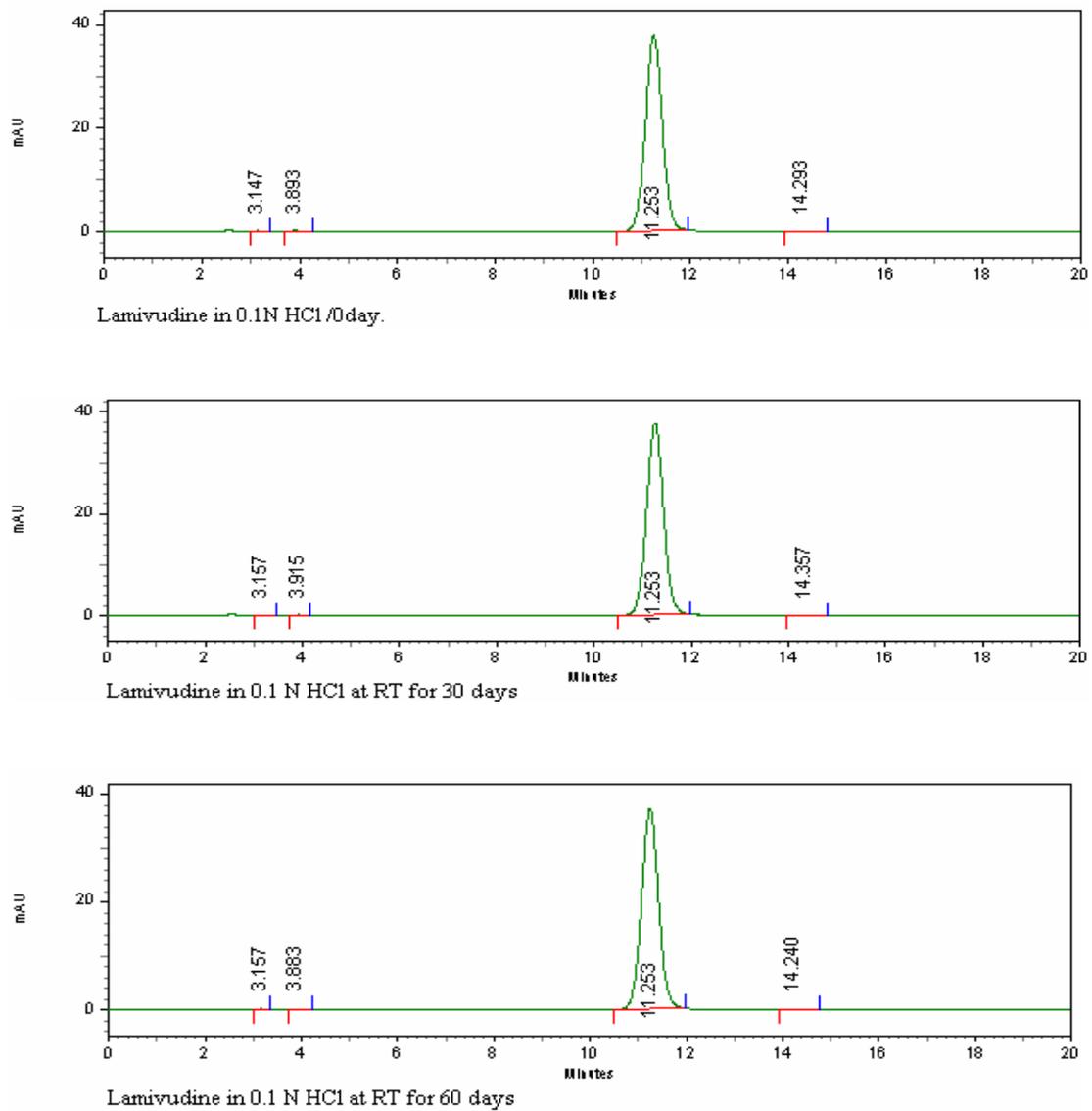


Figure 4.18: HPLC Chromatograms of lamivudine and its degradation products after treatment with 0.1N HCl at RT; obtained with 0.1 M ammonium acetate: methanol: 1 % (v/v) acetic acid (91.9:8:0.1) solvent system at 1ml/min flow rate, C-8 column, at 30 °C, 20 µl injection volume and detection at 270 nm UV-Vis diode array detector (RT = normal working environment)

4.3.2 Alkaline conditions

Lamivudine in 0.1N NaOH was found to decompose rapidly compared to its decomposition in 0.1N HCl on refluxing. Around 78.6% decomposed when it was refluxed with 0.1N NaOH for a bout 2hrs. On subsequent refluxing of lamivudine in 0.1N NaOH for 4, 6, 8, 10, 12 hrs 94.7%, 98.1%, 99.3%, 99.8%, 99.9% degradation were observed, respectively, with formation of yellowish colour solution and a corresponding rise in degradation product peaks which have the same retention time and UV spectrum as of the degradation product formed when refluxed in 0.1N HCl (Figure 4.19 and 4.20). Lamivudine also showed rapid decompositions when treated at 60 °C. The degradation rate in 0.1N NaOH at 60 °C was still high. On heating at this condition for 15, 30, 45 days around 98.5%, 99.7%, 99, 8% of the drug decomposed (Figure4.21) with formation of yellowish color solution and a corresponding rise in degradation peaks indicating the formation of degradation products with chromophoric and non-chrompophoric groups. The stability study of lamivudine in 0.1N NaOH at normal working laboratory condition revealed that about 19.7% and 28% of the drug decomposed within 30 and 60 days respectively (Figure 4.22)

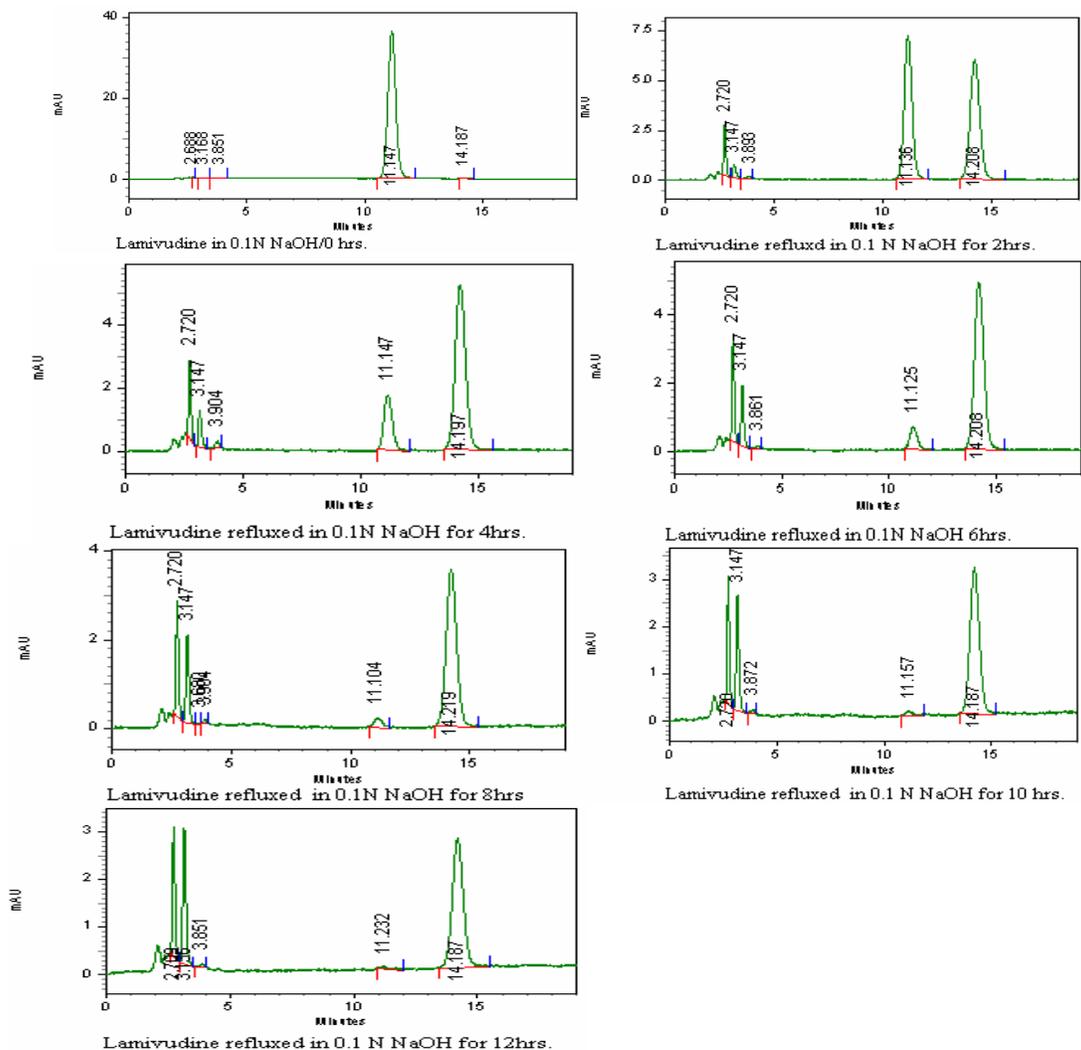


Figure 4.19: HPLC Chromatogram of lamivudine and its degradation products after refluxing in 0.1N NaOH; obtained with 0.1 M ammonium acetate: methanol: 1% (v/v) acetic acid (91.9:8:0.1) solvent system at 1 ml/min. flow rate, C-8 column, at 30 °C, 20 µl injection volume and detection at 270 nm UV-V diode array.

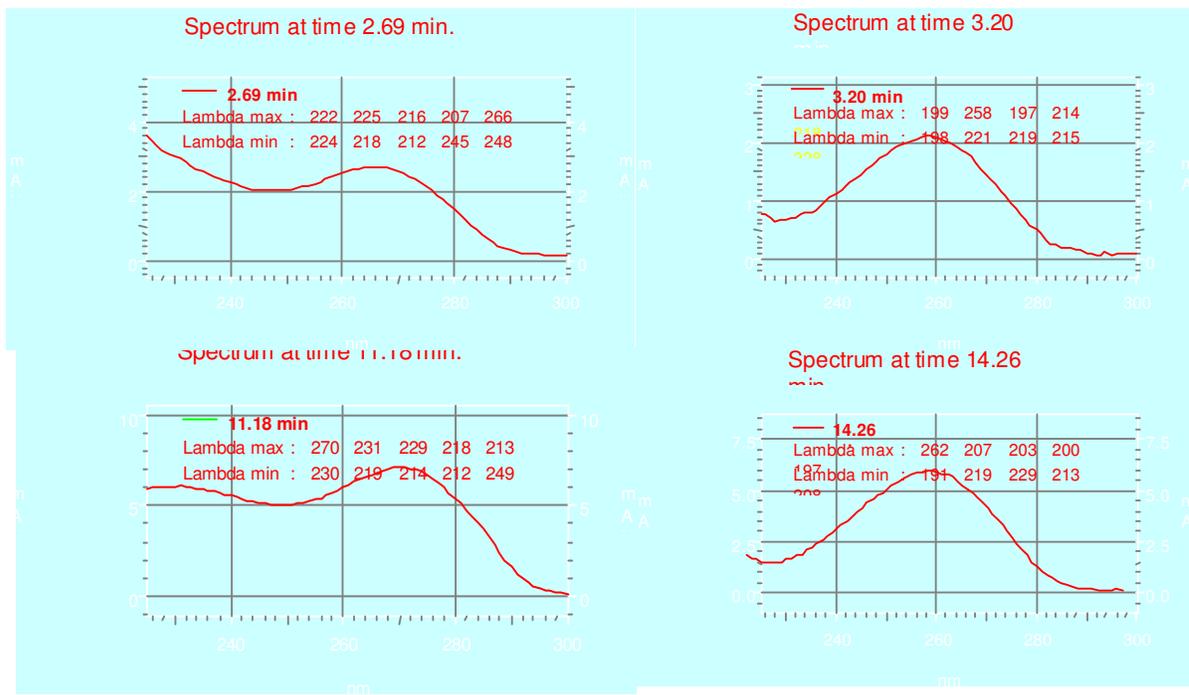


Figure 4.20: UV spectra of lamivudine ($R_T \approx 11.1$) and its degradation products in 0.1 N NaOH; obtained under the chromatographic conditions of 0.1 M ammonium acetate: methanol: 1% (v/v) acetic acid (91.9:8:0.1) solvent system at 1 ml/min. flow rate, C-8 column, at 30 °C, 20 μ l injection volume and detection at 270 nm UV-Vis diode-array detector.

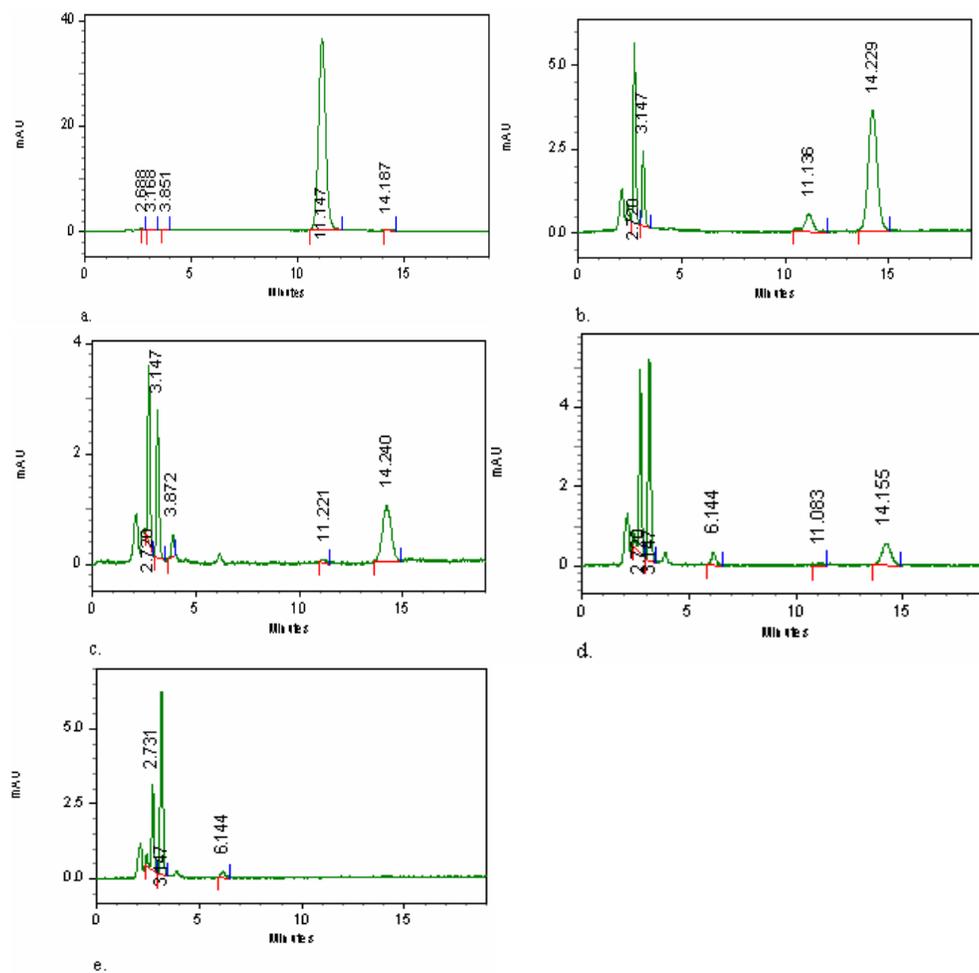


Figure 4.21: HPLC Chromatograms of lamivudine and its degradation products after treatment with 0.1 N NaOH at 60 °C obtained under the chromatographic conditions of 0.1 M ammonium acetate: methanol: 1% (v/v) acetic acid (91.9:8:0.1) solvent system at 1 ml/min. flow rate, C-8 column, at 30 °C, 20 µl injection volume and detection at 270 nm UV-Vis diode-array detector. a) At zero day. b) After 15 days c) After 30 days d) After 45 days e) 60 days

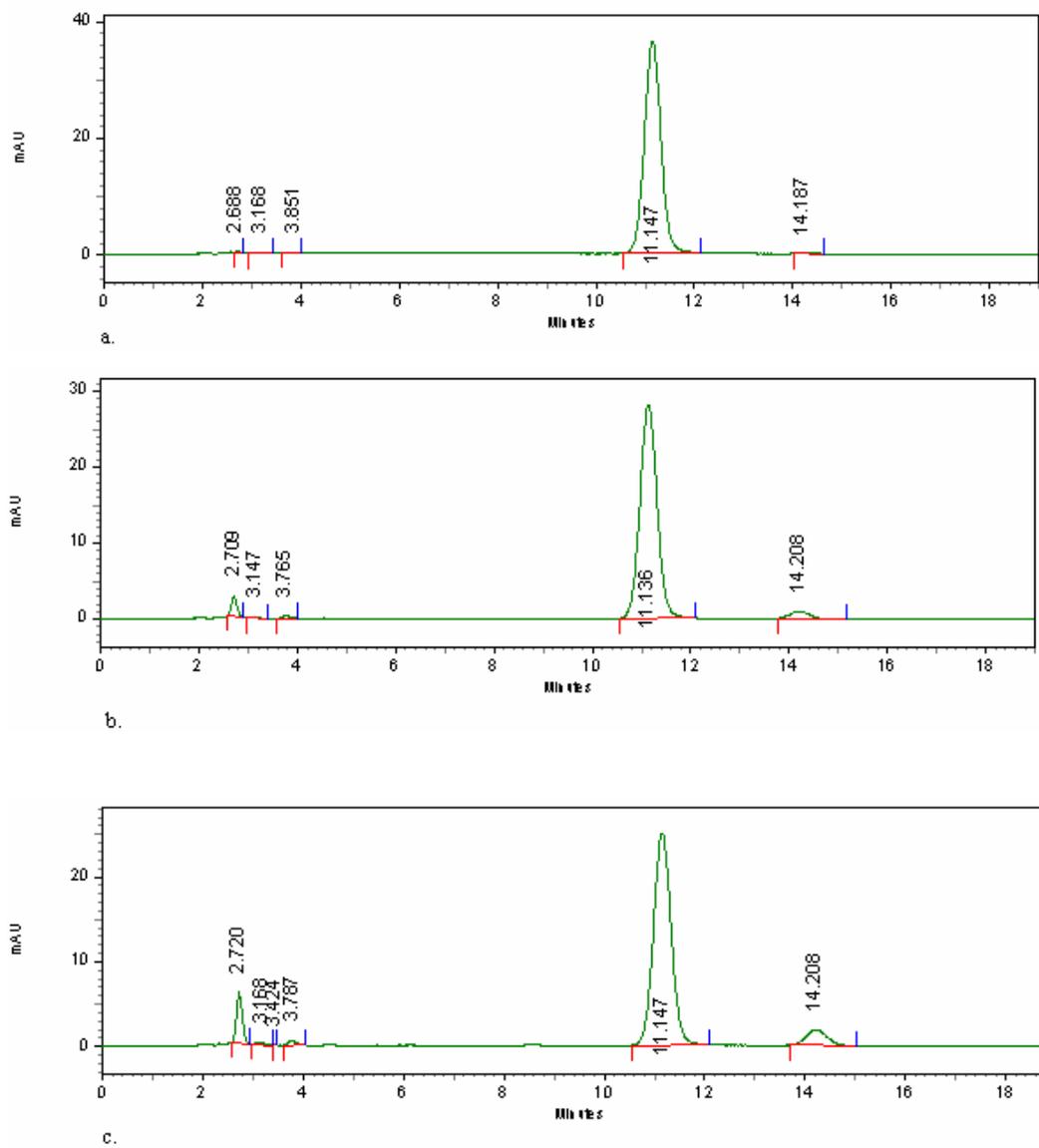


Figure 4.22: HPLC Chromatogram of lamivudine and its degradation products after treatment with 0.1N NaOH at RT; obtained with 0.1 M ammonium acetate: methanol: 1% (v/v) acetic acid (91.9:8:0.1) solvent system at 1 ml/min flow rate, C-8 column, at 30 °C, 20 µl injection volume and detection at 270 nm UV-Vis diode array detector (RT = normal working environment)

4.3.3 Neutral condition

When lamivudine refluxed in distilled water for 24, 60, 72, 84 and 96 hrs a round 1.8%, 19.6%, 26.1%, 48.0% and 82.6% decompositions were observed respectively with a generations of one major peak at around 14.2 min (Figure4.23). On heating the same solution at 60 °C for 15, 45 and 60 days there were about 1.5 %, 2.15 % and 2.3 % decomposition, respectively (Figure 4.24). About 1,2%and1,9% decompositions were observed (Figure 4.25) with corresponding rise in a minor peak at about 14.2 min. when it was kept at room temperature for 30 and 60 days, respectively. The degradation product showed similar UV spectra to that of the degradation product obtained after refluxing with 0.1N HCl and 0.1 N NaOH (Figure 4.26)

4.3.4. Oxidative condition

Lamivudine was found to decompose in H₂O₂ at room temperature. It decomposed to an extent of 30.4 % in 3 % H₂O₂ in 1hr. The extent of degradation increased to 53.8 %, 69.7%, 80.87%, 87.1%, 90.7%, 94.8% in 2, 3, 4, 5, 6, 7 hrs at room condition (Figure 4.27). As the peak of the drug decreased there was a corresponding rise in a new peak which appeared at about 3.87 min. As shown in (Figure4.28) the λ -max of the degradation product with a retention time of about 3.85 min. was 267 (with in 240 nm to 300 nm). As shown in Figure 4.29 subsequent studies were conducted in 0.03 %, 0.09 % and 0.9 % H₂O₂ . It was found that 65.2 % degradation of the drug was seen in 14 days in 0.03 % H₂O₂, 67.8 % in less than 8 days (187 hrs) in 0.09 % H₂O₂ and same percentage in 0.9 % H₂O₂ in 11 hrs. The degradation increased to 99.5% in less than 8 days (187 hrs) in 0.9% H₂O₂. The drug in 0.3% H₂O₂ was also found to decompose completely in three month storage at -20 °C.

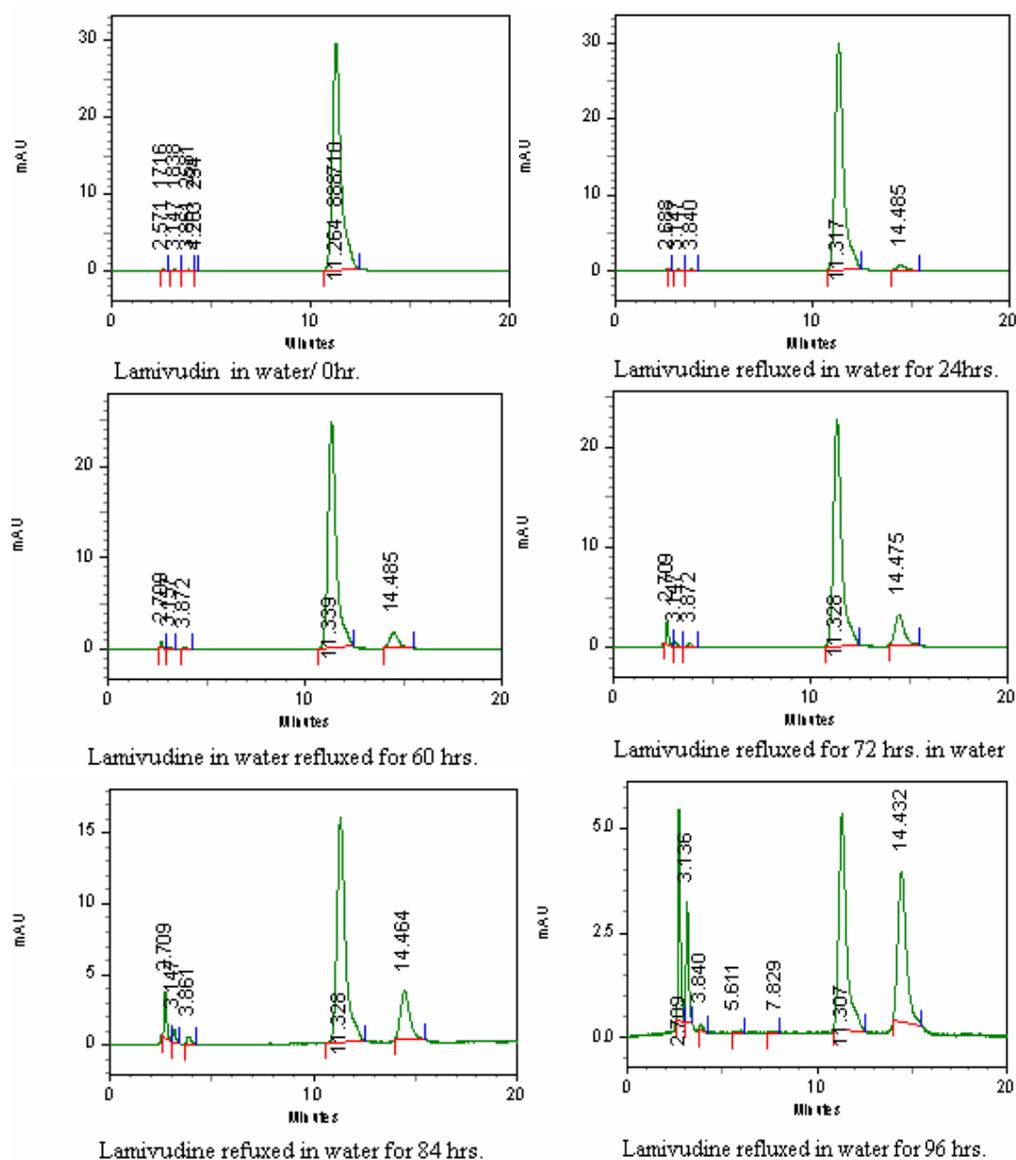


Figure 4.23: HPLC Chromatograms of lamivudine and its degradation products after refluxing with distilled water; obtained under the chromatographic conditions of 0.1 M ammonium acetate: methanol: 1% (v/v) acetic acid (91.9:8:0.1) solvent system at 1 ml/min. flow rate, C-8 column, at 30 °C, 20 µl injection volume and detection at 270 nm UV-Vis diode-array detector.

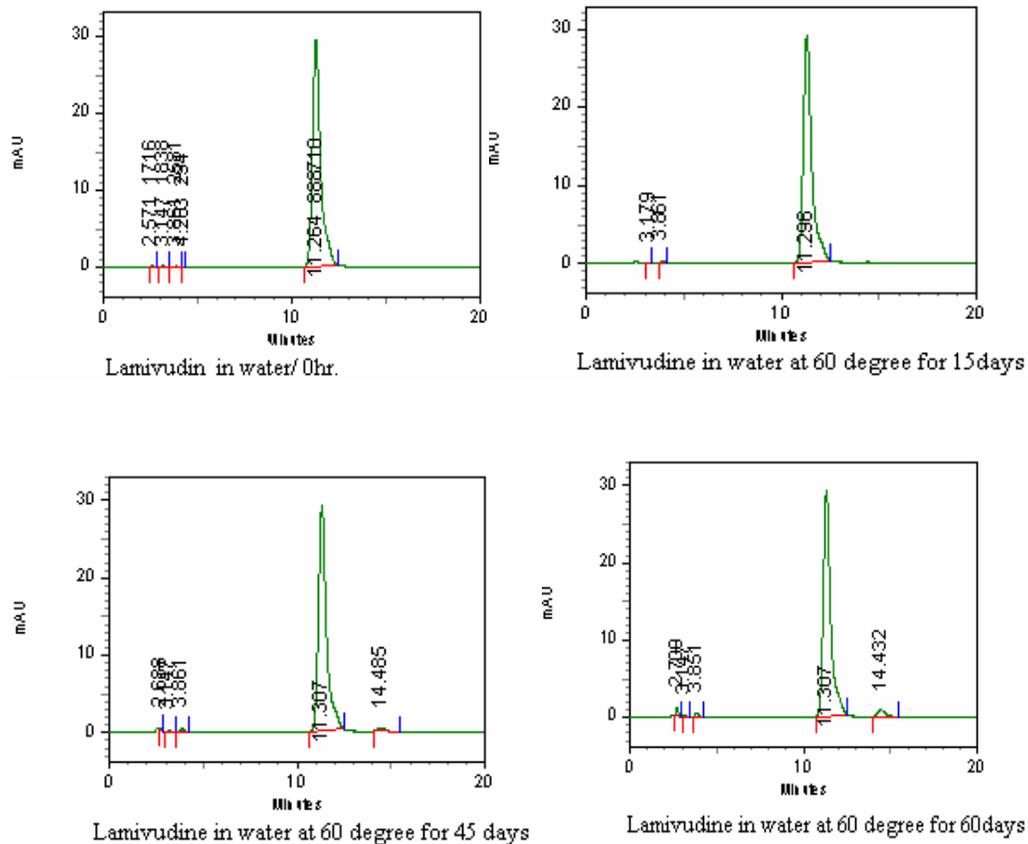


Figure 4.24: HPLC Chromatograms of lamivudine and its degradation product after treatments in water at 60 °C; obtained under the chromatographic conditions of 0.1 M ammonium acetate: methanol: 1%v/v acetic acid (91.9:8:0.1) solvent system at 1 ml/ml flow rate C-8 column, at 30 °C, 20 µl injection volume, and detection at 270 nm UV–Vis diode-array detector.

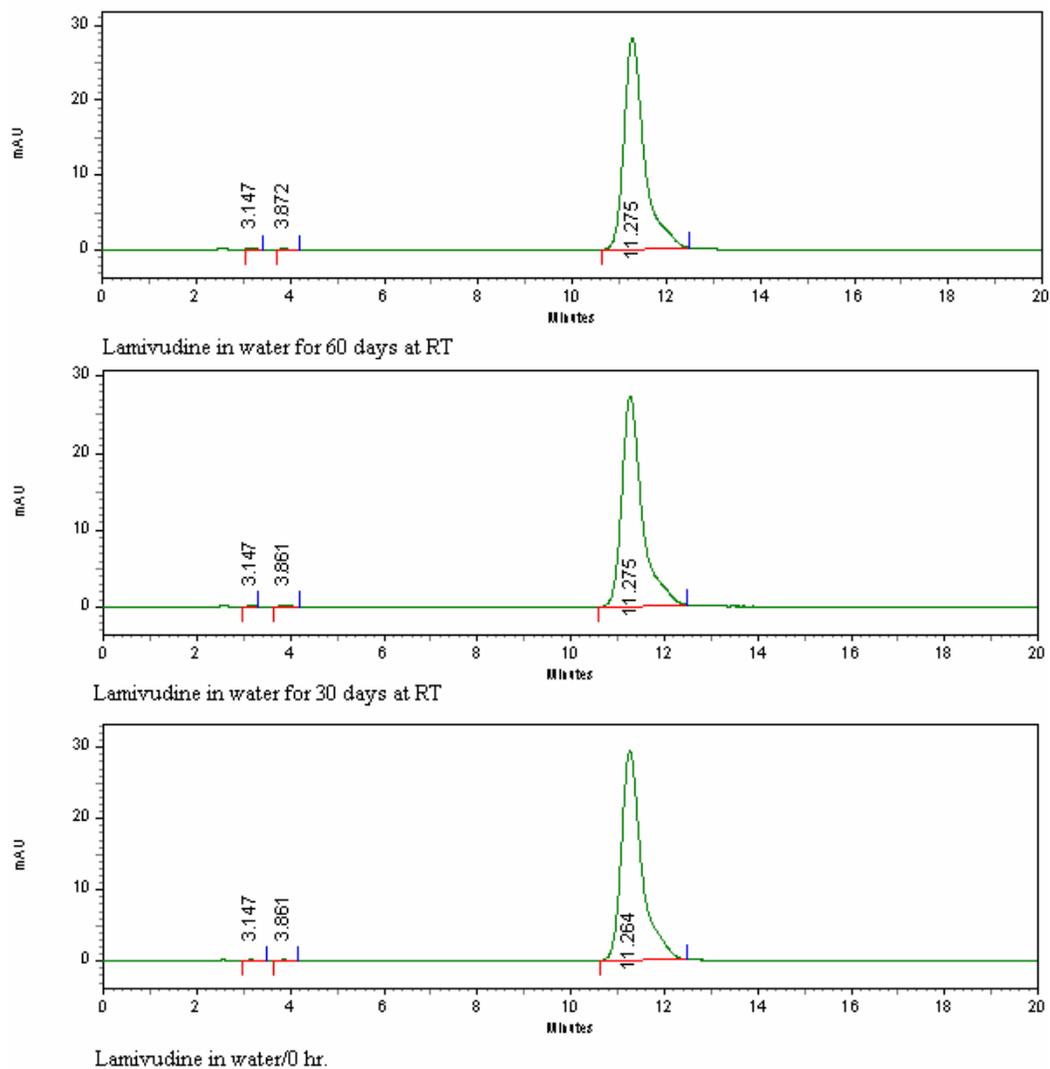


Figure 4.25: : HPLC Chromatogram of lamivudine and its degradation products after treatment with distilled water at RT; obtained with 0.1 M ammonium acetate: methanol: 1 % acetic acid (91.9:8:0.1) solvent system at 1 ml/min flow rate, C-8 column, at 30 °C, 20 µl injection volume and detection at 270 nm UV-Vis diode array detector (RT = normal working environment)

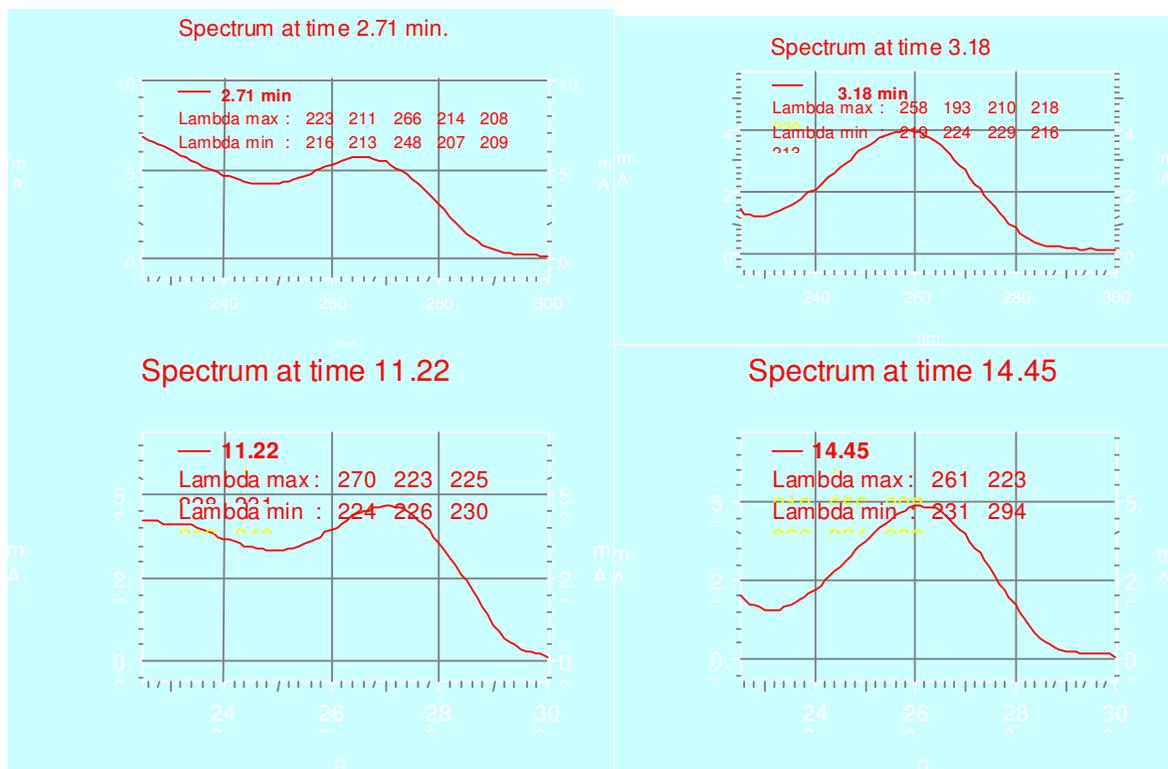
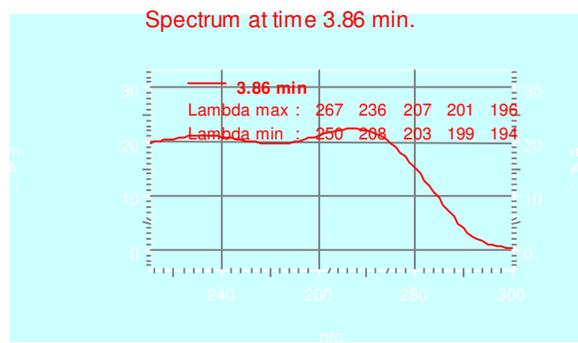


Figure 4.26. : UV spectra of lamivudine ($R_T \approx 11.1$) and its degradation products in distilled water; obtained under the chromatographic conditions of 0.1 M ammonium acetate: methanol: 1% (v/v) acetic acid (91.9:8:0.1) solvent system at 1 ml/min flow rate, C-8 column, at 30 °C, 20 μ l injection volume and detection at 270 nm UV-Vis diode-array detector.



a.



b.

Figure 4.28: a) UV spectra of degradation products of lamivudine in 3.0 % H₂O₂ b) UV Spectra of lamivudine in the presence of its degradation product (a) in 3.0 % H₂O₂ ; obtained under the chromatographic conditions of 0.1 M ammonium acetate: methanol: 1 % (v/v) acetic acid (91.9:8:0.1) solvent system at 1 ml/min. flow rate C-8 column, at 30 °C, 20 µl injection volume and detection at 270 nm UV-Vis diode-array detector.

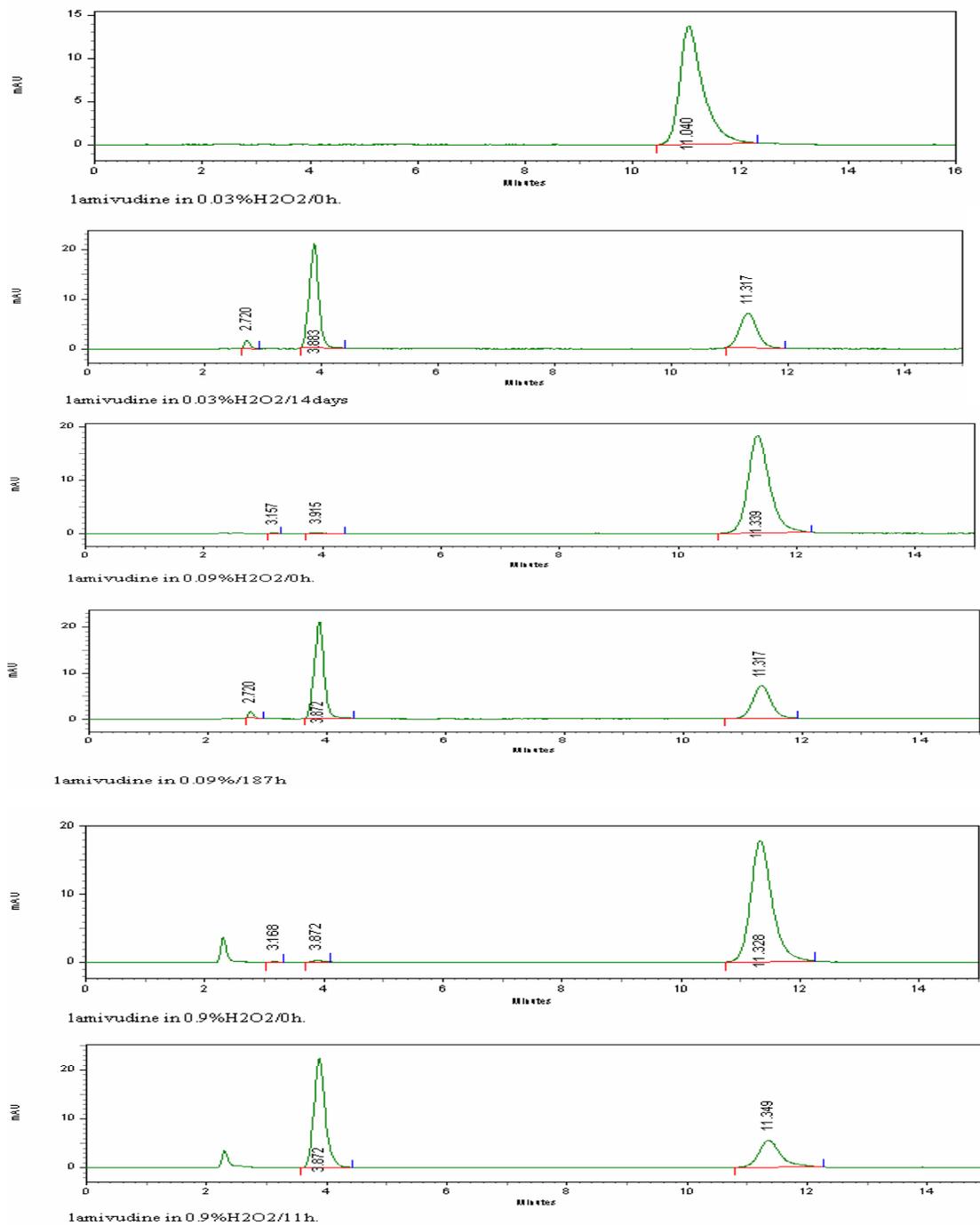


Figure 4.29: HPLC Chromatogram of lamivudine and its degradation products decomposed in 0.03 %, 0.09 % and 0.9 % H₂O₂ obtained under the chromatographic conditions of 0.1 M ammonium acetate: methanol: 1 % (v/v) acetic acid (91.9:8:0.1) solvent system at 1 ml/min. flow rate, C-8 column at 30 °C, 20 µl injection volume and detection at 270 nm UV-Vis diode-array detector.

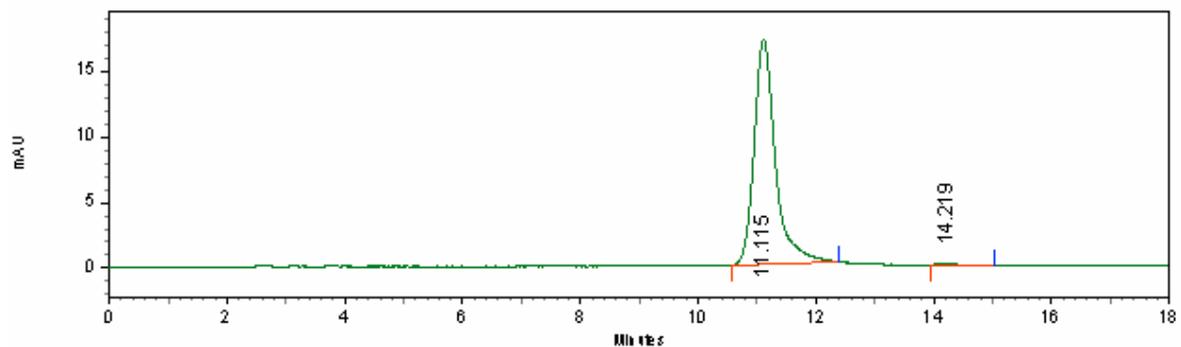
4.3.5. Photolytic condition

The photolytic studies were done in 0.1N HCL, 0.1N NaOH, and water. In acidic solution almost 5.39% degradation was seen on exposure to 1.2 million luxhour visible light, 24.19% degradation on exposure to 200W/m² UV, and 3.73% degradation in dark room (control) indicating that the drug degraded 1.66% on exposure to 1.2 million lux in visible light, 20.46% degradation on exposure to 200 W/m² exposure to UV light (Fig 4.30).

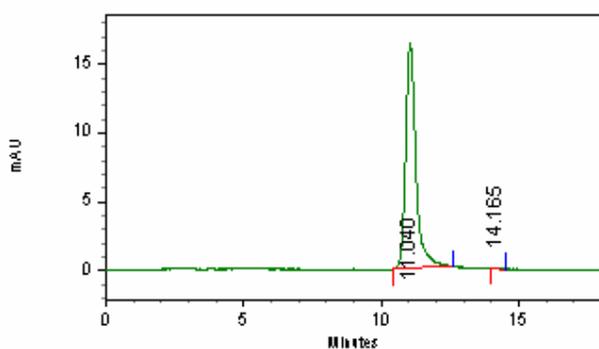
Photolytic degradation of the drug in alkaline solution was also observed. The degradation in UV and visible light was also studied in 0.1N NaOH by increasing the intensity of UV and visible light from 200 W/m² to 1000 W/m² for UV light exposure study and from 1.2 million lux to 6.0 million lux for visible light exposure study. About 55.87-90.98% of the drug degraded in visible light exposure under the study condition, 41.89%-95.27% of the drug as a result of exposure to UV-light, 36.27-87.53% in dark room which was used as control for Visible and UV light exposure study indicating that degradation of the drug as a result of exposure to UV and visible lights were 5.62%-7.72% and 3.45%-19.6% respectively (Fig 4.31).

In neutral condition, as a result of exposure of the drug to 400W/m² UV light and 2.4 million lux visible light, about 2.57% and 8.1% degradations were observed, respectively with 2.07% degradation of the drug in the control group (kept in dark room) indicating, that the degradation of the drug due to the exposed to intensity of UV light and visible light was 0.5% and 6.03% respectively (Fig 4.32).

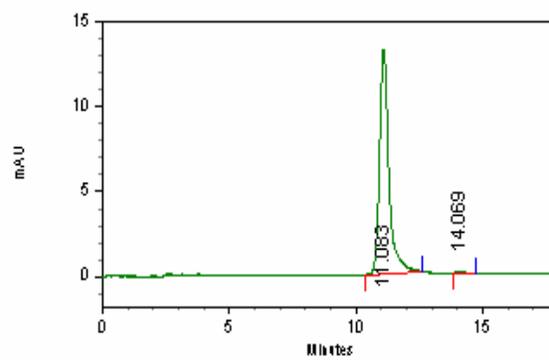
As shown in Figures 4.30-4.32 the drug decomposed with the formation of only hydrolytic products and no specific photolytic peaks (products) indicating that in the presence of light the drug may be degraded to non-chromophoric products.



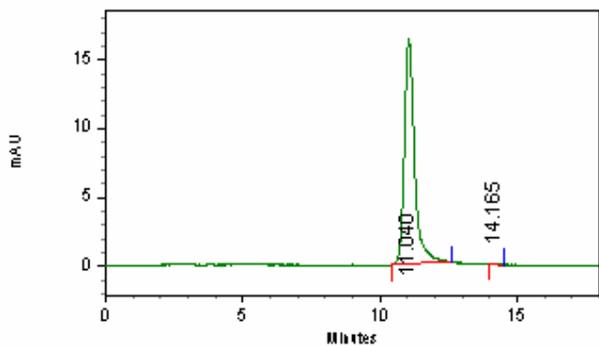
3TC/0.1HCl/0 day



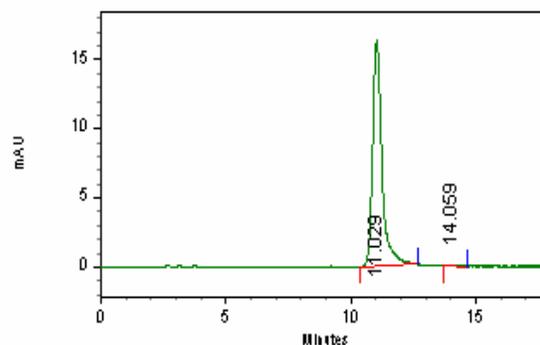
3TC/0.1HCl/Control/7 day.



3TC/0.1HCl/UV light/7 day.

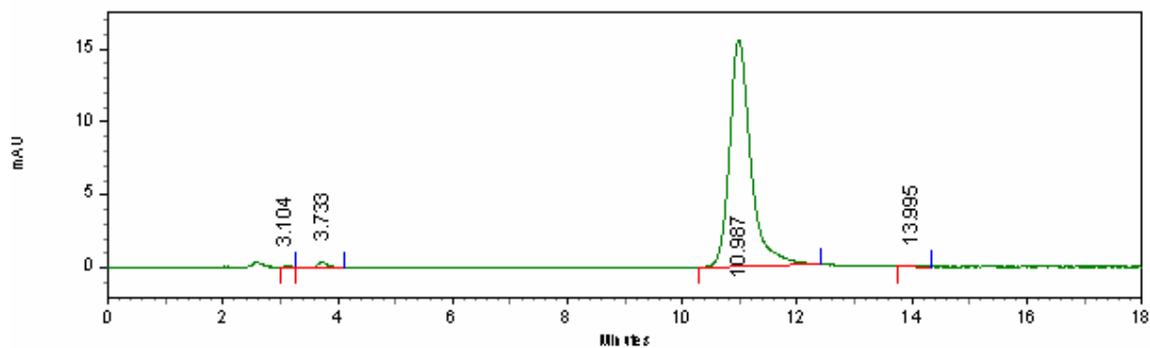


3TC/0.1HCl/Control/7 day.

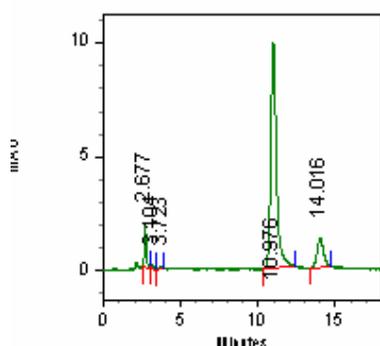


3TC/0.1HCl/Vis light/7 day.

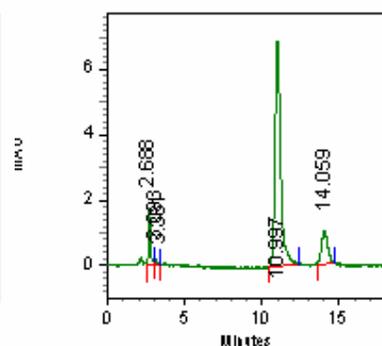
Figure 4.30: HPLC Chromatograms in photolytic decomposition study of lamivudine in 0.1 N HCl in comparison with control samples obtained under the chromatographic conditions of 0.1 M ammonium acetate: methanol: 1% (v/v) acetic acid (91.9:8:0.1) solvent system at 1 ml/min flow rate C-8 column, at 30 °C, 20 µl injection volume, and detections at 270 nm UV-Vis diode-array detector.



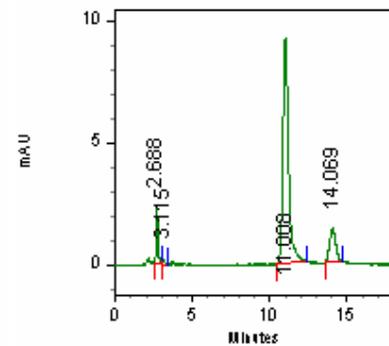
3TC/0.1NN aOH/0 day



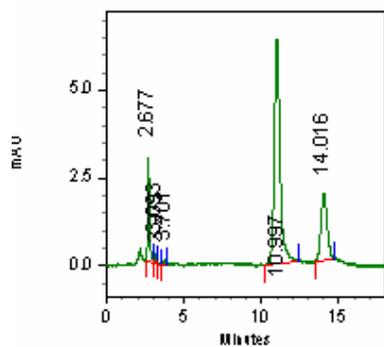
3TC/0.1NN aOH/Control/7 day



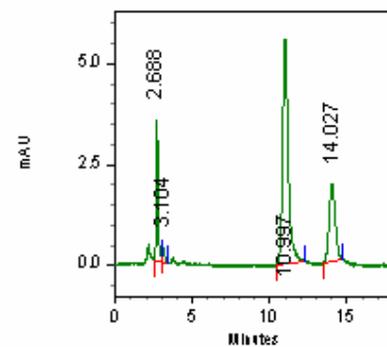
3TC/0.1NN aO/Vis light/7 day



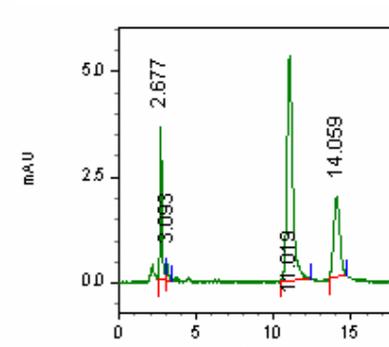
3TC/0.1NN aO/UV light/7 day



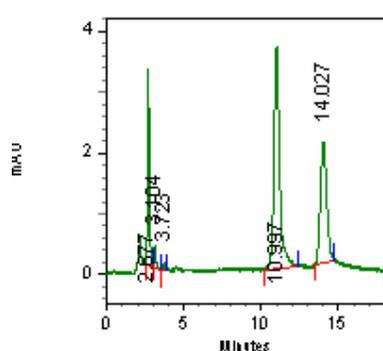
3TC/0.1NN aOH/Control/14 day



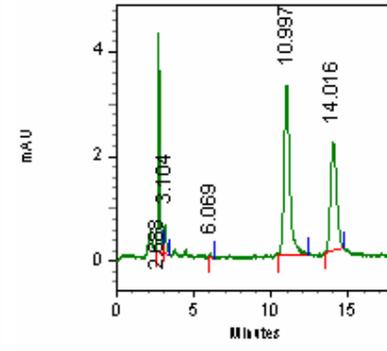
3TC/0.1NN aO/Vis light/14 day



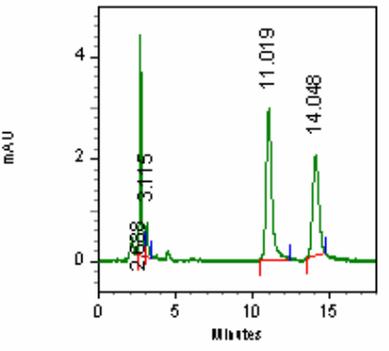
3TC/0.1NN aO/UV light/14 day



3TC/0.1NN aOH/Control/21 day



3TC/0.1NN aO/Vis light/21 da



3TC/0.1NN aOH/UV light/21 day

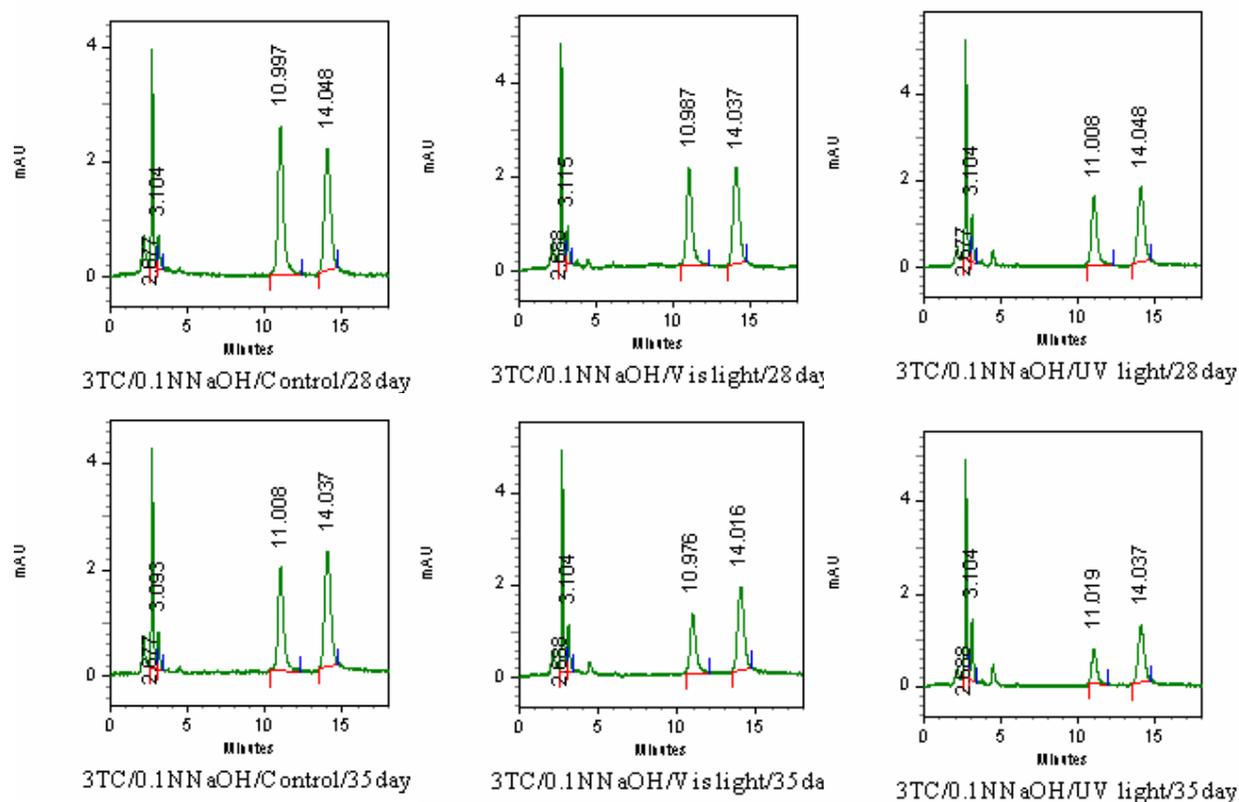


Figure 4.31: HPLC Chromatogram in photolytic decomposition study of lamivudine in 0.1 N NaOH in comparison with control samples obtained under the chromatographic conditions of 0.1M ammonium acetate: methanol: 1%(v/v) acetic acid (91.9:8:0.1) solvent system, 1 ml/min flow rate C-8 column, at 30 °C, 20 µl injection volume and detection at 270 nm UV–Vis diode-array detector.

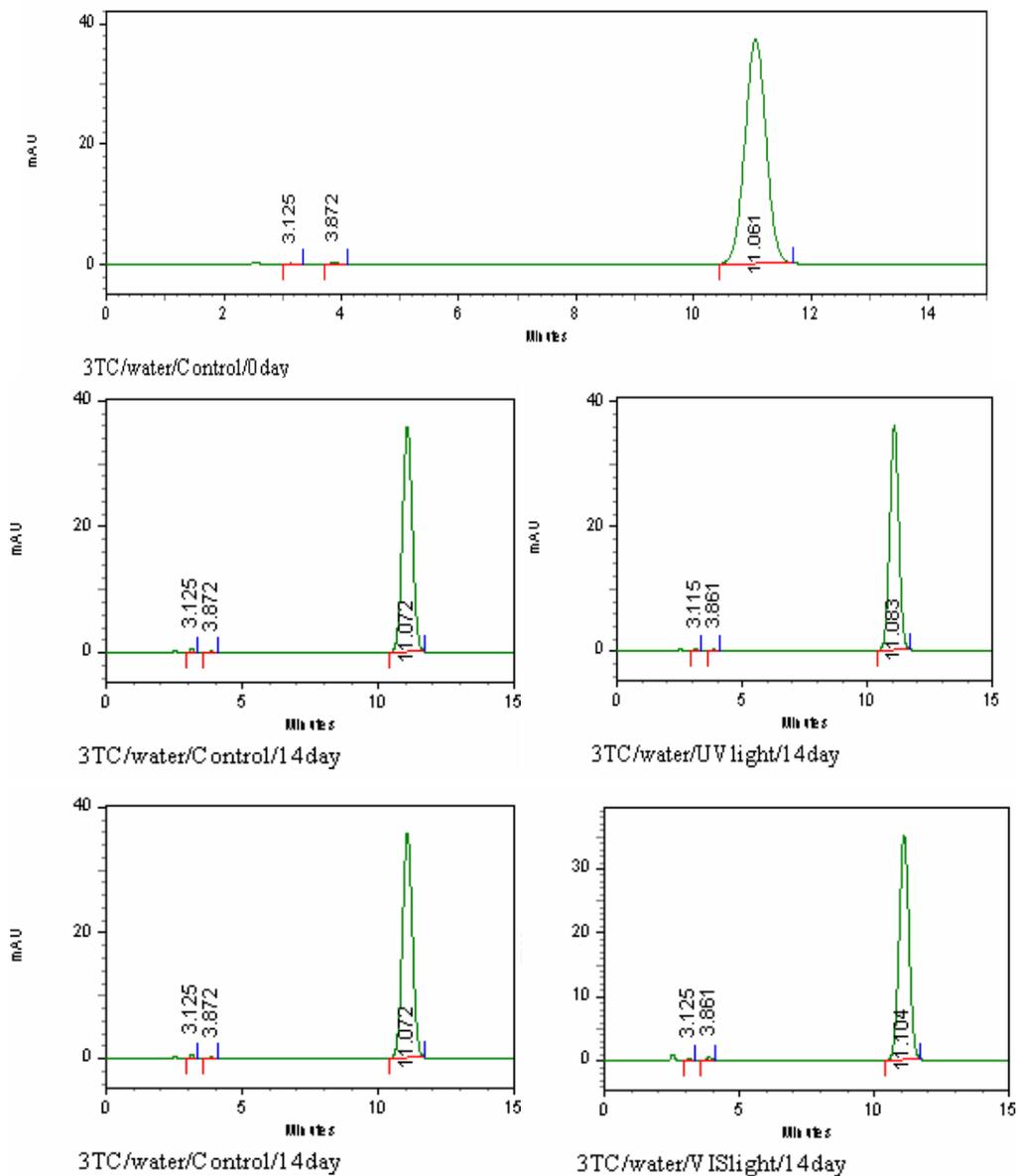


Figure 4.32: HPLC Chromatograms in photolytic decomposition study of lamivudine in water in comparison with control samples obtained under the chromatographic conditions of 0.1M ammonium acetate: methanol: 1 % (v/v) acetic acid (91.9:8:0.1) solvent system, 1 ml/min flow rate C-8 column, at 30 °C, 20 µl injection volume and detection at 270 nm UV-Vis diode-array detector.

4.3.6 Solid-state study

Solid-state studies showed that when the drug powder was exposed to dry heat at 60 °C for a month, no major decomposition of the drug was seen (Fig 4.33). The solid drug exposed to 1000 w/m² intensity UV light and 6 million lux visible light showed only about 0.4% -2.87% degradation.

4.4. Relative rate of degradation in acid, alkali, and hydrogen peroxide.

Kinetics studies were carried out in 0.1N HCl acid, 0.1N NaOH alkali, and 3.0% H₂O₂ by following the fall in drug concentration with time. The plots of log percent remaining versus time are shown in Fig 4.34. As evident, rectilinear curves ($r^2 > 0.98$) were obtained for all three stresses condition; 0.9985 in 0.1N HCl, 0.9877 in 0.1N NaOH, 0.9977 in 3.0 % H₂O₂ indicating that the reactions followed pseudo-first-order kinetics. Rate constant were determined from the slope and the calculated values were -0.01264, -0.2501, -0.1811h⁻¹ in 0.1N HCl, 0.1N NaOH, 3.0 % H₂O₂ respectively. Apparently, two inferences can be made out from these rate constant values. First is that the rate of hydrolysis is for lamivudine is faster in alkaline solution than in acidic solution. Second the rate of oxidation in 3.0 % H₂O₂ is faster than the rate of hydrolysis in acidic condition (0.1N HCl).

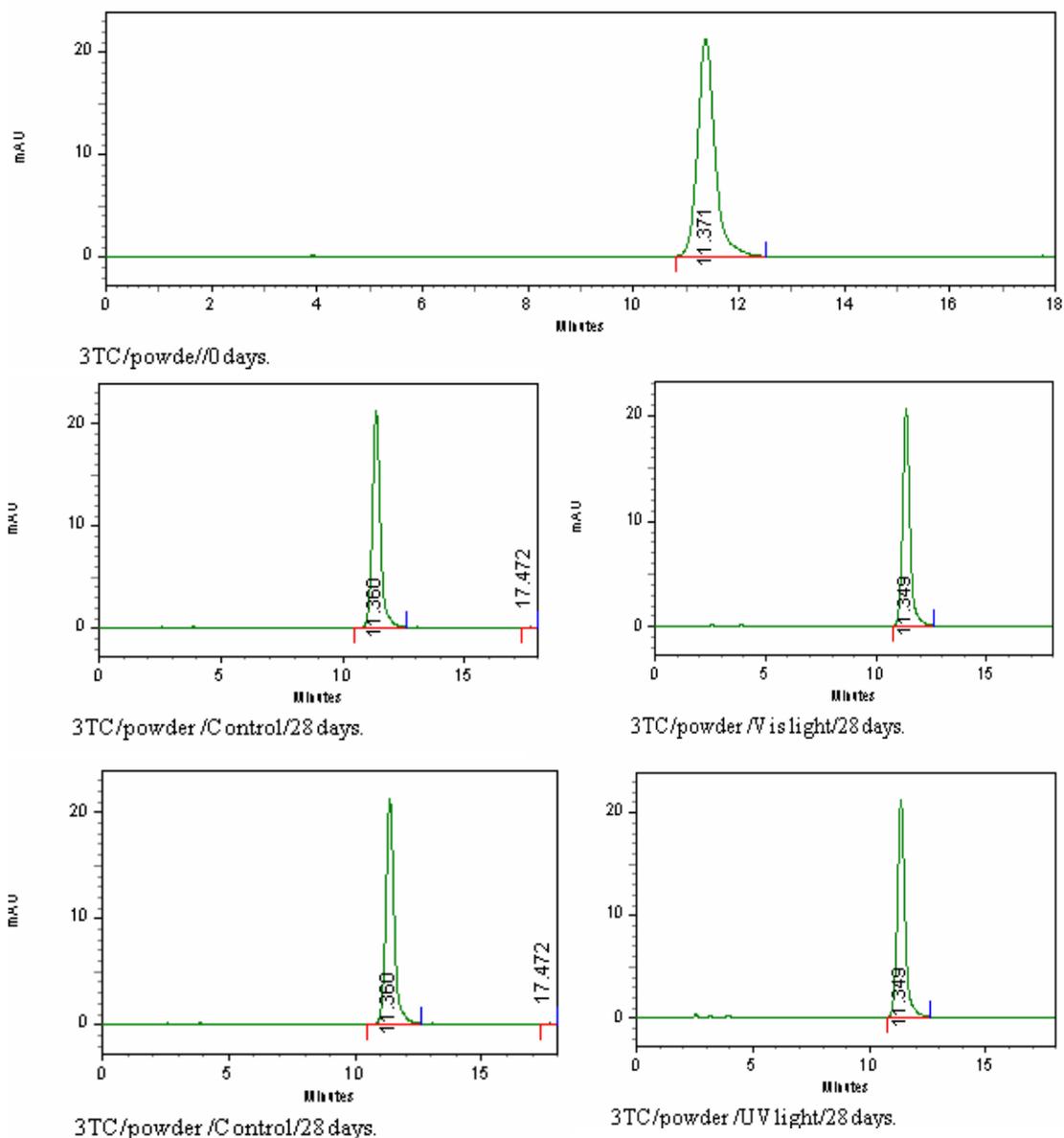
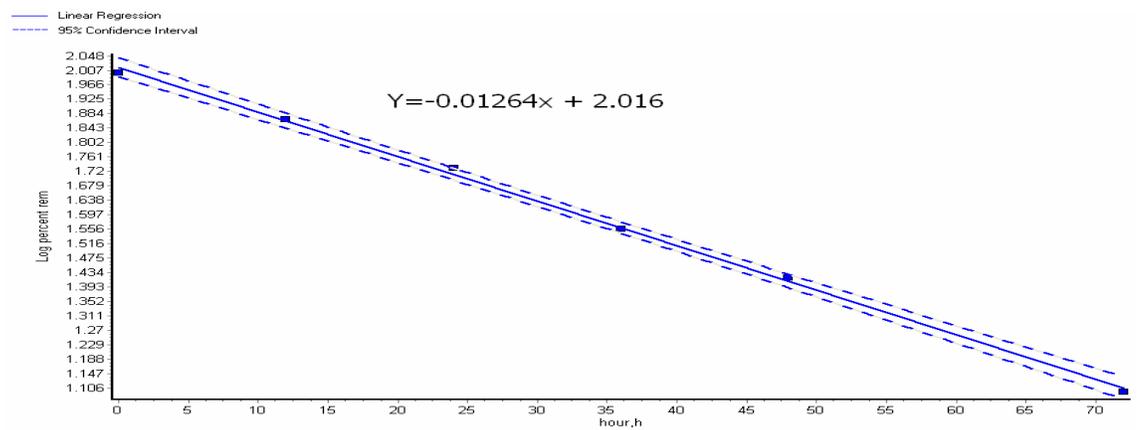
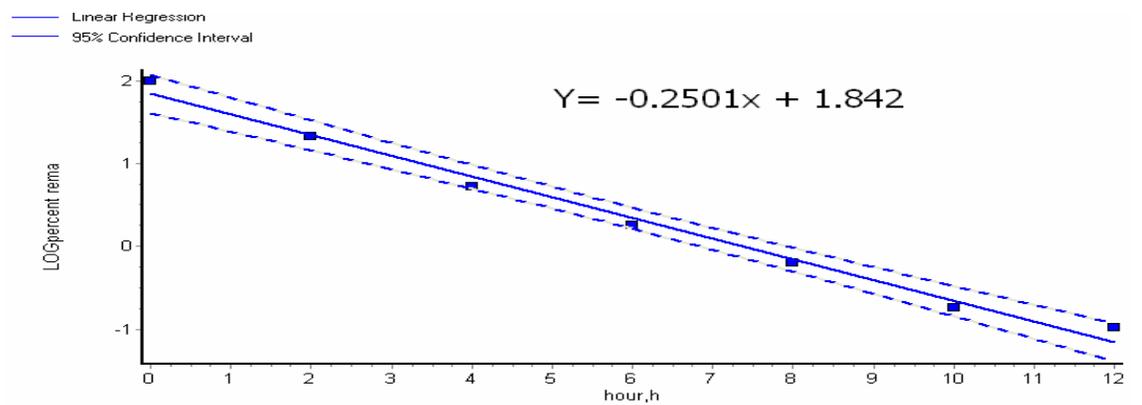


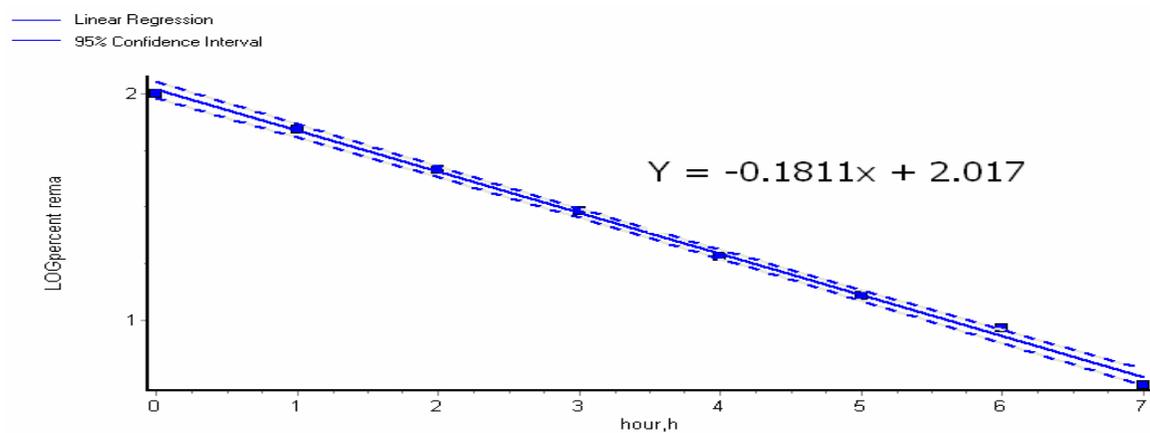
Figure: 4.33. HPLC Chromatogram in photolytic decomposition study of lamivudine in solid state in comparison with control samples obtained under the chromatographic conditions of 0.1 M ammonium acetate: methanol: 1% (v/v) acetic acid (91.9:8:0.1) solvent system at 1 ml/min flow rate C-8 column, at 30 °C, 20 µl injection volume and detection at 270 nm UV-Vis diode-array detector.



a.



b.



c.

Figure 4.34: Kinetics plot for decomposition of lamivudine in 0.1N HCl (a) in 0.1N NaOH (b) at refluxing temperature and in 3.0% H₂O₂ (c) at room temperature.

5. DISCUSSION

5.1. Development of the stability indicating assay method.

Initially separation studies were performed using new reversed-phase octadecyl column (Nucleosil 100C18, TR 011349, N29789) and 50:50, water-methanol, as mobile phase in attempt to obtain well-separated and good quality peaks for the drug of interest. However, it was noticed that with this ratio of organic phase as the mobile phase satisfactory retention time was not obtained. Therefore, increasing the retention time of lamivudine to 10-15 minutes was necessary to separate the degradation products from the parent drug since degradation products from drugs are generally polar in nature and their retention time are generally very short in reverse phase chromatography (Bakshi and Singh,2000). Therefore, separation studies were further conducted with same column and various mixtures of methanol and water were tried until satisfactory retention time for the drug of interest was obtained. The separation of the parent drug from the degradation products in reaction solution was studied using 15% methanol in water as a mobile phase, and which slightly improved the separation. However, the resolution of the drug from the nearest resolving peak and the shape of the peak was not acceptable.

The concentration of methanol was decreased from 15% to 10% to see if there was an improvement in separation. However it was observed that the retention times of the parent drug and its degradation product were pushed towards 12 and 13 min. without showing any improvement in separation. This could be when 10% and 15% aqueous methanolic solution were used as a mobile phase the parent drug and the degradation products elute together because of their similar affinity to the stationary phase.

Before switching to another stationary phase, the assay method described by Harker *et al.* (1994) was tested to see the separation behavior of the individual products in the reaction solution. As shown in Figure 4.4 separation was improved but the shape of the peaks was not satisfactory. The reason for this separation could be the change in mobile phases towards 0.1 M ammonium acetate : methanol: acetonitrile: 1%(v/v) acetic acid (90.9:8:1:0.1), since the buffer cation(NH₄)⁺ may have decreased the silanol effect of the column on the retention time of lamivudine by blocking the ionized silanol (Snyder *et al.*, 1997). Finally separation of lamivudine from its degradation products and the shapes of the peaks were greatly improved

when the stationary phase was changed to C-8 and the mobile phase to 0.1 M ammonium acetate: methanol: 1 % (v/v) acetic acid (90.9:8:0.1).

5.2. Degradation behavior

Degradation studies under different stress condition were monitored by HPLC using 0.1 M ammonium acetate: methanol: 1 % (v/v) acetic acid (90.9:8:0.1) as a mobile phase and C-8 column as stationary phase. It is one of the rare studies where forced decomposition studies were done under all different suggested conditions. In literature, most of studies on the development of stability indicating assay involve either forced decomposition studies under only one or two conditions (Kariem, 1993; Padval, 2003) or separation of drug from major degradation product whose standards were available (Brightman, 1999; Vasselle, 1999; Rao, 2000). Degradation of lamivudine in all stressed conditions was observed but the extent of degradation varied significantly depending up on the stressing condition employed.

The kinetic plot of lamivudine degradation in 0.1N HCl, 0.1N NaOH and 3.0% H₂O₂ revealed that hydrolytic degradation of the drug in alkaline solution taken place at faster rate than its hydrolytic degradation in acid solution and its oxidative degradation, the plot of Log percent remaining Vs. hr. indicates clearly that lamivudine followed pseudofirst order kinetics in its decomposition in refluxing (in 0.1N HCl, 0.1N NaOH and water) and at room temperature in 3.0% H₂O₂. These indicate that lamivudine has functional groups which are liable for oxidation and hydrolysis. The oxidative degradation of lamivudine may account to its thioether functional group which is liable to oxidation and the hydrolytic degradation most probably may account to its amide group.

It was observed that lamivudine degraded when it was exposed to light indicating that this drug contains functional group which is liable to photodecomposition. In literature it is reported that compound like olefins, aryl halo derivatives, aryl acetic acid and those with aromatic nitro groups, N-Oxides undergo photodecomposition (Bakshi and Singh). Therefore the photodecomposition behavior of lamivudine may be due to its olefins group. As it is shown in figure 4.30-4.32 drug degraded as result of its exposure to light without formation of a specific photolytic peaks (products) indicating that the drug degraded to the non chromophoric products.

The degradation studies conducted at 60 °C and room temperature also revealed that lamivudine is more prone to degradation in alkaline pH than in acidic or neutral pH. It was also observed that the degradation of lamivudine in 0.1N HCl, 0.1N NaOH, and water, gave

product with similar retention times ($\approx 2.7, 3.1$ and 14.1 min.) and λ_{max} indicating that the degradation products obtained under these different conditions are most probably identical irrespective of the reagents used for stressing. Moreover a degradation product with a retention time at about 3.8 min. was observed in these solutions (in hydrolytic degradation) as minor component. On the other hand the degradation product with a retention time at about 3.8 min. was the major component when $3.0\% \text{H}_2\text{O}_2$ was used as stressing reagent, indicating that this product is formed mainly as results of oxidation.

6. CONCLUSION

Stress degradation studies were carried out on lamivudine, following the condition prescribed in the parent drug stability testing guideline (Q1A) issued by International Conference on Harmonization (ICH). The inherent stability of the drug substance, its degradation products under the various stress condition has been established. Lamivudine was found to be unstable at 60°C and at refluxing temperature in different stressed solution. The drug was found to be more liable to decompositions in alkaline solution than in acidic solution and neutral condition. Relative rate of degradation of the drug in hydrolytic and oxidative condition shows that the rate of hydrolytic degradation in acidic solution (0.1NHCl) was less than the rate of oxidative degradation in hydrogen peroxide (3.0%). The degradation product formed in acidic alkali, and neutral condition showed similar retention time and UV spectrum.

Separation of the drug and the degradation products under various conditions was successfully achieved on C-8 column utilizing 0.1 M ammonium acetate: methanol: 1% (v/v) acetic acid in the ratio of 91.9:8:0.1. The method was validated with respect to linearity, precision, accuracy, selectivity, specificity and ruggedness. The response was linear ($r=0.9998$) in the drug concentration range of 5-500 μgml^{-1} . The mean values ($\pm\text{RSD}$) of slope and intercept were 46376(± 0.006975) and 200049(± 0.4009) respectively. The RSD values for intra- and inter-day precision studies were $<0.292\%$ and $<1.781\%$ respectively. The recovery of the drug ranged between 98.3 - 101.16% from the mixture of degradation products. The method was specific to the drug and also selective to degradation products. The developed method is simple accurate, precise, specific and selective and rugged and thus it can be used for analysis of the drug and its degradation product in the stability samples.

Suggestion for future

- Identification and characterization of the degradation products
- Identify the degradation product formed as a result of normal storage condition and accelerated stability study and compare them with the degradation product as result of stress condition
- Study the quality of the marketed drug with this method
- Study the applicability of the method for formulation containing lamivudine and zidovudine in combination

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