HPTLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS DETERMINAITON OF LAMIVUDINE AND TENOFOVIR IN FIXED DOSE COMBINATION TABLET

BY MESFIN MOGES

A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES OF ADDIS ABABA UNIVERSITY, SCHOOL OF PHARMACY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN PHARMACEUTICAL ANALYSIS AND QUALITY ASSURANCE

Under supervision of

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June, 2009

ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

HPTLC method development and validation for simultaneous determination of lamivudine and tenofovir in fixed dose combination tablet

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DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

SCHOOL OF PHARMACY

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I am pleased to acknowledge that this work has been the result of an institutional effort involving Addis Ababa University for their financial aid and Ethiopian Drug Administration and Control Authority (DACA), Department of Drug Quality Control and Toxicology Laboratory for providing laboratory space and facilities throughout the whole work.

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I feel a deep sense of gratitude to my family to their support in my work and the whole my life

- To my mother (Almaz wolde) for her constant love, interests, support and encouragements.
- To my eldest brother (Tadesse Moges) for his invaluable and unforgettable support in my life whom I lost in this year.
- To Debebe Moges, Emnet Teshome and whole my family for their excellent suggestions, support and encouragements.

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Abstract

Lamivudine and tenofovir disoproxil fumarate are antiretroviral drugs available in a fixed dose combination tablet. They are nucleoside analogues and an increasingly important member of antiretroviral drugs.

HPTLC-densitometry method was developed and validated for simultaneous determination of antiretroviral drugs tenofovir disoproxil fumarate and lamivudine in fixed dose combinations. The method was based on HPTLC separation of the two drugs followed by densitometric measurements of their spots at 257 nm. Toluene-methanol (6:4, v/v) was used as mobile phase and HPTLC aluminum sheets of silica gel 60 F_{254} as stationary phase and detection of the spots were made by observation under short wavelength (254 nm) UV light. The system was found to give compact spot for lamivudine ($R_f = 0.32 \pm 0.02$) and tenofovir disoproxil fumarate ($R_f = 0.57 \pm 0.02$). The method was validated for precision, accuracy and robustness. The linear regression analysis data for the calibration plots showed good linear relationship with $r^2 = 0.998$ in the concentration range 100-900 ng/spot for tenofovir disoproxil fumarate and linear relationship with $r^2 = 0.994$ in the concentration range 200-800 ng/spot for lamivudine. The mean value of determination coefficient, slope and intercept were 0.998 ± 0.0012 and 0.994 ± 0.002, 4.80 ± 0.076 and 8.12 ± 0.21, 232.80 ± 36.21 and 478.16 ± 72.85 for tenofovir disoproxil fumarate and lamivudine, respectively. The intra-day and inter-day precision was less than 3% relative standard deviation (RSD <3%). The LOD and LOQ were 24.89 and 75.40 ng/spot for tenofovir disoproxil fumarate and 29.60 and 89.72 for lamivudine, respectively. The percentage assay of tenofovir disoproxil fumarate and lamivudine was found 98.63 ± 2.17 and 100.93 ± 1.32, respectively.

The described method has the advantage of being rapid, simple and inexpensive. No chromatographic interferences between the excipients and active ingredients were found. The method therefore could be applied for routine quality control analysis of lamivudine and tenofovir disoproxil fumarate in fixed dose combination tablet.

Key words: Lamivudine; Tenofovir Disoproxil Fumarate; HPTLC-Densitometry; Method validation

Acronyms

λ_{max}	Wavelength of maximum absorbance
2D	Two dimensional
3D	Three dimensional
3TC	Lamivudine
AIDS	Acquired Immunodeficiency Syndrome
AU	Absorption unit
ART	Antiretroviral therapy
BP	British Pharmacopeia
CATS	CAMAG TLC soft ware
C_{18}	Octyl silane
D_2	Deuterium lamp
DF	Disoproxil Fumarate
F_{254}	UV fluorsence under wave length 254 nm
FDA	Food and drug administration
FDC	Fixed dose combination
HAART	Highly active antiretroviral therapy
HIV	Human Immunodeficiency virus
HPLC	High performance liquid chromatography
HPLC-UV	High pressure liquid chromatography with ultraviolet detection
HPTLC	High performance thin layer chromatography
ICH	International Conference on Harmonization
IUPAC	International Union of Pure and Applied Chemistry
LCMS	Liquid chromatography mass spectrometry
LCMS-MS	Liquid chromatography tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantitation
NNRTI	Non nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
PI	Protease inhibitor
r^2	Determination coefficient

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R_f	Retention factor
RP-HPLC	Reversed phase high performance liquid chromatography
RSD	Relative standard deviation
SD	Standard Deviation
TDF	Tenofovir disoproxil fumarate
TLC	Thin layer chromatography
USP	United State Pharmacopeia
UV	Ultraviolet
UV-VIS	Ultraviolet and Visible

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List of Tables

List of Figures

Table of contents

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1. Introduction

Science responded to the challenge of AIDS by rapidly identifying aetiology, describing pathogenesis and transmission routes, and developing diagnostic tests and treatment. However, this did not prevent the global spread of HIV with 25 million fatal cases so far, another 33 million infected, and disastrous socioeconomic and demographic consequences, AIDS is now a pandemic disease (1). The estimated number of persons living with human immunodeficiency virus (HIV) worldwide in 2007 was 33.2 million, a reduction of 16% compared with the report estimate of 39.5 million people in 2006 (2). Every day, over 6800 persons become infected with HIV and over 5700 persons die from AIDS, mostly because of inadequate access to HIV prevention and treatment services. The HIV pandemic remains the most serious of infectious disease challenges to public health. Sub-Saharan Africa remains the most affected region in the world and two thirds of all people living with HIV live in this region. The estimated number of people with HIV/AIDS in Ethiopia was around one million in 2008 (3).

AIDS is the major epidemic that is caused by two variants of the HIV: HIV-1 and HIV-2. There are varieties of antiviral drugs which have proved successful in slowing down the disease, but not eradicating it (4). At present, most drugs that have been developed act on the viral enzymes reverse transcriptase and protease. However, a serious problem with treatment of HIV is the fact that virus undergoes mutation extremely easily and this results in rapid resistance to antiviral drugs. Experience has shown that treatment of HIV with a single drug has a short-term benefit, but in a long term the drug serves only to selected mutated viruses which are resistant. As a result,

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current therapy involves combination of different drugs acting on both reverse transcriptase and protease (4, 5).

Combination antiretroviral therapy (ART) is the most effective approach to manage HIV infections (4, 5). As HIV develops resistance rapidly, highly active antiretroviral therapy (HAART) is a combination of greater than three or equal to three drugs with grater efficacy than one drug penetrating the blood-brain barrier is essential to avoid resistance. Current treatment guidelines states that antiretroviral regimens should contain at least two nucleoside reverse transcriptase inhibitors (NRTIs) and either a non-nucleoside reverse transcriptase inhibitor (NNRTI) such as nevirapine, or a protease inhibitor. Fixed dose combinations of appropriate antiretroviral drugs improve adherence and efficacy and may reduce the development viral resistance (6).

The available drugs for highly active antiretroviral therapy (HAART) include:

Nucleoside reverse transcriptase inhibitors (NRTIs)– zidovudine, didanosine, zalcitabine, stavudine, lamivudine, amodoxovir, emitricitabine, and abacavir.

Non nucleoside reverse transcriptase inhibitors (NNRTIs)– nevirapine, delavirdine, efavirenz

Protease inhibitors (PIs)- saquinavir, ritonavir, inidnavir, nelfinavir, lopinavir, tipranavir, atazanavir, amprenavir and fosamprenavir (7).

Nucleoside reverse transcriptase inhibitors (NRTIs) were the first antiretroviral shown to be clinically effective against HIV infection and remain components of preferred treatment regimen to this day. Lamivudine is a novel member of this family, where as

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tenofovir is a new nucleoside analogue and an increasingly important member of the ARV drugs (6, 8).

1.1 Pharmacological and physicochemical properties of the drugs

1.1.1 Lamivudine

Lamivudine (Fig. 1) is a synthetic analog with activity against HIV (9) and it was initially developed for the treatment of HIV infection. Lamivudine (3TC) belongs to the class of dideoxynucleoside reverse transcriptase and has got a potent activity in *vitro* and *vivo*. Since monotherapy with NRTIs for treatment against HIV-1 results in rapid development of resistance of HIV strains, coadminstration of other ART is necessary. Treatment of HIV-1 infection with an antiviral regimen that includes 3TC is desirable since 3TC has been shown to be somewhat less toxic than other NRTIs (10). The US Department of Health and Human Services current guideline for the treatment of established HIV infection strongly recommends 3TC in combination with another NRTIs and either a protease inhibitor or efavirenz (11). It is a potent and selective inhibitor of type 1 and 2 human immunodeficiency virus. Although, generally lamivudine is less potent than zidovudine in inhibiting HIV-1 and HIV-2 replication in *vivo*, lamivudine has very low cellular cytotoxicity. It is rapidly absorbed with bioavailability of approximately 80% (9).

Lamivudine is a white to off-white crystalline solid with a solubility of approximately 70 mg/mL in water at 20 ^{o}C (12, 13). It has a molecular formula of $C_8H_{11}N_3O_3S$ and a molecular weight of 229.25. Its IUPAC name is (2R,cis)-4-amino-1-(2-hydroxyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidne-2-one or 4-amino-1-[(2R,5S)-2-(hydroxymethyl)-

1,3-oxathiolan-5-yl]pyrimidin-2-one. Lamivudine, 2'-deoxy-3'-thiacytidne, a novel dideoxy-nucleoside analog, has a potent activity against HIV-1 through inhibition of reverse transcriptase activity (10). Lamivudine the (-) enantiomer of 2'-deoxy-3'-thiacytidne, is a nucleoside analog in which the 3 carbon of the ribose of zalcitabine has been replaced by sulfur. The

(-) enantiomer of racemic mixture shows much less cytotoxicity than a positive enantiomer (9, 14). It exhibits polymorphism and can be obtained either as acicular crystals or as bipyramidal crystals. However, only bipyramidal crystals are appropriate to be used in the manufacture of tablets because they have adequate fluidity and are stable. Lamivudine has a pK_a of 4.3 and exists primarily in the unionized form when dissolved in distilled water. It is very stable to light and temperature in both the solid state and in aqueous solution. Moreover, it is soluble in water. Solid dosage forms for oral administration are widely prescribed in clinical practice because they are practical, stable, economical, and usually safe (15).

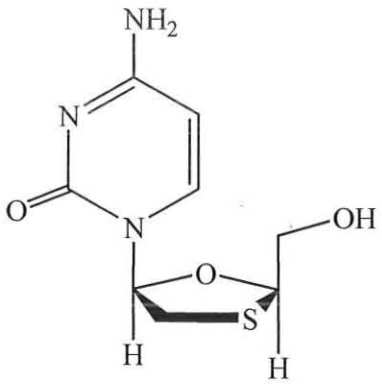

Fig. 1. The chemical structure of lamivudine

1.1.2 Tenofovir disoproxil fumarate

Tenofovir, marketed by Gilead Sciences under the trade name Viread®, belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors (NRTIs), which block reverse transcriptase, an enzyme crucial to viral production in HIV-infected people. It exhibits activity against HIV-1 reverse transcriptase. The dose of tenofovir with other antiretroviral drugs is available in the market for treatment of HIV infected patients. To improve its low bioavailability, a prodrug of tenofovir, tenofovir disoproxil fumarate (tenofovir DF or TDF) is used (16, 17). Tenofovir disoproxil fumarate (TDF) is an increasingly important member of this family of ART drugs. (18). TDF is indicated in combination with other ART drugs for the treatment of patients above 18 years of age infected with HIV who failed or are tolerant to nucleoside analog therapy or are not controlled by their current ART regimen (17). It has also been studied for the treatment of lamivudine-resistant hepatitis B virus (HBV) infection in patients who are coinfected with HIV and HBV. NRTIs were the first ART shown to be clinically effective against HIV infection and remain components of preferred treatment regimens to this day (19). The drug has an elimination half-life of 15 h and is metabolized intracelluraly to tenofovir diphosphate which is an inhibitor of HIV-1 reverse transcriptase.

Tenofovir (Fig. 2a) is white crystalline solid with melting point of 276-280 ^{o}C. The chemical formula of tenofovir is $C_9H_{14}N_5O_4P$ and the molecular weight is 287.21. The chemical name of tenofovir is [(2R)-1-(6-aminopurin-9-yl)propan-2-yl]oxymethylphosphonic acid. Tenofovir is an acyclic nucleotide analogue of deoxyadenosine 5'-monophosphate (19, 20). Tenofovir .disoproxil fumarate (tenofovir

DF) is the water-soluble diester prodrug of the active ingredient tenofovir (20). Tenofovir, {(2'R)-9-[2'-phosphoryl methoxy)-propyl] adenine} or 9-[(R)-2-(phosphonomethoxy)-propyl] adenine is a new nucleoside ART drugs used in many HIV-1 infection.

Tenofovir disoproxil fumarate (a prodrug of tenofovir) which is a fumaric acid salt of bis-isopropoxycarbonyloxymethyl ester derivative of tenofovir. In vivo tenofovir disoproxil fumarate is converted to tenofovir, an acyclic nucleoside phosphonate (nucleotide) analog of adenosine 5'-monophosphate. The chemical name of tenofovir disoproxil fumarate is 9-[(*R*) [[bis[[(isopropoxycarbonyl)oxy]methoxy]phosphinyl]methoxy]propyl]adenine fumarate (1:1). It has a molecular formula of $C_{19}H_{30}N_5O_{10}P \cdot C_4H_4O_4$ and a molecular weight of 635.52. Tenofovir disoproxil fumarate is a white to off-white crystalline powder with a solubility of 13.4 mg/mL in distilled water at 25 °C. It has an octanol/phosphate buffer (pH 6.5) partition coefficient (log p) of 1.25 at 25 °C. Specifically, tenofovir DF is a fumaric acid salt of a bis-isopropxycarbonyloxymethyl ester derivative of tenofovir (21). The synonyms tenofovir disoproxil fumarate is PMPA prodrug, tenofovir DF and TDF. Synonyms of TDF are D,L-Tenofovir, PMPA and tenofovir disoproxil.

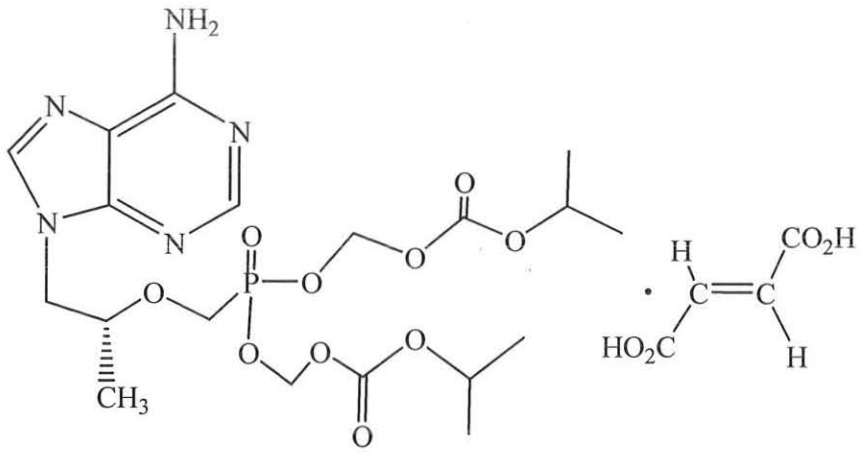

Fig. 2. The chemical structure of tenofovir disoproxil fumarate

1.3 Analytical determination of lamivudine and tenofovir disoproxil fumarate

1.3.1 Lamivudine

Several HPLC methods for determination of 3TC in biological fluids have been published (8, 10, and 15). These assays utilize a variety of techniques including plasma protein precipitation, solid phase extraction, and column switching. While each of these HPLC methods achieves suitable assay sensitivity, none utilize an internal standard (10). The other published report showed that the quanitiation of lamivudine which incorporated the use of 3-isobutyl-methylxanthine as internal standard using HPLC with UV absorbance detection (10). An HPLC assay with UV detection for the quantification of this compound has been reported in human urine and serum. A radioimmunoassay for the quantification of lamivudine in sub-

nanogram per milliliter concentration has been described, which may be of use for the determination of intracellular phosphorylated lamivudine (8). It has been also determined in human plasma, saliva, and cerebrospinal fluid using HPLC-UV detection (8).

Lamivudine has been determined with other ART drugs in blood plasma. Zidovudine (AZT)/lamivudine (3TC)/nevirapine were determined simultaneously using ion-pair HPLC UV detection at 265 nm in human plasma. Plasma samples were treated using a solid-phase extraction procedure. Aprobarbital was chosen as the internal standard (22). Reversed-phase high-performance liquid chromatography assay for the 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 were reported. The new polarity DC C_{18} silica column four different UV wavelengths were used (5). Lamivudine were simultaneously determined with NRTIs, NNRTIs and PIs totally eleven drugs in human plasma with solid–liquid extraction procedure without internal standard coupled with two separate reversed-phase HPLC systems (23). Two methods by reversed-phase liquid chromatography were developed for the analysis of 19 antiretroviral molecules belonging to these three therapeutic classes in human plasma. Both of these HPLC techniques use a C_{18} column and UV detection. The first method is for NRTIs family analysis and allows eight molecules to be separated: zalcitabine, lamivudine, amdoxovir, emtricitabine, didanosine, stavudine, zidovudine and abacavir. The second method is for NNRTIs and IP family analysis and allows 11 molecules to be separated: fosamprenavir, nevirapine,

indinavir, amprenavir, saquinavir, atazanavir, ritonavir, lopinavir, efavirenz, nelfinavir and tipranavir (24).

Three methods have been reported for determination of lamivudine and zidovudine in binary mixtures using first derivative spectrophotometric, first derivative of the ratio spectra and HPLC-UV detection. The described methods can be readily utilized for analysis of pharmaceutical formulation without the necessity of sample pretreatment (9). Lamivudine with stavudine and nevirapine has been simultaneously determined by UV spectroscopy, reverse phase HPLC and HPTLC in tablets (25). In the UV multi-component spectral method, stavudine, lamivudine and nevirapine were quantified at 266, 271 and 315 nm, respectively. In the RP-HPLC method, the drugs were resolved using a mobile phase of 20 mM sodium phosphate buffer (containing 8 mM 1-octanesulphonicacid sodium salt): acetonitrile (4:1, v/v) with pH adjusted to 3.5 using phosphoric acid on a C18-ODS-Hypersil (5 μm, 250 mm × 4.6 mm) column in isocratic mode. The retention time of stavudine, lamivudine and nevirapine was 2.85, 4.33 and 8.39 min, respectively. In the HPTLC method, the chromatograms were developed using a mobile phase of chloroform: methanol (9:1, v/v) on precoated plate of silica gel 60 F_{254} and quantified by densitometric absorbance mode at 265 nm. The R_f of stavudine, lamivudine and nevirapine were 0.21–0.27, 0.62–0.72 and 0.82–0.93, respectively (25). The same combinations of these drugs were determined by UV spectrophotometric and RP-HPLC in pharmaceutical dosage forms. The UV spectrophotometric determinations were performed at 270, 265 and 313 nm for lamivudine, stavudine and nevirapine (14). Lamivudine is not official in BP or USP.

1.3.2 Tenofovir disoproxil fumarate

Very few methods appeared in literatures for the determination of TDF in biological fluids using HPLC-UV, HPLC-tandom mass spectrophotometric and HPLC with spectrofluorimetirc detection, because it is a newly HIV reverse transcriptase inhibitor approved by FDA in 2001 (16, 17, and 18). HPLC method with spectrofluorimetirc detection was also reported for the determination of tenofovir in human plasma. The detection was performed at excitation and emission wavelength set at 236 and 420 nm (16). Sensitive determination of tenofovir in human plasma samples have been reported using reversed-phase liquid chromatography using UV detection (17). The other reported method for determination of tenofovir diphosphate (tenofovir-DP) in human peripheral blood mono nuclear cells describes an indirect methodology for the intracellular quantification of tenofovir-DP that describes more fully the inherent challenges and necessary validation steps associated with this type of intracellular LCMS-MS quantitiaiton (18). Literature survey revealed, few analytical methods which include liquid chromatography with tandem mass spectrometry (18) and simultaneous quantification of emtricitabine and tenofovir in human plasma using high performance liquid chromatography after solid phase extraction (5). Tenofovir DF is not official in BP or USP. However, there is no reported method for simultaneous determination of tenofovir disoproxil fumarate and lamivudine using HPLC or HPTLC in blood plasma or pharmaceutical formulations.

1.4. HPTLC densitometry in drug analysis

Thin layer chromatography (TLC) is a liquid-solid adsorption technique where the mobile phase ascends the thin layer of stationary phase coated unto a backing support

such as glass or aluminum sheet, where as HPTLC is an analytical technique based on TLC, but with enhancements intended to increase the resolution of the compounds to be separated and to allow quantitative analysis of the compounds. Some of the enhancements include: more accurate sample loading, use of a densitometer and computer analysis to determine the size, intensity and R_f of the separated compounds, use of higher quality TLC plates with finer particle sizes in the resolution (26, 27). HPTLC can also be coupled with such techniques as HPLC, gas chromatography or mass spectrometry to increase the analytical power available.

Nowadays, HPTLC is emerging as an important tool (28) and becoming a routine analytical technique due to its advantages of reliability in quantitation of analytes at micro and even in nanogram levels and cost effectiveness (29). The major advantage of HPTLC is that several samples can be analyzed simultaneously using a small quantity of mobile phase unlike HPLC. This reduces the time and cost of analysis and possibilities of pollution of the environment. HPTLC also facilitates repeated detection (scanning) of the chromatogram with same or different parameters. Simultaneous assay of several components in a multi-component formulation is possible. HPTLC is powerful analytical technique, especially useful to analysis of plant material because large number of samples can be chromatographed simultaneously and the samples without any pretreatment can be applied (28) and especially for herbal drug preparations, being multi-component systems, HPTLC seems to have some advantages over HPLC: the sample preparation is simple, the detection by dipping reagents enables specific color reactions and the consumption of organic solvents as well as the analysis time is lower (29). HPTLC densitometry is becoming a powerful analytical technique for evaluation of herbal drugs on phytochemical base and for the analysis of different types of drugs (28-32).

TLC is still largely regarded as a simple and quick technique. Planar chromatography (TLC and HPTLC) is affected by many factors. Although one major advantage of the technique, its enormous flexibility, is based on this, the same flexibility easily can become a major obstacle to obtaining reproducible qualitative and quantitative results. Most pharmacopoeias and other official or semi-official collections of analytical methods include a general description of TLC that reflects the state of the art in the late sixties. Even though the use of high-performance plate material and instrumentation is mentioned and permitted as an option, HPTLC as a synonym for modern thin-layer chromatography is not specifically endorsed (33).

The advantage of TLC is that the mobile phase is evaporated before detection, it does not interfere with the determination of the position of solute spots and this will increase the choice of solvents (27). HPTLC has advantage over HPLC due to its flexibility, application, cost, ease of use, simplicity, validation and multiple scans. It facilitates automatic application and scanning in situ (33-36). Although the initial investment is high for HPTLC, the running cost is lower than HPLC (29).

2. Objectives

2.1. General objective

To develop, optimize and validate an analytical method for qualitative and quantitative determination of fixed dose combination tablets of lamivudine and tenofovir disoproxil fumarate by HPTLC with densitometric measurement.

2.2 Specific objectives

2.2.1. To develop simple, rapid, cost effective and selective analytical method for simultaneous determination of fixed dose combination tablets of lamivudine and tenofovir disoproxil fumarate.

2.2.2. To determine analytical figures of merit: limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy, precision, and specificity, robustness time per analysis and to optimize sample preparation techniques.

2.2.3. To compare the analytical figures of merit of the proposed method with that of reported methods if any in literature.

3. Experimental

3.1 Materials

3.1.1 Chemicals

A. Working standards and drug products

Lamivudine USP standard (Batch number; MLN/WS08/018, validity; 10/04/09, purity; 99.7 %) was imported from Matrix Laboratories limited, India.

Tenofovir disoproxil fumarate standard (Batch number; MLN/WS08/022, validity; 15/06/09, purity; 98 %) was imported from Matrix Laboratories limited, India.

Tenofovir disoproxil fumarate/lamivudine tablets 300mg/300mg (Batch number; 1001519, manufacturing date; September, 2007, expiry date; August, 2009, manufactured by Matrix Laboratories limited, India).

B. Solvents and reagents

Analytical grade: acetone (Fisher, England), acetonitrile (Fisher, England), chloroform (BDH, England), glacial acetic acid (Fulka, Germany), hexane (BDH, England), methanol (Fisher, England), toluene (Fisher, England) and distilled water obtained from Drug Quality Control and Toxicology Laboratory Department, Drug Administration and Control Authority.

3.1.2 Instrumentation, equipments and glass wares

Micro syringe (Linomat syringe 659.004, Hamilton-Bonaduz Schweiz, Camag, Switzerland), precoated silica gel 60 F_{254} aluminum HPTLC plate (20 x 20 cm with

200 µm thickness; Merck, Germany), linomat 5 applicator (Camag, Muttenz, Switzerland), twin trough chamber 20 x 20 cm (Camag, Muttenz, Switzerland), saturation pad (Camag, Muttenz, Switzerland), hair dryer (Philip lady 1000, Type HP 4312, Hong Kong), UV chamber (Camag, Muttenz, Switzerland), TLC scanner III (Camag, Muttenz, Switzerland), thermolyne TLC heater (Iowa, USA), digital camera (8 Megapixel, Pentax, Vietnam), winCATS version 1.4.0 software (Camag, Muttenz, Switzerland), Chemoffice 2005 and Microsoft office excel 2003 were used in the study.

Volumetric flasks of 100 (± 0.2 ml), 50 (± 0.06 ml), 25 (± 0.04 ml), 10 (± 0.02 ml); pipettes of 1 (± 0.02 ml), 2 (± 0.02 ml), 5 (± 0.05 ml), mortar and pestle; sonicator (Bandelin Sonorex Super, Model RK 514, India); analytical balance (electronic microbalance, model AAA 250L, Adams co., U.K.); 0.45 µm nylon membrane filter (Whatman International, Maidstone, England) were used during the study.

3.2 Methods

3.2.1 Preparation of stock standard solution, binary mixture and sample solutions

A. Preparation of stock standard solution

A stock standard solution of (1 mg/ml) of lamivudine and tenofovir disoproxil fumarate was prepared by weighing 25 mg of the working standard and dissolving in 25 ml of methanol and sonicated for about 5 minutes. Different working solutions were prepared during method development and validation process.

B. Preparation of binary mixtures of standard lamivudine and tenofovir disoproxil Fumarate

A mixture of lamivudine and tenofovir disoproxil fumarate working standard solution containing 100 µg/ml of each drug was prepared by mixing 1 ml of both stock solutions and dilution of the resulting mixture to 10 ml using methanol. The binary mixture working solution was freshly prepared before use.

C. Preparation of sample solutions

Twenty tablets were weighed and finely powdered using mortar and pestle. A quantity of the powder equivalent to 50 mg of lamivudine or 50 mg tenofovir disoproxil fumarate respectively was weighed and transferred to 50 ml volumetric flask. About thirty milliliter of methanol was added to the volumetric flask and was sonicated for about 15 minutes. Volume was adjusted to the mark using the above solvent and mixed by shaking. To remove undissolved excipients each solutions was filtered through a 0.45 µm filter paper. After discarding the insoluble residues, 1 ml of the filtrate transferred into a 10 ml volumetric flask and was further diluted to 10 ml using methanol then mixed and used for determinations.

3.2.2 HPTLC instrumentation and chromatographic conditions

A. Stationary phase

In this study, HPTLC aluminum plates 10 cm × 10 cm or 10 cm × 20 cm with 200 µm thickness coated with silica gel were used.

B. Mobile phase

Developing solvents are prepared by measuring the required volume of each component separately and transferring into a solvent graduated volumetric pipette of appropriate size. After testing many mobile phase compositions were tested to optimize and select the most selective solvent, toluene-methanol was used as mobile phase for lamivudine and tenofovir disoproxil fumarate for the standard and sample solutions because of the most suitable R_f value for the target compound and the smoothest base line for quantification.

Twelve milliliters of toluene and 8 ml of methanol was measured separately using 10 and 20 ml of graduated volumetric pipette, transferred to 20 ml volumetric flask and closed with a lid and shaken properly to ensure proper mixing.

C. Sample application

For spotting, the sample should be dissolved in the solvent of lowest suitable polarity to minimize the effects of spot broadening during application. To avoid volume errors, highly volatile solvents should not be used either to prepare or apply standards and samples for quantitative analysis (26). Methanol was used to dissolve both the standards and tablet forms. HPTLC-Camag system was used for quantitation purposes because of its accuracy (up to a nanoliter) and the reproducibility of the autosampler. Automated TLC sampler III® devices take into account defined parameters such as the volume, size of the sprayed band and accurate positioning on the chromatography plate (41). It should be noted that an important advantage of

using an automated sample applicator is that variable volumes of a standard solution and the sample can be applied; to obtain equalized initial zones that lead to a precise densitometric determination (42*)*. These parameters were computerized by winCATS version 1.4.0 software.

Different volumes of standard and sample solutions (0.5 µl to 10 µl) were applied on the plate to obtain concentration ranges of 100 to 1000 ng/spot. Solutions were streaked to give a 4 mm band; 10 mm form the bottom, 15 mm from the side edges of the plate, 7.7 mm apart. Solutions were applied at constant rate of 200 nl/s using nitrogen aspiration. Appropriate concentrations of sample expressed in nanogram per spots were applied from the same solution by varying the application volume using microsyringes. The microsyringe was cleaned by drawing up methanol with plunger and displacing it. After application, the plates were dried using a steam of cold air.

D. Saturation and development

Filter paper with 20 cm × 10 cm was placed in 20 x 20 cm TTC rear trough and the mobile phase was poured into the chamber so the filter paper is thoroughly wetted and adheres to rear wall of the TTC. The chamber was tilted to the side so the solvent volume in both troughs equalizes and chamber was equilibrated for 20 min. The solvent front was marked 80 mm from the lower edge of the plate which is equivalent to 70 mm from the point of application and the plates were developed until the solvent front. The plates were inserted into the front trough of TTC and the layer faced the filter paper and the back of the plate rested against front wall of the TTC. Plates were developed to the mark within 17 minutes. The plate was dried (vertically in the

direction of chromatography for 5 min in a stream of cold air. After each development remaining mobile phase and the filter paper were discarded.

E. Detection of the spots

Spots identification of the standards and the samples were determined by exposing the plate under UV light at 254 nm because the spots were observed in the wavelength and the R_f values of the drugs were determined by taking the ratio of the distance travelled by the drug to the distance travelled by the solvent (solvent front).

F. Densitometric measurement of the chromatograms

Densitometric scanning was performed on Camag TLC scanner III in the reflectance-absorbance mode at 257 nm for all measurements. Data processing was performed by winCATS version 1.4.0 software. The source of radiation utilized was deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm. Concentrations of the compounds chromatographed were determined from the intensity of diffusely reflected light (43). Evaluation was via peak areas with linear regression. Densitometric scanning was performed with a TLC scanner III with the silt dimension settings of 5.00 mm x 0.45 mm, data resolution of 100 µm/step, filter factor of Savitsky – Golay 7 with spectral scan speed of 100 nm/sec and data resolution of 10 nm/step and a scanning rate of 20 mm/s. The maximum absorption wavelength was determined by the measurement of the *in-situ* UV absorption spectrum of a zone. The plate was scanned in the absorbance-reflection mode and the absorption spectra were recorded between 200 and 400 nm; maximum responses were obtained at 257 nm for TDF and at 271 nm for lamivuidne. Though the best sensitivity was obtained in maximum absorption (42), the compromise was made so that both drugs may be

detected and use single wavelength (41). Concentration profiles of the development tracks for 3TC and TDF were recorded under UV light using D_2 lamp in absorbance mode with reflectance type at 257 nm simultaneously.

3.3. Analytical Method Validation

The analytical method was validated according to ICH and IUPAC guidelines for all parameters (36, 38).

3.3.1. Selectivity

Selectivity refers to the extent to which the method can be used to determine particular analyte in mixtures or matrices without interferences from other components of similar behavior (44). The selectivity of the assay was determined in relation to interferences from formulation ingredients and was evaluated by base line resolution of the chromatogram of pure drugs and the sample solution (41).

3.3.2. Linearity

Linearity of HPTLC method was obtained by determining the detector responses against a series of varying concentrations of standards of both 3TC and TDF. It should be noted that an important advantage of using an automated sample applicator is that variable volumes of a standard solution and the sample can be applied on the same plate; obtaining equalized initial zones that lead to an accurate and precise densitometric determination (42). Variable volumes from 0.5 µl to 10 µl were applied

using automated sample applicator to obtain concentration range of 100 ng/spot to 1000 ng/spot for TDF and 200-800 ng/spot for 3TC. Five determinations or analyses per concentration were conducted and calibration plots were constructed. The linear relationship between peak area of the spots and concentration of the drugs were evaluated over the range of concentrations expressed in ng/spot. For 3TC, the concentration ranges of 200-800 ng/spot with seven concentration levels were used. For TDF, the concentration of 100-1000 ng/spot with nine concentration levels was used. The calibration curves were constructed by plotting the peak area (y-axis) against the concentration of the drug in ng/spot (x-axis). The linear regression equation, correlation coefficients, y-intercepts were determined from above determinations. According to International Commission for Harmonization, ICH, tests the minimum specified range was 80-120% of the target concentration.

3.3.3. Precision

In accordance with ICH Guidelines (ICH Q2A and Q2B), precision includes three components: repeatability, intermediate precision and reproducibility. In the current study, reproducibility was not studied. The precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day) with three different days and was reported as % R.S.D. The repeatability was determined with three determinations with in three different concentrations (400 ng/spot, 500 ng/spot and 600 ng/spot) for both 3TC and TDF and intermediate precision was determined with the same concentrations as above with three different days for both 3TC and TDF. The intermediate precision was studied by comparing the assays on 3 different days and the results documented as standard deviation and % R.S.D.

3.3.4. Sensitivity

The sensitivity of the method was determined with respect to LOD and LOQ (31). Limit of detection (LOD) and limit of quantification (LOQ) of the method were calculated using the formula LOD = 3.3σ/S and LOQ = $10\sigma/S$, where σ is the standard deviation of blank determination and can be expressed by the y-intercepts of the regression lines and S is the sensitivity parameter and can be expressed by the slope of the standard calibration curve. The standard solutions were spotted from 100 to 900 ng/spot for TDF and 200 to 800 ng/spot for 3TC (n = 5). The standard deviation of the y-intercepts and the slope were calculated from regression lines of the calibration curve.

3.3.5. Accuracy

Accuracy was determined by the percent of analyte recovered by assay from a known added amount. Data from nine determinations over three concentration levels covering the specified range was determined. The recovery study was carried out by applying the method to drug sample to which known amount of lamivudine and tenofovir disproxil fumarate corresponding to 80, 100 and 120% of the calibration curve (standard addition method). At each level of the amount three determinations were performed and the results obtained were compared with expected results and the recovery was calculated by comparing the resultant peak areas with those obtained from pure standards in methanol at the same concentrations (35).

3.3.6. Robustness

The evaluation of robustness of the method will be considered during the development phase and depends on the type of procedure under study. If the measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled and precautionary statement should be included in the procedure according ICH guidelines. In the robustness study, the composition of mobile phase, the amount of mobile phase, time from spotting to chromatography and from chromatography to scanning was considered. By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different composition like toluene–methanol (6.5:3.5, v/v), toluene–methanol (5.5:4.5, v/v) were tried and chromatograms were run. The amount of mobile phase was varied from 20 ml to 15 ml. Time from spotting to chromatography and from chromatography to scanning was varied from 0, 20, 40 and 60 min. Robustness of the method was done at three different concentration levels 400, 500, 600 ng/spot for both lamivudine and tenofovir disoproxil fumarate, respectively (43). Robustness was determined with comparing the R_f values and the variation of peak area as % R.S.D

3.4 Analysis of marketed formulation

The developed method was applied for determination of lamivudine and tenofovir disoproxil fumarate, which is marketed as oral solid dosage form. The tablets contain 300 mg/300 mg tenofovir disoproxil fumarate/lamivudine was the only available marketed formulation manufactured by Matrix Laboratories limited and this formulation was analyzed using the proposed method. The standard and sample solutions prepared as described in section 3.2.1 were used for the analysis. Five

microliters of the sample solution having 500 ng/spot on duplicate and six standard solutions were applied simultaneously on the same HPTLC plate followed by development and scanned as described in section 3.2.3. Six standard solutions from 200 ng/spot to 700 ng/spot were applied on the same plate with the sample solution to construct the calibration curve, so that the samples can be quantified against standard calibration curve using winCATS version 1.4.0 software. The analysis was repeated in triplicate.

4. Results and discussion

4.1. Method development and optimization

Method development, optimization, and validation must be considered as an integrated process. A very important quality criterion of an analytical method is its capability to deliver signals that are free from interferences and give "true results" (44). Method development starts with suitable sample preparation. In HPTLC, chromatographic plates are disposable. Matrix does not have to be eluted as in column chromatography. This may simplify the separation. A selective solvent for the extraction of target compounds may be desirable. Different extraction solvents and extraction methods or times can be compared on the same HPTLC plate (26).

4.1.1 Stationary phase selection

Silica gel is a good starting point as a stationary phase because it can separate different compounds (26). Silica gel as stationary phase is most widely used because it can separate many substance classes based on type and number of functional groups. As both 3TC and TDF are weak polar compounds, polar stationary phase is logical choice. Silica gel stationary phase (TLC and HPTLC) aluminum plates were used by considering the chemical nature of the drugs and the results on this stationary confirmed that the silica gel F_{254} is the appropriate choice for tenofovir disoproxil fumarate and lamivudine determination in standard drugs and tablets.

4.1.2. Mobile phase selection and optimization

Whenever possible, it is more reliable to dissolve the sample in the mobile phase. The desired mobile phase would provide the greatest solubility, while providing affinity for the stationary phase. Both the working standards and tablets were dissolved in methanol and methanol was used as one of the component comprising the mobile phase. Solutions prepared as described in section 3.2.1 were used in the method development and optimization process. The selection of mobile phases was based on the nature of the compounds, solvent elution strength order, boiling point, viscosity and toxicity. Solvents with low boiling points, low viscosity and low toxicity are preferable for TLC application (27). Mobile phases comprising more than one component were tried in the method development process. Different solvent mixtures were tried using normal TLC and HPTLC. The composition of the mobile phase for development of chromatographic method was optimized by testing different solvent mixtures of varying polarity. Various mobile phases were evaluated (Table 1).

Table 1. R_f values of lamivudine and tenofovir disoproxil fumarate in different mobile phases using normal TLC

S. No	Composition (v/v)	Proportion	R_f of 3TC	R_f of TDF
1	Methanol	100	0.69	0.75
2	Toluene-methanol	3:7	0.64	0.73
3	Toluene-methanol	6:4	0.44	0.61
4	Toluene-methanol	7:3	0.3	0.43
5	Chloroform-methanol	8:2	0.75	0.72

Table 2. R_f values of lamivudine and tenofovir disoproxil fumarate in different mobile phases using HPTLC plate

S. No	Composition (v/v)	Proportion	R_f of 3TC	R_f of TDF
1	Toluene-methanol	5.5:4.5	0. 38	0.59
2	Toluene-methanol	6:4	0.32	0.57
3	Toluene-methanol	6.5:3.5	0.30	0.56

The normal TLC plates were used for initial mobile phase selection and optimization process, because it was less expensive and easily available than HPTLC. The R_f values were summarized as in Table 1. Use of methanol as single component and short saturation time of 30 min give necklace effect. The spots were dense, compacted but there was no separation and the R_f value was near to the solvent front for both drugs. Methanol as mobile phase was used as starting point, because it was used as solvent to dissolve both the standard drugs and tablets dosage forms. The spots were dense and compacted but there was no resolution and R_f value was also near to the solvent front with toluene-methanol (7:3, v/v) as a TLC solvent system. Mobile phase comprising toluene-methanol with (5:5, v/v) showed good improvement in resolution. The TLC spots with toluene-methanol (6:4 v/v) showed dense and compacted having good resolution. Chloroform-methanol with different compositions {(8:2, v/v), (5:5, v/v), (7:3, v/v)} were tried, but there was overlapping of spots and the R_f value was near to solvent front.

After using normal TLC plate for mobile phase selection and optimization, the optimized parameters such as saturation time and solvent combinations and their R_f values were further optimized using HPTLC plate. The R_f values were summarized as under Table 2. Toluene-methanol using different compositions {(5.5:4.5, v/v), (6:4, v/v), (6.5:3.5) when used as mobile phase using HPTLC plate. The best result was obtained using toluene-methanol (6:4, v/v).

Mobile phase consisting of toluene-methanol (6:4 v/v) gave dense, compacted spots with good resolution and R_f values of 0.32 ± 0.02 and 0.57 ± 0.02 for lamivudine and tenofovir disoproxil fumarate, respectively as it is shown in the Fig. 3 and also gave sharp, symmetrical in nature and well resolved peaks for lamivudine and tenofovir disoproxil fumarate without tailing when HPTLC plates were scanned at 257 nm for both standard drugs and tablet dosage forms, respectively (Fig. 4 and Fig. 5). The chromatograms of the standard drugs and sample solutions were dense and compacted for lamivudine and tenofovir disoproxil fumarate, respectively as it is shown in the Fig. 6 and Fig. 7. Because of the above merits, toluene-methanol (6:4 v/v) was chosen as ultimate mobile phase for subsequent study, since methanol was used as solvent, chemical inertness, detector compatibility, reasonable price of methanol and toluene made them preferable.

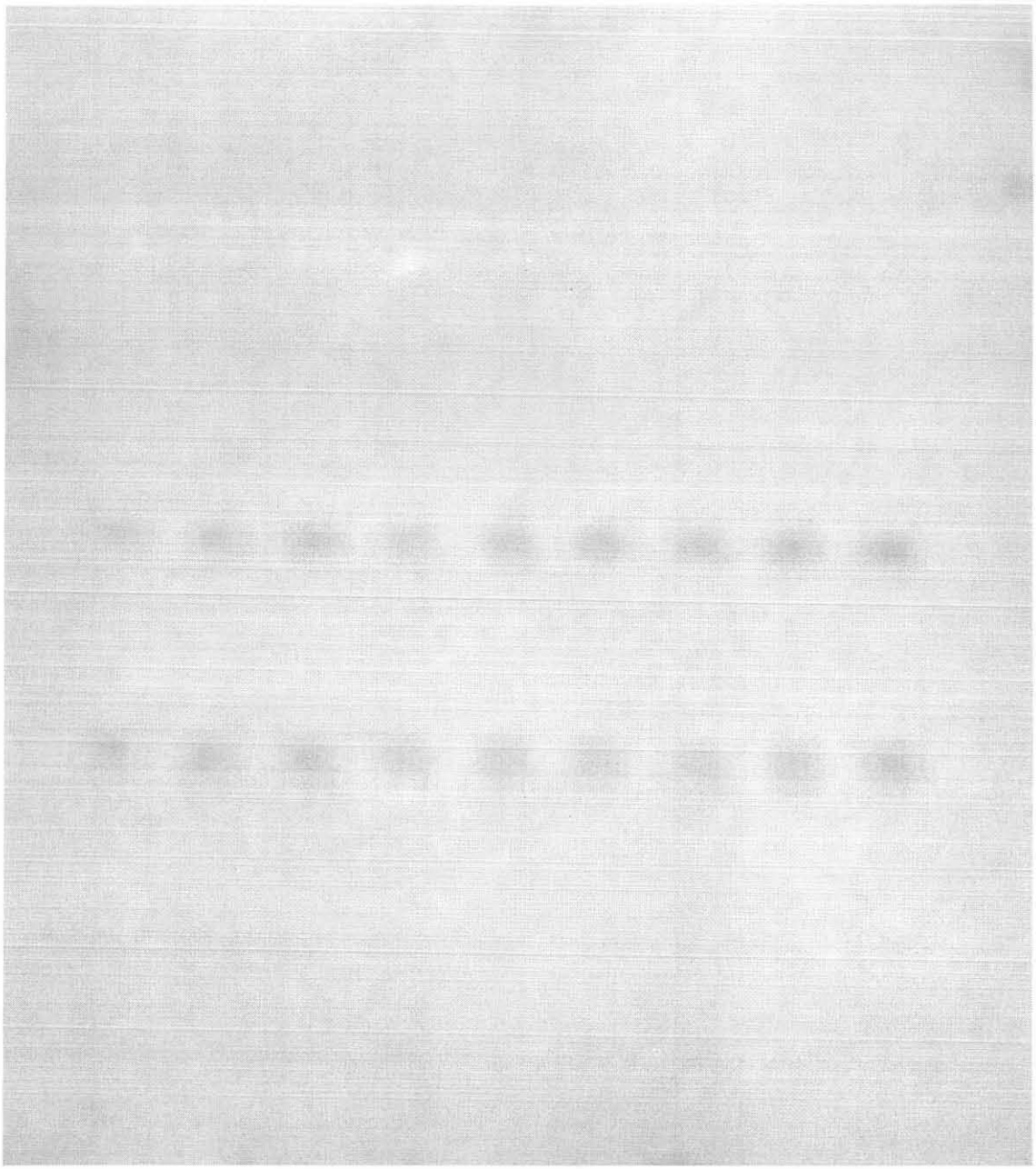

Fig. 3. Spots of standard drugs and sample solution on HPTLC plate. Mobile phase toluene-methanol (6:4, v/v) documentation UV light at 254 nm using digital camera.

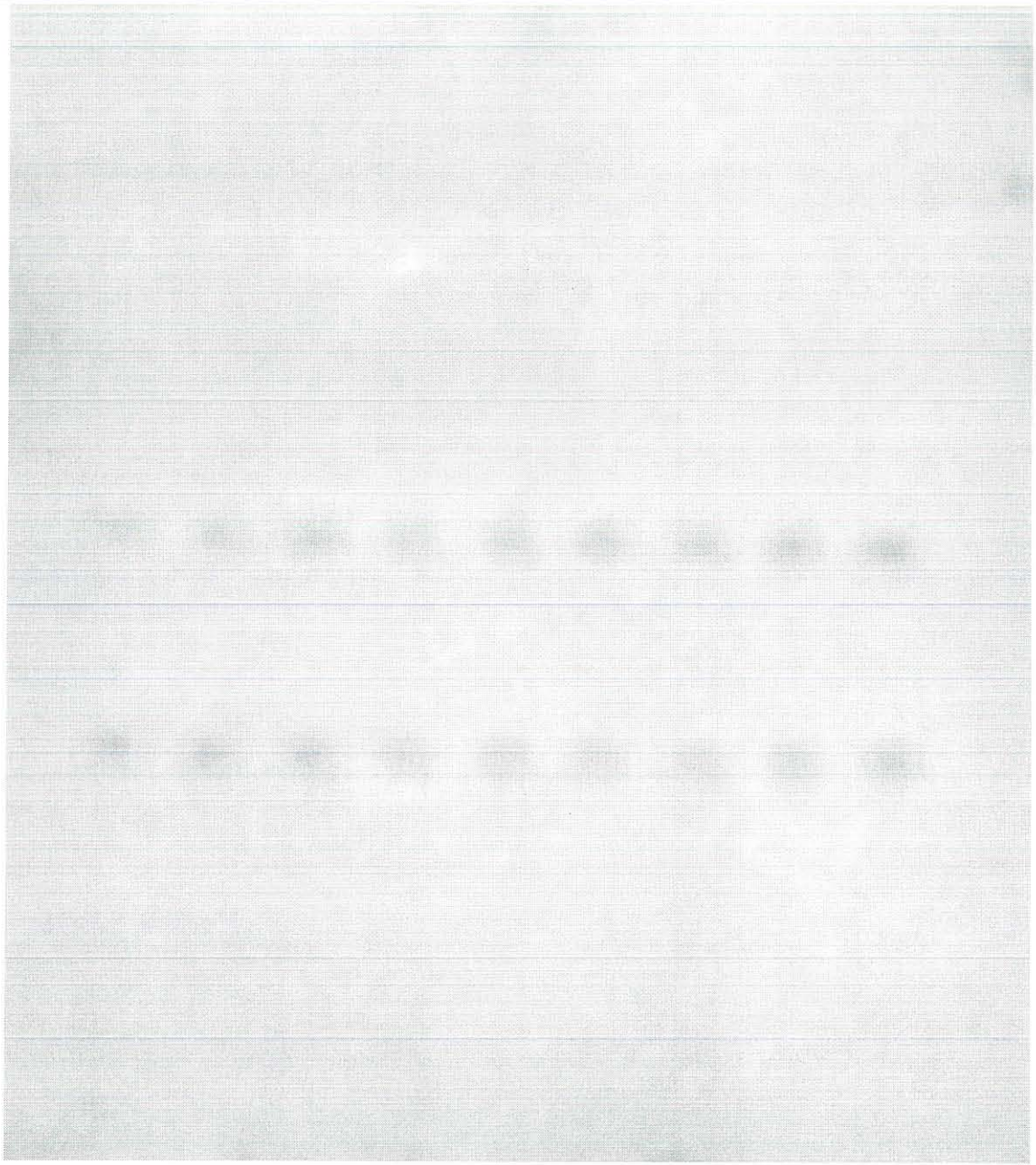

Fig. 3. Spots of standard drugs and sample solution on HPTLC plate. Mobile phase toluene-methanol (6:4, v/v) documentation UV light at 254 nm using digital camera.

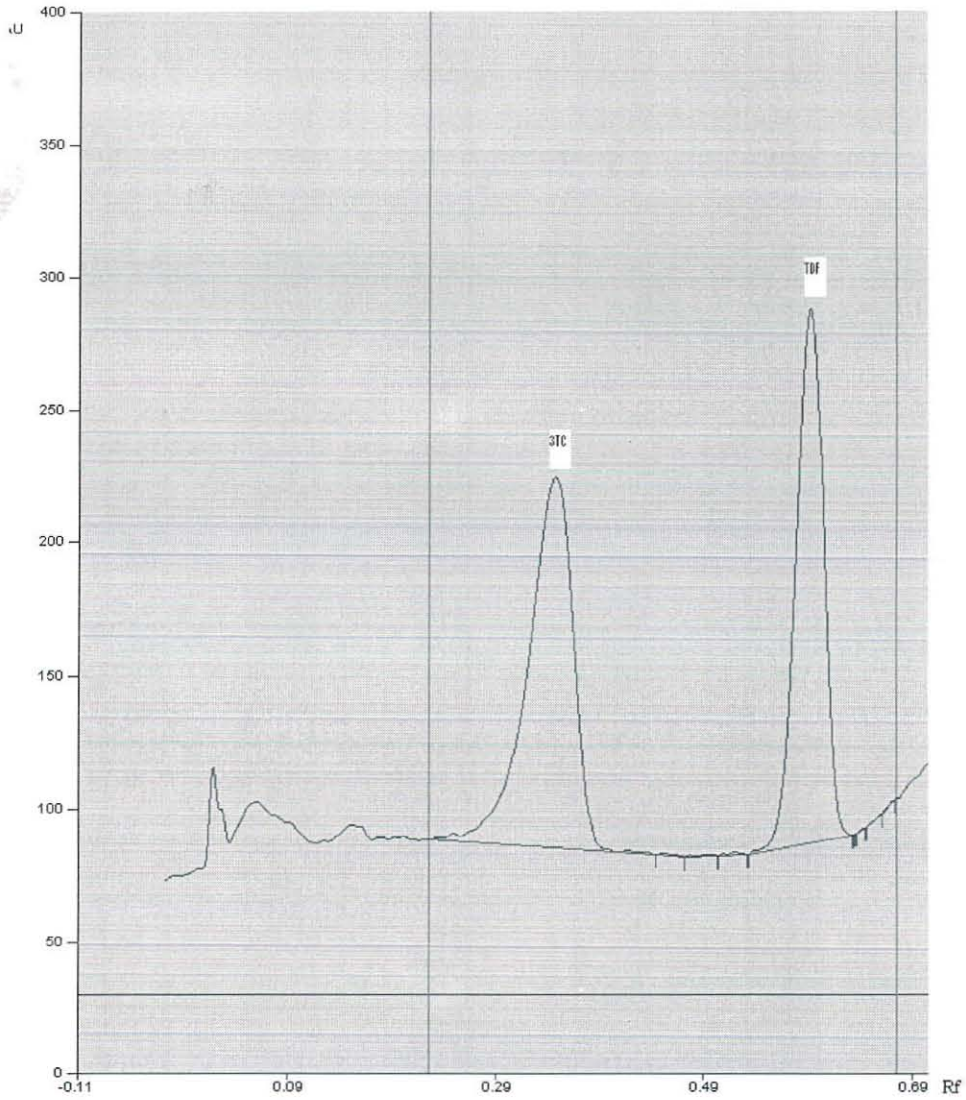

Fig. 4. Densitogram peaks of standard solutions of 3TC (500 ng/spot); (R_f: 0.32 ± 0.02) and TDF (500 ng/spot); (R_f: 0.57 ± 0.02), measured at 257 nm, the mobile phase, toluene–methanol (6:4, v/v).

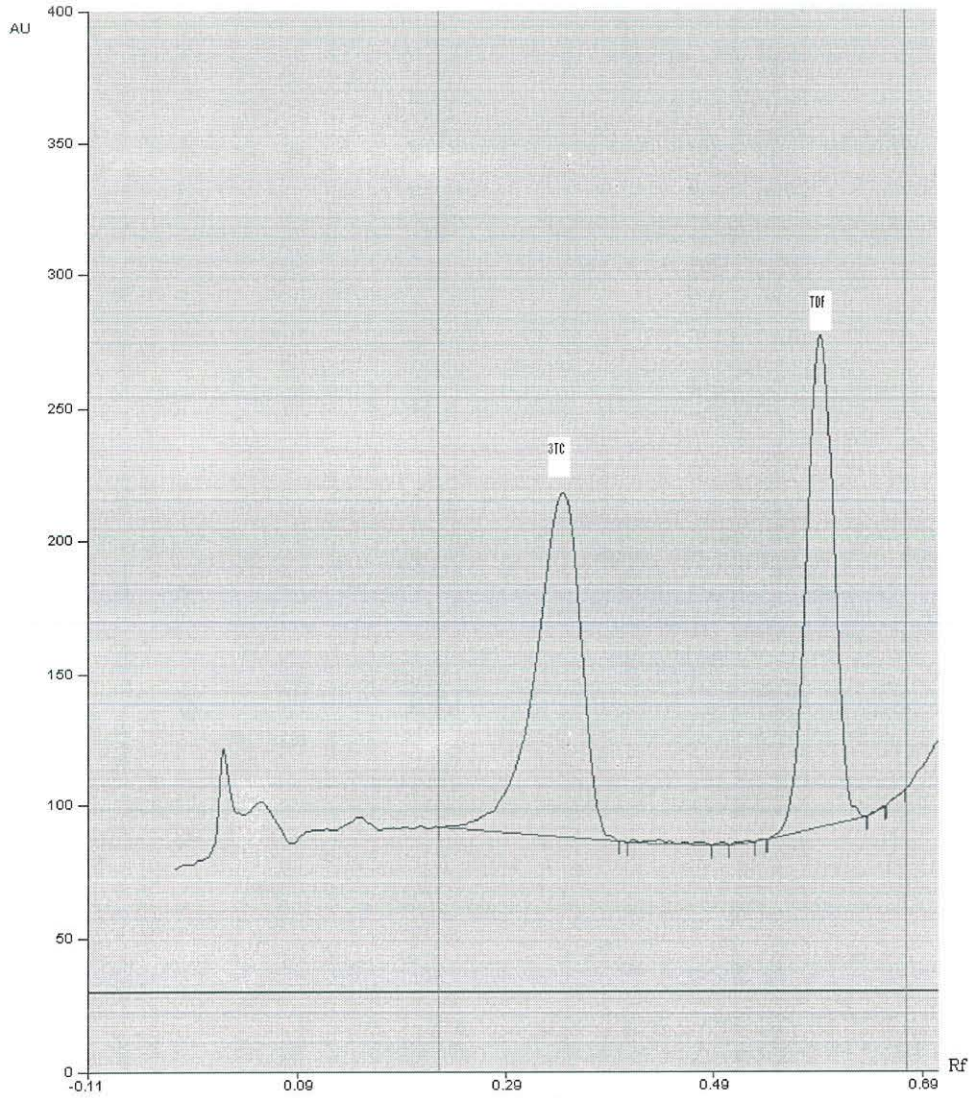

Fig. 5. Densitogram peaks of sample solutions of 3TC (500 ng/spot); (R_f: 0.32 ± 0.02) and TDF (500 ng/spot); (R_f: 0.57 ± 0.02), measured at 257 nm, the mobile phase, toluene–methanol (6:4, v/v).

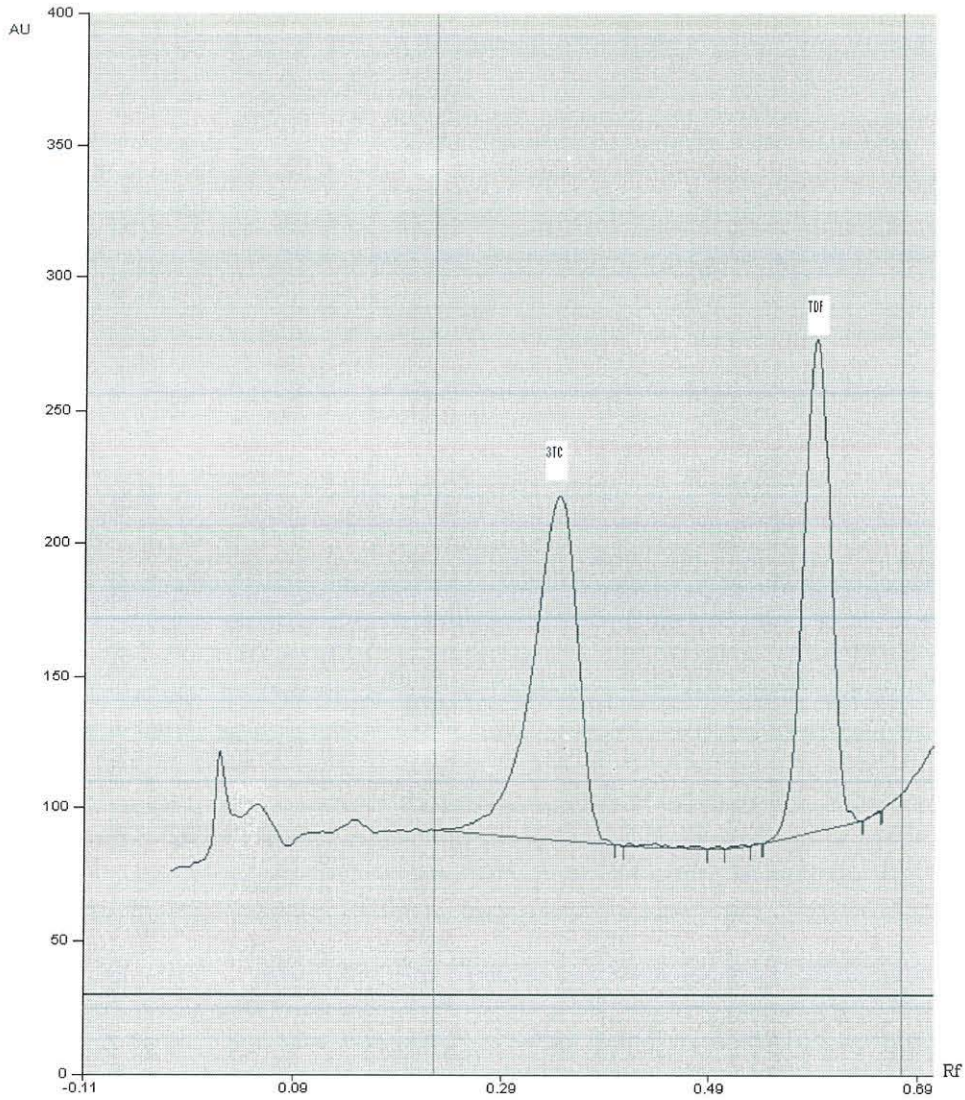

Fig. 5. Densitogram peaks of sample solutions of 3TC (500 ng/spot); (R_f: 0.32 ± 0.02) and TDF (500 ng/spot); (R_f: 0.57 ± 0.02), measured at 257 nm, the mobile phase, toluene–methanol (6:4, v/v).

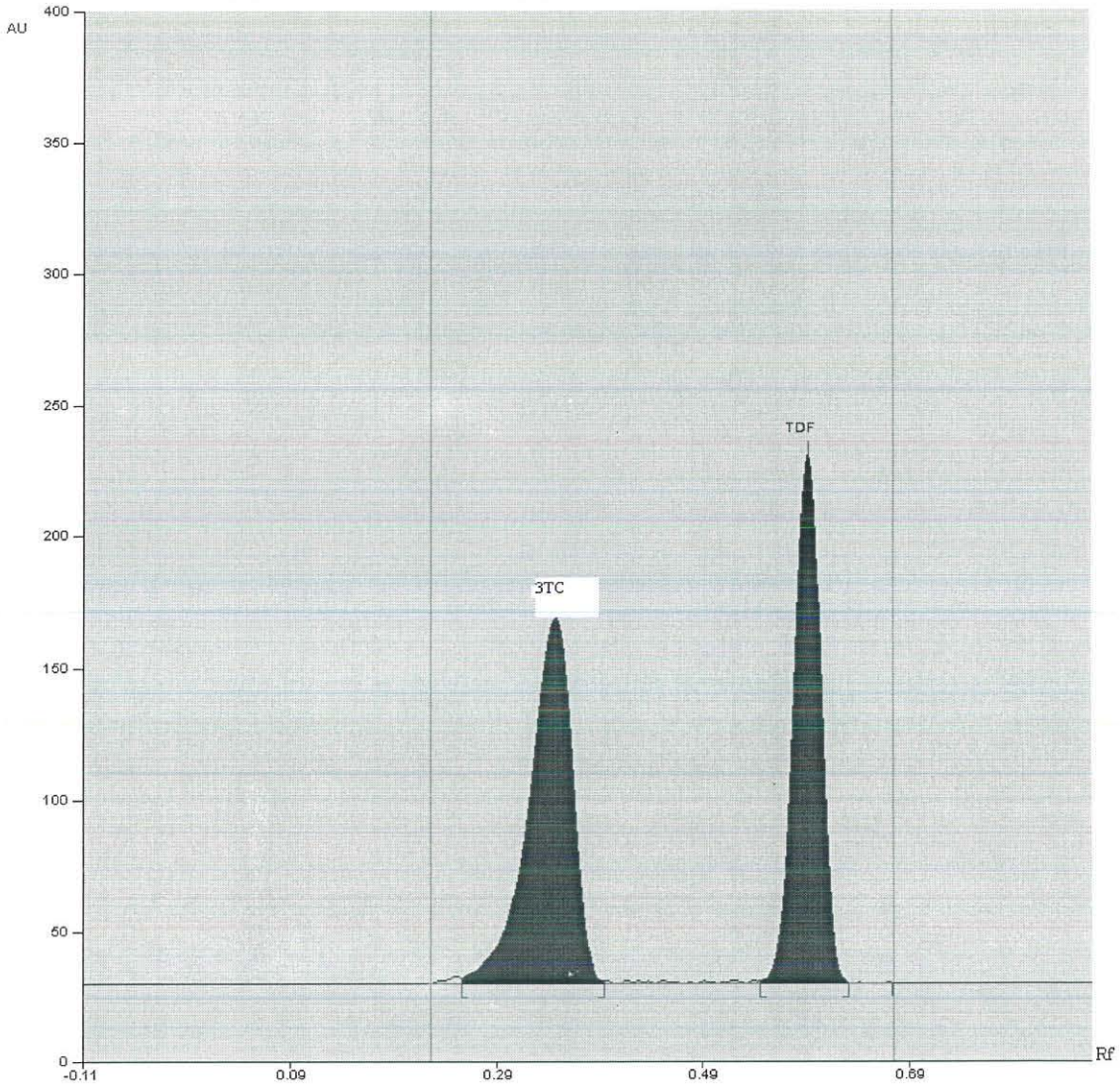

Fig. 6. HPTLC chromatogram of standard solutions of 3TC (500 ng/spot); (R_f: 0.32 ± 0.02) and TDF (500 ng/spot); (R_f: 0.57 ± 0.02), measured at 257 nm, mobile phase, toluene–methanol (6:4, v/v).

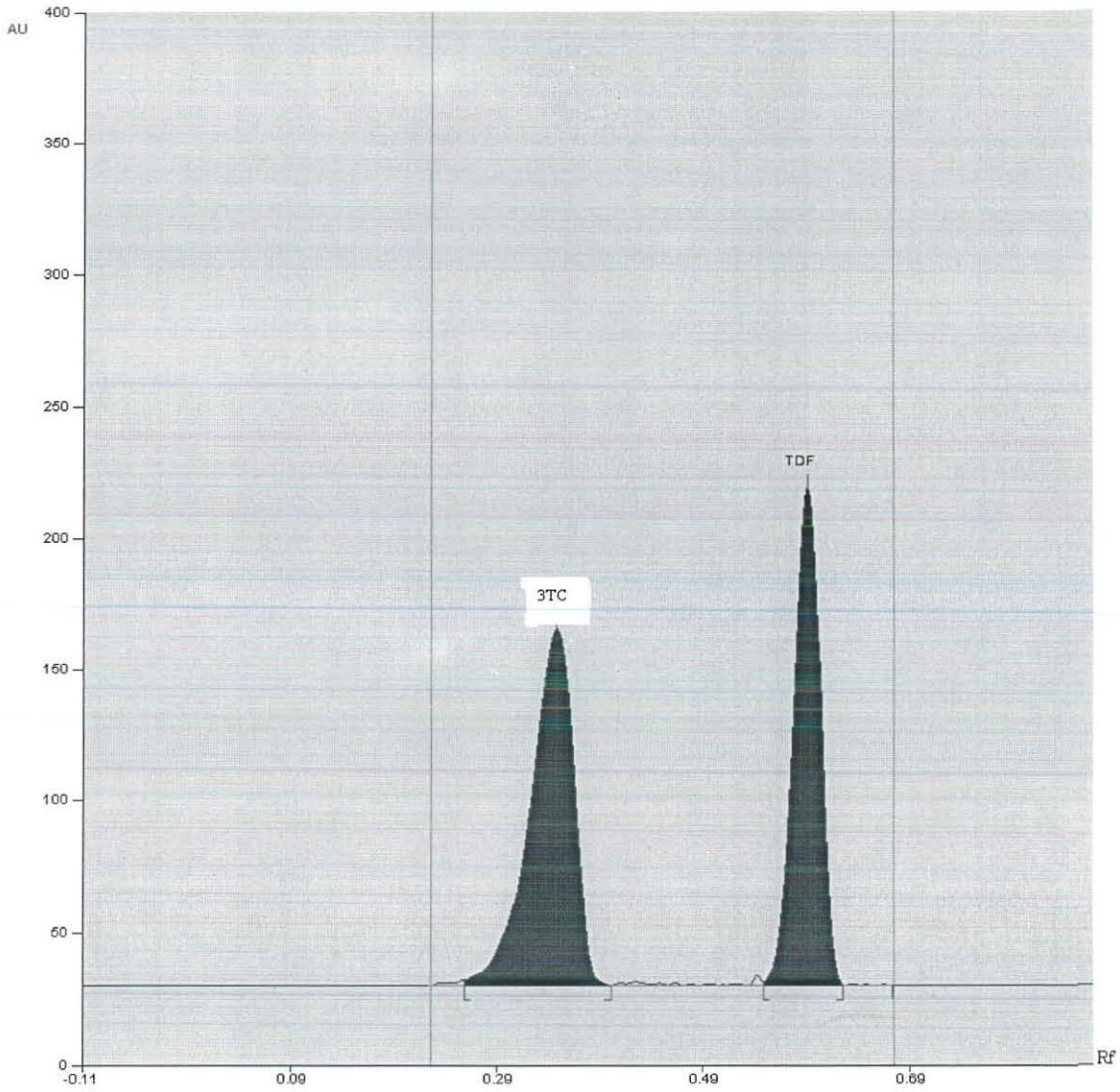

Fig. 7. HPTLC chromatogram of sample solutions of 3TC (500 ng/spot); (R_f: 0.32 ± 0.02) and TDF (500 ng/spot); (R_f: 0.57 ± 0.02), measured at 257 nm, mobile phase toluene–methanol (6:4, v/v).

4.1.3. Saturation time

The saturation of the chamber atmosphere with eluent vapor is recommended for crucial mobile phases to obtain reproducible migration distances. In order to establish the optimum conditions for the analysis, the saturation time was studied, and a strong influence of this parameter on the band shapes was observed. The optimum saturation time is 20 minutes. In order to maintain a constant saturation degree in the chamber, the mobile phase was replaced after washing and developing one plate. As consecutive development carried out with in the day, the R_f increment was observed for both drugs unless the chamber stay for about half an hour after discarding the used mobile phase. The relative humidity and temperature were in the range of 41-46 % and 19-23 ^{o}C, respectively, in laboratory atmosphere.

4.1.4. Wave length selection and optimization

The choice of detection wavelength is crucial for developing reliable and accurate qualitative and quantitative analysis of the drugs (33). Both drugs contain UV absorbing chromophore so that the detection and quantification may be carried out in this region. HPTLC facilitates in *situ* scanning of a plate using multiple wavelengths (35), so that appropriate wavelength may be chosen. Though the maximum response was obtained at 271 nm for lamivudine and at 257 nm for tenofovir DF, a compromise was made to use single wavelength for both drugs. Densitometric scanning was performed at 257 nm, symmetrical and well-resolved peaks (Figures 4 and 5) were obtained and this wavelength was used for both qualitative and quantitative analysis. Since there are no other spots than the two drugs were observed in toluene-methanol

(6:4, v/v) as mobile phase at 257 nm as in Fig. 3, this wave length is selective and appropriate for identification and quantification of the lamivudine and tenofovir DF.

4.2. Method validation

4.2.1. Selectivity

Selectivity is related with development of more sensitive and discriminating methods that have a capability to identify and quantify analytes with less interference from other components, similar or dissimilar, than earlier methods were able to do (44). The selectivity of the assay was determined in relation to interferences from formulation ingredients and was evaluated by base line resolution of the chromatogram of the standard drugs and the sample solution (41). Selectivity of the method was achieved by employing sample preparation, appropriate extraction techniques, mobile phase selection and optimization, saturation time optimization and selection of appropriative wavelength. Since there were no other spots than the two drugs in the mobile phase toluene-methanol (6:4, v/v) observed under UV light and the spots were resolved as shown in the Fig. 3. Selectivity was carried out by comparing base peak resolution between lamivudine and tenofovir DF standards and tablets. As it was shown in the Fig. 4-7, the peaks and chromatograms of the standard drugs and tablet form were separated and resolved well and there are no interferences from excpients. The two standard drugs and tablets were resolved well with R_f 0.32 and 0.57 for 3TC and TDF respectively, as it is shown in the Fig. 4 and 5. The base line of the peaks which were displayed in 3D presentation as in the Fig. 8 and 9 showed the peaks and the chromatogram were separated and resolved well. The proposed method can identify and quantify TDF in the presence of 3TC and excpients, can also identify and quantify 3TC in the presence of TDF and excpients.

Since selectivity refers to the extent to which the method can be used to determine active ingredients in the presence of excpients (44), the proposed method can selectively identify and quantify the lamivudine and tenofovir DF in the presence of other components or excpients.

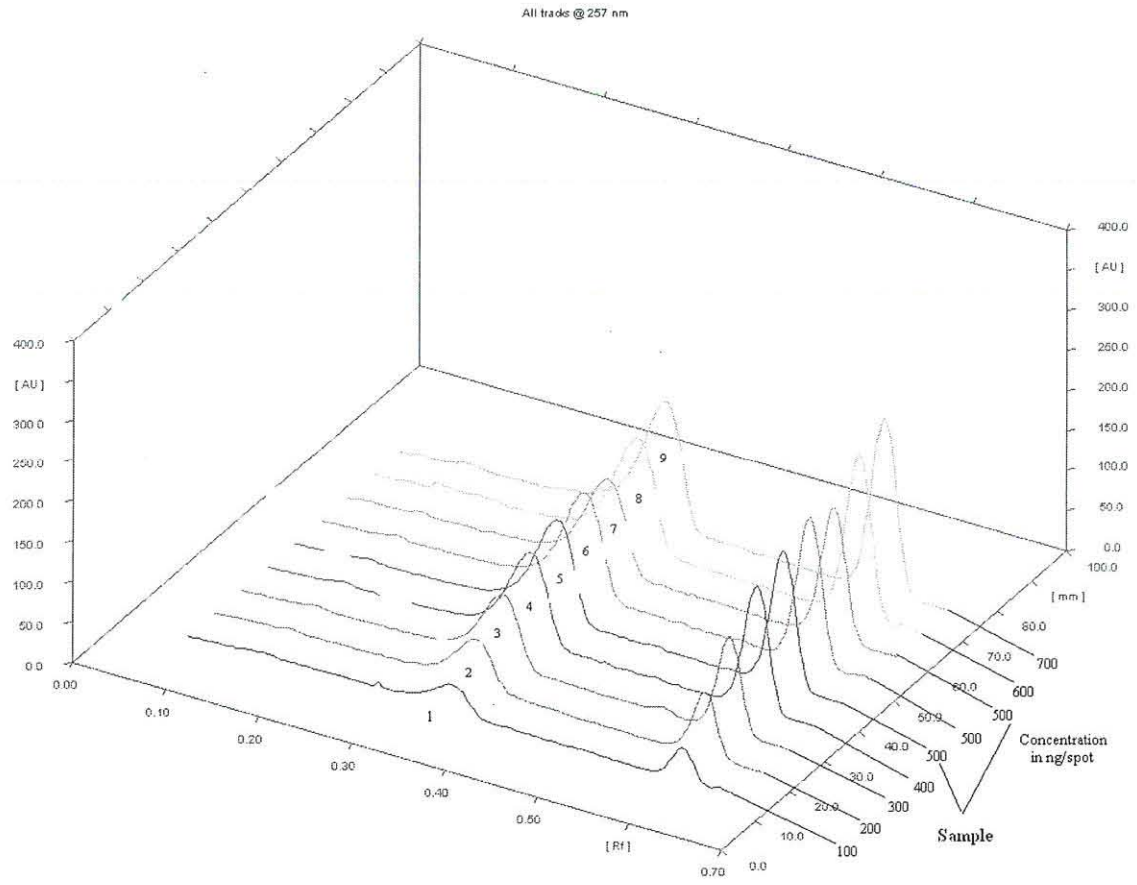

Fig. 8. 3D overlay of 9 chromatograms of lamivudine and tenofovir DF of standard and sample solutions and using toluene-methanol (6:4 v/v) and densitogram analysis was performed at 257 nm on a TLC scanner 3. Chromatogram 4 and 6 were sample solutions and the other 7 chromatograms were of standard solutions.

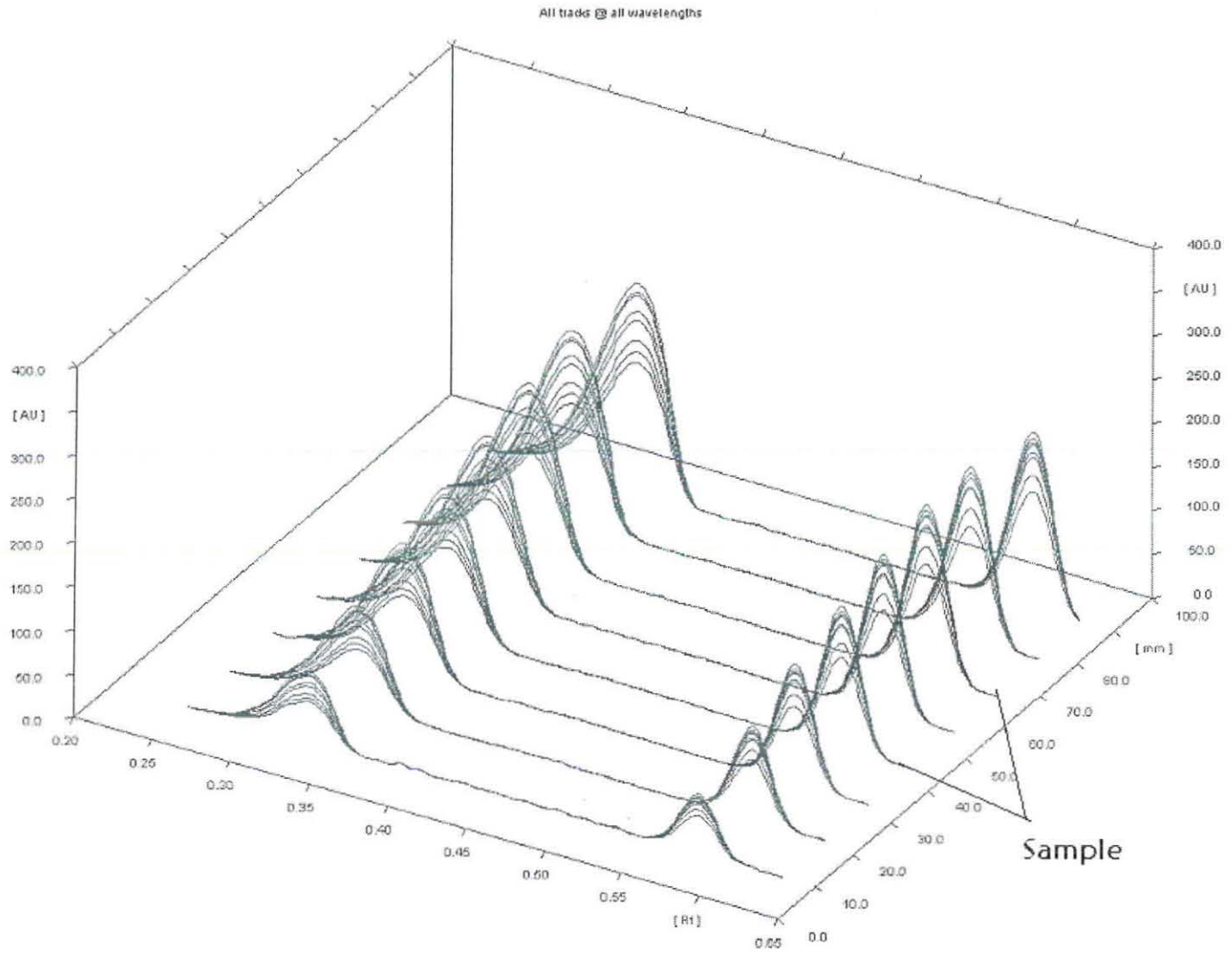

Fig. 9. 3D overlay of 9 chromatograms of lamivudine and tenofovir DF of binary standard and sample solutions and using toluene-methanol (6:4 v/v) and densitogram analysis was performed at multiple wavelengths on a TLC Scanner 3. Chromatogram 4 and 6 were sample solutions and the other 7 chromatograms were standard solutions.

Table 3. Summary of linear regression data for calibration curve (n = 5)

Parameters	Lamivudine	Tenofovir DF
Linearity range	200 – 800 ng/spot	100-900 ng/spot
$R^2 \pm SD$	0.994 ± 0.002	0.9978 ± 0.0012
Slope ± SD	8.12 ± 0.21	4.80 ± 0.076
Intercept ± SD	478.16 ± 72.85	232.80 ± 36.21
Linear regression equation	Y = 8.12 X + 478.16	Y = 4.80X + 230.03
	x shows conc. ng/spot	x shows conc. Ng/spot
LOD	29.60 ng/spot	24.89 ng/spot
LOQ	89.72 ng/spot	75.4 ng/spot

Table 4. Variation of standard peak area of lamivudine and tenofovir DF (n = 5)

Concentration in ng/spot	% RSD of the peak area of TDF (n = 5)	% RSD of the peak area 3TC (n = 5)
100	1.10	
200	1.80	1.79
300	2.07	2.15
400	1.80	1.54
500	1.76	2.1
600	1.43	2.03
700	0.88	2.1
800	1.26	1.87
900	1.25	

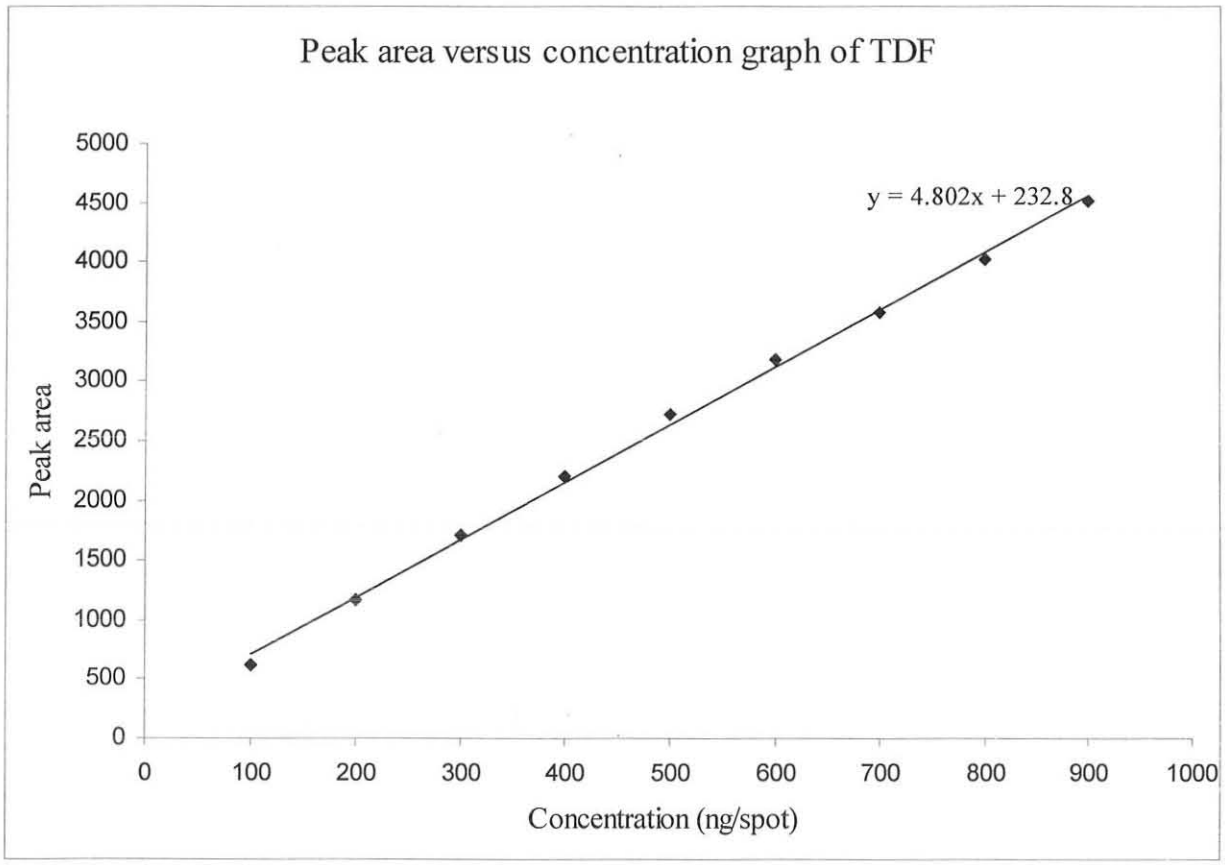

Fig. 10. Peak area versus concentration graph of tenofovir disoproxil fumarate.

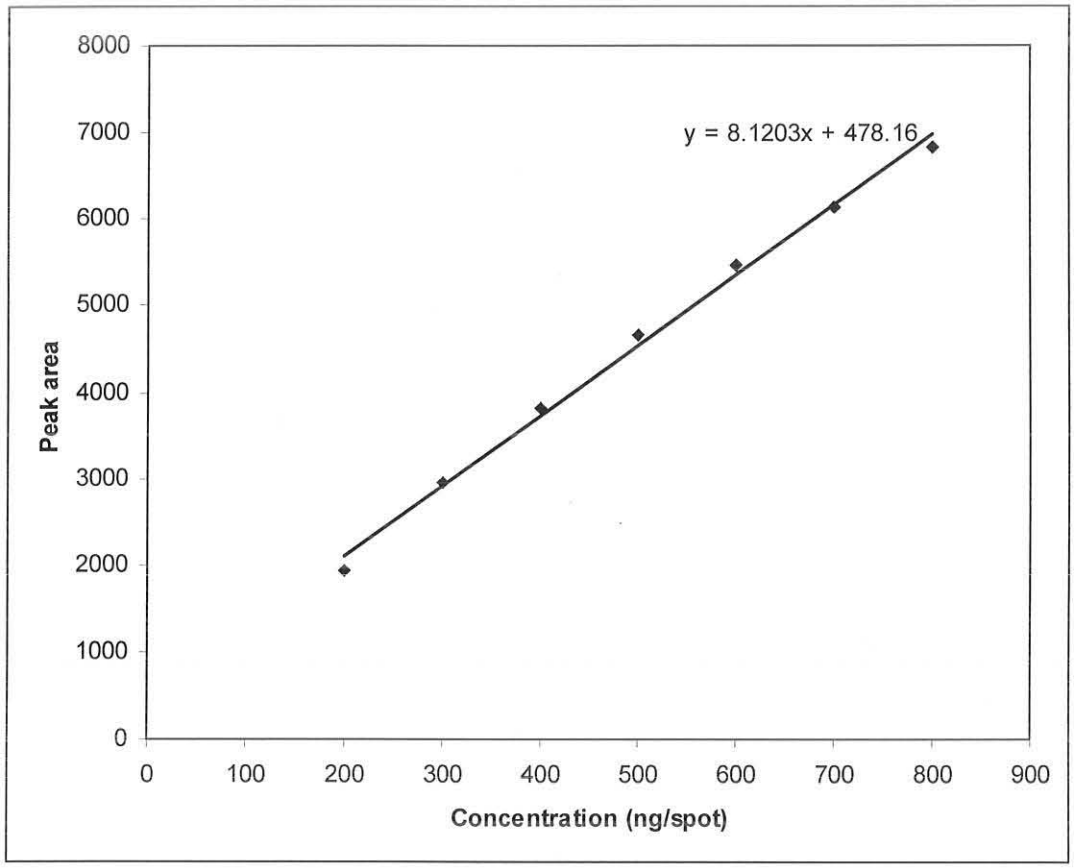

Fig. 11. Peak area versus concentration graph of lamivudine.

4.2.3 Precision

Repeatability and intermediate precision of the developed method were expressed in terms of percent relative standard deviation (% RSD) of the peak area. The results revealed that intra-day and inter-day variation of the results at three concentration levels of 400, 500 and 600 ng/spot for both lamivudine and tenofovir disoproxil fumerate was within acceptable range. The % RSD values for intra-day and inter-day precision was summarized in (Table 5). The methods can be considered as precise since all % RSD values were below 3% (41). The proposed method is more precise for lamivudine than tenofovir DF. The precision of lamivudine was found to be better than tenofovir disoproxil fumarate.

Table 5. Intra-day (repeatability) and inter-day (intermediate) precision

Compound	Amount (ng/spot)	Intra-day precision (n = 3)		Inter-day precision (n = 3)	
		SD	% RSD	SD	% RSD
Tenofovir DF	400	36.85	1.17	78.43	2.9
	500	114.03	0.99	44.93	1.67
	600	196.84	0.86	109.31	2.60
Lamivudine	400	116.43	0.35	123.76	2.30
	500	164.65	0.48	107.12	2.09
	600	136.78	0.90	107.43	1.77

4.2.4 Sensitivity

The LOD and LOQ of tenofovir disoproxil fumarete was found to be 24.89 and 75.4 ng/spot respectively, where as the LOD and LOQ of lamivudine was found to be 29.60 and 89.72 ng/spot respectively. Based on the LOD and LOQ values, the developed method suggested to be more sensitive for TDF compared 3TC.

4.2.5 Accuracy

Accuracy was determined by the percent of analyte recovered by assay from a known added amount. Data from nine determinations over three concentration levels covering the specified range were determined. The recovery study was carried out by applying the method to drug sample to which known amount of lamivudine and tenofovir disproxil fumarate corresponding to 80, 100 and 120% of the standard calibration curve. At each level of the amount added three determinations were performed and the results obtained were compared with expected results and the recovery was calculated by comparing the resultant peak areas with those obtained from pure standards in methanol at the same concentrations (35). The % recovery was summarized in Table 6 both for 3TC and TDF, respectively. The result showed that the method for each spiked standard was satisfactory.

Table 6 Recovery study of the method using standard addition method

Tenofovir Disoproxil Fumarate (n = 3)				Lamivudine (n = 3)			
Percent added	Amount added (ng/spot)	% recovered	% RSD	Percent added	Amount added (ng/spot)	% recovered	% RSD
80%	480	101.70	1.75	80%	450	101.53	1.03
100%	600	101.90	2.13	100%	500	99.95	0.53
120%	660	101.89	0.89	120%	550	98.95	0.56

4.2.6 Robustness

Robustness is a measure of the method which remains unaffected by small variations in the method conditions and is an indication of the method reliability (31). The evaluation of robustness of the method was considered during the development phase. For the proposed method, robustness was validated by varying composition of mobile phase, the amount of mobile phase, time from spotting to chromatography and from chromatography. The peak area and R_f values were considered to indicate the method robustness and % RSD was calculated for each parameter. Robustness of the method was done at three different concentration levels 400, 500, 600 ng/spot for both lamivudine and tenofovir disoproxil fumarate, respectively.

Except for mobile phase composition using toluene-methanol (5.5, 3.5, v/v), the standard deviation of peak areas was calculated for each parameter and % R.S.D. was found to be less than 5%. By introducing small variation of in the volume of methanol, the R_f value increased from 0.32 to 0.38 for lamivudine and from 0.56 to 0.59 for tenofovir disoproxil fumarate. Hence the R_f value is more sensitive when the polarity of the mobile phase increased. The amount of mobile phase can be varied from 15 ml to 20 ml with out affecting the method. This showed that the method was less susceptible for amount of mobile phase than mobile phase composition. Time from spotting to chromatography was more susceptible than time from chromatography to scanning. Increasing the proportion of methanol as mobile phase was susceptible to variations beyond the acceptable limit, this parameter should be suitably controlled.

4.3 Analysis of the dosage form

The applicability of the method was verified by determination of tenofovir disoproxil fumarate/lamivudine in pharmaceutical formulation. The marketed formulation tablets with label claim 300 mg/300 mg tenofovir disoproxil fumarate/lamivudine (manufactured by Matrix Laboratories limited) was determined using the proposed method, because it was the only formulation available in the local market of Ethiopia. Five microliters of the sample solution (500 ng/spot for both 3TC and TDF) was applied on the HPTLC plate followed by development and scanning. Calibration curve was constructed by using standard solutions and two sample solutions on the same plate to minimize error. The samples were quantified against standard calibration curve using winCATS version 1.4.0. The results for triplicate analysis of the both drugs using the proposed method were displayed in Table 7. The analysis of marketed formulation of 300 mg/300 mg of tenofovir disoproxil fumarate/lamivuidne showed assay 98.63 ± 2.17 and 100.93 ± 1.32 for tenofovir disoproxil fumarate and lamivuidne, respectively. The variation for determination of lamivudine was found to be better than tenofovir disoproxil fumarate. The employed method has been successfully applied in the analysis of marketed formulations. No chromatographic interferences from the tablet excipients were found. Hence it can be applied for routine quality control analysis of tenofovir disoproxil fumarate and lamivuidne.

Table 7 Summary of the analysis of lamivudine/tenofovir disoproxil fumarate in tablet (n = 3)

Substance	Label claim (ng/spot)	Amount detected (ng/spot)	Assay	%RSD
TDF	500	495.45	99.09 %	2.02
LAM	500	503.20	100.64	0.52
TDF	500	490.54	98.11	3.47
LAM	500	500.78	100.15	1.68
TDF	500	493.5	98.7	1.01
LAM	500	510.04	102.01	1.75

5. Conclusion

HPTLC technique offers an important advantage due to the fact that chromatographic plates are disposable and matrix does not have to be eluted as in column chromatography. The proposed method for screening and quantitative determination of lamivudine and tenofovir disoproxil fumarate results in a compacted spot, symmetrical and well resolved peaks. The major advantage of this method several samples can be analyzed simultaneously using a small quantity of mobile phase, unlike HPLC. This reduces the time, cost of analysis and the possibilities of environmental pollution. The developed method represents a good alternative to save money and time because the quantity of solvents used is lower than HPLC; the procedure requirements to obtain the results are fewer. Its main advantages are low cost of reagents, high speed, and simplicity of the technique. The proposed method was validated and found to be selective, linear, repeatable and accurate within the established range. The precision and accuracy achieved is sufficient for general quality control.

The proposed method is simple, cost effective, eco friendly, easily adaptable for simultaneous screening and quantitative determination of lamivudine and tenofovir disoproxil fumarate in pharmaceutical formulations, and appropriate for quality control of both drugs.

6. Recommendations

It is recommended that the method should be revalidated for use in area where there is variation in humidity and temperature because humidity and temperature has got an impact on the analytical results. Automatic development chamber (ADC) was not available at the laboratory in the studied period of the research; so additional research could be carried out by including (ADC) which can minimize the effect of humidity on the method.

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DECLARATION

I, the undersigned, declare that this thesis is my original work and has not been presented for a degree in any other university.

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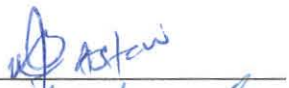
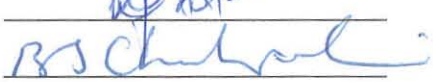
Dr. Asfaw Debela	
Prof B.S. Chandravanshi	

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