

**SIMULTANEOUS DETERMINATION OF
LAMIVUDINE AND ZIDOVUDINE IN
PHARMACEUTICAL FORMULATIONS
BY
HIGH PERFORMANCE THIN LAYER
CHROMATOGRAPHY (HPTLC)-
DENSITOMETRIC METHOD**



*A THESIS PRESENTED TO THE SCHOOL OF GRADUATE STUDIES OF
ADDIS ABABA UNIVERSITY IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS OF THE DEGREE OF MASTER OF SCIENCES IN
PHARMACEUTICAL ANALYSIS AND QUALITY ASSURANCE*

*BY
GIRUM HABTE*

*UNDER THE SUPERVISION OF DR. ARIAYA HYMETE AND DR. FATHY
KANDEEL EL-FIKY*

*DEPARTMENT OF PHARMACEUTICAL CHEMISTRY
SCHOOL OF PHARMACY
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ACRONYMS

TLC:	Thin Layer Chromatography
HPTLC:	High Performance Thin Layer Chromatography
HPLC:	High Performance Liquid Chromatography
R _f :	Retention factor
UV:	Ultraviolet
USP:	United State Pharmacopoeia
BP:	British Pharmacopoeia
ICH:	International Conference on Harmonization
FDA:	Food and Drug Administration
UNAIDS:	United Nations programme on HIV/AIDS
WHO:	<u>World Health Organization</u>

Abstract

A high performance thin layer chromatographic (HPTLC) method for the simultaneous determination of lamivudine and zidovudine in binary mixture has been developed.

The method developed was based on HPTLC separation of the two drugs followed by densitometric measurements of spots at 276 and 271 nm for lamivudine and zidovudine respectively. Separation was carried out on Merck HPTLC silica gel 60 F₂₅₄ plates, using toluene:chloroform:methanol (1:6:3 v:v) as mobile phase.

Validation of the method was performed based on ICH guide line in terms of linearity, accuracy, precision, limit of detection, limit of quantification and robustness.

Second order polynomial equation was obtained for the regression line in the range of 250–1400 and 250–1700 ng/spot for lamivudine and zidovudine, respectively. Correlation coefficient (r) values were 0.9998 for both analytes. The method provides sufficient accuracy indicated by % recoveries ranged from 98.68 to 100.73 for lamivudine and from 98.56 to 100.61 for zidovudine. For system precision study the low coefficient of variation values (< 2%) for both lamivudine and zidovudine insured reproducible performance of the instrument. In the method precision study coefficient of variation < 2% was obtained which showed that the proposed method provides acceptable intra-day and inter-day variation. The detection and quantification limits for lamivudine and zidovudine were found to be 3.06 and 9.28 and 3.34 and 10.13 ng/spot, respectively. Mobile phase composition, amount of mobile phase, time from spotting to development and time from development to scanning parameters were employed while testing for robustness of the method and the standard deviation of peak areas was calculated for each parameter. The low coefficient of variation value (< 2%) indicated the robustness of the method. Statistical manipulation did not show any significant effect of one parameter over the others on the robustness of the method.

No chromatographic interference from the tablet excipients was observed. The described method can be readily utilized for analysis of both drugs in pharmaceutical formulations.

Statistical evaluations were performed to correlate the accuracy and dosage form analysis results of lamivudine and zidovudine determined by means of the proposed HPTLC and the reported HPLC method. The calculated t-test and *F* ratio value did not exceed the theoretical values at the 95% confidence level, indicating no significant difference between the performance of the two methods regarding the mean values and standard deviations.

No degradation product was found in the sample analyzed by this method. The time and cost effectiveness of the developed HPTLC method was also compared with the reported HPLC method. It was found that the HPTLC method is both time and cost effective for the determination of lamivudine and zidovudine mixtures in pharmaceutical formulations compared to the reported HPLC method.

1. INTRODUCTION

1.1 HIV/AIDS

More than twenty years after the first clinical evidence of acquired immunodeficiency syndrome (AIDS) was reported it has become one of the most devastating diseases human kind has ever faced. Since the epidemic began, more than 60 million people have been infected with the virus. HIV/AIDS has become the fourth-largest cause of death worldwide (AVERT I, 2006).

Most researchers believe that HIV originated in sub-Saharan Africa during the twentieth century; it is now a [pandemic](#), with an estimated 38.6 million people living with the disease worldwide (AVERT II, 2006). As of January 2006, the Joint UNAIDS and the WHO estimate that AIDS has killed more than 25 million people since it was first recognized on June 5, 1981, making it one of the most destructive [epidemics](#) in recorded history. In 2005 alone, AIDS claimed an estimated 2.4 - 3.3 million lives, of which more than 570,000 were children, and over 4 million new HIV infections. One third of these deaths occurred in sub-Saharan [Africa](#), retarding economic growth and destroying human capital (UNAIDS, 2006). About one-third of those currently living with HIV/AIDS are aged 15-24. Many of them do not know they carry the virus. Many millions more are vulnerable to HIV as they know nothing or too little about the virus to protect themselves against it (AVERT III, 2006).

People have been warned about HIV and AIDS for over twenty years now, and there's no cure - so AIDS will be around for a while yet. However, some people still don't know exactly what HIV and AIDS actually are (U.S DHHS, 2005).

HIV stands for the 'Human Immunodeficiency Virus'. Someone who is infected with HIV is said to be “HIV+” or “HIV positive” (The AIDS InfoNet, 2007).

HIV is a virus. Viruses infect the cells of living organisms and replicate (make new copies of themselves) within those cells. A virus can damage the cells it replicates in, which is one of the things that can make an infected creature become ill (WHO, 1990). People can become infected with HIV from other people who already have it, and when they are infected they can then go on infecting other people. Basically, this is how HIV is spread (CDC, 1999).

As time goes by, a person who has been infected with HIV is likely to become ill more and more with one of a number of severe illnesses, usually several years after infection. It is at this point that they are said to have AIDS - when they first become seriously ill, or when the number of immune system cells left in their body drops below a particular point. AIDS is an extremely serious condition, and at this stage the body has very little defense against any sort of infection (Schneider, *et. al.*, 2005; Paul, 1999).

[Antiretroviral medication](#) can prolong the time between HIV infection and the onset of AIDS and hence reduces both the [mortality](#) and the [morbidity](#) of HIV infection. Without drug treatment, HIV infection usually progresses to AIDS in an average of ten years. Modern combination therapy is highly effective and, theoretically, someone with HIV can live for a long time before it becomes AIDS. These medicines, however, are not widely available and routine access to antiretroviral medication is still a problem in many poor countries around the world (CDC, 2005).

Introduction of highly active antiretroviral treatment (HAART) in industrialized countries has considerably reduced disease progression to AIDS and transformed HIV infection from a lethal disease to an effectively manageable chronic disease (Girard, 2006).

1.1.1 Therapeutic advances in the management of HIV infection

In the eighties and early nineties, HIV infection was a synonym of death; at that time period, no highly active treatment or strategy to fight the infection was available and medical effort was aimed essentially to treat opportunistic infections and malignancies resulting from the acquired immunosuppression (Girard, 2006).

The introduction of protease inhibitors in 1996 was a major step forward and the real turning point in the treatment and management of HIV infection. It defined the launch of the highly active antiretroviral therapy era, when, for the first time, physicians had access to active and potent drugs against HIV replication with the objective of sustained response. Death from HIV infection dramatically decreased and morbidity was significantly lowered thereafter. Progressively, HIV infection evolved from a lethal sub acute infection in to a chronic disease, in some way similar to diabetes, cardiovascular diseases and many other chronic pathologies (Richman, 2006).

In 2006, 10 years after the introduction of highly active antiretroviral therapy, therapeutic needs in HIV are different from what they were a decade ago. Physicians now handle more than 20 different molecules, all active against HIV virus. However, they still need safe, tolerable and simple regimens in order to improve patient's compliance to therapy, as well as more potent drugs in cases of emergence of multi-resistant viral strains (Saliba and Yeni, 2006).

1.1.2 Groups of antiretroviral drugs

There are four groups of anti-HIV drugs. Each of these groups attack HIV in different ways.

1.1.2.1 Nucleoside analog reverse transcriptase inhibitors

The first group of antiretroviral drugs is the nucleoside/nucleotide reverse transcriptase inhibitors. These were the first type of drug available to treat HIV infection in 1987. Nucleoside reverse transcriptase inhibitors (also known as nucleoside analogues or nukes) interfere with the action of HIV protein called reverse transcriptase, which the virus needs to make new copies of it self. Nucleoside reverse transcriptase inhibitors are sometimes called the "backbone" of combination therapy because most regimens contain at least two of these drugs.

1.1.2.2 Non-nucleoside reverse transcriptase inhibitors

The second group of antiretroviral drugs is the non-nucleoside reverse transcriptase inhibitors, for which the first member was approved in 1997. Like the nucleoside analogues, non-nucleoside reverse transcriptase inhibitors (also known as non-nucleosides or non-nukes) stop HIV from replicating within cells by inhibiting the reverse transcriptase protein.

1.1.2.3 Protease inhibitors

The third type of antiretrovirals is the protease inhibitor group. The first protease inhibitor (saquinavir) was approved in 1995. Protease inhibitors, as the name indicates, inhibit protease, which is another protein involved in the HIV replication process.

1.1.2.4 Fusion or entry inhibitors

The fourth group of antiretrovirals is comprised of entry or fusion inhibitors. One of these drugs - commonly called T-20 - has been licensed both in the US and in Europe since 2003, but only for use by people who have already tried other treatments. The T-20 fusion inhibitor differs from the other antiretrovirals in that it needs to be injected (otherwise it would be digested in the stomach). Entry inhibitors prevent HIV from entering human cells (AVERT, 2007).

1.1.3 Combination therapy

Antiretroviral treatment for HIV infection consists of combination drugs which work against HIV infection in their own way but synergistically slowing down the replication of HIV in the body. The drugs are often referred to as antiretrovirals or anti-HIV drugs (Bernard, *et. al.*, 2007).

For antiretroviral treatment to be effective, it has been found that one need to take a combination of antiretroviral drugs to tackle the resistance developed by the virus against individual drugs and hence slow down its replication. In fact using a combination of antiretrovirals reduced progression to AIDS or death by 40% compared to single antiretroviral (Jordan, *et. al.*, 2002).

Combination therapy is therefore considered to be the standard care of HIV infected patients and taking combined antiretrovirals vastly reduces the rate at which resistance develops. These therapeutic regimens result however in a great number of daily doses of tablets/capsules (Ben, 1995).

1.2 Challenge of combined drug analysis

Many analytical methods are designed to determine quantitatively a specific component in a mixture. These analyses are often only possible after time consuming sample preparation involving each component of interest being extracted, separated, chemically transformed or even purified. Methods may also require different measuring parameters for each component in a mixture and in which case the simultaneous analysis of complex multi-component mixtures is not possible (Lim, 1991).

Pharmaceutical formulations comprise, in addition to one or more medicinally active ingredients, a number of inert materials, such as diluents, colors, flavors and preservatives (Gennario, 2000). Most products generally are quite complex in the number of the formulation components (Lieberman, *et. al.*, 1998).

The determination of two or more components in a mixture is therefore a difficult task for the analyst and the problem is even more complicated if these compounds are included in the dosage form where excipients represent other interfering materials (Sacndulescu, *et. al.*, 2000).

There is therefore the need for a simple, fast and robust method which does not require extensive sample pretreatment, particularly in industries that need continuous process control.

To ensure quality and stability of active ingredients in the final product, the pharmaceutical analyst must be able to separate these ingredients individually prior to quantitative analysis. Chromatographic methods, especially HPTLC have been widely adopted for the analysis of such drug mixtures and pharmaceuticals due to its high sensitivity and adequate accuracy over the traditional methods of analysis (Szepesi and Nyiredy, 1992).

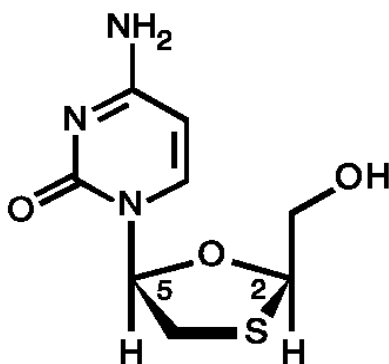
1.3 Combivir and its major components

Each film-coated combivir tablet contains 150 mg of lamivudine, 300 mg of zidovudine (FDA, 2006). It was the first fixed dose combination containing two known antiretroviral agents belonging to the reverse transcriptase inhibitors class. They are synthetic nucleoside analogues with activity against HIV (GlaxoSmithKline, 2006). Lamivudine/zidovudine was approved by the FDA on September 27, 1997, for use in combination with other antiretroviral agents (e.g. with abacavir). These tablets are an alternate regimen to lamivudine and zidovudine given as separate formulations (FDA, 2007).

Lamivudine and zidovudine each inhibit viral reverse transcriptase, an enzyme HIV requires in order to replicate, by incorporating into viral DNA and terminating the viral DNA chain. *In vitro*, lamivudine with zidovudine had synergistic antiretroviral activity (AIDSinfo, 2007).

1.3.1 Lamivudine

Lamivudine (3TC, LMV) has a molecular formula of $C_8H_{11}N_3O_3S$, a molecular weight of 229.3 and the following structural formula:



(2R,cis)-4-amino-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one.

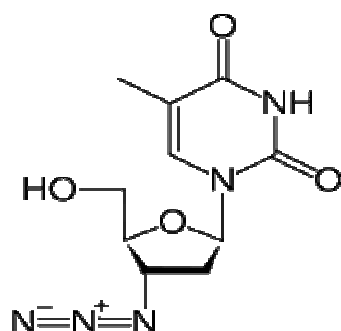
It is a white to off-white crystalline solid with a solubility of approximately 70 mg/ml in water at 20°C (GlaxoSmithKline, 2004).

Lamivudine is a synthetic nucleoside analogue that is phosphorylated intracellularly to its active 5'-triphosphate metabolite, lamivudine triphosphate. Lamivudine triphosphate is a

structural analogue of deoxycytidine triphosphate, the natural substrate for viral reverse transcriptase. Lamivudine triphosphate competes with naturally occurring deoxycytidine triphosphate for incorporation into viral DNA by reverse transcriptase and once incorporated, causes premature termination of viral DNA synthesis (GlaxoSmithKline, 2006).

1.3.2 Zidovudine

Zidovudine (AZT, ZDV) has a molecular formula of $C_{10}H_{13}N_5O_4$ and a molecular weight of 267.24 and has the following structural formula:



1-[(2R,4S,5S)- 4-azido-5-(hydroxymethyl) oxolan-2-yl]- 5-methyl-pyrimidine-2,4-dione

It is a white to beige, odorless, crystalline solid with a solubility of 20.1 mg/ml in water at 25°C (GlaxoSmithKline, 2004).

Zidovudine is also a synthetic nucleoside analogue that is phosphorylated intracellularly to its active 5'-triphosphate metabolite, zidovudine triphosphate. Zidovudine triphosphate appears to compete with thymidine triphosphate for the reverse transcriptase enzyme and incorporation into viral DNA. After incorporation of Zidovudine triphosphate, DNA synthesis is prematurely terminated because the 3'-azido group in the zidovudine molecule prevents further 5' to 3' phosphodiester linkages (GlaxoSmithKline, 2006).

1.3.3 Literature review on the studied mixture

No official analytical method is reported on both British (2004) and US (2005) pharmacopoeias for the quantification of combivir tablets.

But few non official methods for the simultaneous determination of lamivudine and zidovudine mixtures in both pharmaceutical formulations and biological fluids have been reported as:

Simultaneous determination of lamivudine and zidovudine in binary mixtures using first derivative spectrophotometric, first derivative of the ratio spectra and high-performance liquid chromatography–UV methods were reported. For first derivative spectrophotometric method the values of the first derivative amplitudes were measured at 265.6 nm (zero-crossing of zidovudine) and 271.6 nm (zero-crossing of lamivudine) for the determination of lamivudine and zidovudine, respectively. First derivative of the ratio spectra method depends on first derivative of the ratio spectra by measurements of the amplitudes at 239.5 and 245.3 nm for lamivudine and 225.1 and 251.5 nm for zidovudine. In the HPLC, a reversed-phase column with a mobile phase of methanol:water:acetonitrile (70:20:10) at 0.9 ml/min flow rate was used to separate both compounds followed by UV detection at 265.0 nm. Finasteride (a chemical used to improve the resolution of the mixture during chromatography) was used as an internal standard (Uslu and Ozkan, 2002).

The accuracy as well as the dosage form analysis result of the above reported HPLC method was used to compare with the data obtained from this newly proposed HPTLC method.

Simultaneous determination of zidovudine and lamivudine in human serum employing high performance liquid chromatography coupled with tandem mass spectrometry was reported. The method was based on a fully automated ultra filtration sample preparation step that replaces the solid-phase extraction step typically used for HPLC with UV detection. Chromatography was performed using a mobile phase comprised of acetonitrile:water (15:85) on a Keystone Aquasil (C₁₈ 150×2 mm, 5µm) column (Kenney, 2000). Simultaneous determination of lamivudine and zidovudine in small volumes of human seminal plasma (25µl) using high-performance liquid chromatography and tandem mass spectrometry was reported. The method was based on isotopically labeled lamivudine and zidovudine as internal standards and ultrafiltration through a molecular mass cut-off membrane.

Acetonitrile:water (15:85) was used to elute the samples through an Aquasil (C₁₈ 5 µm, 150×2.1 mm) analytical column (Pereira, 2000).

Simultaneous measurement of zidovudine and lamivudine in maternal and fetal mice using liquid chromatography–mass spectrometry method was reported. The method was based upon solid phase extraction, gradient liquid chromatography and electrospray mass spectrometry to measure serum concentrations in maternal and fetal mice of the nucleoside analogs zidovudine, lamivudine and several metabolites. Chromatographic separation was achieved on a Polaris C₁₈ analytical column (150mm×2.0mm, 3 µm particles) and the mobile phase consisted of formic acid (0.1%) and acetonitrile. Initial conditions were set to 0% acetonitrile increasing to 31% over 4 min and then a gradient to 90% acetonitrile at 8.9 min was employed for separation (Lee, *et. al.*, 2003).

A number of methods have been reported for the determination of lamivudine and zidovudine in biological fluids when they are present as individual drugs:

Some four high performance liquid chromatographic methods for the quantitative determination of the HIV reverse transcriptase inhibitor lamivudine in human serum, plasma, saliva and cerebrospinal fluid was reported. All those methods employed different extraction techniques and mobile and stationary phases during chromatographic separation (Bahrami, *et. al.*, 2005; Kazue, *et. al.*, 2005; Zheng, *et. al.*, 2001; Hoetelmans, *et. al.*, 1998). A reversed phase HPLC method for the quantification of lamivudine in rat plasma, amniotic fluid, placental and fetal tissues was reported. The method was validated and applied to the study of the placental transport of this drug in the pregnant rat (Alnouti, *et. al.*, 2004).

Two different methods have been reported for the analysis of zidovudine in biological fluid: a reversed phase high-performance liquid chromatographic method for the separation and quantification of zidovudine in rat plasma (Zhang, *et. al.*, 2007) and a spectrophotometric flow-injection method for the determination of zidovudine in plasma samples. The paper described the development of analytical method to monitor the drug plasma levels as an alternative to chromatographic

procedures (Checa, *et. al.*, 2007). A method is also reported for stability study of zidovudine. Stability studies based on forced decomposition under hydrolysis, oxidation, photolysis and thermal stress conditions using HPLC method. The drug decomposed under hydrolytic stress upon refluxing, and also on exposure to light. It was stable to oxidation and thermal stress (Dunge, *et. al.*, 2005).

Different methods have also been reported for the analysis of lamivudine and/or zidovudine together with other drugs:

Simultaneous determination of lamivudine and stavudine in combined pharmaceutical tablets using first derivative spectrophotometry and high performance liquid chromatography were reported. The spectrophotometric method depends on first derivative UV-spectrophotometry with zero-crossing measurement technique (Kapoor, *et. al.*, 2006). Simultaneous quantification of stavudine, lamivudine and nevirapine in tablets using UV spectroscopy, reverse phase HPLC and HPTLC methods were also reported (Anbazhagan, *et. al.*, 2005).

Simultaneous determination of zidovudine and its monophosphate in mouse plasma and peripheral red blood cells using high-performance liquid chromatography was reported to investigate the bioconversion and pharmacokinetic profiles of the compounds (Tan and Boudinot, 2000). Two chromatographic methods have been reported for the simultaneous determination of zidovudine and nevirapine in human plasma: reversed-phase liquid chromatography with UV detection (Marchei, *et. al.*, 2002) and reversed-phase high performance liquid chromatographic method (Ramachandran, *et. al.*, 2006). Both methods employ different extraction techniques and different mobile and stationary phase during chromatographic separation.

Flow-injection spectrophotometric determination of reverse transcriptase inhibitors zidovudine, zalcitabine and nevirapine was reported. The paper describes the quantification of these reverse transcriptase inhibitors based on a pH-gradient flow-

injection system with diode array detection and further data analysis using multivariate curve resolution (Checa, *et. al.*, 2006). Micellar electrokinetic chromatography methods were reported to separate and quantitate anti-HIV drug mixtures containing zidovudine, didanosine, nevirapine (mixture A) and zidovudine, didanosine, ritonavir (mixture B) in human serum (Fan and Stewart , 2002a).

Two reversed phase high performance liquid chromatographic methods have been reported. Simultaneous quantitative determination of the nucleoside reverse transcriptase inhibitors lamivudine, didanosine, stavudine, zidovudine and abacavir in plasma (Verweij-van, *et. al.*, 2005) and simultaneous quantitative determination of the nucleoside reverse transcriptase inhibitors zalcitabine, lamivudine, didanosine, stavudine, zidovudine, and abacavir with the non-nucleoside reverse transcriptase inhibitor nevirapine in human blood plasma (Naser, *et. al.*, 2003). Both methods employ different extraction techniques and mobile and stationery phases for chromatographic separation.

Simultaneous determination of zidovudine, lamivudine and nevirapine in human plasma using high-performance liquid chromatography method was also reported (Fan and Stewart, 2002b).

Among the above reported methods, the HPLC method described by Usulu and Ozkan, (2002) that was used to determine lamivudine and zidovudine simultaneously in pharmaceutical formulations (Uslu and Ozkan, 2002) required more time to equilibrate the column from one analytical run to another. In addition to that the internal standard (Finasteride) used to assay of the drug is not easily available.

As far as an individual drug is concerned there is one method reported for the determination of lamivudine together with stavudine and nevirapine using HPTLC (Sockalingam, *et. al.*, 2005).

To the knowledge of the investigator, no HPTLC (high performance thin layer chromatography) method is available for the simultaneous determination of lamivudine and zidovudine in pharmaceutical formulations.

1.4 Objectives

1.4.1 General objective

To develop a relatively simple method for the simultaneous quantification of lamivudine and zidovudine mixtures (combivire tablets) in pharmaceutical formulations by using high performance thin layer chromatographic-densitometric technique.

1.4.2 Specific objectives

- ◇ Quantitative determination of both drugs in a mixture, one in the presence of another.
- ◇ To select appropriate mobile phase combination for the analysis of the mixture.
- ◇ To compare the accuracy as well as the dosage form analysis result of this proposed HPTLC method with that of the reported method (HPLC).
- ◇ Detecting the degradation products of both drugs in commercial formulations if present.
- ◇ To compare the cost as well as time effectiveness of the method to that of the reported method
- ◇ Comparing the different types of HPTLC plates that can be used in the routine analysis of the combivir mixture.

2. EXPERIMENTAL

2.1 Materials and methods

2.1.1 Equipments and instruments

Computerized Camag HPTLC system (Camag, Muttens, Switzerland) consisting of a Camag Linomat V semiautomatic spotting device connected to a nitrogen tank and WinCATS 4 software (version 4.05, Camag), a Camag TLC scanner III densitometer equipped with mercury, tungsten and deuterium lamp driven by the same WinCATS 4

software, a Camag 100 μ l HPTLC sample syringe (Hamilton, Bonaduz, Switzerland) and a Camag glass twin-trough (20 cm $\times$ 10 cm) development chamber, different size pipettes, volumetric flasks, measuring cylinders, micro syringes and ruler .

2.1.2 Chemicals and solvents

Lamivudine (GlaxoSmithKline product No: GR 109714X purity: 99.7% W/W(HPLC) and 99.9% W/W(UV)), zidovudine (GlaxoSmithKline product No: AWS 1736B purity: 99.4% W/W(HPLC) and 99.9%W/W(UV)) reference standards, HPTLC plates (silica gel60 F₂₅₄, glass backing).

HPLC grade methanol (Fisher Scientific-Leicestershire, UK), HPLC grade chloroform (BDH Laboratory Supplies, Poole, BH 15 1TD, England), analytical grade toluene (E. Merck-Darmstadt, Germany).

2.1.3 Methods

2.1.3.1 Standard solution

Stock solution of the reference standard was prepared by dissolving 50mg of lamivudine and 100mg of zidovudine in 100ml of methanol to get a concentration of 0.5mg/ml and 1mg/ml for lamivudine and zidovudine, respectively.

Working standard solution was prepared by dilution of 25 ml of the stock solutions with 75 ml methanol to give a concentration of 125 μ g/ml and 250 μ g/ml for lamivudine and zidovudine, respectively. The final concentration ratio of lamivudine to zidovudine was made to be the same as that of the dosage form.

2.1.3.2 Sample solution (dosage form)

Twenty tablets containing 150 mg of lamivudine and 300 mg of zidovudine were accurately weighed and powdered. A portion of the powdered tablets equivalent to 25mg

of lamivudine and 50 mg of zidovudine was weighed accurately and transferred in to 100 ml volumetric flask. The powder was mixed with 80 ml of methanol. After 15 minutes of mechanical shaking and 5-10 minutes of sonication the solution was filtered and the volume was made to the mark with methanol to give a 0.25mg/ml of lamivudine and 0.5mg/ml of zidovudine.

Working sample solution was prepared by dilution of 25 ml of the above sample stock solution with 25 ml of methanol to give a concentration of 125µg/ml and 250µg/ml of lamivudine and zidovudine respectively.

2.1.3.3 Chromatographic conditions

The mobile phase (15 ml per run) consisting of toluene:chloroform:methanol (1:6:3) was used for method development. The samples were spotted in the form of bands of width 6mm with a Camag 100 µl sample syringe on precoated silica gel 60 F₂₅₄ (10 cm×10 cm with 200µm thickness) plate using a Camag Linomat V sample applicator. A constant application rate of 0.1µl/s was employed.

Linear ascending development was carried out in a twin trough glass chamber (20 cm×10 cm) saturated with the mobile phase. The optimized chamber saturation time was 50 minutes at ambient temperature (24⁰C ± 2) with relative humidity of 35–40%. The mobile phase was allowed to cover a distance of 8 cm and that took approximately 13 min. Subsequent to development; the plates were dried in a current of air.

Densitometric scanning was performed on Camag TLC scanner III in the absorbance mode at 276 and 271 nm for lamivudine and zidovudine respectively and operated by WinCATS 4 software (version 4.05, Camag). The slit dimension of the scanner was kept at 5mm×0.45mm and 20 mm/s scanning speed was employed. Concentrations of the compounds chromatographed were determined from the intensity of diffusely reflected light. Evaluation was *via* peak areas with polynomial regression by the WinCATS 4 software.

2.2 Validation and the procedures followed during validation

Once chromatographic conditions were established, the method was validated in compliance with ICH guidelines (ICH, 1994; ICH, 1996). The following parameters were performed for validation:

2.2.1 Linearity

Linearity of the peak area response was determined by making six measurements at five concentration points in the range of 250-1400 and 250-1700 ng/spot for lamivudine and zidovudine respectively.

2.2.2 Accuracy/recovery studies

In order to examine the accuracy of the method and to check the interference from excipients used in the studied formulation, recovery studies were carried out by standard addition method. These recovery studies were carried out by applying the proposed method on the drug sample to which known amount of lamivudine and zidovudine standard corresponding to 80, 100 and 120 % of label claim had been added (standard addition method). At each level of the amount six determinations were performed and the results obtained were compared with the theoretical amounts. The recovery of the new method was also compared with the reported HPLC method.

2.2.3 Precision

Precision of the method was checked by using the following system and method precision parameters. Precision of the proposed method was determined with the product.

2.2.3.1 System precision

The repeatability of sample application and the repeatability of measurement of peak area were performed by the proposed method for system precision studies to determine variations due to the instrument. The repeatability of sample application was carried out by making six measurements on a single stock solution (lamivudine = 500 ng/spot and zidovudine = 1000 ng/spot). Repeatability of measurement of peak area was determined by scanning of the developed spot (lamivudine = 500 ng/spot and zidovudine = 1000

ng/spot) six times without changing the plate position. In both cases, i.e. the repeatability of sample application and the repeatability of measurement of the peak area, the coefficient of variation of measurement of the peak area were taken to evaluate the system precision.

2.2.3.2 Method precision

Method repeatability was carried out by repeating the assay six times in the same day for intra-day precision and by repeating the assay on three different days, three times on each day, for intermediate precision (inter-day precision) to determine variations arising due to the method itself. And based on the inter-day precision the reproducibility of the three day result was checked. The intra-day and inter-day variation for determination of lamivudine and zidovudine was carried out at three different concentration levels 400, 500, 600 and 800, 1000, 1200 ng/spot for lamivudine and zidovudine, respectively. In both cases the coefficient of variation of the peak area of the spots was used to evaluate method precision.

2.2.4 Limit of detection and limit of quantification

Determination of the detection and quantitation limits was performed based on the standard deviation of the response and the slope. The slope was estimated from the calibration curve of the analyte and the estimate of the standard deviation was carried out from the standard deviation of y-intercepts of regression lines. Signal/noise ratios of 3.3:1 and 10:1 were considered as limit of detection and limit of quantification, respectively.

2.2.5 Robustness

Effects of small but deliberate changes in the mobile phase composition, total volume of mobile phase, time between application of the sample and insertion in to the development chamber and time between development of the chromatograms to scanning on the results were examined. Mobile phases having different composition of toluene–chloroform–methanol viz (1.0:6.5:2.5); (1.5:6.0:2.5); (1.0:5.5:3.5); (1.5:5.5:3.0); (0.5:6.0:3.5) and (0.5:6.5:3.0) were used to develop chromatograms. The amount of mobile phase was

varied in the range of $\pm 10\%$ and both time variations: time from spotting to development and from development to scanning, were varied in the range of $\pm 20\%$ (Kaul, *et. al.*, 2004). On each of the effects three concentration levels (400, 500, 600 ng/spot and 800, 1000, 1200 ng/spot for lamivudine and zidovudine, respectively) and three determinations on each concentration were performed to study the robustness of the method. The relative standard deviations of the peak area of the spots were taken to document the robustness of the method. The optimized method, lower range and upper range values of each parameter used in the study are given in Table 2.1.

Table 2.1. Experimental variables used in the robustness study

Variable	Level		
	Lower range	Upper range	Medium range (used during validation)
Mobile phase combination			
(Toluene:chloroform:methanol)	0.5:5.5:2.5	1.5:6.5:3.5	1:6:3
Amount of mobile phase (ml)	13.5	16.5	15.0
Time from spotting to development (min.)	4.0	6.0	5.0
Time from development to scanning (min.)	6.4	9.6	8.0

3. RESULTS AND DISCUSSION

Due to its versatility and speed of analysis, the HPTLC technique was considered to be suitable, for the analysis of lamivudine and zidovudine concentrations in combivir tablets.

After evaluation of various solvent systems to arrive at an optimum resolution of the two drugs, the solvent systems consisting of toluene:chloroform:methanol (1:6:3 v/v) gave dense, compact and well separated spots of the drugs from the mixture at R_f of 0.28 and 0.57 (Fig. 3.1).

It was observed that pre-saturation of HPTLC chamber with the mobile phase for 50 min ensured good reproducibility and symmetrical peak shape of lamivudine and zidovudine.

Chamber saturation periods less than 50 min were tried but the chromatograms obtained were curved and not reproducible and consequently gave poor/unsymmetrical peak shape. Densitometric evaluation was performed at 271 and 276 nm for zidovudine and lamivudine, respectively, wavelength of maximum absorbance (λ_{max}), in absorbance/reflectance mode. Hence, for separation and simultaneous determination of both drugs, a high performance thin layer chromatographic system was adopted.

The basis for high quality data is a reliable analytical method. Therefore, new analytical methods to be used for the intended purpose require careful method development to be followed by a thorough validation of the final method. At this point, it must be stated that the quality of an analytical method largely depends on the process of development as compared to its validation. Therefore, it is imperative that the method itself is also fit for the purpose. Though the process of development has a significant role over validation for quality analytical method, it is only validation which can objectively demonstrate the inherent quality of an analytical method by fulfillment of minimum acceptance criteria and thus prove its applicability for a certain purpose. Since such objective performance data are essential for assessment of a method's quality, the importance of validation should not be underestimated.

3.1 Method optimization for the HPTLC–densitometric method

3.1.1 Mobile phase composition

Various solvent systems were evaluated to arrive at an optimum resolution of the two drugs. Since both drug mixtures are polar (lamivudine being more polar compared to zidovudine) the mixture needs a mobile phase combination which is polar for good separation of the two drugs.

The first combination of mobile phase which was tried in a one to one ratio was chloroform:methanol (1:1, V/V). The compounds are separated but both move a maximum distance, lamivudine traveled a distance close to the solvent front and zidovudine traveled almost together with the solvent front. R_f was equal to 0.83 and 1.0

for lamivudine and zidovudine respectively, which was not suitable for quantification process. So it was necessary to reduce the polarity of the mobile phase. By reducing the polarity of the mobile phase combination a number of trials were performed as shown in Table 3.1. But many of them made one of the compounds either to remain on a short distance from the point of application or to travel a close distance with the solvent front. In either case scanning is not a favored task and consequently might give a poor quantitation result.

Table 3.1. The different mobile phase combinations tried during method development

Trial No.	Mobile phase combination (V/V)		R_f	
			Lamivudine	zidovudine
1	Chloroform:methanol	(1:1)	0.83	1.00
2	Chloroform:methanol	(6.2:3.8)	0.68	0.98
3	Chloroform:methanol	(7:3)	0.67	0.91
4	Chloroform:methanol	(9:1)	0.15	0.72
5	Chloroform:methanol	(4:1)	0.37	0.89
6	Toluene:chloroform:methanol	(1:6:3)	0.28	0.57
7	Chloroform:propanol:methanol	(6:1:3)	0.44	0.97
8	Toluene:chloroform: propanol	(1:6:3)	0.06	0.73

Among the above mobile phase combinations tried, trial number six (toluene:chloroform:methanol in a 1:6:3 ratio) gave well separated as well as resolved spots of the drug mixture. Both lamivudine and zidovudine traveled a suitable distance, appropriate position for scanning and hence good for quantitation purpose. The R_f values were 0.28 and 0.57 for lamivudine and zidovudine respectively. The mobile phase combinations in trial number four and five also gave better R_f values. But in case of trial number four lamivudine is a little closer to the point of application as compared to the distance traveled by lamivudine in trial number six, in which case scanning is more favored. In trial number five, zidovudine is closer to the solvent front as compared to the distance traveled by zidovudine in trial number six, in which case again scanning is much easier.

Other mobile phase combinations were tried just to check if there was other combination of mobile phase which is as good as the one in trial number six.

In trial number seven toluene was replaced by propanol (chloroform:propanol:methanol, in a 6:1:3 ratio) which has higher polarity than toluene and in this combination the result obtained was not good ($R_f = 0.44$ and 0.97 for lamivudine and zidovudine, respectively). Even though lamivudine is located at a good position, zidovudine traveled almost with the solvent front making scanning process difficult. This might prove that a mobile phase combination which has higher polarity than trial number six combination would be inappropriate. In trial number eight methanol was replaced by propanol (toluene:chloroform:propanol in a 1:6:3 ratio) which is less polar as compared to methanol. In this combination the result was not good as well; ($R_f = 0.06$ and 0.73 for lamivudine and zidovudine, respectively). While zidovudine covered a good distance lamivudine remained more or less at the origin which was not appropriate for quantitation purpose. This might again suggest that a mobile phase combination which has less polarity than trial number six combination would not be appropriate.

And hence the polarity of the mobile phase combination of toluene:chloroform:methanol (1:6:3) is considered to be the best combination for separation of lamivudine/zidovudine mixture among the ones tested.

3.1.2 Conditions used during validation

The optimal conditions in which the method was validated were:

- ◆ Precoated silica gel glass plate 60 F₂₅₄ (10 cm×10 cm with 200µm thickness).
- ◆ Toluene:chloroform:methanol (1:6:3, V/V), 15 ml/run.
- ◆ Application rate 0.1µl/s.
- ◆ Chamber saturation time 50 minutes at room temperature ($24^{\circ}\text{C} \pm 2$) with relative humidity of 35–40%.
- ◆ Length of chromatogram run 8 cm for approximately 13 minutes.

- ◆ Detection wave length 271 and 276 nm for zidovudine and lamivudine, respectively.

Figure 3.1, indicates the HPTLC densitogram of the two drug mixture by using the optimal conditions stated and Figure 3.2, shows the UV-spectra of the two drug mixture at their maximum absorbance (λ_{max}), 271 and 276nm for zidovudine and lamivudine.

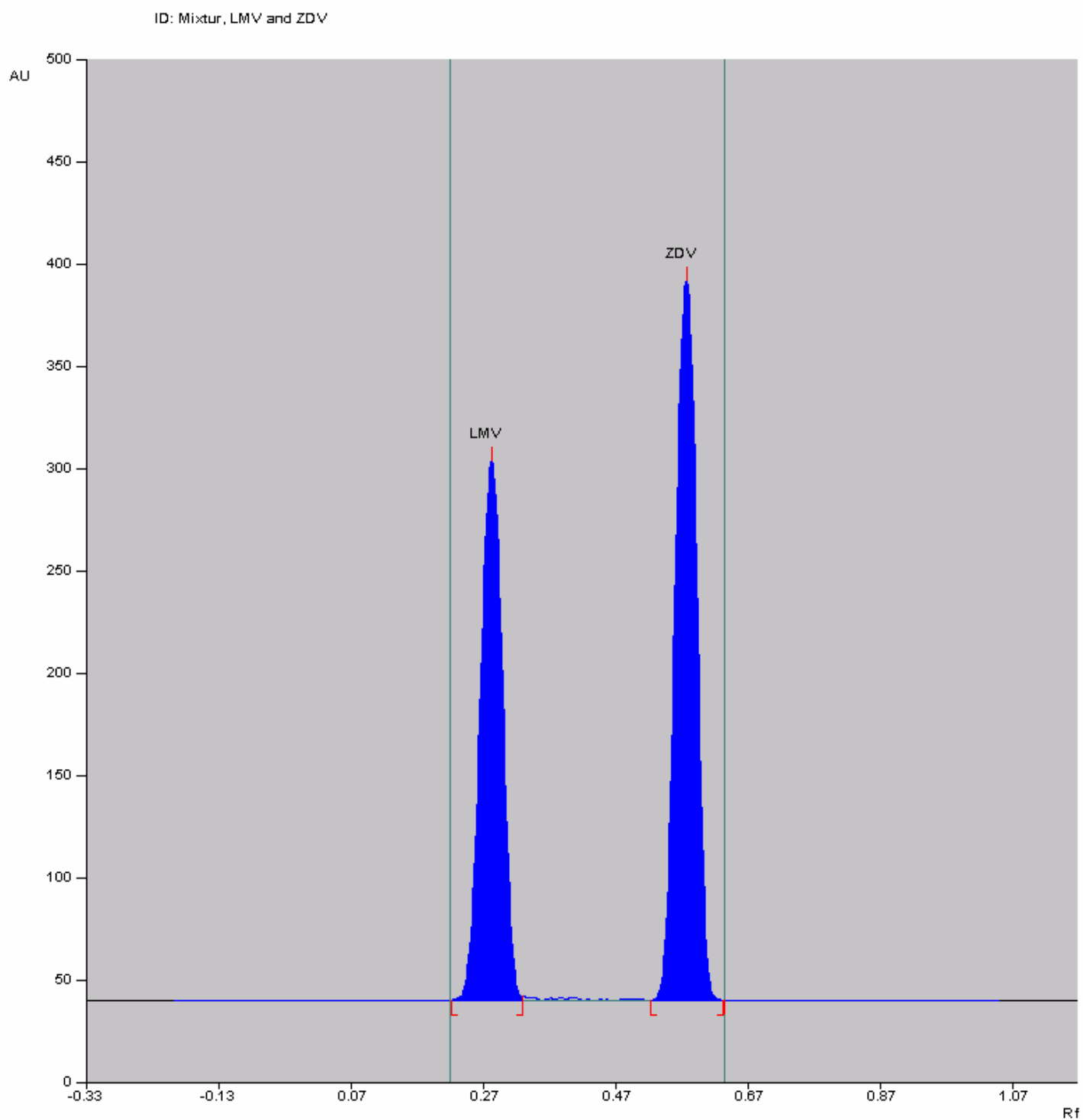
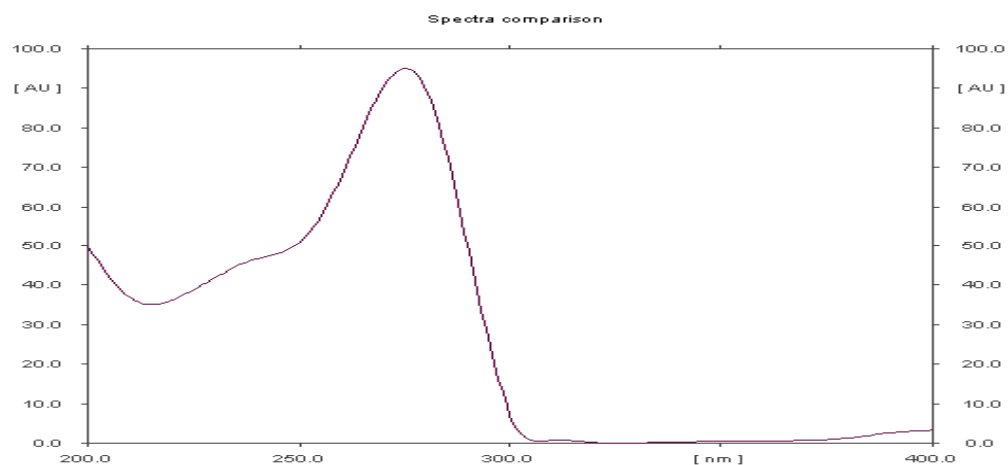
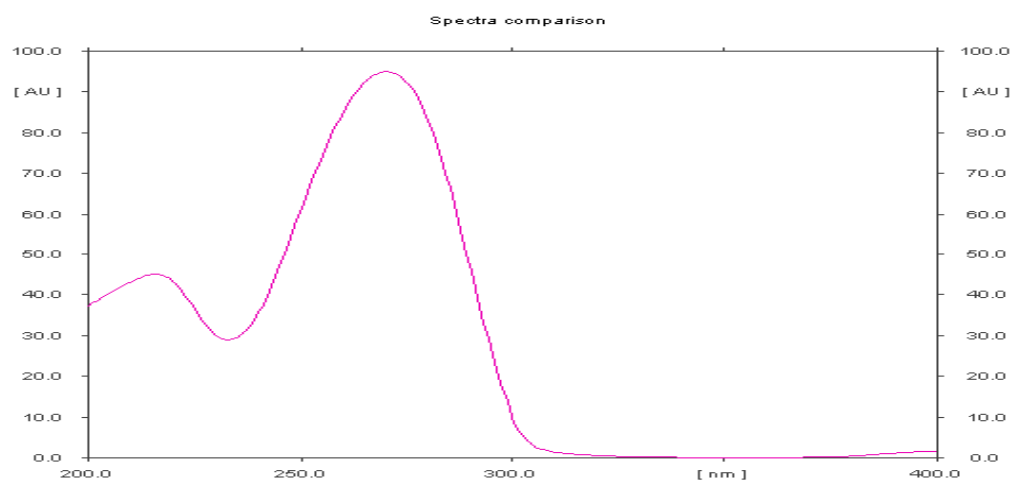


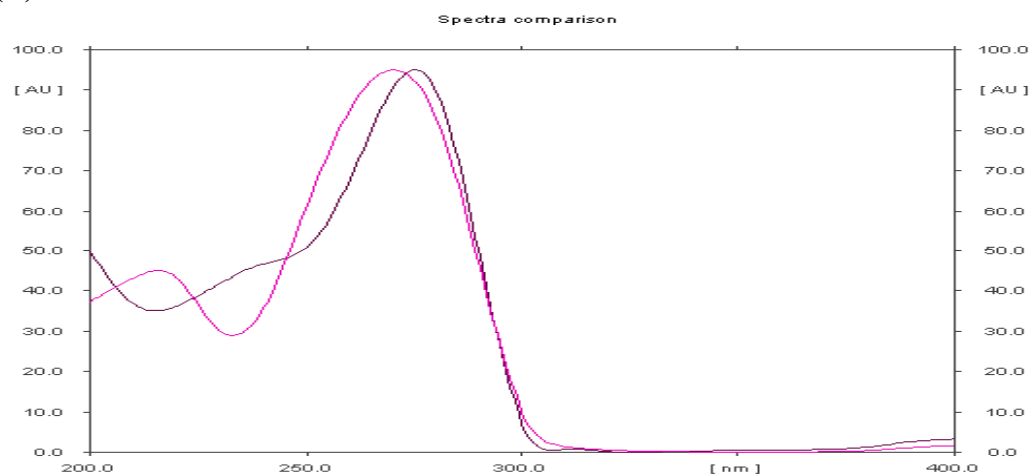
Fig. 3.1. The HPTLC densitogram of lamivudine (500 ng/spot) and zidovudine (1000ng/spot) using the optimal conditions stated.



(A)



(B)



(C)

Fig. 3.2. UV spectra of lamivudine (500ng/spot) (A), zidovudine (1000ng/spot) (B) and their mixture (500 and 1000 ng/spot for lamivudine and zidovudine, respectively) (C).

3.2 Method validation result

3.2.1 Linearity

The relationship between the concentration of each of lamivudine and zidovudine; and peak area of the spot was investigated. The linear relationship was tested and found to be less accepted due to the minimal fitting of the residuals on the calibration line (Fig. 3.3. (A), Fig. 3.4. (A)), indicating lower precise correlations ($r = 0.9887$ and 0.9806 and $r^2 = 0.9776$ and 0.9616 for lamivudine and zidovudine, respectively). So the linear model for fitting the data can be used but with less precise calculations. The characteristic parameters of the linear regression equation of the two drugs are shown in Table 3.2. The second order polynomial fit was found to be more suitable, its residuals plot showed a much better fitting (Fig. 3.3.(B), Fig. 3.4.(B)) than that of linear model, indicating good correlation ($r = 0.9998$ and 0.9998 and $r^2 = 0.9997$ and 0.9996 for lamivudine and zidovudine, respectively). The calibration graphs were constructed in the range of 250–1400 ng/spot for lamivudine and 250–1700 ng/spot for zidovudine. The characteristic parameters of the second order polynomial regression equation of the two drugs are shown in Table 3.3.

Table 3.2. Characteristic parameters for the linear regression equations obtained from the HPTLC- densitometric method for the studied drug ($n=6$)

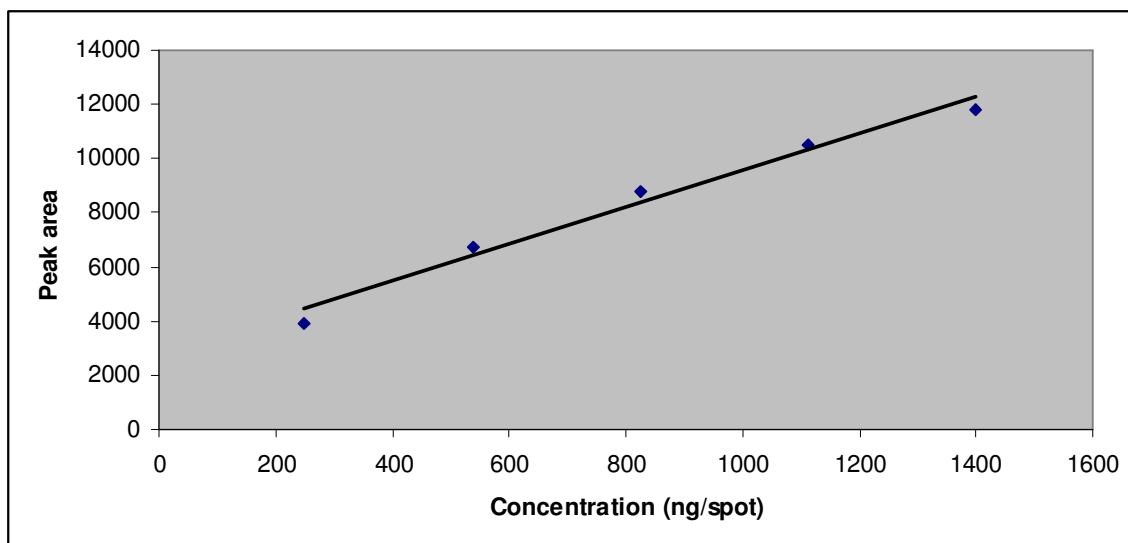
Parameters	Lamivudine	Zidovudine
Linearity range (ng/spot)	250–1400	250-1700
Coefficient of $x \pm$ S.D.	6.7978 ± 0.012182	4.5974 ± 0.005575
Intercept $\pm$ S.D.	2754.2 ± 6.93	2048.1 ± 7.73
Determination coefficient (r^2)	0.9776	0.9616
Correlation coefficient (r)	0.9887	0.9806

All calculations were done under 95% confidence limit.

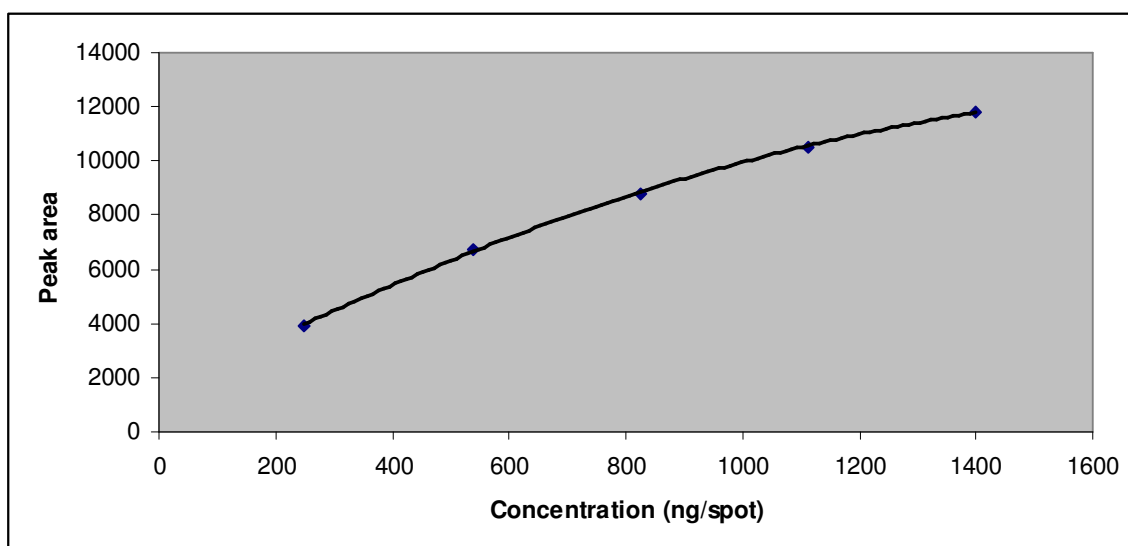
Table 3.3. Characteristic parameters for the second order polynomial regression equations obtained from the HPTLC-densitometric method for the studied drug ($n=6$)

Parameters	Lamivudine	Zidovudine
Calibration range (ng/spot)	250–1400	250-1700
Coefficient of $x^2 \pm$ S.D.	-0.003 ± 0.000041	-0.0021 ± 0.000052
Coefficient of $x \pm$ S.D.	11.756 ± 0.05	8.7158 ± 0.03
Intercept $\pm$ S.D.	205.60 ± 12.07	568.09 ± 12.70
Determination coefficient (r^2)	0.9997	0.9996
Correlation coefficient (r)	0.9998	0.9998

All calculations were done under 95% confidence limit.

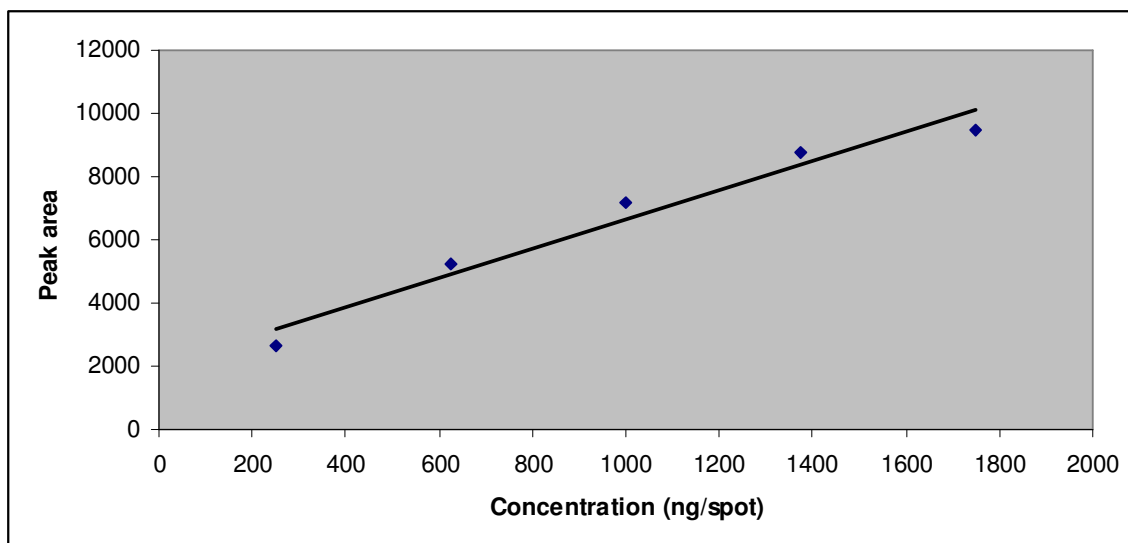


(A)

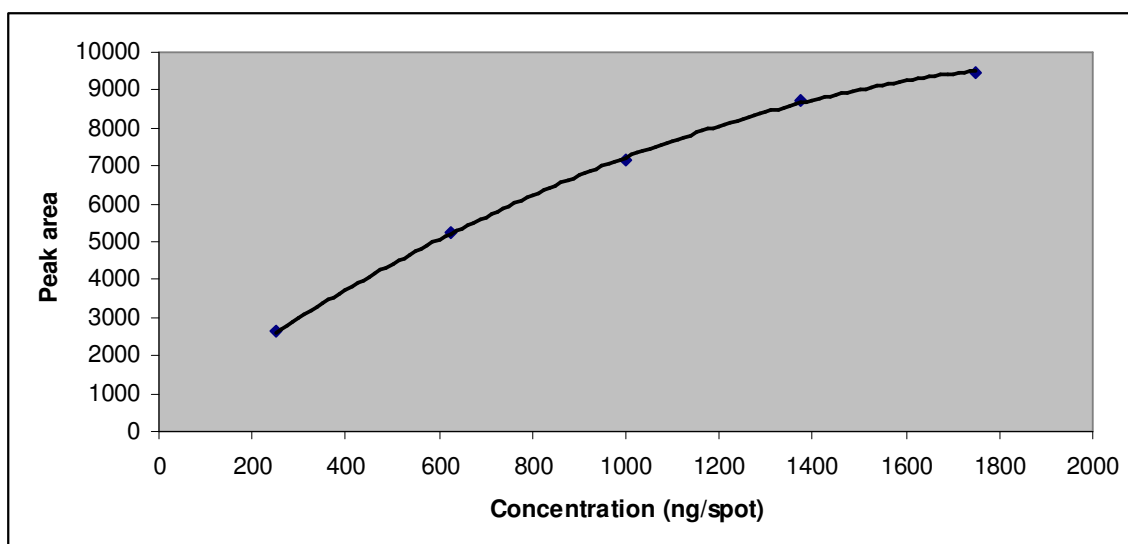


(B)

Fig. 3.3. Calibration curves for the determination of lamivudine using the linear fit (A) and the second order polynomial fit (B), for the same data given.



(A)



(B)

Fig. 3.4. Calibration curves for the determination of zidovudine using the linear fit (A) and the second order polynomial fit (B), for the same data given.

3.2.2 Accuracy/recovery studies

Satisfactory recoveries ranged from 98.68 to 100.73 for lamivudine and from 98.56 to 100.61 for zidovudine were obtained using the HPTLC method. These satisfactory recoveries with small coefficient of variation ($\leq 1.41\%$, for both drugs) indicate that, the method provides sufficient accuracy. The degree of recovery increased as the percentage of the pure drug added to sample increased for both lamivudine and zidovudine as shown in Fig 3.5, which again indicates the accuracy of the method. The accuracy of the HPTLC method was also compared with the reported HPLC method and the results are given in Table 3.4. For each percentage recovery the calculated t-test did not exceed the theoretical values (2.57) at the 95% confidence level for both lamivudine and zidovudine, indicating no significant difference between the two methods.

Remark: The internal standard, finasteride, used in the reported method to enhance the resolution of the two compounds was not available. Because of that the resolution of the compounds using the mobile phase reported in the method, methanol:water:acetonitrile (70:20:10), was found not good. So to enhance the resolution of the two compounds there was a need to adjust the proportion of the mobile phase combination. And after a certain trial the mobile phase combination of methanol:water:acetonitrile in a 50:40:10 proportion gave a well resolved lamivudine and zidovudine mixture. Therefore the work was done using that mobile phase proportion, but all other things were done according to the reported method.

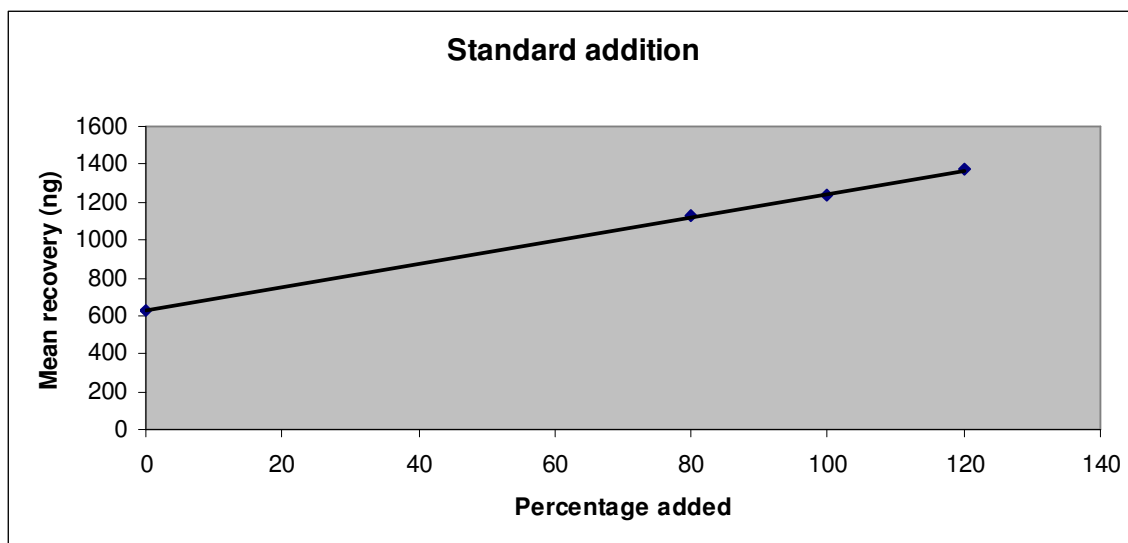
Table 3.4. Recovery study (using standard addition technique) for determination of lamivudine (a) and zidovudine (b) by HPTLC-densitometry and HPLC method ($n=6$, for both)

HPTLC		HPLC				t-value	
Amount of standard drug added to the analyte (%)*	Theoretical content (ng)	Recovery (%)	C.V. **	Amount of standard drug added to the analyte (%)	Theoretical content (µg)	Recovery (%)	C.V. **
(a) Lamivudine							
0	625	99.85	1.41	0	20	98.70	1.57
80	1125	100.73	0.66	80	36	101.19	1.20
100	1250	98.68	0.87	100	40	99.45	1.41
120	1375	99.69	0.47	120	44	100.16	0.73
(b) Zidovudine							
0	750	100.61	1.17	0	21.43	98.60	1.42
80	1350	100.14	1.07	80	38.57	99.46	1.23
100	1500	99.73	0.45	100	42.86	99.84	1.40
120	1650	98.56	0.58	120	47.14	100.85	0.76

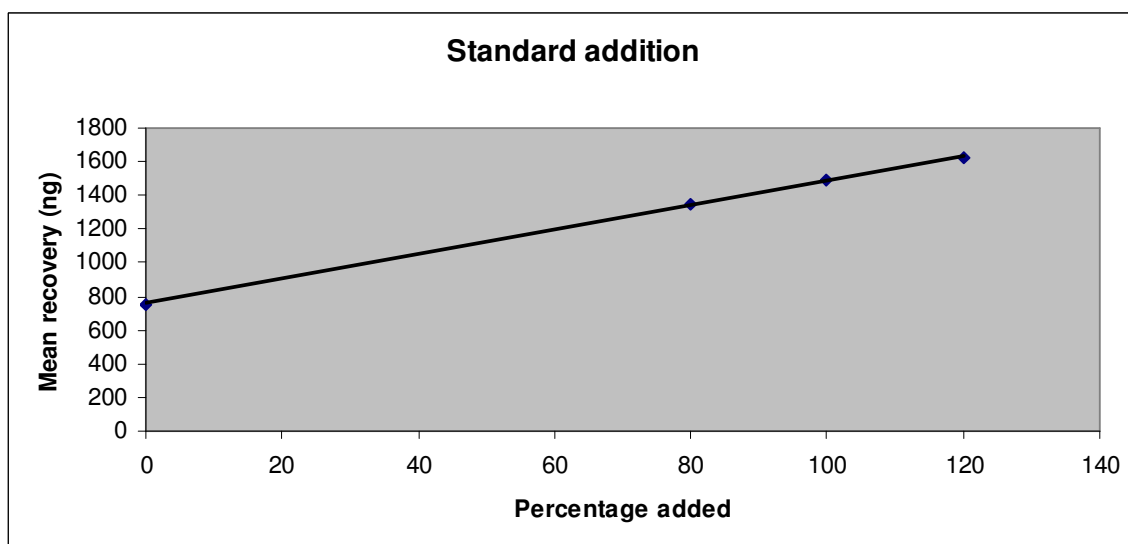
* % of the label claim amount

** Coefficient of variation

*** The calculated t-value is 2.57 (P = 0.05)



(A)



(B)

Fig. 3.5. Standard addition technique for lamivudine (A) and zidovudine (B) showing the increment of the recovered amount as the percentage of the pure drug added to the sample increased

3.2.3 Precision

As shown in Table 3.5 the repeatability of sample application and measurement of peak area were expressed in terms of coefficient of variation and were found to be very low (0.26 and 0.28 and 0.08 and 0.07 for lamivudine and zidovudine, respectively) which, in turn, insured reproducible performance of the instrument. The coefficient of variation values depicted in Table 3.6 shows that the proposed method provides acceptable intra-day and inter-day variation of lamivudine and zidovudine, which indicates the reproducible performance of the method itself. Table 3.7 shows the reproducibility of the method within three different days. As depicted in Table 3.5, 3.6 and 3.7 the coefficient of variation less than 2% suggesting system suitability and precision of the developed method.

Table 3.5. System precision studies of the developed method

No.	Repeatability of sample application		Repeatability of measurement of peak area	
	Area of LMV ^a (500 ng/spot)	Area of ZDV ^b (1000 ng/spot)	Area of LMV (500 ng/spot)	Area of ZDV (1000 ng/spot)
1	6459.54	7135.95	6440.30	7143.68
2	6428.95	7169.50	6435.88	7153.60
3	6425.02	7132.93	6446.06	7155.78
4	6454.57	7178.66	6439.46	7154.56
5	6420.41	7145.18	6439.85	7153.38
6	6451.41	7170.02	6449.35	7145.24
Mean area	6439.98	7155.37	6441.48	7151.37
S.D.*	17.06	19.70	4.36	5.52
C.V. **	0.26	0.28	0.07	0.08

* Standard deviation

** Coefficient of variation

^a and ^b Lamivudine and zidovudine, respectively

Table 3.6. Method precision studies of the developed method

Compound (ng/spot)	Amount	Intra-day			Inter-day								
		Precision (n=6)			precision (n=3)								
		Day-one			Day-two			Day-three					
		Mean area	S.D. *	C. V. **	Mean area	S.D.	C. V.	Mean area	S.D.	C. V.	Mean area	S.D.	C. V.
LMV ^a	400	5390.19	16.70	0.31	5391.03	19.50	0.36	5392.30	27.92	0.52	5394.53	29.50	0.55
	500	6439.36	18.75	0.29	6438.21	29.88	0.46	6436.58	34.44	0.54	6439.92	39.19	0.61
	600	7115.52	24.10	0.32	7411.02	20.48	0.27	7412.96	23.93	0.32	7411.69	20.80	0.28
ZDV ^b	800	6022.58	21.22	0.35	6023.23	35.59	0.59	6027.25	24.57	0.41	6020.91	25.66	0.43
	1000	7153.98	23.42	0.33	7139.81	25.78	0.36	7140.72	45.77	0.64	7142.62	29.54	0.41
	1200	8002.04	35.72	0.45	8006.12	36.83	0.46	8004.35	41.52	0.52	8003.68	31.18	0.39

* Standard deviation

** Coefficient of variation

^a and ^b Lamivudine and zidovudine, respectively

Table 3.7. Reproducibility testing of the developed method (n=3)

Compound	Amount (ng/spot)	Mean area (of the three day)	S.D. *	C.V. **
Lamivudine	400	5392.62	1.77	0.03
	500	6438.24	1.67	0.03
	600	7411.89	0.99	0.01
Zidovudine	800	6023.80	3.21	0.05
	1000	7141.05	1.43	0.02
	1200	8004.72	1.26	0.02

* Standard deviation

** Coefficient of variation

3.2.4 Limit of detection and limit of quantification

The limit of detection and limit of quantification of the proposed method were calculated according to $3.3 \sigma/S$ and $10 \sigma/S$ criteria, respectively, where σ is the standard deviation of y-intercepts of regression lines (n=6) of the sample and s is the slope of the corresponding calibration curve. The limit of detection of lamivudine and zidovudine were found to be 3.06 and 3.34 ng/spot, respectively. These were the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. And the limit of quantification of lamivudine and zidovudine were 9.28 and 10.13 ng/spot, respectively. These were the lowest concentration of drugs that were accurately detected and integrated by the instrumentation used.

3.2.5 Robustness

The standard deviation of peak areas was calculated for each parameter and the coefficient of variation was found to be less than 2%. The low values of coefficient of variation as shown in Table 3.8 indicated robustness of the method. Among the parameters chosen to evaluate robustness experiments very little variation was made on mobile phase composition, which was found to be a very sensitive parameter during the optimization process.

But to identify statistically significant effects from the recommended variables, a t-test was performed. The absolute value of the effect of a factor X is considered to be significant if the t-test value is larger than a critical value (t-critical). However the t-test value for all the parameters was found to be less than the critical value which indicates that among the variables used to test robustness non has a significant effect over the others. The tabulated t-value with two degrees of freedom ($P = 0.05$) is given as 4.3.

Table 3.8. Robustness testing of the developed method ($n=3$)

Parameter	Lamivudine			Zidovudine		
	S.D. ^a of peak area	C.V. ^b	% change	S.D. ^a of peak area	C.V. ^b	% change
Mobile phase composition	45.44	0.71	89.41	46.17	0.65	83.21
Amount of mobile phase	28.60	0.44	19.22	29.13	0.41	15.60
Time from spotting to development	30.65	0.48	27.76	31.72	0.45	25.87
Time from development to scanning	34.26	0.53	42.81	36.19	0.51	43.61
Using optimal conditions	23.99	0.38	-	25.20	0.37	-

^a Standard deviation of average of three concentrations 400, 500, 600 ng/spot and 800, 1000, 1200 ng/spot for lamivudine and zidovudine, respectively.

^b Coefficient of variation

When the percentage change was considered, changing some parameters, such as amount of mobile phase, time from spotting to development and from development to scanning caused a relatively smaller change in the result obtained (19.22-42.81% and 15.60%-43.61% for lamivudine and zidovudine, respectively). The change caused by variation of mobile phase composition was however relatively higher (89.41% and 83.21% for lamivudine and zidovudine, respectively) as compared to the other parameters and this was the expected as many HPTLC separations largely depend on the percent of mobile phase composition used.

Table 3.9. Summary of method validation parameters

Parameter	Lamivudine	Zidovudine
Linearity range (ng/spot)	250-1400	250-1700
Correlation coefficient	0.9998	0.9998
Limit of detection (ng/spot)	3.06	3.34
Limit of quantitation (ng/spot)	9.28	10.13
Recovery (Mean $\pm$ S.D.)	99.74 $\pm$ 0.84	99.76 $\pm$ 0.88
Precision (C.V)		
Repeatability of application (n=6)	0.26	0.28
Repeatability of measurement (n=6)	0.07	0.08
Intra-day (n = 6)	0.31	0.38
Inter-day (n = 3)	0.43	0.47
Reproducibility (of the three day)	0.02	0.03
Robustness	Robust	Robust

3.2.6 Analysis of the marketed formulation

The proposed method was applied to the determination of lamivudine and zidovudine in commercial tablets of combivir. Six replicate determinations were made. Satisfactory results were obtained for both drugs and were in a good agreement with the label claims. As can be seen from Table 3.10 the drug content was found to be 99.69% $\pm$ 1.54 (coefficient of variation = 0.32) and 99.73% $\pm$ 1.66 (coefficient of variation = 0.17) for lamivudine and zidovudine, respectively. The low values of coefficient of variation indicated the suitability of this method for routine analysis of lamivudine and zidovudine in pharmaceutical dosage forms.

The results of determination of lamivudine and zidovudine in tablets obtained from the HPTLC method were compared with those of the reported HPLC method. Statistical evaluation was performed using Student's *t*-test and the *F* ratio at 95% confidence level

as shown in Table 3.10. And it was found that there is no significant difference between the two methods.

Table 3.10. Applicability of the proposed methods for the determination of lamivudine and zidovudine in commercial tablets ($n=6$, for both HPTLC and HPLC)

Parameters	HPTLC		HPLC	
	Lamivudine	Zidovudine	Lamivudine	Zidovudine
Label claim (mg/tablet)	150	300	150	300
Drug content (%) $\pm$ S.D.	99.69 $\pm$ 1.54	99.73 $\pm$ 1.66	99.57 $\pm$ 0.74	100.14 $\pm$ 0.80
t -value ^a	0.23	0.12		
F -value ^a	4.27	4.31		

^a The theoretical t - and F -values are equal to 2.57 and 5.05, respectively ($P = 0.05$).

Results of analysis of the dosage form containing lamivudine and zidovudine given in Table 3.10 indicate that the proposed method can successfully be used to estimate lamivudine and zidovudine when present in combination in pharmaceuticals. In addition the results of analysis of the commercial tablets (Table-3.10) and the recovery study (standard addition method) (Table 3.4) of both drugs suggested that there is no interference from any excipients, which are normally present in tablets.

3.2.7 Stability in sample solution

The time the sample is left to stand in the solvent prior to chromatographic development may influence the stability of spots and are required to be investigated. The procedure is also useful to check the degradation product of the marketed formulation, if any. So solutions of two different concentrations (500 and 600 ng/spot for lamivudine) and (1000 and 1200 ng/spot for zidovudine) were prepared and stored at room temperature for 0.5, 1.0, 2.0, 4.0 and 24 hr (Kaul, *et. al.*, 2004). They were then applied on the HPTLC plate, after development the densitogram was evaluated as listed in Table 3.11 for additional

spots if any. No other new peak appeared to indicate presence of degradation product. Only the spots at R_f 0.28 (for lamivudine) and 0.57 (for zidovudine) were observed in the densitogram of the drug samples prepared from tablets. It may therefore be inferred that degradation (compound instability in the sample solution) of lamivudine and zidovudine had not occurred in the marketed formulations that were analyzed by this method.

Statistical comparison between the data obtained from the sample spotted immediately after preparing the solution and the rest of the samples (which were spotted after some time the solution was prepared and stored) were also performed. Both the calculated t- and F-values are smaller than their theoretical values (Table 3.11), indicating the absence of significant difference between the two: the sample spotted immediately after preparing the solution and the other samples.

3.2.8 Cost and time comparison

Comparison of time as well as cost effectiveness of the developed HPTLC method was done with that of the reported method. The comparison showed that the proposed HPTLC method was both time and cost effective over the reported HPLC method.

The total time required for the analysis of a sample in HPTLC (per sample bases) was found to be smaller than the time required by the HPLC method as shown in Table 3.12.

Table 3.12 Time comparison between the proposed HPTLC and the reported HPLC method

Parameter	HPTLC (min.)	HPLC (min.)
Solution preparation (dilution + degassing of the solvent (HPLC))	25	65
Pre conditioning of the chromatographic system (chamber saturation for HPTLC)	50	60
Pre treatment of the mobile phase	–	20
Run time	23	12
Total	98	157

The running coast for the analysis of a sample in HPTLC (per sample bases) was again found to be smaller than that of the reported HPLC method (Table 3.13).

Table 3.13 Coast comparison between the proposed HPTLC and the reported HPLC method

Parameter	HPTLC (litter)	HPLC (litter)
Solvent (dilution, mobile phase, pre conditioning (HPLC))	0.315	2.472
That in terms of money (rough conversion)	US \$ 6.17	US \$ 44.64

3.2.9 Testing different stationery phase for routine analysis of the studied drug

Precoated plates of different support materials like glass, aluminum sheets and plastic, and different sorbents are available. Some of the sorbents are silica (unmodified), spherical silica lichrospher, monolithic silica, modified silica, cellulose and aluminium oxide. Unmodified silica gel covers nearly 80 % of HPTLC applications. It enables separation of a large range of very different substances such as alkaloids, anabolics, carbohydrates, fatty acids, glycosides, lipids, mycotoxins, nucleotides, peptides, pesticides, steroids, sulfonamides, surfactants, tetracyclines and many others. These plates offer unsurpassed separation performance and are therefore ideal for quantitative thin layer analysis including: automated applications for quantitative separations, quality control of drugs and medicinal plant and herbal analysis (Merck).

In addition, analysis of basic substances and steroids are done using aluminum oxide as a coating material. Analysis of amino acids, dipeptides and alkaloids are done using cellulose as a coating material, while analysis of non-polar substances, fatty acids, carotenoids, cholesterol are done using modified silica (RP2, RP8 and RP18) as a coating material. Preservatives, barbiturates, analgesic and phenothiazines are analyzed using hybrid plates-RPWF254s as coating material (Kananen, *et. al.*, 1970; Stahlmann, *et. al.*, 2001).

Based on the above ideas when the structure of the studied drugs (lamivudine and zidovudine) are looked the kind of plate which is best suited for use was found to be the unmodified silica gel (silica gel 60 F₂₅₄). Due to that reason testing different plates which is suitable for routine analysis of combivir tablets was found to be an unnecessary task.

Conclusion

For an antiretroviral treatment to be effective for a long time, it has been reported that one needs to take more than one antiretroviral drug at a time formulated in a combined form. Quantitative determination of components of such combined dosage forms is often a difficult task as tedious separation techniques may be involved in the process before the actual quantitation of the drug mixture.

The objective of this study was to develop an analytical method that would enable direct quantitative determination of the two drugs in combivir mixture (lamivudine and zidovudine) without preliminary pretreatment or extraction.

Therefore the developed HPTLC method provides simple, precise and accurate analysis method for the simultaneous determination of lamivudine and zidovudine in pharmaceutical formulations. A good separation of analytes was achieved using toluene:chloroform:methanol (1:6:3, V/V) as a mobile phase on precoated silica gel 60 F₂₅₄ plates. The method was validated as per ICH guidelines. The recovery of active ingredients in drug formulations was $\geq 98.56\%$ with $\leq 1.41\%$ relative standard deviation, which indicated that the method is suitable to quantify the studied drugs in pharmaceutical formulations without any interference from excipients. Statistical tests indicated that the developed HPTLC and the reported HPLC methods (Uslu and Ozkan, 2002) appear to be equally suitable for routine simultaneous determination of lamivudine and zidovudine in pharmaceutical formulation.

Compared with the reported HPLC method, the present method has some advantages such as short analysis time, large sample capacity per run and minimal use of solvent volume (solvent consumption per sample basis in this developed HPTLC method is only about 12% of that required in the reported HPLC method). In addition, HPTLC has the advantage of being a rapid, reliable, economical and less hazardous analysis method. The whole procedure may be extended to pharmaceutical preparations and other applications on the same drugs for routine screening.

Recommendation for further work

- The method developed is useful for the simultaneous determination of lamivudine and ziovine in pharmaceutical formulations. This method could also be useful if tried for the simultaneous determination of clinically relevant levels of lamivudine and zidovudine in human serum to support post-marketing clinical studies for combivir tablet.
- Determination of appropriate shelf life using a stability indicating method could be important for combivir tablet. And hence it would be beneficial to try this developed method as stability indicting for the determination of the shelf life of the drug mixture.

BIBLIOGRAPHY

- AIDSinfo, (2007). Lamivudine/Zidovudine. A service of the U.S department of health and human services, (http://www.aidsinfo.nih.gov/DrugsNew/DrugDetailT.aspx?MenuItem=Drugs&int_id=285&Search=Off&ClassID=&TypeID=#FA1) (Accessed on 10.02.07).
- Alnouti, Y., White, C.A. and Bartlett, G.M. (2004). Determination of lamivudine in plasma, amniotic fluid, and rat tissues by liquid chromatography. *J. of Chromatography B*, **803**: 279–284.
- Anbazhagan, S., Indumathy, N., Shanmugapandiyar, P. and Sridhar, K.S. (2005). Simultaneous quantification of stavudine, lamivudine and nevirapine by UV spectroscopy, reverse phase HPLC and HPTLC in tablets. *J. of Pharmaceutical and Biomedical Analysis*, **39**: 801–804.
- AVERT I. (2006). AIDS around the world. AVERTing HIV and AIDS, (<http://www.avert.org/aroundworld.htm>.) (Accessed on 03.10.06).
- AVERT II. (2006). Introduction to HIV/AIDS Treatment. AVERTing HIV and AIDS, (<http://www.avert.org/introtrt.htm>.) (Accessed on 03.10.06).
- AVERT III. (2006). World wide HIV and AIDS Statistics. AVERTing HIV and AIDS, (<http://www.avert.org/worldstats.htm>.) (Accessed on 09/10/06).
- AVERT. (2007). Introduction to HIV/AIDS treatment. Averting HIV and AIDS, (<http://www.avert.org/introtrt.htm>) (Accessed on 26.05.07).
- Bahrami, G., Mirzaeei, S., Kiani, A. and Mohammadi, B. (2005). High-performance liquid chromatographic determination of lamivudine in human serum using liquid–liquid extraction; application to pharmacokinetic studies. *J. of Chromatography B*, **823**: 213–217.
- Ben, C. (1995). HIV treatment information. Project Inform, Inc. (<http://www.projinf.org/pip/17/pip17a.html>) (Accessed on 03.10.06).
- Bernard, L.A., Padian, N., Barnes, B. and Mark, C. (2007). The obligation to provide antiretroviral treatment in HIV prevention trials. *AIDS*, **21**:1229–1231.

- Bourget, P., Paci, A., Jean, B.R., Mercier, L. and Demirdjian, S. (2003). Contribution of programme in a hospital chemotherapy manufacturing unit. *E. J. of Pharmaceutics and Biopharmaceutics*, **56**: 445-451.
- CDC (1999). HIV and its transmission. Divisions of HIV/AIDS prevention, (<http://www.cdc.gov/HIV/resources/factsheets/transmission.htm>) (Accessed on 03.10.06).
- CDC (2005). Living with HIV/AIDS. Divisions of HIV/AIDS prevention, (<http://www.cdc.gov/hiv/pubs/brochure/livingwithhiv.htm>) (Accessed on 03.10.06).
- Checa, A., Oliver, R., Saurina, J. and Hern'andez, C.S. (2006). Flow-injection spectrophotometric determination of reverse transcriptase inhibitors used for acquired immuno deficiency syndrome (AIDS) treatment. Focus on strategies for dealing with the background components. *Analytica Chimica Acta*, **572**: 155–164.
- Checa, A., Oliver, R., Hern'andez, C.S. and Saurina, J. (2007). Flow-injection determination of zidovudine in plasma samples using multivariate curve resolution. *Analytica Chimica Acta*, **592**: 173–180.
- Dunge, A., Sharda, N., Singh, B. and Singh, S. (2005). Validated specific HPLC method for determination of zidovudine during stability studies. *J. of Pharmaceutical and Biomedical Analysis*, **37**: 1109-1114.
- Fan, B. and Stewart, J.T. (2002a). Determinations of zidovudine/ didanosine/ nevirapine and zidovudine/ didanosine/ ritonavir in human serum by micellar electrokinetic chromatography. *J. of Pharmaceutical and Biomedical Analysis*, **30**: 955-960.
- Fan, B. and Stewart, J.T. (2002b). Determination of zidovudine/ lamivudine/ nevirapine in human plasma using ion-pair HPLC. *J. of Pharmaceutical and Biomedical Analysis*, **28**: 903–908.
- FDA (2005). Guideline for submitting samples and analytical data for methods validation. (<http://www.fda.gov/cder/guidance/ameth.htm>) (Accessed on 25.10.2006).

- FDA (2006). An overview. (<http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm?Fuseaction=Search.Overview&DrugName=LAMIVUDINE%3B%20ZIDOVUDINE.>) (Accessed on 25.10.2006).
- FDA (2007). Drugs used in the treatment of HIV infection. (<http://www.fda.gov/oashi/aids/virals.html>.) (Accessed on 05.08.2007).
- Gennario, A.R. (2000). Remington; *The Science and practice of pharmacy*. 20th ed., The University of Sciences, Philadelphia, pp. 587-596.
- Girard, M.P., Osmanov S.K. and Kieny M.P. (2006). A review of vaccine research and development: The human immunodeficiency virus (HIV). *Vaccine*, **24**: 4062–4081.
- GlaxoSmithKline (2004). COMBIVIR (lamvudine/zidovudine) Tablets. (http://www.fda.gov/cder/foi/label/2004/20857slr014_combivir_lbl.pdf) (Accessed on 10.01.2007).
- GlaxoSmithKline (2006). Combivir product information. (http://us.gsk.com/products/assets/us_combivir.pdf.) (Accessed on 05.01.2007).
- Hoetelmans, R.M.W., Profijt, M., Meenhorst, M.P.L, Mulder, J.W. and Beijnen, J.H. (1998). Quantitative determination of (2)-2'-deoxy-3'-thiacytidine (lamivudine) in human plasma, saliva and cerebrospinal fluid by high-performance liquid chromatography with ultraviolet detection. *J. of Chromatography B*, **713**: 387–394.
- ICH (1994). International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use. Text on validation of analytical procedures. (<http://www.biologicsconsulting.com/docs/ich/ICHQ2A.pdf>). (Accessed on 5.11.06).
- ICH (1996). International conference on harmonization of technical requirements for the registration of pharmaceuticals for human use. Validation of analytical procedures: Methodology. (http://www.biologicsconsulting.com/docs/_ich/ICHQ2B.pdf) (Accessed on 5.11.06).

- Jordan, R., Gold, L., Cummins, C. and Hyde, C. (2002). Systematic review and meta-analysis of evidence for increasing numbers of drugs in antiretroviral combination therapy. *BMJ*, **324**:757-784.
- Kalasz, H. and Bathori, M. (2000). *Separation Methods in Drug Synthesis and Purification*. K. Valko Ed., Elsevier, Amsterdam, pp. 439-501.
- Kananen, G., Sunshine, I. and Monforte, J. (1970). A comparison of thin-layer chromatographic adsorbents, supports and developing units. *J. of Chromatography A*, **52**: 291-303.
- Kapoor, N., Khandavilli, S. and Panchagnula, R. (2006). Simultaneous determination of lamivudine and stavudine in antiretroviral fixed dose combinations by first derivative spectrophotometry and high performance liquid chromatography. *J. of Pharmaceutical and Biomedical Analysis*, **41**: 761–765.
- Kazue, K.E., Helena, D.C., Emiko, M.E., Schramm, A.S. and Porta, V. (2005). Determination of lamivudine in human plasma by HPLC and its use in bioequivalence studies. *International J. of Pharmaceutics*, **297**: 73-79.
- Labcompliance (2006). Validation of Analytical Methods and Procedures. (<http://www.labcompliance.com/seminars/audio142/index.htm>.) (Accessed on 09.10.06).
- Lee, D.W., Linda, S.V., Frederick, A.B. and Daniel, R.D. (2003). Liquid chromatographic–mass spectrometric determination of the metabolism and disposition of the anti-retroviral nucleoside analogs zidovudine and lamivudine in C57BL/6N and B6C3F1 mice. *J. of Chromatography B*, **798**: 55–62.
- Lieberman, H.A., Rieger, M.M. and Banker, G.S. (1998). *Pharmaceutical dosage forms; disperse systems*. Volume 3, 2nd ed., Marcel Dekker Inc., pp. 425-429.
- Lim, S. (1991). Quantitative analysis of complex multi-component mixtures, (<https://www.varianinc.com/media/sci/apps/uv53.pdf>) (Accessed on 27.06.06).
- Marchei, E., Valvo, L., Pacifici, R., Pellegrini, M., Tossini, G. and Zuccaro, P. (2002). Simultaneous determination of zidovudine and nevirapine in human plasma by RP-LC. *J. of Pharmaceutical and Biomedical Analysis*, **29**: 1081–1088.
- Merck. Thin layer chromatography by Merck –traditionally innovative. (<http://www.Merck.de/servlet/PB/show/1705930/TLC.pdf>) (Accessed on 05.05.2007)

- Naser L.R., Richard R.T. and Angela D.M. (2003). Simultaneous determination of six HIV nucleoside analogue reverse transcriptase inhibitors and nevirapine by liquid chromatography with ultraviolet absorbance detection. *J. of Chromatography B*, **791**: 137–147.
- Kaul, N., Dhaneshwar, S.R. , Agrawal, H., Kakad, A. and Patil, B. (2004). Application of HPLC and HPTLC for the simultaneous determination of tizanidine and rofecoxib in pharmaceutical dosage form. *J. of Pharmaceutical and Biomedical Analysis*, **37**: 27-38
- Paul, A.L. (1999). The immune system. An overview. The body, (<http://www.thebody.com/content/art1788.html>) (Accessed on 04.02.07).
- Pereira, A.S., Kenney, K.B., Cohen, M.S., Hall, J.E. and Eron, J.J. (2000). Simultaneous determination of lamivudine and zidovudine concentrations in human seminal plasma using high-performance liquid chromatography and tandem mass spectrometry. *J. of Chromatography B*, **742**: 173–183.
- Pharmainfo.net (2006). Methods of estimation of multi-component formulations: A review. (http://www.pharmainfo.net/exclusive/reviews/methods_of_estimation_of_multi-component_formulations:_a_review/). (Accessed on 5.11.2006).
- Ramachandran, G., Hemanthkumar, A.K. Kumaraswami, V. and Swaminathan S. (2006). A simple and rapid liquid chromatography method for simultaneous determination of zidovudine and nevirapine in plasma. *J. of Chromatography B*, **843**: 339–344.
- Richman, D.D. (2006). Antiviral drug resistance. *Antiviral Research*, **71**: 117–121.
- Sacndulescu, R., Mirel, S. and Oprean, R. (2000). The development of spectrophotometric and electroanalytical methods for ascorbic acid and acetaminophen and their applications in the analysis of effervescent dosage forms. *J. of pharmaceutical and Biomedical Analysis*, **23**: 77-87.
- Saliba, G. and Yeni, P. (2006). Recent and future therapeutic advances in the management of HIV infection. *Pathologie Biologie*, **54**: 545–550.
- Schneider, M. F., Gange, S. J., Williams, C. M., Anastos, K., Greenblatt, R. M., Kingsley, L., Detels, R. and Munoz, A. (2005). Patterns of the hazard of death

- after AIDS through the evolution of antiretroviral therapy. *AIDS*, **19**: 2009-2018.
- Szepesi, G. and Nyiredy, S. (1992). Planar chromatography: Current status and future perspectives in pharmaceutical analysis. Applicability, quantitation and validation. *J. of Pharmaceutical and Biomedical Analysis*, **10**: 1007-1015.
- Tan, X. and Boudinot F.D. (2000). Simultaneous determination of zidovudine and its monophosphate in mouse plasma and peripheral red blood cells by high-performance liquid chromatography. *J. of Chromatography B*, **740**: 281–287.
- The AIDS InfoNet (2007). What is Aids? A project of community partners, (<http://www.aids.org/factSheets/101-what-is-aids.html>) (Accessed on 04.02.07).
- UNAIDS, (2006). The impact of aids on people and societies, Report on the global AIDS epidemic 2006. (http://www.unaids.org/en/HIV_data/2006GlobalReport/default.asp) (Accessed on 15.04.07).
- U.S DHHS (U.S Department of Health and Human Services) (2005). HIV infection and AIDS: an overview. National institute of allergy and infectious diseases, (<http://www.niaid.nih.gov/factsheets/hivinf.htm>) (Accessed on 03.10.06).
- Uslu, B. and Ozkan, S.A. (2002). Determination of lamivudine and zidovudine in binary mixtures using first derivative spectrophotometric, first derivative of the ratio-spectra and high-performance liquid chromatography–UV methods. *Anal. Chim. Acta*, **466**: 175–185.
- Verweij-van, W., Aarnoutse, R.E. and Burger, D.M. (2005). 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. *J. of Chromatography B*, **816** : 121–129.
- WHO (1990). Interim proposal for a WHO staging system for HIV infection and disease. *WHO Wkly Epidem. Rec.*, **65**: 221-228.
- Zhang, L., Deng, Y., Li, Y., Wu, H. and Xu, S. (2007). Investigation of the pharmacokinetics and determination of cholesteryl carbonate zidovudine in rat

plasma by non-aqueous reversed-phase high performance liquid chromatography with UV detection. *J. of Chromatography B*, **853**: 163–167.

Zheng, J.J., Wu, S.T. and Emm, T.A. (2001). High-performance liquid chromatographic assay for the determination of 2'-deoxy-3'-thiacytidine (lamivudine) in human plasma. *J. of Chromatography B*, **761**: 195–201.