ANALYSIS OF SOME ANTIRETROVIRAL DRUGS IN BULK, PHARMACEUTICAL FORMULATIONS AND BIOLOGICAL FLUID

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(FACULTY OF MEDICINE)

BY

Mr. RAJNIKANT BHIKHUBHAI MARDIA
M. PHARM. (QUALITY ASSURANCE)

CO- GUIDE
PROF. (Dr.) B. N. SUHAGIA
(M. PHARM., Ph. D., L. L. B., F. I. C.)

GUIDE
PROF. (Dr.) T. Y. PASHA
(M. PHARM., Ph. D.)

JUNE-2012

CERTIFICATE

This is to certify that the thesis entitled **"Analysis of some antiretroviral drugs in bulk, Pharmaceutical formulations and biological fluid"** represents bonafide and genuine research work of **Mr. Rajnikant Bhikhubhai Mardia** carried out under our guidance and supervision. The work presented in this dissertation was carried out at Faculty of Pharmacy, Dharmsinh Desai University, Nadiad, Gujarat, India and is upto our satisfaction.

Co-Guide

Prof. (Dr.) B. N. Suhagia
Professor and Dean,
Faculty of Pharmacy,
Dharmsinh Desai University,
Nadiad, Gujarat, India.

Guide

Prof. (Dr.) T. Y. Pasha
Professor,
Department of Pharmaceutical Chemistry,
Parul Institute of Pharmacy,
Baroda, Gujarat, India.

Date:
Place: Rajkot

DECLARATION

I hereby declare that thesis entitled **"Analysis of some antiretroviral drugs in bulk, Pharmaceutical formulations and biological fluid"** is a bonafide and genuine research work carried out by me, under the guidance of **Prof. (Dr.) B. N. Suhagia (Co-Guide),** Professor and Dean, Faculty of Pharmacy, Dharmsinh Desai University, Nadiad, Gujarat, India and **Prof. (Dr.) T. Y. Pasha (Guide)**, Professor, Department of Pharmaceutical Chemistry, Parul Institute of Pharmacy, Baroda, Gujarat, India. The results presented in this dissertation are original and has not been submitted in part or full for any degree/diploma to any University.

Date:
Place: Rajkot

Mr. Rajnikant B. Mardia
Reg. No. 3873
Department of Pharmaceutical Sciences, Saurashtra University, Rajkot -360005.

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Mr. Rajnikant B. Mardia
Reg. No. 3873
Department of Pharmaceutical Sciences, Saurashtra University, Rajkot- 360005.

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Mr. Rajnikant B. Mardia

(M.Pharm, Q.A.)

DEDICATED TO

MY GOD , GURUJI AND

BELOVED

FAMILY MEMBERS

ABBREVIATIONS

HIV	:	Human immune deficiency virus
IP	:	Indian Pharmacopoeia
BP	:	British Pharmacopoeia
USP	:	United States Pharmacopoeia
HPLC	:	High Performance Liquid Chromatography
HPTLC	:	High Performance Thin Layer Chromatography
RP-HPLC	:	Reversed Phase High Performance Liquid Chromatography
LC	:	Liquid Chromatography
TLC	:	Thin Layer Chromatography
MS	:	Mass Spectroscopy
LC-MS	:	Liquid Chromatography - Mass Spectroscopy
LC-ESI	:	Liquid Chromatography – Electrospray Ionisation
PDA-MS	:	Photo Diode array – Mass Spectroscopy
R^2	:	Correlation coefficient.
M	:	Molar
mM	:	Millimolar
µL	:	Micro liter
mL	:	Milliliter
ng	:	Nanogram
µg	:	Microgram
mg	:	Milligram
nm	:	Nanometer
mm	:	Millimeter
%	:	Percentage
Min.	:	Minute
Temp.	:	Temperature
CV	:	Coefficient of Variation
LOD	:	Limit of Detection
LOQ	:	Limit of Quantitation
RSD	:	Relative Standard Deviation

SD	:	Standard Deviation
ICH	:	International Conference on Harmonization
USFDA	:	United states food and drugs administration
r(S, M)	:	Peak purity between peak start and peak middle
r(M, E)	:	Peak purity between peak middle and peak end
PLM	:	Plasma
LPV	:	Lopinavir
RTV	:	Ritonavir
TNV	:	Tenofovir Disoproxil Fumarate
EFV	:	Efavirenz

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CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1. Virus

A virus is a small infectious intracellular organism, much smaller than a bacterium that must invade a living cell to reproduce (replicate). The virus attaches to a cell, enters it, and releases its DNA or RNA inside the cell. The virus's DNA or RNA is the genetic code containing the information needed to replicate the virus. The viral genetic material takes control of the cell and forces it to replicate the virus. The infected cell usually dies because the virus keeps it away from performing its normal functions. Before it dies, however, the cell releases new viruses, which go on to infect other cells[1].

Drugs that combat viral infections are called antiviral drugs. Antiviral drugs work by interfering with viral replication. Because viruses are tiny and replicate inside cells using the cells' own metabolic pathways, there are only a limited number of metabolic functions that antiviral drugs can target. Antibiotics are not effective against viral infections, but if a person has a bacterial infection in addition to a viral infection, an antibiotic is often necessary. Human immunovirus (HIV) decreases the immunity called AIDS.

Retrovirus structure[2]:

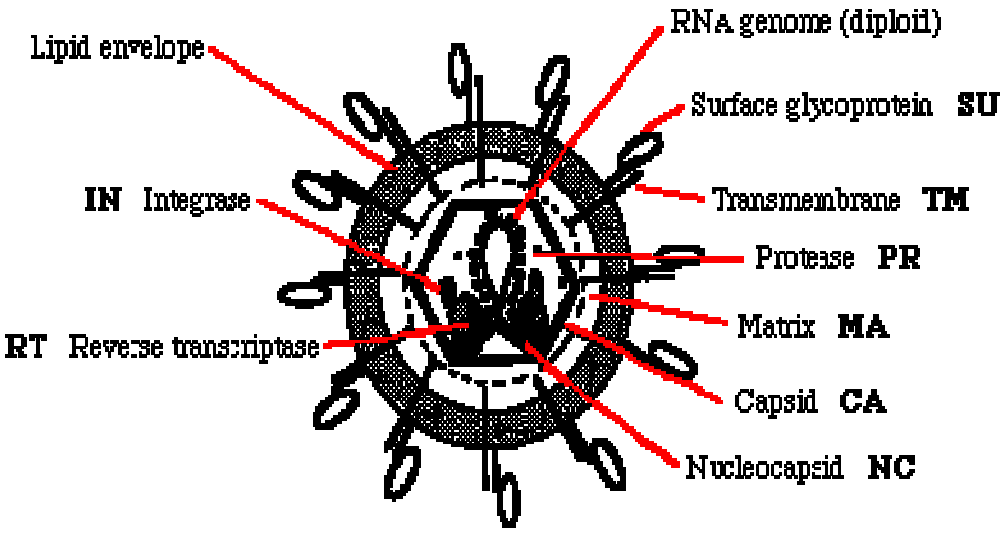
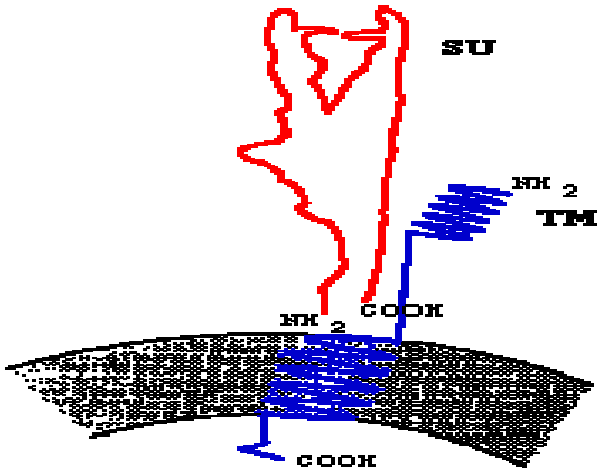


Figure 1.1: Structure of retrovirus

Retroviruses have enveloped particles, somewhat variable in size/shape but~100nm diameter. The envelope carries a virus-encoded glycoprotein, which forms spikes in the membrane. There are certain structural/functional similarities between the envelope glycoprotein and the influenza haemagglutinin . The mature protein is cleaved into 2 polypeptides:

The outer envelope glycoprotein (**SU**), the major antigen of the virus, responsible for receptor binding, linked by disulphide bonds.

The trans-membrane glycoprotein (**TM**), holds the SU protein in the envelope, responsible for membrane fusion.



Inside the membrane is the **matrix (MA)** protein, rather amorphous. This largely obscures the **capsid (CA)**, which is *believed* to be icosahedral. CA is the most abundant protein in the particle (~33% total weight). Inside the capsid is the **core** = RNA genome + NC protein + RT + IN. This is usually a conical, electron-dense structure clearly visible in -ve stained E.M. pictures (matrix and capsid appear amorphous)[3].

1.2 Virus function and life cycle

As viruses have no metabolic machinery of their own, they have to attach to and penetrate a living host cell-animal, plant or bacterial-and use the victim's own metabolic processes to replicate. The first step in this process is facilitated by polypeptide binding sites on the envelope or capsid, interacting with receptors on the host cell. These 'receptors' are normal membrane

constituents-receptors for cytokines, neurotransmitters or hormones, ion channels, integral membrane glycoproteins, etc. The receptor-virus complex enters the cell (often by receptor-mediated endocytosis), during which time the virus coat may be removed by host cell enzymes (often lysosomal in nature). Some bypass this route. Once in the host cell, the nucleic acid of the virus then uses the host cell's machinery for synthesizing nucleic acids and proteins that are assembled into new virus particles. The actual way in which this occurs varies between DNA and RNA viruses.

- Replication in DNA viruses

Viral DNA enters the host cell nucleus, where transcription into mRNA occurs catalyzed by the host cell RNA polymerase. Translation of the mRNA into virus-specific proteins then takes place. Some of these proteins are enzymes that then synthesize more viral DNA, as well as proteins comprising the viral coat and envelope. After assembly of coat proteins around the viral DNA, complete virions are released by budding or after host cell lysis.

- Replication in RNA viruses

Enzymes within the virion synthesize its mRNA from the viral RNA template, or sometimes the viral RNA serves as its own mRNA. This is translated by the host cell into various enzymes, including RNA polymerase (which directs the synthesis of more viral RNA), and also into structural proteins of the virion. Assembly and release of virions occurs as explained above. With these viruses, the host cell nucleus is usually not involved in viral replication, although some RNA viruses (e.g. Orthomyxo viruses) replicate exclusively within the host nuclear compartment.

- Replication in retroviruses

The virion in retroviruses contains a reverse transcriptase enzyme (virus RNA-dependent DNA polymerase), which makes a DNA copy of the viral RNA. This DNA copy is integrated into the genome of the host cell, and it is then termed a provirus. The provirus DNA is transcribed into both new viral genome RNA as well as mRNA for translation in the host into viral proteins, and the completed viruses are released by budding. Many retroviruses can replicate without killing the host cell[4,5].

1.3 Cause and types of viral infection

Human cells are vulnerable to viruses, and when the body is exposed to viral particles, the immune system will try to destroy these particles and eliminate them from the system. A lowered immune system allows the virus to more easily attach itself to available cells, often bringing about general symptoms such as fever, chills, and muscle aches. This also makes it easier for the virus to replicate, and thus advances symptoms until the immune system can fight the virus off.

- Symptoms of Viral Infections

Viral infections come with a variety of symptoms ranging from mild to severe. Symptoms may vary depending on what part of the body is affected, type of viruses, age, and overall health of the affected person[6].

These symptoms can include:

- Fever
- Muscle aches
- Runny nose
- Headache
- Chills
- Diarrhea
- Coughing
- Sneezing
- Vomiting

More severe symptoms include:

- Personality changes
- Neck stiffness
- Dehydration
- Seizures
- Paralysis of the limbs
- Confusion
- Back pain
- Loss of sensation
- Impaired bladder and bowel function

- Sleepiness that can progress into a coma or death

1.4 Antiviral drugs[7]

Because viruses hijack many of the metabolic processes of the host cell itself, it is difficult to find drugs that are selective for the pathogen. However, there are some enzymes that are virus-specific, and these have proved to be useful drug targets. Most currently available antiviral agents are effective only while the virus is replicating.

- Classification of Antiviral drugs based on mechanism of action

1. Nucleoside reverse transcriptase inhibitors:

   eg. Abacavir, Adefovir Dipivoxil, Didanosine, Emtricitabine, Lamivudine, Stavudine, Tenofovir, Zalcitabine, Zidovudine

2. Non-nucleoside reverse transcriptase inhibitors:

   eg.Efavirenz, Nevirapine

3. Protease inhibitors:

   eg. Amprenavir, Atazanavir, Indinavir, Lopinavir, Nelfinavir, Ritonavir, Saquinavir

4. Viral DNA polymerase inhibitors:

   eg. Aciclovir, Cidofovir, Famciclovir, Foscarnet, Ganciclovir, Idoxuridine, Penciclovir

5. Inhibitors of HIV fusion with host cells:

   eg. Enfurvitide

6. Inhibitors of viral coat disassembly and neuraminidase inhibitors:

   eg. Amantadine, Oseltamivir, Zanamivir

7. Biologics and immunomoduators:

   eg. Interferon-a, Pegylated interferon-a, Inosine Pranobex, Palivizumab

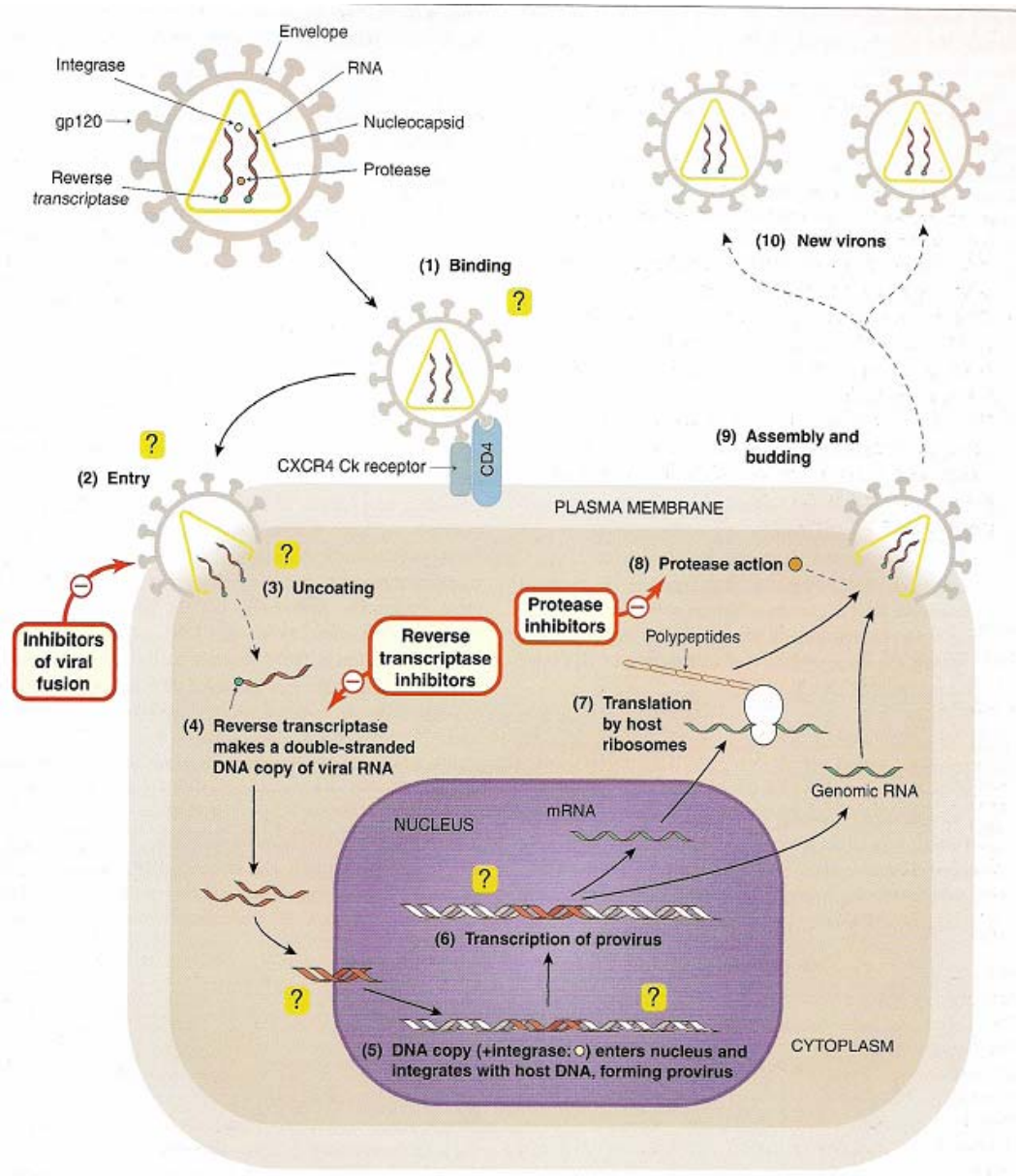


Figure 1.2: Schematic diagram of infection of a $CD4^+$cell by an HIV virion with the series of action of the two main classes of anti-HIV drugs

1.5 Approach to drug therapy

1.5.1 Approaches by life cycle stage

- Before cell entry

One antiviral strategy is to interfere with the ability of a virus to infiltrate a target cell. The virus must go through a sequence of steps to do this, beginning with binding to a specific "receptor" molecule on the surface of the host cell and ending with the virus "uncoating" inside the cell and releasing its contents. Viruses that have a lipid envelope must also fuse their envelope with the target cell, or with a vesicle that transports them into the cell, before they can uncoat.

- Entry inhibitor

A very early stage of viral infection is viral entry, when the virus attaches to and enters the host cell. A number of "entry-inhibiting" or "entry-blocking" drugs are being developed to fight HN. HN most heavily targets the immune system's white blood cells known as "helper T cells", and identifies these target cells through T-cell surface receptors designated "CD4" and flCCRS". Attempts to interfere with the binding of HIV with the CD4 receptor have failed to stop mv from infecting helper T cells, but research continues on trying to interfere with the binding of HN to the CCRS receptor in hopes that it will be more effective.

- Uncoating inhibitor

Inhibitors of uncoating have also been investigated[8, 9]. Amantadine and Rimantadine have been introduced to combat influenza. These agents act on penetration uncoating[10]. Pleconaril works against rhinoviruses, which cause the common cold, by blocking a pocket on the surface of the virus that controls the uncoating process. This pocket is similar in most strains of rhinoviruses and enteroviruses, which can cause diarrhea, meningitis, conjunctivitis, and encephalitis.

1.5.2 During viral synthesis

A second approach is to target the processes that synthesize virus components after a virus invades a cell.

- Reverse transcription

One way of doing this is to develop nucleotide or nucleoside analogues that look like the building blocks of RNA or DNA, but deactivate the enzymes that synthesize the RNA or DNA once the analogue is incorporated. This approach is more commonly associated with the inhibition of reverse transcriptase (RNA to DNA) than with "normal" transcriptase (DNA to RNA).The first successful antiviral, acyclovir, is a nucleoside analogue, and is effective against Herpes virus infections. The first antiviral drug to be approved for treating HIV, Zidovudine (AZT), is also a nucleoside analogue.

- Translation / antisense

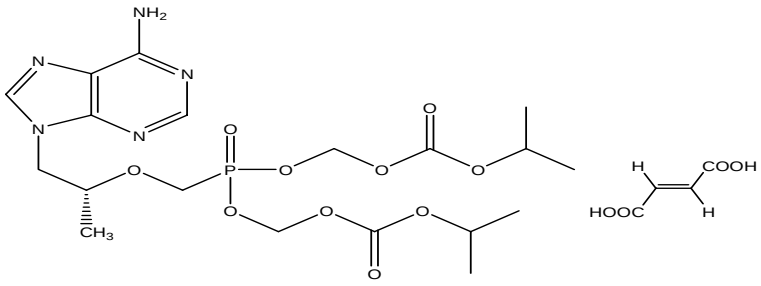
Genomics has not only helped find targets for many antivirals, it has provided the basis for an entirely new type of drug, based on "antisense" molecules. These are segments of DNA or RNA that are designed as complementary molecule to critical sections of viral genomes, and the binding of these antisense segments to these target sections blocks the operation of those genomes. A Phosphorothioate antisense drug named Fomivirsen has been introduced, used to treat opportunistic eye infections in AIDS patients caused by cytomegalovirus, and other antisense antivirals are in development. An antisense structural type that has proven especially valuable in research is Morpholino antisense[11, 12].

- Protease inhibitors

Some viruses include an enzyme known as a protease that cuts viral protein chains apart so they can be assembled into their final configuration. HIV includes a protease, and so considerable research has been performed to find "protease inhibitors': to attack HIV at that phase of its life cycle[13]. Protease inhibitors became available in the 1990s and have proven effective, though they can have unusual side effects, for example causing fat to build up in unusual places[14]. Improved protease inhibitors are now in development.

1.6 DRUG PROFILE

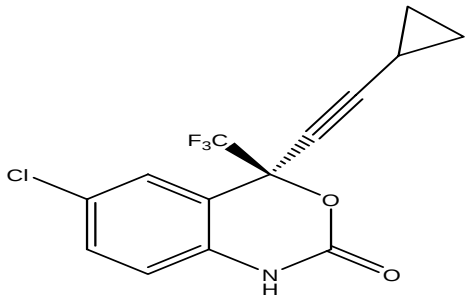
1.6.1 Tenofovir disoproxil fumarate[15, 16, 17]

Structure	
Chemical Name	9 - [(R) -2 - [[bis [[(isopropoxy carbonyl) oxy] methoxy] phosphinyl] methoxy] propyl] adenine fumarate
CAS No.	147127-20-6
Merck Index No.	9146
Molecular Formula	$C_{19}H_{30}N_5O_{10}P$ –$C_4H_4O_4$
Molecular weight	635.52 g/mol
State and Solubility	White to off white crystalline powder. Approximately 13.4 mg/ml, soluble in distilled water at 25°C.
Melting Point	279°C
pKa	3.75
Mechanism of action	Tenofovir belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors (NaRTIs), which block reverse transcriptase, an enzyme crucial to viral production in HIV-infected people. Tenofovir inhibits the activity of HIV reverse transcriptase

	by competing with the natural substrate deoxyadenosine 5'-triphosphate and, after incorporation into DNA, by DNA chain termination.
Pharmacokinetics	➢ The oral bioavailability in fasted patients is approximately 25%. Administration of food (high fat meal containing 40 to 50% fat) increases the oral bioavailability, with an increase in the AUC of approximately 40%. ➢ Tenofovir is eliminated by a combination of glomerular filtration and active tubular secretion. There may be competition for elimination with other compounds that are also renally eliminated.
Uses and Administration	➢ For the treatment of HIV-l or chronic hepatitis B: The dose of tenofovir disoproxil fumarate) is 300 mg once daily taken orally, without regard to food. ➢ In the treatment of chronic hepatitis B, the optimal duration of treatment is unknown.
Side Effects	liver damage - nausea, stomach pain, low fever, loss of appetite, dark urine, clay-colored stools, jaundice, lactic acidosis - muscle pain or weakness, numb or cold feeling in your arms and legs, dizziness, trouble breathing, stomach pain, nausea with vomiting, slow or uneven heart rate, dizziness, or feeling very weak or tired, kidney problems -increased thirst and urination, loss of appetite, weakness, constipation, urinating less than usual or not at all, fever, chills, body aches, flu symptoms.
Contraindications	Tenofovir is indicated in combination with other antiretroviral agents for the treatment of HIV-1 infection in

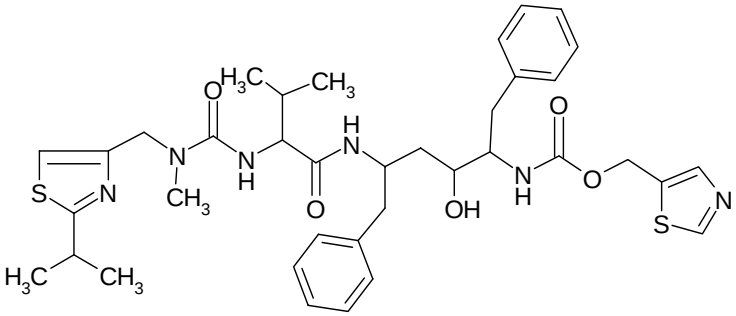
	adults. There are no study results demonstrating the effect of tenofovir on the clinical progression of HIV. It also has activity against lamivudine-resistant HBV.
Storage	Store at room temperature at 77°F (25°C) away from light and moisture.

1.6.2 Efavirenz[18-23]

Structure	
Chemical Name	(4S) – 6 – chloro – 4 - (cyclopropy ethynyl) - I,4 - dihydro-4-(trifluoromethyl)-2H-3,1-benzoxazin-2-one.
CAS No.	154598-52-4
Merck Index No.	3521
Molecular Formula	$C_{14}H_9ClF_3NO_2$
Molecular weight	315.68 g/mol
State and Solubility	White to slightly pink crystalline powder. It is practically insoluble in water, soluble in methanol.
Melting Point	139-141 °C
pKa	10.2
Mechanism of action	Efavirenz is an HIV-1 specific, non - nucleoside reverse transcriptase inhibitor (NNRTl). EFV activity is mediated predominantly by noncompetitive inhibition of HIV-I reverse transcriptase (RT)
Pharmacokinetics	➢ In HIV-I-infected patients at steady state, mean C_{max}, mean C_{min}, and mean AUC were dose proportional

	following 200-mg, 400-mg, and 600-mg daily doses. Time-to-peak plasma concentrations were approximately 3-5 hours and steady-state plasma concentrations were reached in 6-10 days. ➢ Efavirenz is highly bound (approximately 99.5-99.75%) to human plasma proteins, predominantly albumin. ➢ Efavirenz is principally metabolized by the cytochrome P450 system to hydroxylated metabolites with subsequent glucuronidation of these hydroxylated metabolites. These metabolites are essentially inactive against HIV-1. The in vitro studies suggest that CYP3A and CYP2B6 are the major isozymes responsible for Efavirenz metabolism.
Administration	The recommended dosage of Efavirenz is 600 mg, once daily, in combination with a protease inhibitor and/or nucleoside analogue reverse transcriptase inhibitors. It is recommended that Efavirenz be taken on an empty stomach, preferably at bedtime. Dosing at bedtime may improve the tolerability of nervous system symptoms.
Side Effects	Psychiatric symptoms, nervous system symptoms, rash
Contraindications	It is never used alone and is always given in combination with other drugs. The decision on when to start treatment should take into account CD4 count, HIV viral load, treatment history, resistance profiles and patient preference.
Storage	It should be stored at room temperature at 77°F (25°C) away from light and moisture.

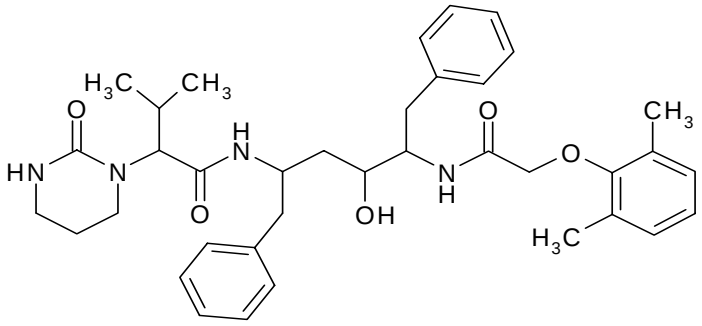
1.6.3 Ritonavir[24-28]

Structure	
Chemical Name	10 – Hydroxy – 2 – methyl – 5 - (I – methyl ethyl) –I - [2 - (l - methy lethyl) – 4 - thiazolyl] - 3, 6 – dioxo - 8, I1 - bis (phenyl methyl) - 2,4,7,12- tetraazatridecan – 13 - oic acid, 5 -thiazolylmethyl ester.
CAS No.	155213-67-5
Merck Index No.	8283
Molecular Formula	$C_{37}H_{48}N_6O_5S_2$
Molecular weight	720.95 g/mol
State and Solubility	A white to light tan powder. Freely soluble in methanol and ethanol and Insoluble in water.
Melting Point	207-209°C
pKa	14.22
Mechanism of action	Ritonavir reversibly binds to the active site of the HIV protease, preventing polypeptide processing and subsequent virus maturation. Virus particles are produced in the presence of ritonavir but are noninfectious.

Pharmacokinetics	➢ T_{max} is approximately 3-5 hours on oral administration. 98-99 % bound to human serum proteins and it is independent of concentration. ➢ The major biotransformation pathways consisted of monooxygenation and dioxygenation. Others are glucuronidation, N-dealkylation, hydrolysis, and oxygenation with dehydrogenation. ➢ Majority of the administered drug is recovered in the feces and urine (79% and 13% respectively). The terminal elimination half-life is approximately 7 hours at steady state.
Administration	By mouth with food or up to 2 hours after food, usually 2 times daily
Side Effects	Gastrointestinal and include nausea, vomiting, diarrhea, anorexia, abdominal pain, and taste perversion. Accidental injury, allergic reaction, back pain, cachexia, chest pain, chills, facial edema, facial pain, flu syndrome, hormone level altered, hypothermia, kidney pain, neck pain, neck rigidity, pelvic pain, photosensitivity reaction, and substernal chest pain
Contraindications	Concomitant therapy of ritonavir with a variety of medications may result in serious and sometimes fatal drug interactions. These interactions can occur with strong inhibitors, strong or moderate inducers or substrates of hepatic cytochrome P450 CYP3A4 isoform. Midazolam and triazolam are contraindicated, carbamazepine decreased metabolism, possible toxicity, Cisapride decreased metabolism, possible prolongation of Q-T interval and life-threatening arrhythmias , disulfiram (with Ritonavir oral preparation) – decreased

	metabolism of Ritonavir.
Storage	It should be stored at 2° to 8°C protected from light.

1.6.4 Lopinavir[29-30]

Structure	
Chemical Name	[1S-[1R*,(R*), 3R*, 4R*]] –N –[4- [[(2,6 –dimethyl phenoxy) acetyl]amino]-3-hydroxy-5-phenyl- 1-phenyl methyl) pentyl] tetrahydro-alpha-(1-methylethyl)-2-oxo-1(2H)-pyrimidine acetamide.
CAS No.	192725-17-0
Merck Index No.	5573
Molecular Formula	$C_{37}H_{48}N_4O_5$
Molecular weight	628.8008 g/mol
State and Solubility	white to light tan powder. freely soluble in methanol and ethanol, soluble in isopropanol and practically insoluble in water.
Melting Point	279°C
pKa	13.98
Mechanism of action	Lopinavir inhibits the HIV viral protease enzyme. This prevents cleavage of the gag-pol polyprotein and, therefore, improper viral assembly results. This

	subsequently results in non-infectious, immature viral particles.
Pharmacokinetics	➢ Administered alone, Lopinavir has insufficient bioavailability; however, like several HIV protease inhibitors, its blood levels are greatly increased by low doses of ritonavir, a potent inhibitor of cytochrome P450 3A4. ➢ Lopinavir is highly bound to plasma proteins (98-99%). ➢ Lopinavir is extensively metabolized by the hepatic cytochrome P450 system, almost exclusively by the CYP3A isozyme.
Uses and Administration	Treatment of HIV infections in combination with other antiviral agents. Administer with food. Not to be administered with the drugs like dihydroergotamine, ergonovine, ergotamine, methylergonovine, triazolam, astemizole, pimozide, cisapride, propafenone, terfenadine, flecainide, midazolam.
Side Effects	Diarrhoea, nausea, vomiting, bloating, headache, dyslipidaemias raised LFTs
Contraindications	Concurrent administration with drugs that are highly dependent on CYP3A or CYP2D6 for clearance and for which elevated plasma levels are associated with serious or life-threatening reactions.
Storage	It should be stored at 2° to 8°C protected from light.

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CHAPTER 2

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1 OFFICIAL COMPENDIA METHODS FOR DETERMINATION OF ANTIRETROVIRAL AGENTS

Sr. No.	Matrix	Method	Parameters	Ref. No.
1.	Tenofovir	HPLC	Stationary phase – Stainless steel column (25 cm x 4.6 mm, 5 µm) packed with octadecyl silane bonded to porous silica Mobile phase - Acetonitrile : 0.05M sodium dihydrogen phosphate (pH 2.3) (40:60 v/v) Flow rate - 1 mL/min. Detection wavelength - 260 nm.	1
2.	Tenofovir tablet	HPLC	Stationary phase – Stainless steel column (15 cm x 4.6 mm, 5 µm) packed with octadecyl silane bonded to porous silica Mobile phase - Acetonitrile : 0.05M sodium dihydrogen phosphate (pH 2.3) (40:60 v/v) Flow rate - 1 mL/min. Detection wavelength - 260 nm.	1
3.	Tenofovir disoproxil fumarate and Emtricitabine tablet	HPLC	Stationary phase – Stainless steel column (5 cm x 4.6 mm, 3µm) packed with octadecyl silane bonded to porous silica Column temperature - 40ºC, Mobile phase - Acetonitrile : monobasic potassium phosphate buffer (pH 3.0) (80:20 v/v) Flow rate – 1.5 mL/min.	1

			Detection wavelength - 260 nm.	
4.	Ritonavir	HPLC	Stationary phase – Stainless steel column (15 cm x 4.6 mm, 5µm) packed with octadecyl silane bonded to porous silica Mobile phase - Acetonitrile : sodium acetate buffer (pH 4.0) (45:55 v/v) Flow rate – 1 mL/min. Detection wavelength - 239 nm.	1
5.	Ritonavir	HPLC	Stationary phase – Stainless steel column (15 cm x 4.6 mm; 3 µm) packed with end capped butyl silyl silica gel Temperature - 60 °C Mobile phase A – Butanol : tetrahydrofuran : acetonitrile : potassium dihydrogen phosphate buffer (5:8:18:69 v/v/v/v) Mobile phase B - Butanol : tetrahydrofuran : acetonitrile : potassium dihydrogen phosphate buffer (5:8:47:40 v/v/v/v) Flow rate 1.0 mL/min. Detection wavelength - 240 nm. Injection volume 50 µL	2
6.	Ritonavir tablet and capsule	HPLC	Stationary phase – Stainless steel column (5 cm x 4.6 mm, 3.5 µm) packed with octadecyl silane bonded to porous silica Column temperature - 45ºC Mobile phase – Acetonitrile : sodium acetate buffer (pH 4.0)	1

			(45:55 v/v) Flow rate 2.5 mL/min. Detection wavelength - 240 nm.	
7.	Ritonavir and Lopinavir capsule	HPLC	Stationary phase – Stainless steel column (5 cm x 4.6 mm, 5 µm) packed with octadecyl silane bonded to porous silica Mobile phase - Acetonitrile : methanol : potassium dihydrogen orthophosphate buffer (pH 3.0) (42.5:12.5:45 v/v/v) Flow rate 1.0 mL/min. Detection wavelength - 210 nm.	3
8.	Ritonavir and Lopinavir tablet	HPLC	Stationary phase – Stainless steel column (15 cm x 4.6 mm, 5 µm) packed with octadecyl silane bonded to porous silica Mobile phase A - Acetonitrile : methanol (80:20 v/v) B - Mobile phase A : potassium dihydrogen ortho phosphate buffer solution (pH 3.0) (55:45 v/v) Flow rate 1.5 mL/min. Detection wavelength - 210 nm.	3
9.	Efavirenz (API, tablet and capsule)	HPLC	Stationary phase – Stainless steel column (25 cm x 4.6 mm, 5 µm) packed with base deactivated octadecyl silyl silica gel Mobile phase - Acetonitrile : 0.86 % w/v ammonium dihydrogen phosphate buffer (pH 3.0 ± 0.05) (50:50 v/v) Flow rate 1.5 mL/min.	3

			Detection wavelength - 254 nm.	
10.	Lopinavir	HPLC	Stationary phase – Stainless steel column (25 cm x 4.6 mm, 5 µm) Packed with octyl silane bonded to porous silica Mobile phase - Solution B : 0.05 M potassium dihydrogen phosphate buffer (pH 3.0) (55:45 v/v) Solution B : Acetonitrile : methanol (80:20 v/v) Flow rate - 1.5 mL/min. Detection wavelength - 210 nm.	3

2.2 REPORTED METHODS FOR DETERMINATION OF ANTIRETROVIRAL AGENTS

2.2.1 Reported methods for determination of Tenofovir disoproxil fumarate with other drugs

Sr. No.	Matrix	Method	Parameters	Ref. No.
1.	Tablet (Tenofovir Disoproxil Fumarate and Emtricitabine)	RP-HPLC	Stationary phase - Agilent C_{18} column Mobile phase – Methanol : Phosphate buffer (pH 7.0) (65:35 v/v) Detection wavelength -260 nm. Flow rate - 1.0 mL/min., Linearity range - 5 - 70 µg/mL,	4
2.	Tablet (Tenofovir Disoproxil Fumarate and Lamivudine)	HPTLC	Stationary phase - Merck HPTLC aluminium plates of silica gel 60 F_{254}, (20 × 10 cm) with 250 µm thickness Mobile phase - Chloroform: methanol: toluene (8:2:2 v/v/v) Detection wavelength - 265nm.	5
3.	Tablet (Tenofovir Disoproxil Fumarate and Emtricitabine)	Spectro-photometry	1^{st} method – wavelength 262 nm as λ_1 (isobestic point) and 281 nm as λ_2 (λ_{max} of Emtricitabine) 2^{nd} method - wavelength 281nm 3^{rd} method – First derivative spectroscop, wavelength 226.5nm for tenofovir and 298.5 nm for emtricitabine Linearity range – 4 – 24 µg/mL	6
4.	Tablet (Tenofovir	RP-HPLC	Stationary phase - Agilent eclipse XDB-C_{18} (5 µm, 4.6 mm	7

	Disoproxil Fumarate and Lamivudine)		× 150 mm) column Mobile phase - Methanol : 0.02 M potassium dihydrogen ortho phosphate (pH 3.0) (60:40 v/v)) Detection wavelength - 260nm. Flow rate: 0.9 mL/min. Linearity range: 5 - 50 µg/mL,	
5.	Tablet (Tenofovir Disoproxil Fumarate, rilpivirine and Emtricitabine)	LC	Stationary phase - Hypersil BDS C_{18} column Mobile phase - Gradient elution using containing acetonitrile, 0.2 M Potassium dihydrogen phosphate and Water. Solvent for active ingredients - DMSO : distilled water (1:1)	8
6.	API	RP-HPLC	Stationary phase - RP-18 column (25 mm × 4.6 mm, porous material) Mobile phase - water (pH 4.0 adjusted with acetic acid) : methanol (60:40 v/v)	9
7.	Tablet (Tenofovir Disoproxil Fumarate and Lamivudine)	Spectro-photometry	Wave length - 259 nm Solvent – Methanol: water (50:50 v/v) Linearity range - 5.0-30.0 µg/mL	10
8.	Tablet (Tenofovir Disoproxil Fumarate and Lamivudine)	First derivative spectro-photometry	Wave length - 249 nm for tenofovir and 281 nm for lamivudine Solvent – Distilled water Linearity range - 10 – 60 µg/mL for tenofovir and 5 -30 µg/mL for lamivudine	11

9.	API and Tablet (Tenofovir Disoproxil Fumarate and Emtricitabine)	RP-HPLC	Stationary phase - Phenomenex Luna C_{18} (150 mm × 4.6 mm, particle size 5 µm) column Mobile phase - Acetonitrile: methanol: water (30:50:20 v/v/v) Detection wavelength – 258 nm. Flow rate - 0.6 mL/min. Retention time – 3.49 min. Linearity range - 1– 6 µg/mL	12
10.	Bulk and Tablet (Tenofovir Disoproxil Fumarate, Efavirenz and Emtricitabine)	UPLC	Column - BEH Ph column Mobile phase - Potassium dihydrogen phosphate buffer (pH 6.5) : methanol : acetonitrile (45:27.5:27.5 v/v/v) Detection wavelength – 260 nm. Linearity range - 0.024-0.088 µg/mL Retention time - less than 1.5 min	13
11.	Tablet (Tenofovir Disoproxil Fumarate and Emtricitabine)	Spectro-photometry	Wavelength - 259 nm (Tenofovir) and 281 nm (Emtricitabine) Linearity range - 6-48 µg/mL Solvent - Methanol	14
12.	Tablet (Tenofovir Disoproxil Fumarate and Emtricitabine)	RP-HPLC	Stationary phase - Luna C_{18} (25 cm× 4.60 mm, particle size 5 µm) column Mobile phase – Acetonitrile : potassium dihydrogen phosphate buffer (pH 3.0±0.05) : triethyl amine (70:30:0.5 v/v/v)	15

			Detection wavelength - 260nm Retention time - 2.27 min Linearity range - 5-50 µg/mL	
13.	Tablet (Tenofovir Disoproxil Fumarate, Efavirenz and Emtricitabine)	RP-HPLC	Stationary phase - Inertsil ODS 3V column Mobile phase - Gradient composition of 0.02M Sodium dihydrogen orthophosphate as mobile phase A and mixture of methanol and water (85:15 v/v) as mobile phase B Flow rate - 1.5 mL/min Detection wavelength - 265nm Retention time - 8.8 min Linearity range - 12-180 µg/mL	16
14.	Rat Plasma (with several antiretroviral nucleosides)	HPLC	Stationary phase - Atlantis C_{18} column Mobile phase - 5 mM acetic acid-hydroxylamine buffer (pH 7) : acetonitrile gradient elution Linearity range - 30-10,000 ng/mL Detection wavelength - 260nm	17

2.2.2 Reported methods for determination of Tenofovir disoproxil fumarate

Sr. No	Matrix	Method	Parameters	Ref .No
1.	Tablet	Spectro-photometry	1st method - Complexation with ammonium molybdate Wavelength - 495 nm Molar absorptivity - 1234.09 mol/L/cm 2nd method - Complexation with picric acid Wavelength – 465 nm Molar absorptivity - 12,330.92 mol/L/cm	18
2.	Human Plasma	Isotope dilution MALDI-triple quadrupole tandem mass spectrometry	m/z 288.0 → 176.2 and m/z 293.2 → 181.2 for TNV LOD - 0.10 µmol/L LOQ - 0.04 µmol/L Observed plasma TNV concentrations ranged between 0.11 and 0.76 µmol/L	19
3.	API and Tablet	UV spectroscopy	Wavelength - 261nm. Solvent – 0.1N HCl Linearity range - 5 – 90 µg/mL	20
4.	Tablet	Spectro-photometry	1st method Reduction in presence of 1,10-phenanthroline Wavelength – 500.2 nm. Linearity range - 2-10 µg/mL. 2nd method Reduction in presence of 2, 2-bipyridyl	21

			Wavelength - 511.2 nm. Linearity range - 5-25 µg/mL. 3rd method – oxidation Wavelength - 640 nm Linearity range - 5-25 µg/mL.	
5.	API and tablet	RP-HPLC	Stationary phase - Base deactivated C_{18} column (Hypersil BDS 25 cm × 4.6 mm, 5 µm) maintained at 30°C. Mobile phase – Acetonitrile : tetrabutylammonium phosphate buffer (pH 6) : water: A – (2:20:78 v/v/v) B – (65:20:15 v/v/v). Flow rate - 1.0 mL/min Linearity range - 0.1µg/mL - 0.15 mg/mL.	22
6.	Tablet	RP-HPLC	Stationary phase - Luna C_{18} (25 cm × 4.6 mm, 5 µm) Mobile phase - 0.1% formic acid : acetonitrile (50:50 v/v) Flow rate - 0.8 mL/min Retention time: 6.718 min. Linearity range - 5-30 µg/mL Detection wavelength - 305nm.	23
7.	Human Plasma	RP-HPLC	Stationary phase - C_{18} (25 cm × 4.6 mm, 5 µm) Mobile phase – Acetonitrile : Water (75:25 v/v) Flow rate - 1mL/min. Linearity range - 0.2-10 µg/mL Detection wavelength - 259nm. Internal standard - Piroxicam	24

2.2.3 Reported methods for determination of Ritonavir with other drugs

Sr. no.	Matrix	Method	Parameters	Ref. no.
1.	Human Plasma (Atazanavir and Ritonavir)	LC-MS/MS	Stationary phase – Ascentis C_{18} column (50 mm ×4.6 mm, 5 µm) Elution mode - Gradient Linearity range - 19.1 ng/mL - 2066.3 ng/mL Imprecision - < 13.8% Inaccuracy - ± 7.1%	25
2.	Solid Lipid Nanoparticle (Ketoconazole, Ritonavir and Lopinavir)	RP-LC	Stationary phase - Kromasil C_{18} column (250 mm × 4.5 mm, 5 µm) Mobile phase – Acetonitrile : 0.2% triethylamine (pH 6.5) (60:40 v/v) Detection wave length - 210 nm Flow rate - 1 mL/min.	26
3.	API (Intermediate of Ritonavir and Lopinavir)	Chiral LC	Stationary phase - chiralcel OD-H column (250mm × 4.6 mm, 5 µm) Mobile phase - n-Hexane : ethanol : trifluoroacetic acid : triethylamine (950:50:1:1 v/v) Detection wave length - 210 nm Flow rate - 1.0 mL/min.	27
4.	Human Plasma (Ritonavir, Etravirine, and 9 Other Antiretroviral	LC-MS/MS	Stationary phase: C_{18} HPLC column (Waters Sunfire 100 mm × 2.1 mm, 3.5 µm) Elution mode - Gradient Flow rate - 0.3 mL/min. Precision - ±20%	28

	Agents)		Accuracy – 80 – 120%	
5.	Human Plasma (Lopinavir and Ritonavir)	Ultrafast LC-MS/MS	Stationary phase - HT column (30 mm × 2.1 mm, 1.8 µm) Mobile phase – Acetonitrile : water (55:45 v/v) Flow rate - 0.5 mL/min.	29
6.	Human Plasma (with other HIV protease inhibitors)	HPLC-MS	Stationary phase - ODS3 column (50 mm x 2.0 mm, 5 µm) Mobile phase - Acetate buffer (pH 5) : methanol Elution mode - Gradient Flow rate - 0.5 mL/min.	30
7.	Soft Gelatin Capsule (Lopinavir and Ritonavir)	LC-MS	Stationary phase - LiChrospher 100 RP-18 (250 mm × 4.6 mm, 5 µm) Mobile phase – Acetonitrile : water : methanol (53:37:10 v/v/v) Flow rate - 1.0 mL/min. Detection wavelength - 210 nm.	31
8.	Tablet (Atazanavir sulfate and Ritonavir)	Spectro-photometry	Solvent - Methanol Wavelength - 238.5 nm as λmax of ritonavir and 249.5 nm for atazanavir sulfate Linearity range - 10-50 µg/mL	32
9.	Tablet (Ritonavir and Lopinavir)	Spectro-photometry	Solvent - Methanol Wavelength - 239 nm and 259 nm as λmax of ritonavir and lopinavir respectively. Isoabsoptive point - 257.4 nm. Linearity range - 5-30 µg/mL	33
10.	Tablet (Ritonavir and Lopinavir)	Spectro-photometry	Solvent - Acetonitrile Wavelength - 239 nm and 259 nm as λmax of ritonavir and	34

			lopinavir respectively. Simultaneous equation method Linearity range - 5-50 µg/mL for ritonavir and 20-120 µg/mL for lopinavir	
11.	Tablet (Ritonavir and Lopinavir)	Spectro-photometry	Wavelength - 240 nm and 259 nm as λmax of ritonavir and lopinavir respectively. Simultaneous equation method Solvent - Methanol Linearity range - 15-55 µg/mL for ritonavir and 10-50 µg/mL for lopinavir	35
12.	Human Plasma (16 anti -HIV agents)	HPLC	Stationary phase - C_{18} Symmetry column (250 mm × 4.6 mm, 5 µm) Mobile phase : 0.01 M KH_2PO_4 buffer : acetonitrile Elution mode - Gradient Flow rate - 1.0 mL/min Detection wave length - 240 nm	36
13.	Human Plasma (Saquinavir and Ritonavir)	RP- HPLC	Stationary phase - C_{18} column (150 mm ×4.6 mm, 5 µm) Mobile phase - Acetonitrile : 70 mM KH_2PO_4 buffer (pH 5) (46:54 v/v) Detection wave length - 210 nm. Retention time - 8.3 min.	37
14.	Human Plasma (nine antiretroviral agents)	HPLC	Stationary phase - C_8 column. Mobile phase - Gradient mobile phase 25 mM KH_2PO_4 : methanol : acetonitrile	38

			Detection wavelength - 210 nm LOQ - 50 ng/mL	
15.	Human Plasma (with other antiproteases)	HPLC	Stationary phase - Two Ph columns (100 mm × 4.6 mm, and 250 mm × 4.6 mm) Mobile phase - Acetonitrile : 0.03 M disodium hydrogen phosphate (55:45 v/v) Detection wavelength - 240 nm Linearity range - 0.05 to 10 µg/mL	39
16.	Human Serum (Ritonavir and Saquinavir)	HPLC	Stationary phase - C_8 column after solid phase extraction with C_{18} cartridge Detection wavelength – 240 nm. Linearity range - 0.5-32 µg/mL	40
17.	Human Plasma (Indinavir, Amprenavir, Nelfinavir and its active metabolite M8, Saquinavir, Ritonavir, Lopinavir, Nevirapine and Efavirenz)	HPLC	Stationary phase - C_{18} column. Mobile phase - Binary gradient elution with solvent A and B A - Acetonitrile : 0.025M tetra methyl ammonium perchlorate (55:45 v/v) B - Methanol : 0.025M tetra methyl ammonium perchlorate (55:45 v/v) Detection wavelength - 259 nm, LOQ - 0.10 mg/L	41
18.	Human Plasma (Indinavir, Ritonavir and	HPLC	Stationary phase - Stainless steel column packed with 5 µm Phenomenex phenyl hexyl material operated at 40 ^{0}C	42

	Lopinavir)		Mobile phase – Acetonitrile : 10 mM Potassium phosphate buffer (50:50 v/v). LOQ - 100 µg/mL	
19.	Tablet (Lopinavir and Ritonavir)	HPTLC	Stationary phase - Aluminium backed silica gel $60F_{254}$ HPTLC plate Mobile phase - Ethyl acetate : ethanol : toluene: diethylamine (7:2.0:0.5:0.5 v//v/v/v) Detection wavelength - 266 nm Linearity range - 2-10 µg/mL	43
20.	Capsule (Lopinavir and Ritonavir)	HPTLC	Stationary phase - Aluminium backed silica gel $60F_{254}$ HPTLC plate Mobile phase – Toluene : ethyl acetate : methanol : ammonia (6.5:2.5:0.5:0.5 v/v/v/v) LOD - 1.5 ng/spot and 4.6 ng/spot LOQ – 21.00 ng/spot and 5.10 ng/spot for lopinavir and ritonavir respectively Linearity range - 6.5 - 20.00 µg /spot and 1.5 - 5.00 µg /spot for lopinavir and ritonavir respectively	44
21.	Capsule (Lopinavir and Ritonavir)	HPTLC	Stationary phase – Aluminum backed silica gel $60F_{254}$ HPTLC plate Mobile phase – Toluene : ethyl acetate : methanol : glacial	45

			acetic acid (7.0:2.0:0.5:0.5 v/v/v/v) Linearity range - 6.67-20.00 and 1.6- 5.00 µg/spot for lopinavir and ritonavir, respectively LOQ – 7.00 ng/spot and 21.00 ng/spot for lopinavir and ritonavir respectively Detection wavelength – 263 nm	
22.	Human Plasma (Indinavir, Amprenavir, Saquinavir, Ritonavir, Nelfinavir and Efavirenz)	HPLC	Stationary phase – Nucleosil (100 mm x 4.6 mm, 5 µm) C_{18} AB column Mobile phase - Acetonitrile : phosphate buffer (pH 5.15) Elution mode - Gradient Detection wavelength - 201 nm Retention time: 26.5 min for ritonavir Internal standard - Clozapine	46
23.	Human Plasma (Indinavir, Amprenavir, Saquinavir, Ritonavir and Nelfinavir)	RP-HPLC	Stationary phase - Narrowbore C_{18} column Gradient elution Detection wavelength - Dual detection at 265 and 210 nm Linearity range - 25-10000 ng/mL	47

2.2.4 Reported methods for determination of Ritonavir

Sr. No	Matrix	Method	Parameter	Ref No.
1.	API and tablet	RP-HPLC	Stationary phase - HiQSil C_{18} column (25 cm × 4.60 mm, 5 µm) Mobile phase – Methanol : acetonitrile : water (87:10:3 v/v/v) Detection wavelength - 240 nm retention time : 3.6 min. Flow rate : 1.0 mL/min. Linearity range - 25-200 µg/mL	48
2.	API	LC-MS/MS	Stationary phase - Waters XTerra C_{18} column (250 mm × 4.6 mm, 5 µm) Mobile phase – Water : methanol : acetonitrile (40:20:40 v/v/v)	49
3.	Capsule	Spectro-photometry	Method – 2^{nd} derivative spectroscopy Wavelength - 222.3 nm Solvent - Methanol Linearity range - 10.0-30.0 µg/mL Correlation coefficient – 0.9995	50
4.	API and tablet	HPLC	Stationary phase - Hypersil BDS C_{18} column (25 cm × 4.6 mm, 5 µm), Mobile phase – Acetonitrile : phosphate buffer (pH 4.0) : water, gradient mobile phase Detection wavelength - 240 nm	51
5.	Soft Gelatin Capsule	LC- MS	Stationary phase – Reverse phase C_8 (125mm × 4.0 mm , 5 µm) Mobile phase - Methanol : water	52

			(67:33 v/v) Detection wavelength - 210 nm Linearity range - 100-300 µg/mL	
6.	Tablet	Spectro-photometry	Wavelength - 510 nm Linearity range - 20-100 µg/mL Reagent for chemical derivatization - Methanol and ferric chloride	53
7.	API and tablet	RP-HPLC and HPTLC	**HPLC Method** Stationary phase - Eclipse XBD C_{18} RP column (150mm x 4.6mm, 15µm) Mobile phase – Acetonitrile : water (60:40 v/v) Detection wavelength - 209 nm Flow rate - 1.4mL/min. **HPTLC Method** Stationary phase - Aluminum backed silica gel 60F254 HPTLC plate Mobile phase – Toluene : ethyl acetate : methanol : glacial acetic acid) (7.0:2.0:0.5:0.5 v/v/v/v) Detection wavelength - 263 nm	54

2.2.5 Reported methods for determination of Efavirenz with other drugs

Sr. No.	Matrix	Method	Parameter	Ref No.
1.	Tablet (Zidovudine, Lamivudine and Efavirenz)	RP-HPLC-DAD	Stationary phase - Phenomenex C_{18} column (250 mm × 4.6 mm, 5µm) Mobile phase - Binary gradient acetonitrile : water Detection wave length - 248nm Linearity range - 40µg/mL	55
2.	API and tablet (Saquinavir mesylate and Efavirenz)	Spectro-photometry	Wavelength - 239 and 247 nm for saquinavir mesylate and efavirenz respectively Solvent - Methanol Linearity range - 1.25-10 µg/mL for saquinavir mesylate and 2.5-12.5 µg/mL for efavirenz	56
3.	Human Plasma (Six HIV protease inhibitors and two non-nucleoside reverse transcriptase inhibitors)	HPLC	Method for extraction – Liquid-liquid extraction with diethyl ether Stationary phase – RP C_{18} column, Mobile phase - Acetonitrile : 50 mM phosphate buffer (pH 5.65) gradient mobile phase Detection wavelength - 240 nm Linearity range - 100-10000 ng/mL LOQ – 50 ng/mL	57

2.2.6 Reported methods for determination of Efavirenz

Sr. No	Matrix	Method	Parameter	Ref .No
1.	Tablet	RP-HPLC	Stationary phase - Hypersil BDS C_{18} (250 mm × 4.6 mm, 5µm) column Mobile phase – Acetonitrile : 0.03M KH_2PO_4 buffer (pH 3.2) (60:40 v/v) Flow rate - 0.8mL/min Detection wavelength - 260 nm Retention time - 10.549 min Linearity range - 12-144 µg/mL, LOD - 0.12 µg/mL, LOQ - 0.36 µg/mL	58
2.	Tablet	RP-HPLC	Stationary phase - C_{18} (250 mm × 3.9 mm, 10 µm) column Mobile phase – Acetonitrile : water : orthophosphoric acid (70:30:0.1 v/v/v) Detection wavelength - 252 nm	59
3.	API and tablet	RP-HPLC	Stationary phase - Sunfire C_{18} (250 mm × 4.6 mm, 5µm) column Mobile phase - Methanol : acetonitrile (7:3 v/v) Detection wavelength - 249 nm Linearity range - 10-40µg/mL	60
4.	Saliva	LC-MS/MS	Stationary phase - Phenomenex Kinetex C_{18} (150 mm × 3 mm, 2.6 µm) column Mobile phase – Gradient elution with increasing the proportion of methanol	61

			Flow rate - 0.4 mL/min., Retention time - 6.5 min Linearity range - 3.125-100 µg/L LOD - 1.84 µg/L LOQ - 6.11µg/L, Ionization mode: electrospray positive ionization	
5.	API	HPLC	Stationary phase - Chiralpak-IA, Detection wavelength - 252 nm Flow rate - 2.5 mL/min, Resolution - > 4.0 Correlation coefficient - 0.9999	62
6.	API	HPLC	Stationary phase: Chiralcel OD (250 mm × 4.6 mm, 10 µm) column containing tris- (3,5-dimethyl phenyl carbomate). Mobile phase - n-Hexane : iso propyl alcohol (80:20 v/v) Detection wavelength - 254 nm Flow rate - 1.0 mL/min. Linearity range - 0.1- 6 µg/mL LOD - 0.03 µg/mL LOQ - 0.1 µg/mL	63
7.	API and tablet	Spectro-photometry	Wavelength - 245 nm Solvent – Methanol : water (80:20) Linearity range - 5 - 50µg/mL.	64
8.	API and tablet	Spectro-photometry	Wavelength - 247 nm Solvent - Sodium lauryl sulfate (SLS) (1%w/v).	65
9.	API and capsule	Spectro-photometry	Wavelength - 247nm for normal UV spectroscopy and 258 nm for first order derivative spectroscopy Solvent - Acetonitrile,	66

			Linearity range - 4-24 µg/mL	
10.	API and tablet	HPTLC	Stationary phase - Aluminum plate precoated with silica gel 60 F_{254} Mobile phase – Dichloromethane : methanol (5:0.3 v/v) Detection wavelength - 247 nm Linearity range - 400-2000 ng/spot R_f value - 0.72 ± 0.03	67
11.	API and tablet	HPLC	Stationary phase - Novak ph column Mobile phase - Phosphate buffer : acetonitrile (50:50 v/v) Detection wavelength - 247 nm Linearity range - 0.05-0.15 mg/mL	68

2.2.7 Reported methods for determination of Lopinavir with other drugs

Sr. No.	Matrix	Method	Parameter	Ref. No.
1.	Human Plasma (HIV non-nucleoside reverse transcriptase inhibitors and protease inhibitors)	UPLC	Stationary phase - Acquity UPLC BEH C_{18} (150 mm × 2.1 mm, 1.7 µm) column Mobile phase - Acetonitrile : 5mM trimethyl ammonium phosphate buffer (pH 3.0) in gradient mode Run time - 9.5 min. Linearity range – 0.1 -10 µg/mL Accuracy – 94.9 - 103.5% LOQ – 0.1µg/mL	69
2.	Tablet (Lopinavir and Ritonavir)	RP-HPLC	Stationary phase - Phenomenex Gemini C_{18} (250 mm × 4.6 mm, 5 µm) column Mobile phase - Potassium dihydrogen phosphate buffer (pH 6.0 ± 0.1) : acetonitrile : methanol (50:35:15 v/v/v) Flow rate - 1.0 mL/min Detection wavelength - 254 nm Retention time - 6 min for lopinavir and 3.7 min for ritonavir	70
3.	Plasma	HPLC-MS/MS	Internal standard: Quinoxaline Stationary phase - RP C_{18} (250 mm × 4.6 mm, 5 µm) column Mobile phase - Acetonitrile : 0.05% formic acid in gradient mode Detection - Via selective reaction monitoring (SRM) on a triple-quadrupole mass spectrometer	71

			Run time - 5 min. Correlation coefficient - 0.998	
4.	Syrup (Lopinavir and Ritonavir)	HPLC	Stationary phase - RP C_{18} (250 mm × 4.6 mm, 5 µm) column Mobile phase - 10 mM ammonium acetate buffer (pH 7) : acetonitrile (50:50 v/v) Flow rate - 1mL/min Detection wavelength - 245 nm Retention time - 14.7 min. and 12.4 min. for lopinavir and ritonavir respectively. Correlation coefficient - 0.999	72
5.	Dry plasma spot (Nine anti-HIV drugs)	HPLC-MS	Internal standard - Quinoxaline Extraction - With 4 mL tert-butyl methyl ether (TBME) and 500 µL ammonia (15%) Supernatant dissolved in water : acetonitrile (60:40 v/v) Stationary phase – Atlantis T3 (150 mm x 2.1 mm, 3µm) column Mobile phase - Water (0.05% formic Acid) : acetonitrile (0.05% formic acid) in gradient mode Detection - By ESI single quadrupole mass spectrometry	73
6.	Human Plasma (Lopinavir and Ritonavir)	UPLC-ESI-MS/MS	Extraction- Solid phase by using Waters Oasis HLB cartridge Stationary phase – Waters acquity UPLC BEH C_{18} (50 mm × 2.1 mm, 1.7 µm) column Mobile phase - 10 mM ammonium formate (pH 4.0) : methanol (10:90	74

			v/v) Run time - 1.2 min. Detection – By triple quadrupole mass spectrometer Linearity range - 29.6-14379 ng/mL	
7.	Human Plasma (Raltegravir, Lopinavir and 11 Other Antiretroviral Agents)	HPLC - PDA	Internal standard - Quinoxaline Stationary phase - RP C_{18} (150 mm × 2.0 mm, 5 µm) column Mobile phase - Acetonitrile : phosphate buffer in gradient mode Run time - 28 min Correlation coefficient - 0.998	75
8.	Dried Blood Spot (Protease inhibitors and non-nucleoside reverse transcriptase inhibitors)	HPLC –MS	Stationary phase - RP C_{18} (150 mm × 2.0 mm, 5 µm) column Mobile phase - Acetonitrile,: methanol : 0.2M zinc sulphate in water (1:1:2 v/v/v) Run time - 10 min Detection - Triple quadrupole mass spectrometry Linearity range 0.1 – 20 mg/mL	76
9.	Human Plasma (Lopinavir, Darunavir, and 10 other antiretroviral agents)	HPLC-MS	Internal standard - Quinoxaline Stationary phase - RP C_{18} (150 mm × 2.1 mm, 5 µm) column Mobile phase - Acetonitrile : water with 0.05% formic acid in gradient mode Run time - 25 min Detection – Mass spectrometry	77
10.	Human Plasma (HIV protease	LC-MS	Stationary phase - RP C_{18} (150 mm × 2.1 mm, 5 µm) column Mobile phase - Acetate buffer (pH	78

	inhibitors and non-nucleoside reverse transcriptase inhibitors)		5) : Methanol in gradient mode Flow rate - 0.25mL/min Run time - 10 min Detection - Triple quadrupole mass spectrometry Linearity range - 0.1 to 20 µg/mL	
11.	Peripheral blood mononuclear cells and human plasma (Lopinavir and Ritonavir)	LC/MS/MS	Extraction method – Liquid - liquid extraction Internal standard - 2H5-saquinavir Stationary phase – Phenomenex Jupiter Proteo C_{12} 90A (100 mm × 2mm, 4µm) column Mobile phase - Acetonitrile : 20 mM ammonium acetate buffer : aqueous acetic acid (55:45:0.1 v/v) Run time – 6 min. Detection – Electrospray tandem mass spectrometry LOQ - 4.0 ng/mL in plasma, 0.2 ng/mL in ultrafiltrate and 0.1 ng/cell pellet in PBMCs	79
12.	Human Plasma (Non-nucleoside reverse transcriptase inhibitor and Protease inhibitor)	HPLC	Stationary phase - S-3 (150 mm × 2.1 mm, 5 µm) column Mobile phase – 25 mM potassium phosphate (pH 4.9) : acetonitrile (52:48 v/v) Detection - 212 nm Accuracy - 91.0 - 112.8%	80
13.	Human Plasma (Amprenavir,	HPLC with UV and fluorimetric	Stationary phase - Allsphere hexyl (150 mm × 4.6 mm, 5 µm) column Extraction method - Liquid liquid	81

	Indinavir, Atazanavir, Ritonavir, Lopinavir, Saquinavir, Nelfinavir, and M8-Nelfinavir metabolite)	detection	extraction with Hexane from buffered samples with borate buffer pH 9.0 Mobile phase – Acetonitrile : methanol : 15 mM sodium dihydrogen phosphate buffer (pH 4.5) (35:20:45 v/v/v) Flow rate – 1.0 mL/min. Detection wavelength – 215 nm in UV and 280 and 340 nm in fluorimetry as excitation and emission wavelengths respectively Linearity range - 0.025–10 mg/L	
14.	Human Plasma (Lopinavir and Nevirapine)	HPLC after solid phase extraction	Internal standard - Clozapine Extraction - Solid-phase extraction on a solid phase cartridge Stationary phase – Nucleosil (100 mm, 5 µm) column Mobile phase - Acetonitrile : phosphate buffer (pH 5.07) : 0.02% sodium heptane sulfonate in gradient elution mode Detection wavelength - 201 and 282 nm	82
15.	Human Plasma (Indinavir, Amprenavir, Ritonavir, Lopinavir, Saquinavir, Nelfinavir and the Nelfinavir	HPLC	Stationary phase – Nova-Pak C_{18} (150 mm × 3.9 mm, 5 µm) column Mobile phase – Acetonitrile : 5mM sodium dihydrogen phosphate buffer (pH 6.0) containing triethylamine Detection wavelength - 210 and 239 nm Linear range: 25–5000 ng /mL	83

	active metabolite M8)			
16.	Human Plasma (HIV protease inhibitors and non-nucleoside reverse transcriptase inhibitors)	HPLC	Stationary phase – Zorbax C_{18} (150 mm × 4.6 mm, 3.5 µm) column Mobile phase – 50 mM phosphate monobasic buffer (pH 4.5) : methanol : acetonitrile with TFA (25:15:60 v/v/v) Flow rate - 0.9-1.1 mL/min Run time - 30 min Linearity range - 25–5000 ng/mL	84
17.	Human Plasma (16 anti-HIV drugs)	HPLC	Stationary phase – Symmetry C_{18} (250 mm × 4.6 mm, 5 µm) column Mobile phase - 0.01 M KH_2PO_4 : acetonitrile in gradient mode Detection wavelength - 240 and 260 nm Flow rate - 1 mL/min Run time - 35 min	85
18.	API and tablet (Lopinavir, Ritonavir and Efavirenz)	HPLC	Stationary phase – RP C_{18} (250 mm × 4.6 mm, 5 µm) column Mobile phase – Acetonitrile : methanol : tetramethyl ammonium perchlorate (TMAP) in dilute aqueous trifluoroacetic acid (45:5: 50 v/v/v) Column temperature - 30ºC	86
19.	Tablet (Lopinavir and Ritonavir)	RP-HPLC	Stationary phase– RP phenomenex - luna C_{18} (250 mm × 4.6 mm, 5 µm) column Mobile phase - Acetonitrile :	87

			triethylamine (67:33 v/v) Internal standard - Ambroxol LOD - 30 ng/mL Recovery - 99.9%	
20.	API (Lopinavir)	MALDI – TOF mass spectro-metry	Matrix – Meso tetrakis (pentafluoro phenyl) porphyrin Spotting technique - Brushing with prestructured target plates Samples cleaning - By solid-phase extraction plate Samples spotting - By a pipetting robot	88
21.	Human Plasma (Six HIV protease inhibitors, one metabolite, and two non-nucleoside reverse transcriptase inhibitors)	RP-HPLC	Extraction method - Liquid - liquid extraction by diethyl ether From 250 µL plasma samples Stationary phase – X-TERRA column Mobile phase – Water (with 3 mM pyrolidine) : acetonitrile (58:42 v/v) Detection - UV detection with diode array detector Linearity range - 25 ng/mL - 9000 ng/mL	89
22.	Human Plasma (Amprenavir, Indinavir, Lopinavir, Nelfinavir, M8 Nelfinavir metabolite, Ritonavir, Efavirenz,	Column liquid chromato-graphy	Extraction method – Solid phase extraction Stationary phase – Novapak RP C_{18} (150 mm × 2.1 mm, 4 µm) column Mobile phase A - Buffer (pH 5) : acetonitrile : methanol (42.5:28:29.5 v/v/v) over first 32 min. and 22 min after mobile phase B	90

	and Saquinavir)		(Buffer consisted of 0.5% 5.8M orthophosphoric acid and 0.02% triethylamine adjusted at pH 5.0 with 10 mol sodium hydroxide Mobile phase B – Acetonitrile : water (75:25 v/v) for 38 min. Flow rate – 0.45 mL/min. Detection wavelength – Dual detection at 265 nm and 210 nm Linearity range - 25 - 5,000 ng/mL	
23.	Human Plasma (Lopinavir , Indinavir, Amprenavir, Saquinavir, Ritonavir and Nelfinavir)	HPLC	Extraction - With 500µl 0.1M ammonium hydroxide solution and 5 mL tertiary butyl ether Stationary phase – C_{18} (250 mm x 4.6 mm, 5µm) column Mobile phase - 50 mM phosphate buffer (pH 5.40) : acetonitrile (50:50 v/v) Detection wavelength - 215 nm	91
24.	Peripheral blood mononuclear cells (Fourteen antiretroviral agents)	HPLC-MS	Internal standard - Quinoxaline Extraction - With methanol : water (70:30 v/v) Supernatant dissolved in water : acetonitrile (60:40 v/v) Stationary phase – Atlantis T3 (150 mm x 2.1 mm, 3µm) column Mobile phase - Water (0.05% formic Acid) : acetonitrile (0.05% formic acid) in gradient mode Detection - By ESI single quadrupole mass spectrometry Linearity range - 0.1 to 32 ng/mL	92

25.	Peripheral blood mononuclear cells (10 anti-retrovirals)	LC-MS/MS	Extraction - With methanol : water (60:40 v/v) Stationary phase – Symmetry Shield RP 18 (50 mm x 2.1 mm) column Eluent composition A – 10 mM ammonium acetate : 10 mM formic acid (50:50 v/v) B - Acetonitrile : 10 mM formic acid (50:50 v/v) Mobile phase – A : B (95:5 v/v) from 0 – 2 min. Linear gradient elution upto A : B (10:90 v/v) at 12 min. Detection - Electro spray positive ionization Linearity range - 0.25-125 ng/mL Correlation coefficient - > 0.99	93

2.2.8 Reported methods for determination of Lopinavir

Sr. No	Matrix	Method	Parameter	Ref. No.
1.	Tablet	Spectro-photometry	1st derivative spectroscopy Wavelength - 220 nm Solvent - Methanol Linearity range - 5 – 35 µg/mL LOD - 0.844 µg/mL LOQ - 2.558 µg/mL	94
2.	API and tablet	RP-HPLC	Stationary phase – Zorbax SB C_{18} 5 µm column Mobile phase - Phosphate buffer (pH 4.0): acetonitrile (55:45 v/v) Flow rate - 1.5 mL /min Detection wavelength - 210 nm	95
3.	API	HPLC	Stationary phase – YMC Pack ODS-AQ column Mobile phase - 0.02 M KH_2PO_4 (pH 2.5) : acetonitrile in gradient mode Temperature - 45^0C Detection wavelength - 210 nm LOD - 0.028 µg/mL - 0.063 µg/mL LOQ - 0.084 µg/mL - 0.192 µg/mL	96
4.	Cerebo spinal fluid and plasma	LC-MS	Stationary phase – Waters Symmetry C_{18} (250 mm × 4.6 mm, 5 µm) column Mobile phase – 5 mM ammonium acetate buffer (pH 3.5) : acetonitrile (95:5 v/v) Linearity range - 0.313-25.0 ng/mL	97

			Detection - By triple quadrupole mass spectrometer	
5.	API and tablet	HPLC	Stationary phase – C_8 column Mobile phase - 50 mM potassium phosphate buffer : acetonitrile : methanol (40:50:10 v/v/v) Flow rate - 1 mL/min Detection wavelength - 210 nm	98
6.	Human Plasma	HPLC	Extraction – Liquid solid extraction on OASIS HLB column Stationary phase – Xterra, C_8 (150mm x 3.9 mm) column Mobile phase – acetonitrile : water (41:59 v/v) Detection wavelength - 210 nm Linearity range - 0.187-10.0 µg/ mL LOQ - 0.187 µg/mL	99

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CHAPTER 3

AIM OF PRESENT WORK

3. AIM OF PRESENT WORK

Most of the antiviral agents are used in HIV, herpes, hepatitis B and C and influenza A and B viruses.

Objective of the present work:

All the manufacturing industries are using sophisticated instruments like HPLC, HPTLC and LC-MS; hence the aim of the present work was to develop and validate sensitive and selective methods for the determination of novel antiretroviral agents in bulk and their pharmaceutical formulations. Literature review reveals that very few methods are reported for estimation of Lopinavir, Ritonavir, Efavirenz and Tenofovir disoproxil fumarate. Therefore it was thought of interest to develop and validate stability indicating RP-HPLC, HPTLC and UV spectroscopic methods for determination of selected drugs in bulk, their dosage forms and biological fluid.

Specific aim of the present work:

- To develop and validate stability indicating reverse phase high performance liquid chromatographic method for estimation of Efavirenz and Tenofovir disoproxil fumarate.

- To develop and validate reverse phase high performance liquid chromatographic method for estimation of Lopinavir and Ritonavir in bulk and their combined tablet dosage form.

- To apply the developed RP-HPLC methods for determination of Efavirenz, Tenofovir disoproxil fumarate, Lopinavir and Ritonavir in plasma and to validate the developed RP-HPLC methods for determination of selected drugs in plasma.

- To develop and validate high performance thin layer chromatographic (HPTLC) methods for estimation of Tenofovir disoproxil fumarate,

Efavirenz, Lopinavir and Ritonavir in bulk and their combined tablet dosage form.

- To develop and validate difference and derivative UV Spectrophotometric methods for the estimation of Efavirenz, Tenofovir disoproxil fumarate and Ritonavir in bulk and tablet dosage form.

- Statistical comparision of the developed methods by applying *F*-test (ANOVA) and paired *t*-test.

CHAPTER 4

STABILITY INDICATING RP-HPLC METHODS FOR DETERMINATION OF ANTIRETROVIRAL AGENTS

4. STABILITY INDICATING RP-HPLC METHODS FOR DETERMINATION OF ANTIRETROVIRAL AGENTS

4.1. STABILITY INDICATING RP-HPLC METHOD FOR DETERMINATION OF EFAVIRENZ

4.1.1 EXPERIMENTAL:

4.1.1.1 Instrumentation

- A Perkin Elmer (USA) HPLC system (series 200) equipped with Perkin Elmer series 200 pump system having back pressure 5000 psi, manual injector of 20 µL loop, UV-Visible detector and Brownlee C_{18} column (250 mm x 4.6 mm i.d., 5 µm)
- BP211D,Sartorious Gottingen AG (Germany), analytical balance
- An ultra-sonic cleaner (TEC-4, Roop Telesonic Ultrasonix)
- A Shimadzu model 1800 double beam UV/Vis. spectrophotometer with a pair of 10 mm matched quartz cells

4.1.1.2 Reagents and Materials

- Efavirenz (EFV) was kindly gifted by Aurobindo Pharmaceuticals Ltd., Hyderabad, India.
- Acetonotrile and water HPLC grade (Rankem, RFCL Ltd., New Delhi)
- Tablets (ESTIVA 600®, Genix Pharma) containing efavirenz (600 mg) were purchased from local market.
- Nylon membrane filter 0.45 µm (Gelman laboratory, Mumbai, India)
- Hydrogen peroxide, sodium hydroxide and hydrochloric acid (36%) AR grade (Finar Chemicals Pvt. Ltd, Ahmedabad, India)
- Ammonium acetate crystalline pure (E. Merck, Mumbai, India)

4.1.1.3 Chromatographic Conditions

The chromatographic separation was achieved on Brownlee C_{18} column, using mobile phase comprised of acetonitrile : 10mM ammonium acetate buffer (pH 6.5 ± 0.05) (80:20 v/v), at a flow rate of 1.0 mL/min. The mobile phase was filtered through nylon 0.45 µm membrane filter and was degassed before use. The determination was carried out at 254 nm wavelength by UV-

Visible detector. The injection volume was 20 µL and total run time was 10 min. The analysis was performed at 25 ± 2 ^{0}C temperatures.

4.1.1.4 Preparation of the Mobile Phase

The mobile phase was prepared by mixing 80 mL acetonitrile and 20 mL 10mM ammonium acetate buffer (pH 6.5 ± 0.05) previously filtered through 0.45 µm nylon membrane filter. The mobile phase was degassed for 15 minutes by sonicating the solution before use.

4.1.1.5 Preparation of standard solution

Accurately weighed EFV (25 mg) was transferred to a 25 mL volumetric flask, dissolved in and diluted to the mark with acetonitrile to obtain a standard stock solution (1 mg/mL).

4.1.1.5A Preparation of working standard solution (10 µg/mL)

Standard solution (0.1 mL) was transferred in a 10 mL volumetric flask and diluted up to the mark with mobile phase.

4.1.1.5B Preparation of hydrochloric acid (0.1N)

Accurately transferred 0.85 mL concentrated hydrochloric acid (36%) to 100 mL volumetric flask and diluted up to the mark with distilled water.

4.1.1.5C Preparation of sodium hydroxide (0.1N)

Accurately weighed and transferred 0.4 gm sodium hydroxide to 100 mL volumetric flask, dissolved in 60 mL distilled water and diluted up to the mark with distilled water.

4.1.1.6 Selection of Wavelength for Determination

The working standard solution of EFV (10 µg/mL) was scanned in the range of 200-400 nm using mobile phase as blank. Maximum absorbance was observed at 254 nm which was selected for the determination.

4.1.1.7 Analysis of Tablet Dosage Form

Twenty tablets were weighed and average weight was calculated. The tablets were finely powdered; a quantity of powder equivalent to 25 mg EFV was weighed accurately and transferred to a 25 mL volumetric flask containing 15 mL acetonitrile, and sonicated for 15 minutes. Allowed to stand at room temperature for 5 min and the volume was made up to the mark with acetonitrile to obtain the sample stock solution (1 mg/mL). The solution was filtered through 0.45 µm membrane filter. Aliquot (1 mL) was taken and transferred to 10 mL volumetric flask and volume was made up to the mark with acetonitrile to give a solution containing 100 µg/ml EFV. The solution (2 mL) was transferred to 10 mL volumetric flask and diluted up to the mark with mobile phase to give a solution containing 20 µg/mL EFV. An aliquot (20 µL) was injected and the chromatogram was recorded. The peak area was noted and the amount of EFV was calculated from the regression equation.

4.1.1.8 FORCED DEGRADATION STUDY

EFV was subjected to various forced degradation conditions to effect partial degradation of the drug preferably in 20-80% range. The study provides information about the conditions in which the drug is unstable so that measures can be taken during formulation to avoid potential instabilities.

4.1.1.8.1 Effect of Acid, Alkaline and Neutral Hydrolysis

Accurately weighed EFV (10 mg) was transferred to three different 50 mL volumetric flasks and dissolved in acetonitrile (10 mL). Hydrochloric acid (0.1N, 5 mL), sodium hydroxide (0.1N, 5 mL) and water (5 mL) were added to separate flasks containing drug samples and mixed properly for acidic, alkaline and neutral degradation respectively and stored at room temperature for 72 h.

The samples were neutralized with base or acid as appropriate and diluted up to the marks with acetonitrile to obtain stock solutions (200 µg/mL). Dilutions were made with mobile phase to obtain the degraded EFV solutions (25 µg/mL).

4.1.1.8.2 Effect of Oxidation

Accurately weighed EFV (10 mg) was transferred to a 50 mL volumetric flask and dissolved in acetonitrile (10 mL). Hydrogen peroxide solution (3%, 5 mL) was added, mixed properly, and stored at room temperature for 72 h. The sample was diluted up to the mark with acetonitrile to obtain stock solution (200 µg/mL). Dilution was made with mobile phase to obtain the degraded EFV solution (25 µg/mL).

4.1.1.8.3 Effect of Heat

EFV (10 mg) was distributed over a glass plate and kept in an oven at 60°C for 72 h, then EFV was transferred in a 50 mL volumetric flask and dilutions were made with mobile phase to obtain the degraded EFV solution (25 µg/mL).

4.1.1.8.4 Effect of Light

EFV solution (prepared by dissolving 10 mg EFV in 10 mL acetonitrile in 50 mL volumetric flask) was exposed to sun light for 48 h, while EFV (10 mg) in powder state was exposed to UV light for 48 h. After exposure, dilutions were made to obtain the degraded EFV solutions (25 µg/mL). Aliquots (20 µL) of the stressed samples were injected into the HPLC system as described under chromatographic conditions (4.1.1.3), and the chromatograms were recorded.

4.1.1.9 METHOD VALIDATION

As per the ICH guideline Q2 (R1), the method validation parameters like specificity, linearity, accuracy, precision, limit of detection, limit of quantitation and robustness were studied.

4.1.1.9.1 Solution Stability

Sample solutions were kept at 25 ± 2°C (24 hours) and 2 - 8^0C (3 days), respectively. Assay percentage of initial time period was compared with these two time periods. The change in the assay percentage was calculated. The difference between assay results should not be more than 2 % for formulation, and 0.5% for API.

4.1.1.9.2 Specificity

Specificity of an analytical method is its ability to measure the analyte accurately and specifically in presence of component that may be expected to be present in the sample matrix. Chromatograms of EFV solutions and degraded samples were studied in order to provide an indication of the stability indicating properties and specificity of the method. The stress conditions employed were acidic, alkaline, neutral, oxidative, thermal and photolytic, the degraded samples were analyzed against freshly prepared sample solutions using UV-visible detector. Specific conditions are described in 4.1.1.8.

4.1.1.9.3 Linearity (Calibration Curve)

Standard solutions (0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 mL equivalent to 5.0, 10.0, 15.0, 20.0, 25.0 and 30.0 µg/mL of EFV) were transferred in a series of 10 mL volumetric flasks and diluted to the mark with mobile phase. An aliquot (20 µL) of each solution was injected under the operating chromatographic conditions as described earlier. Calibration curve was constructed by plotting peak areas versus concentrations, and the regression equation was calculated. Each response was average of three determinations.

4.1.1.9.4 Accuracy (% Recovery)

Accuracy of the method was determined by calculating percentage recovery of EFV by the standard addition method. Known amount of standard solutions of EFV (0, 5, 10 and 15 µg/mL) were added to a pre-analyzed sample solution of EFV (10 µg/mL). Each solution was injected in triplicate and the percentage recovery was calculated by measuring the peak areas and fitting these values into the regression equation of the calibration curve.

4.1.1.9.5 Precision

Repeatability was checked by repeatedly (n = 6) injecting EFV solution (10 µg/mL) and recording the chromatogram. Intra-day and inter-day precisions of the developed method was determined by measuring the corresponding responses 3 times on the same day and on 3 different days over a period of 1

week for 3 different concentration of EFV (10.0, 20.0 and 30.0 µg/mL). The results were reported in terms of relative standard deviation.

4.1.1.9.6 Limit of Detection and Limit of Quantification

Limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the standard deviation of response (σ) and slope (S) of the calibration curve.

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

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

4.1.1.9.7 Robustness

Robustness was studied by analyzing the samples of EFV by deliberate variation in the method parameters. The change in the response of EFV was noted. Robustness of the method was studied by changing the extraction time of EFV from tablet dosage form by ± 2 min, composition of mobile phase by ± 2% of organic solvent, wavelength by ± 2 nm, flow rate by ± 0.2 mL/min and column oven temperature by ± 2ºC. The changes in the response of EFV were noted and compared with the original one.

4.1.1.9.8 System-Suitability Test

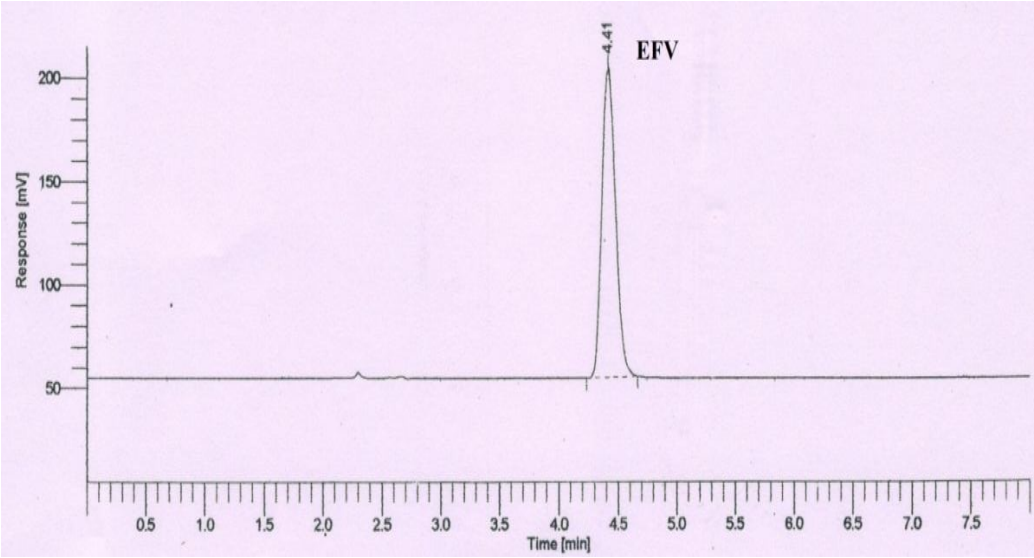
System suitability tests were used to verify that the resolution and repeatability of the system were adequate for the analysis intended. The parameters used in this test were retention time, tailing factor and theoretical plates of chromatographic peak as RSD of peak area for replicate injections.

4.1.2 RESULTS AND DISCUSSION:

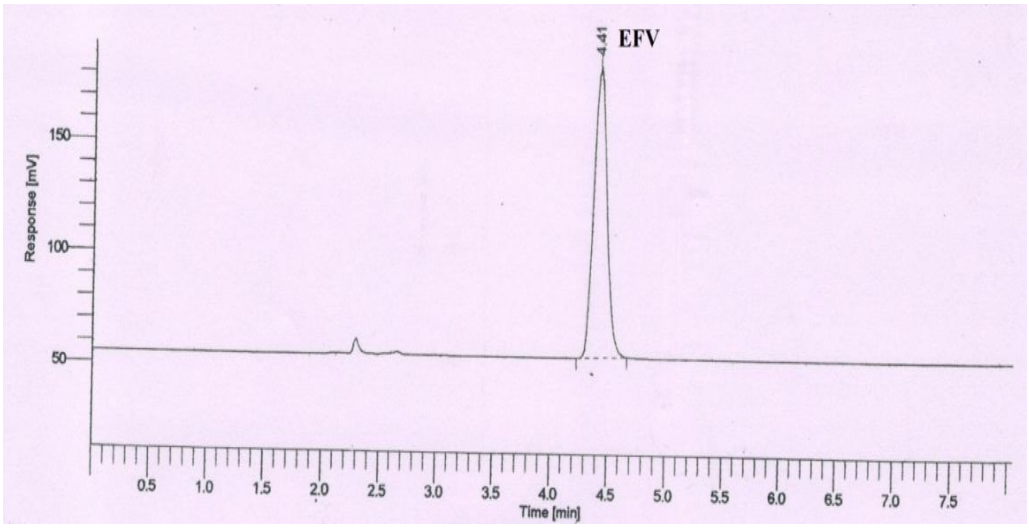
4.1.2.1 Selection of Column and Mobile Phase

As per the published literature and knowledge of the molecule, reverse phase liquid chromatography (RP-HPLC) is suitable for analysis of EFV. In case of RP-HPLC various columns are available, but as the main aim of the method was to resolve the compound from degraded products, C_{I8} column (250 mm x 4.6 mm i.d., 5 µm particle size) was preferred over the other columns. Resolution is the most important criteria for the method, it is imperative to achieve good resolution among the compound and degraded products. As per the value of pKa and solubility of compound various composition of mobile phase were tried.

The chromatographic conditions were optimized with a view to develop a stability indicating assay method, which can separate the drug from its degradation products with good resolution. Mobile phase consisting of acetonitrile : 10mM ammonium acetate buffer (pH 6.5 ± 0.05) (80:20 v/v) at a flow rate of 1.0 mL/min, was found to be satisfactory to obtain well-resolved peaks with better reproducibility and repeatability for EFV.



(a)



(b)

Figure 4.1.1: Chromatogram of EFV with retention time of 4.41 min from (a) standard (30 µg/mL); and (b) tablet dosage form (20 µg/mL)

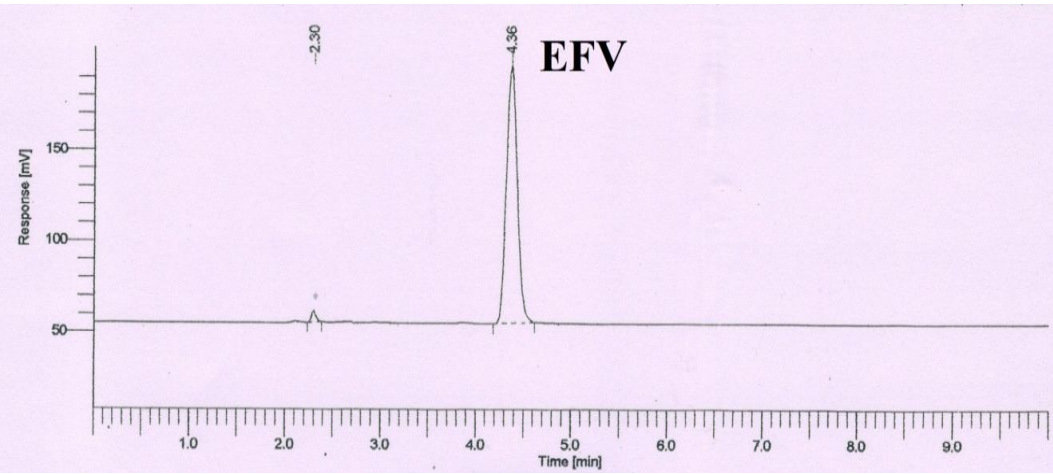
4.1.2.2 METHOD VALIDATION

4.1.2.2.1 Solution Stability

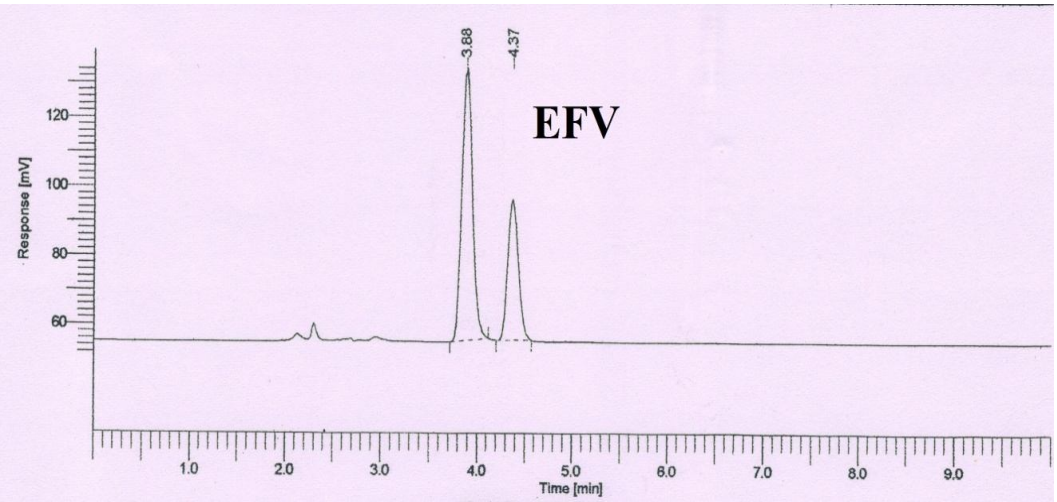
The change in assay results after storage at 25°C (24 hours) and 2-8°C (3 days) was evaluated. It was found that the difference in assay results was not more than 2 % for formulation, and 0.5% for API, indicating stability of EFV solution.

4.1.2.2.2 Specificity

The developed analytical method was found to be specific as there was no inference of any related impurities after the stress degradation study (Figure 4.1.2). It was shown that the EFV peaks were free from excipients and co-eluting impurities.


2.30
4.36
EFV
Response [mV]
150
100
50
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
Time [min]

(a)


3.88
4.37
EFV
Response [mV]
120
100
80
60
1.0
2.0
3.0
4.0
5.0
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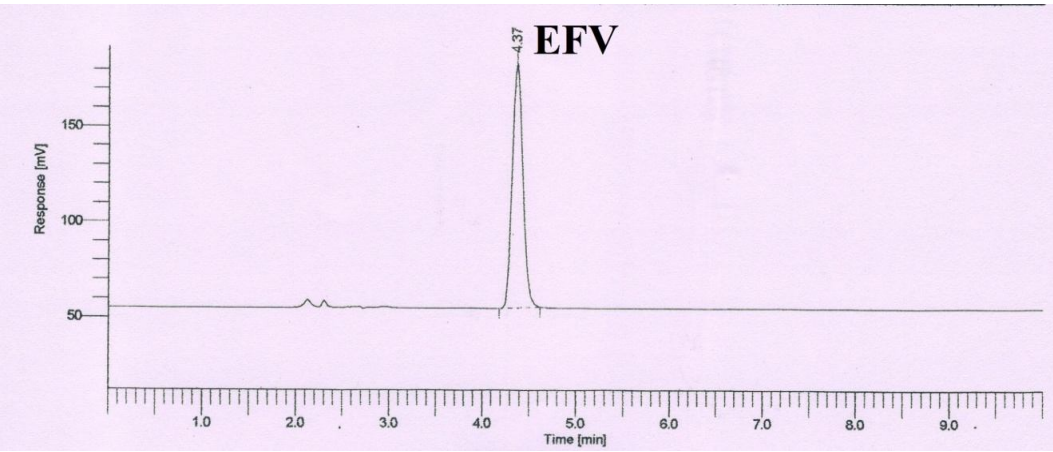
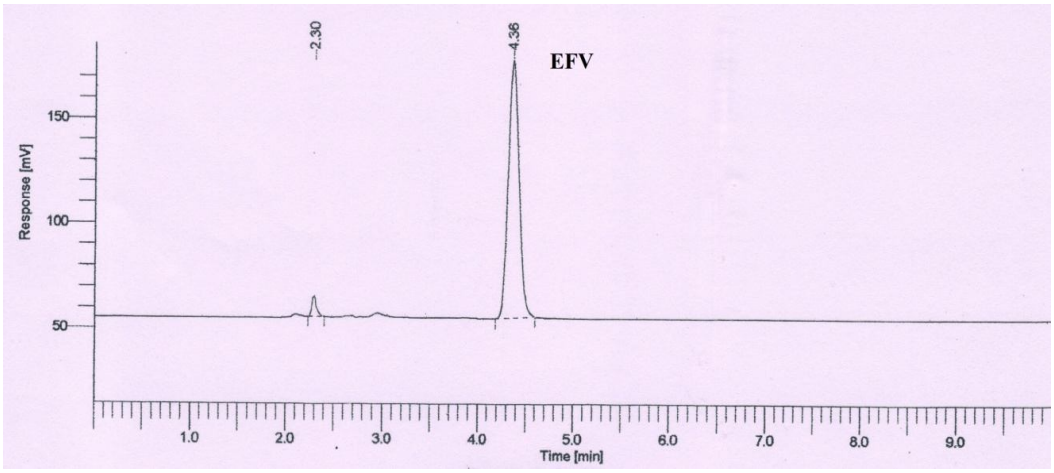
7.0
8.0
9.0
Time [min]

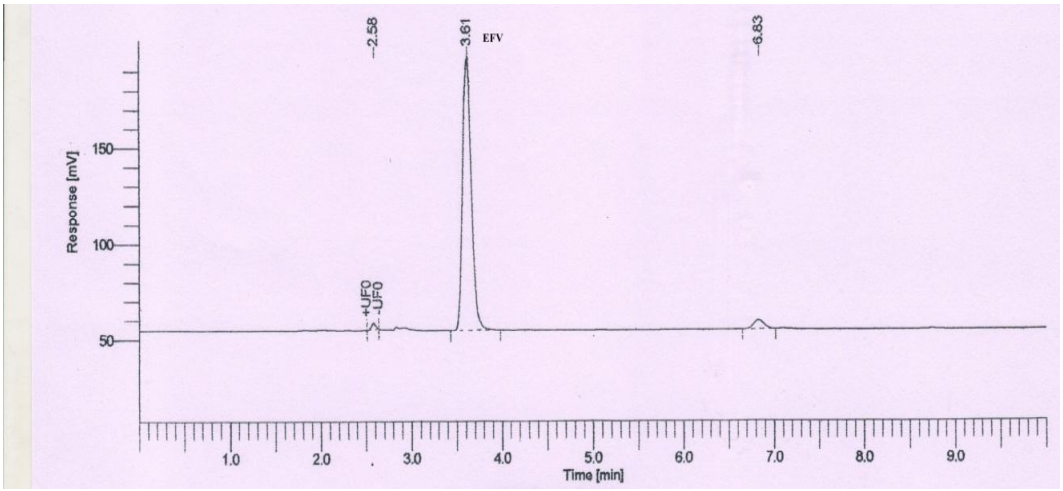
(b)


4.37
EFV
Response [mV]
150
100
50
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
Time [min]

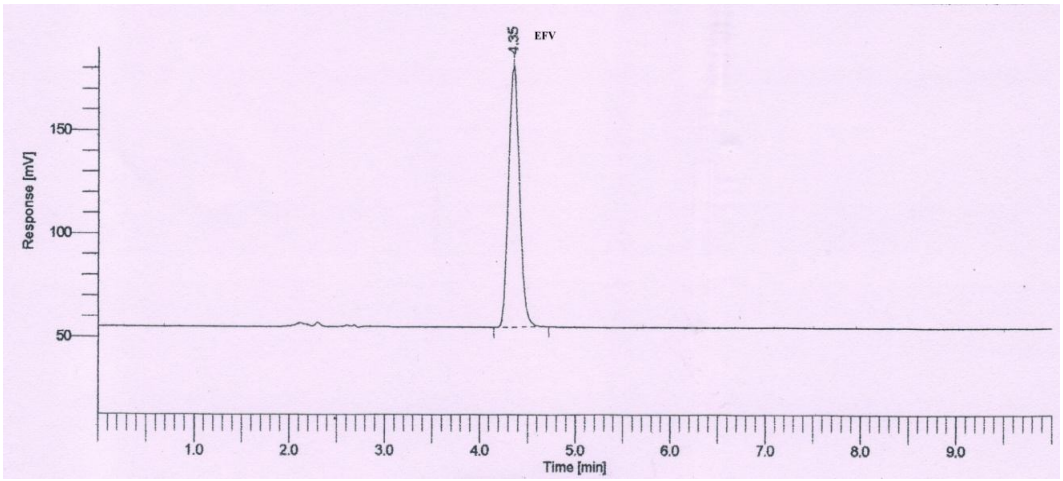
(c)


2.30
4.36
EFV
Response [mV]
150
100
50
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
Time [min]

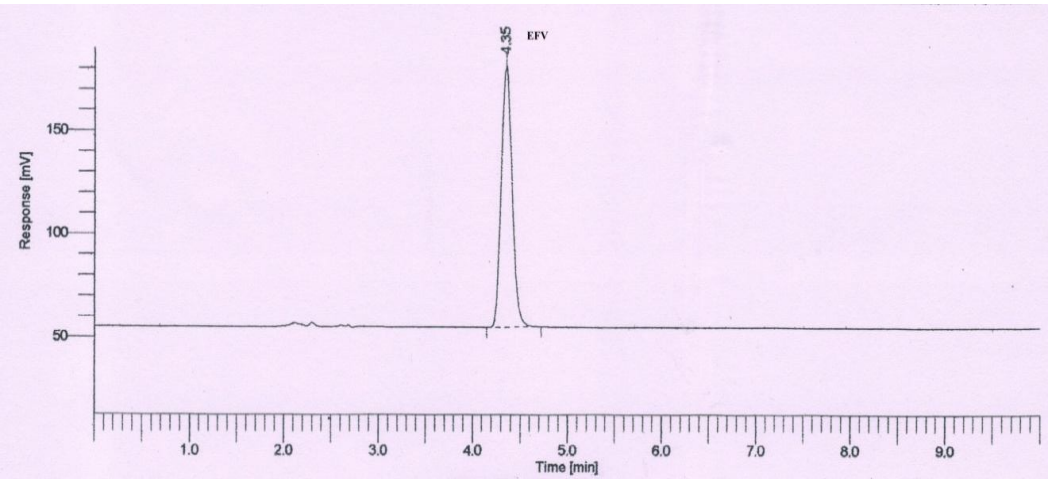
(d)


2.58
3.61
EFV
6.83
+UF0
-UF0
Response [mV]
150
100
50
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
Time [min]

(e)


4.35
EFV
Response [mV]
150
100
50
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
Time [min]

(f)



(g)

Figure 4.1.2: Chromatograms of EFV after (a) acidic hydrolysis; (b) basic hydrolysis; (c) neutral hydrolysis; (d) oxidative degradation; (e) thermal degradation; (f) photolytic (Sun light) degradation, (g) photolytic (UV light) degradation

4.1.2.2.3 Linearity

The linear correlation was obtained between peak area and concentration of EFV in the range of 5-30 µg/mL, the linearity of the calibration curve was validated by the value of correlation coefficient of the regression (r), the regression analysis of the calibration curves is listed in Table 4.1.1

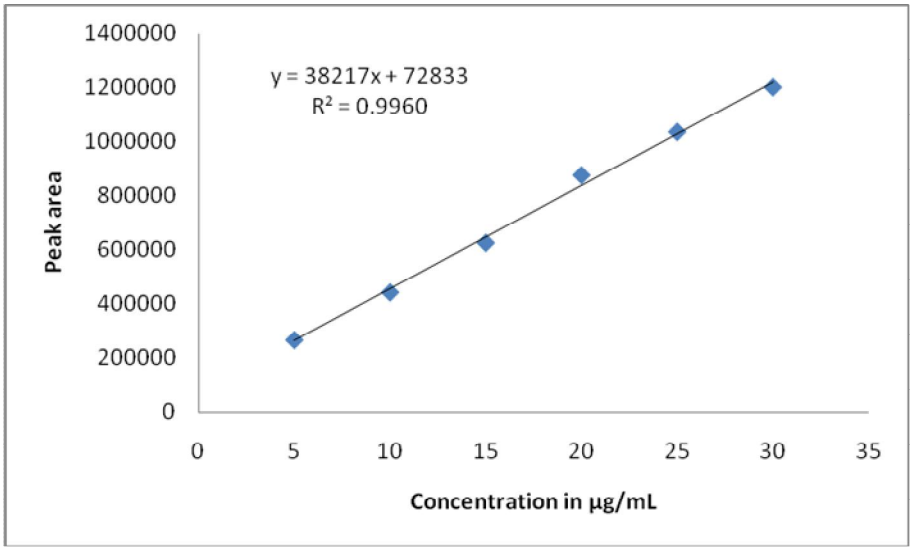


Figure 4.1.3: Calibration curve of EFV

Table 4.1.1: Optical and regression characteristics (n=3)

Parameter	EFV
Linearity range (µg/mL)	5-30
Linearity equation	y = 38217x + 72833
LOD (µg/mL)	0.062
LOQ (µg/mL)	0.187
Correlation coefficient (r)	0.9960

4.1.2.2.4 Accuracy (% Recovery)

The accuracy study was carried out by the standard addition method. The percent recoveries were found in the range of 98.86-101.12 %, which indicated accuracy of the method.

Table 4.1.2: Results of recovery study (n=3)

Amount Taken (µg/mL)	Amount added (µg/mL)	Amount found (µg/mL)	Recovery ± S.D, %	% RSD
10	0	10.05	100.50 ± 0.92	0.92
10	5	14.83	98.86 ± 1.65	1.65
10	10	19.90	99.50 ± 1.37	1.37
10	15	25.28	101.12 ± 0.77	0.77

4.1.2.2.5 Precision

The % RSD for repeatability of EFV was found to be 1.25.

The value of % RSD for intra-day precision was found to be in the range of 0.93 - 1.15% and inter-day precision was found to be in the range of 1.07 - 1.22 %, which indicated that the method was precise.

Table 4.1.3 Results of repeatability (n=6)

Drug	**EFV Peak area**
1	443614.0
2	454953.1
3	443275.4
4	445228.7
5	448743.8
6	438564.2
Mean	445729.9
SD	5590.19
% RSD	1.25

Table 4.1.4 Results of Intra-day and Inter-day precision (n=3)

EFV (µg/mL)	**Intra-day precision**		**Inter-day precision**	
	Mean peak area ± SD	**% RSD**	**Mean peak area ± SD**	**% RSD**
10	443614.0 ± 4152.12	0.93	448745.3 ± 4836.18	1.07
20	875777.0 ± 9638.35	1.10	878418.6 ± 9858.21	1.12
30	1200410.0 ± 13864.16	1.15	1201059.6 ± 14728.26	1.22

4.1.2.2.6 Limit of detection and limit of quantification

The Limit of detection (LOD) for EFV was found to be 0.062 µg/mL. while the Limit of quantification (LOQ) was 0.187 µg/mL.

4.1.2.2.7 Robustness

The method was found to be robust as the results were not significantly affected by slight variation in extraction time, composition of mobile phase, wavelength and flow rate of the mobile phase.

4.1.2.2.8 System-Suitability Test

The % RSD of system-suitability test parameters was found satisfactory. The results are listed in Table 4.1.5

Table 4.1.5: System suitability test parameters (n = 6)

No.	Retention time, Min.	Tailing factor	Theoretical plates
1	4.41	1.49	9282.12
2	4.41	1.49	9254.23
3	4.35	1.48	9237.48
4	4.27	1.49	9187.75
5	4.38	1.47	9265.58
6	4.41	1.45	9176.38
Mean	4.37	1.48	9233.92
SD	0.055	0.016	42.88
% RSD	1.26	1.08	0.46

4.1.2.3 Analysis of Tablet Dosage Form

The proposed RP-HPLC method was successfully applied for determination of EFV from tablet dosage form. The percentage of EFV was found to be satisfactory; which was comparable with the corresponding label claim.

Table 4.1.6: Analysis results of tablet dosage form (n=3)

Formulation	Labelled amount (mg)	Amount found (mg)	Assay ± SD, %
ESTIVA 600®	600	597.90	99.65 ± 1.72

4.1.2.4 RESULTS OF DEGRADATION STUDY

Forced degradation study of EFV was carried out under various stress conditions and resultant chromatograms are depicted in Figure 4.1.2.

4.1.2.4.1 Effect of Acid, Alkaline and Neutral Hydrolysis

EFV was found to undergo minute decomposition about 2.4% under acidic stress condition with a degradation product at retention time of about 2.30 min and 74.88 % decomposition under alkaline stress condition with a major degradation product at retention time of about 3.88 min and minor degradation product at retention time of about 2.30 min (Figure. 4.1.2 (a, b) respectively. Under neutral degradation condition, no degradation was observed.(Figure 4.1.2 (c)). Hence, EFV was found to be highly degradable in basic condition, and very minute degradable in acidic condition but not degradable in neutral condition.

4.1.2.4.2 Effect of Oxidation

In oxidation stress condition, almost 7.5 % of EFV was degraded and degradation peak appeared in chromatogram at 2.30 min retention time. (Figure 4.1.2 (d)),

4.1.2.4.3 Effect of Heat

Under dry thermal stress condition, EFV was degraded about 8.3 % with degradation product at retention time of about 2.58 and 6.83 min. (Figure 4.1.2 (e)).

4.1.2.4.4 Effect of light

When EFV in solution state was exposed to sun light; and EFV in powder state was exposed to UV light, no degradation was observed, respectively (Figure 4.1.2 (f,g)).

The samples exposed to acidic, alkaline, neutral, oxidative, thermal and photolytic conditions were colorless. In Photolytic stability, EFV was found to be stable showing no degradation. All degradates were resolved from EFV peak and the percentage degradation for each condition indicated that there was no interference from degradates in determination of the EFV in tablet dosage form. Thus, the proposed, method was found to be "Stability Indicating".

Table 4.1.7: Results of stress degradation study

Stress conditions/duration	% Degradation
Acidic/0.1N HCl 72 h	2.40
Alkaline/ 0.1N NaOH / 72 h	74.88
Neutral/water/ 72 h	0.00
Oxidative/ 3% H_20_2/ 72 h	7.50
Photolysis/ Sun light/ 48 h	0.00
Photolysis/ UV light/48 h	0.00
Thermal 60°C / 72 h	8.30

4.1.3 CONCLUSION:

An isocratic stability indicating reverse phase liquid chromatographic method has been developed and validated for the estimation of EFV in tablet dosage form, the method was found to be specific as there was no interference of any co-eluting impurities after stress degradation study. The proposed method was found to be simple, accurate, precise, sensitive and robust. Hence, it can be used successfully for the routine analysis of EFV in pharmaceutical dosage forms, and for analysis of stability samples obtained during accelerated stability study

4.2 STABILITY INDICATING RP-HPLC METHOD FOR DETERMINATION OF TENOFOVIR DISOPROXIL FUMARATE

4.2.1 EXPERIMENTAL:

4.2.1.1. Instrumentation

Same as described under 4.1.1.1

4.2.1.2 Reagents and Materials

- Tenofovir disoproxil fumarate (TNV) was kindly gifted by Aurobindo Pharmaceuticals Ltd., Hyderabad, India.
- Acetonotrile and water HPLC grade (Rankem, RFCL Ltd., New Delhi).
- Tablets (TENOF®, Genix Pharma) containing tenofovir disoproxil fumarate (300 mg) were purchased from local market.
- Nylon membrane filter 0.45 µm (Gelman laboratory, Mumbai, India)
- Hydrogen peroxide, sodium hydroxide and hydrochloric acid (36%) AR grade (Finar Chemicals Pvt. Ltd, Ahmedabad, India)
- Ammonium acetate crystalline pure (E. Merck, Mumbai, India)

4.2.1.3 Chromatographic conditions

Same as described under 4.1.1.3

4.2.1.4 Preparation of the Mobile Phase

Same as described under 4.1.1.4

4.2.1.5 Preparation of standard solution

Accurately weighed TNV (25 mg) was transferred to a 25 mL volumetric flask, dissolved in and diluted to the mark with acetonitrile to obtain a standard stock solution (1mg/mL).

4.2.1.5A Preparation of working standard solution (10 µg/mL)

Standard solution (0.1 mL) was transferred in a 10 mL volumetric flask and diluted up to the mark with mobile phase.

4.2.1.5B Preparation of hydrochloric acid (0.1N)

Same as described under 4.1.1.5B

4.2.1.5C Preparation of sodium hydroxide (0.1N)

Same as described under 4.1.1.5C

4.2.1.6 Selection of Wavelength for Determination

The working standard solution of TNV (10 µg/mL) was scanned in the range of 200-400 nm using mobile phase as blank. Significant absorbance was observed at 254 nm which was selected for the determination.

4.2.1.7 Analysis of Tablet Dosage Form

Twenty tablets were weighed and average weight was calculated. The tablets were finely powdered; a quantity of powder equivalent to 25 mg TNV was weighed accurately and transferred to a 25 mL volumetric flask containing 15 mL acetonitrile, and sonicated for 15 minutes. Allowed to stand at room temperature for 5 min and the volume was made up to the mark with acetonitrile to obtain the sample stock solution (1 mg/mL). The solution was filtered through 0.45 µm membrane filter. Aliquot (1 mL) was taken and transferred to 10 mL volumetric flask and volume was made up to the mark with acetonitrile to give a solution containing 100 µg/ml TNV. The solution (2 mL) was transferred to 10 mL volumetric flask and diluted up to the mark with mobile phase to give a solution containing 20 µg/mL TNV. An aliquot (20 µL) was injected and the chromatogram was recorded. The peak area was noted and the amount of TNV was calculated from the regression equation.

4.2.1.8 FORCED DEGRADATION STUDY

TNV was subjected to various forced degradation conditions to effect partial degradation of the drug. The study provides information about the conditions in which the drug is unstable so that measures can be taken during formulation to avoid potential instabilities.

4.2.1.8.1 Effect of Acid, Alkaline and Neutral Hydrolysis

Accurately weighed TNV (10 mg) was transferred to three different 50 mL volumetric flasks and dissolved in acetonitrile (10 mL). Hydrochloric acid (0.1N, 5 mL), sodium hydroxide (0.1N, 5 mL) and water (5 mL) were added to separate flasks containing drug samples and mixed properly for acidic, alkaline and neutral degradation respectively and stored at room temperature for 72 h. The samples were neutralized with base or acid as appropriate and diluted up to the marks with acetonitrile to obtain stock solutions (200 µg/mL). Dilutions were made with mobile phase to obtain the degraded TNV solutions (25 µg/mL).

4.2.1.8.2 Effect of Oxidation

Accurately weighed TNV (10 mg) was transferred to a 50 mL volumetric flask and dissolved in acetonitrile (10 mL). Hydrogen peroxide solution (3%, 5 mL) was added, mixed properly, and stored at room temperature for 72 h. The sample was diluted up to the mark with acetonitrile to obtain stock solution (200 µg/mL). Dilution was made with mobile phase to obtain the degraded TNV solution (25 µg/mL).

4.2.1.8.3 Effect of Heat

TNV (10 mg) was distributed over a glass plate and kept in an oven at 60°C for 72 h, then TNV was transferred in a 50 mL volumetric flask and dilutions were made with mobile phase to obtain the degraded TNV solution (25 µg/mL).

4.2.1.8.4 Effect of Light

TNV solution (prepared by dissolving 10 mg TNV in 10 mL acetonitrile in 50 mL volumetric flask) was exposed to sun light for 48 h, while TNV (10 mg) in powder state was exposed to UV light for 48 h. After exposure, dilutions were made to obtain the degraded TNV solutions (25 µg/mL). Aliquots (20 µL) of the stressed samples were injected into the HPLC system as described under chromatographic conditions (4.1.1.3), and the chromatograms were recorded.

4.2.1.9 METHOD VALIDATION

As per the ICH guideline Q2 (R1), the method validation parameters like specificity, linearity, accuracy, precision, limit of detection, limit of quantitation and robustness were studied.

4.2.1.9.1 Solution Stability

Sample solutions were kept at 25 ± 2°C (24 hours) and 2 - 8^0C (3 days), respectively. Assay percentage of initial time period was compared with these two time periods. The change in the assay percentage was calculated. The difference between assay results should not be more than 2 % for formulation, and 0.5% for API.

4.2.1.9.2 Specificity

Specificity of an analytical method is its ability to measure the analyte accurately and specifically in presence of component that may be expected to be present in the sample matrix. Chromatograms of TNV solutions and degraded samples were studied in order to provide an indication of the stability indicating properties and specificity of the method. The stress conditions employed were acidic, alkaline, neutral, oxidative, thermal and photolytic, the degraded samples were analyzed against freshly prepared sample solutions using UV visible detector. Specific conditions are described in 4.2.1.8.

4.2.1.9.3 Linearity (Calibration Curve)

Standard solutions (0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 mL equivalent to 5.0, 10.0, 15.0, 20.0, 25.0 and 30.0 µg/mL of TNV) were transferred in a series of 10 mL volumetric flasks and diluted to the mark with mobile phase. An aliquot (20 µL) of each solution was injected under the operating chromatographic conditions as described earlier. Calibration curve was constructed by plotting peak areas versus concentrations, and the regression equation was calculated. Each response was average of three determinations.

4.2.1.9.4 Accuracy (% Recovery)

Accuracy of the method was determined by calculating percentage recovery of TNV by the standard addition method. Known amount of standard solutions of TNV (0, 5, 10 and 15 µg/mL) were added to a pre-analyzed sample solution of TNV (10 µg/mL). Each solution was injected in triplicate and the percentage recovery was calculated by measuring the peak areas and fitting these values into the regression equation of the calibration curve.

4.2.1.9.5 Precision

Repeatability was checked by repeatedly (n = 6) injecting TNV solution (10 µg/mL) and recording the chromatogram. Intra-day and inter-day precisions of the developed method was determined by measuring the corresponding responses 3 times on the same day and on 3 different days over a period of 1 week for 3 different concentration of TNV (10.0, 20.0 and 30.0 µg/mL). The results were reported in terms of relative standard deviation.

4.2.1.9.6 Limit of Detection and Limit of Quantification

Limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the standard deviation of response (σ) and slope (S) of the calibration curve.

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

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

4.2.1.9.7 Robustness

Robustness was studied by analyzing the samples of TNV by deliberate variation in the method parameters. The change in the response of TNV was noted. Robustness of the method was studied by changing the extraction time of TNV from tablet dosage form by ± 2 min, composition of mobile phase by ± 2% of organic solvent, wavelength by ± 2 nm, flow rate by ± 0.2 mL/min and column oven temperature by ± 2ºC. The changes in the response of TNV were noted and compared with the original one.

4.2.1.9.8 System-Suitability Test

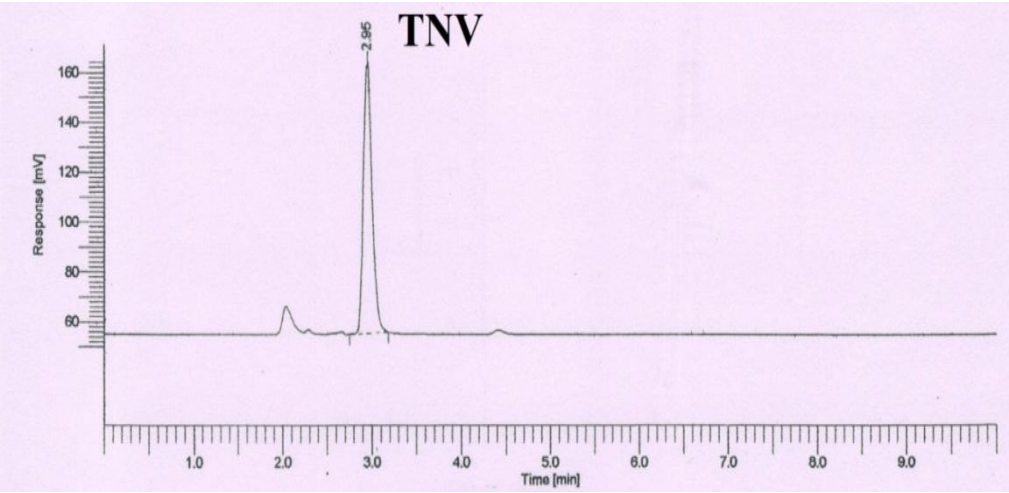
System suitability tests were used to verify that the resolution and repeatability of the system were adequate for the analysis intended. The parameters used in this test were retention time, tailing factor and theoretical plates of chromatographic peak as RSD of peak area for replicate injections.

4.2.2 RESULTS AND DISCUSSION:

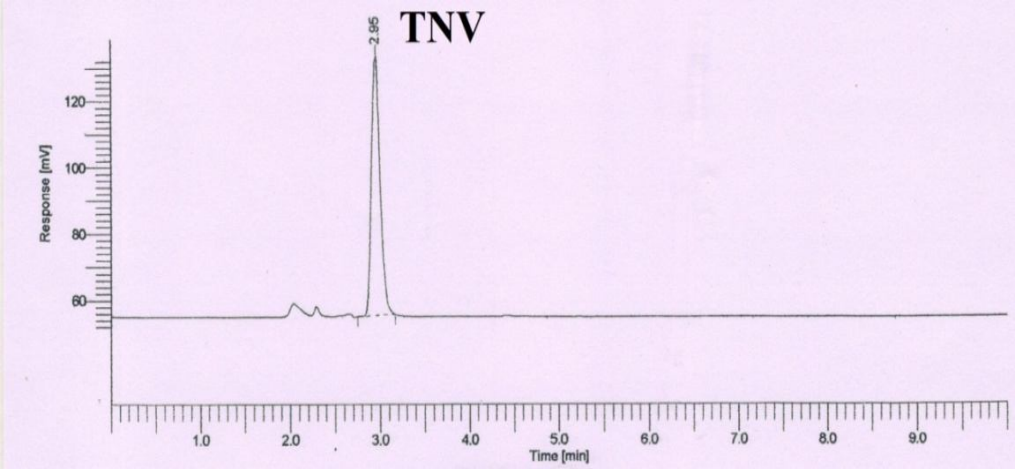
4.2.2.1 Selection of Column and Mobile Phase

As per the published literature and knowledge of the molecule, it suggested reverse phase liquid chromatography (RP-HPLC) is suitable for analysis of TNV. In case of RP-HPLC various columns are available, but as the main aim of the method was to resolve the compound from degraded products, C_{18} column (250 mm x 4.6 mm i.d., 5 µm particle size) was preferred over the other columns. Resolution is the most important criteria for the method, it is imperative to achieve good resolution among the compound and degraded products. As per the value of pKa and solubility of compound various composition of mobile phase were tried.

The chromatographic conditions were optimized with a view to develop a stability indicating assay method, which can separate the drug from its degraded products with good resolution. Mobile phase consisting of acetonitrile : 10mM ammonium acetate buffer (pH 6.5 ± 0.05) (80:20 v/v) at a flow rate of 1.0 mL/min, was found to be satisfactory to obtain well-resolved peaks with better reproducibility and repeatability for TNV.



(a)



(b)

Figure 4.2.1: Chromatogram of TNV with retention time of 2.95 min from (a) standard (30 µg/mL) and (b) tablet dosage form (20 µg/mL)

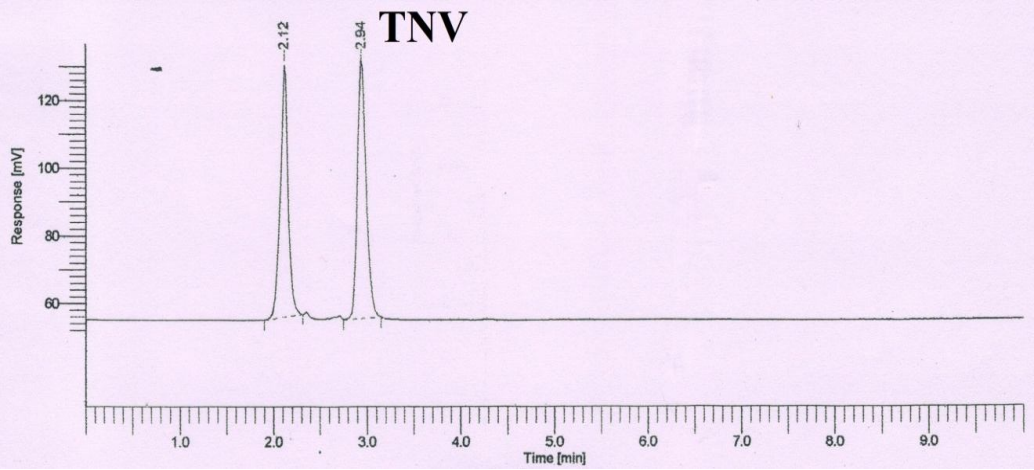
4.2.2.2 METHOD VALIDATION

4.2.2.2.1 Solution Stability

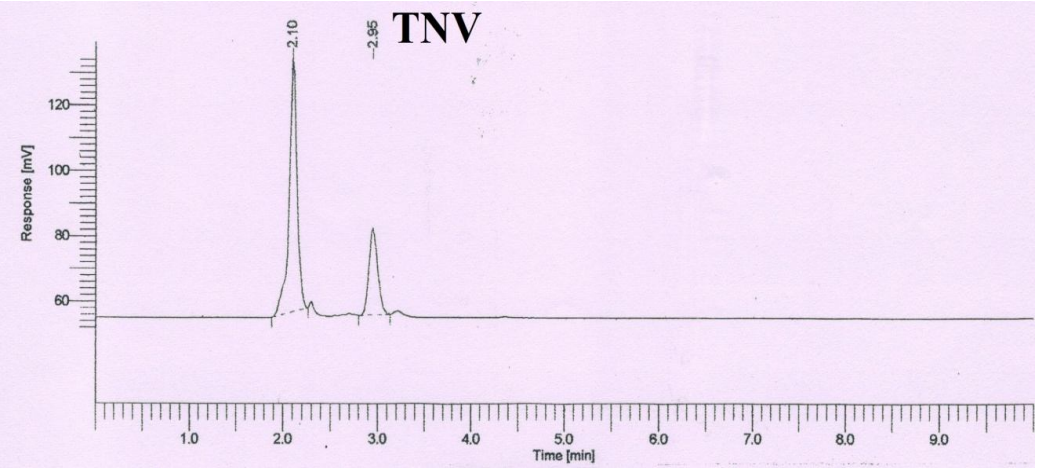
The change in assay results after storage at 25°C (24 hours) and 2-8°C (3 days) was evaluated. It was found that the difference in assay results was not more than 2 % for formulation, and 0.5% for API, indicating stability of TNV solution.

4.2.2.2.2 Specificity

The developed analytical method was found to be specific as there was no inference of any related impurities after the stress degradation study (Figure 4.2.2). It was shown that the TNV peaks were free from excipients and co-eluting impurities.


TNV
2.12
2.94
Response [mV]
120
100
80
60
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
Time [min]

(a)


TNV
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2.95
Response [mV]
120
100
80
60
1.0
2.0
3.0
4.0
5.0
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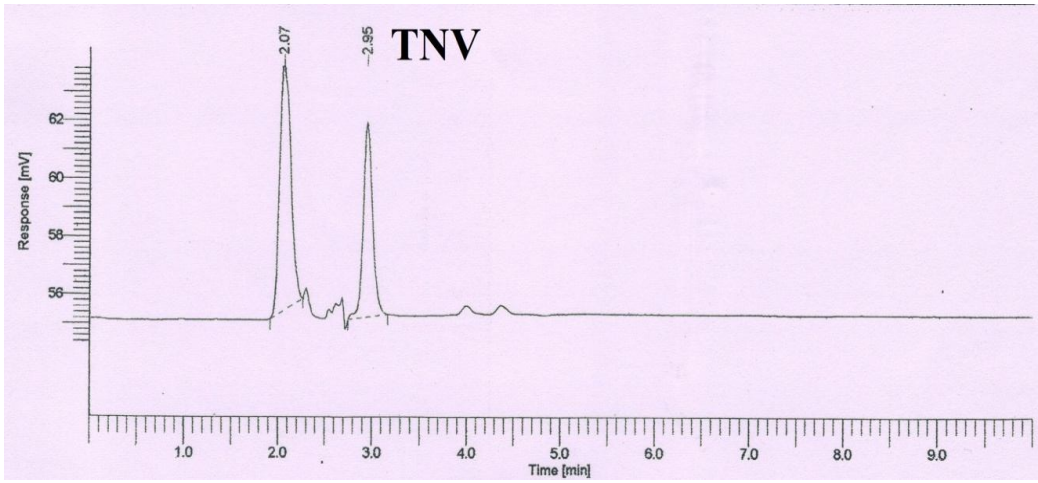
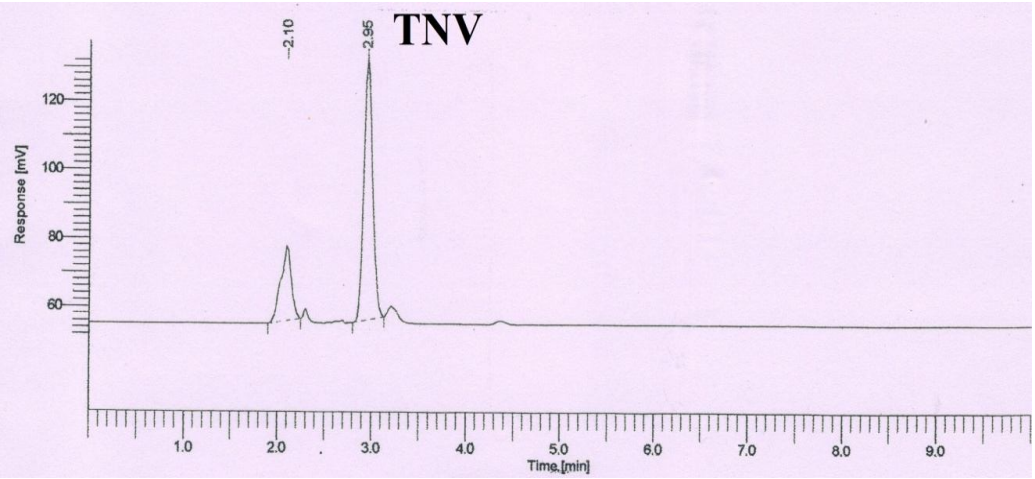
8.0
9.0
Time [min]

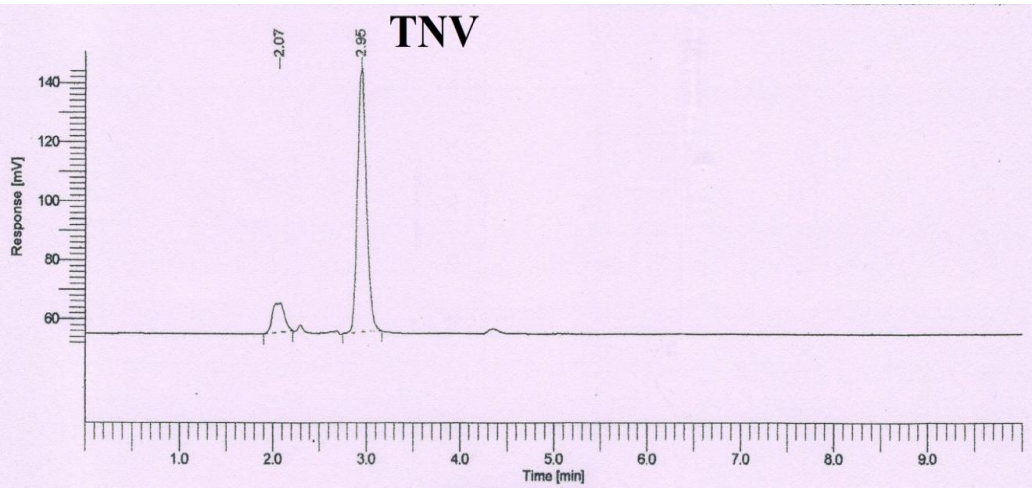
(b)


TNV
2.07
2.95
Response [mV]
62
60
58
56
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
Time [min]

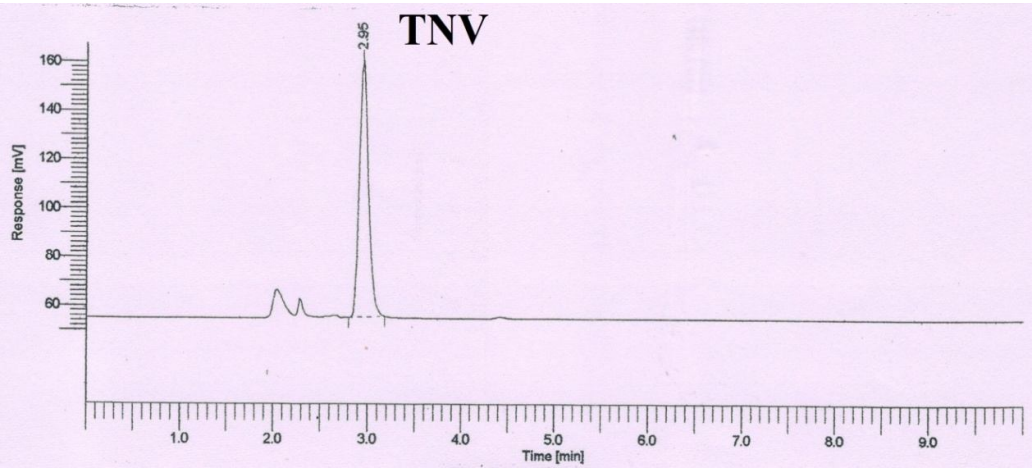
(c)


TNV
2.10
2.95
Response [mV]
120
100
80
60
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
Time [min]

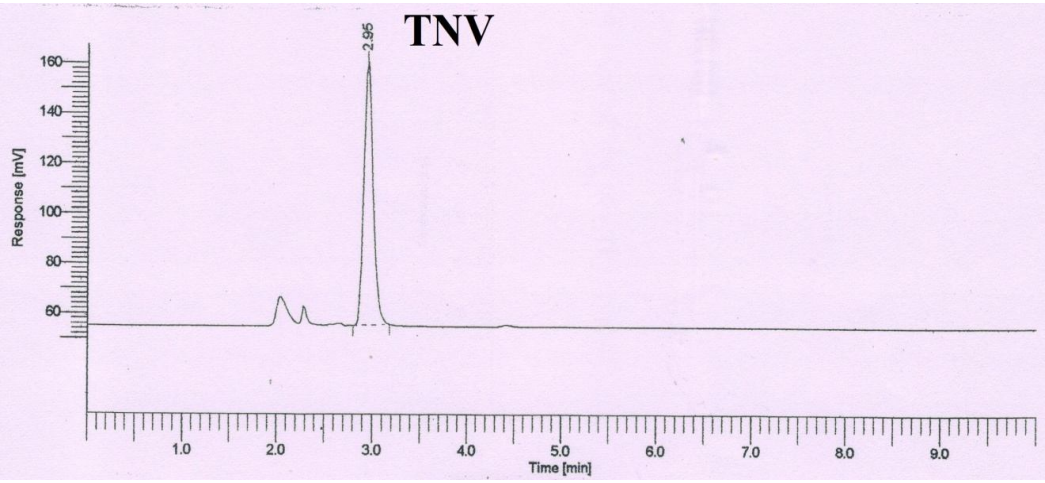
(d)


TNV
2.07
2.95
Response [mV]
140
120
100
80
60
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
Time [min]

(e)


TNV
2.95
Response [mV]
160
140
120
100
80
60
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
Time [min]

(f)



(g)

Figure 4.2.2: Chromatograms of TNV after (a) acidic hydrolysis; (b) basic hydrolysis; (c) neutral hydrolysis; (d) oxidative degradation; (e) thermal degradation; (f) photolytic (Sun light) degradation, (g) photolytic (UV light) degradation

4.2.2.2.3 Linearity

The linear correlation was obtained between peak area and concentration of TNV in the range of 5-30 µg/mL, the linearity of the calibration curve was validated by the value of correlation coefficient of the regression (r), the regression analysis of the calibration curves is listed in Table 4.2.1

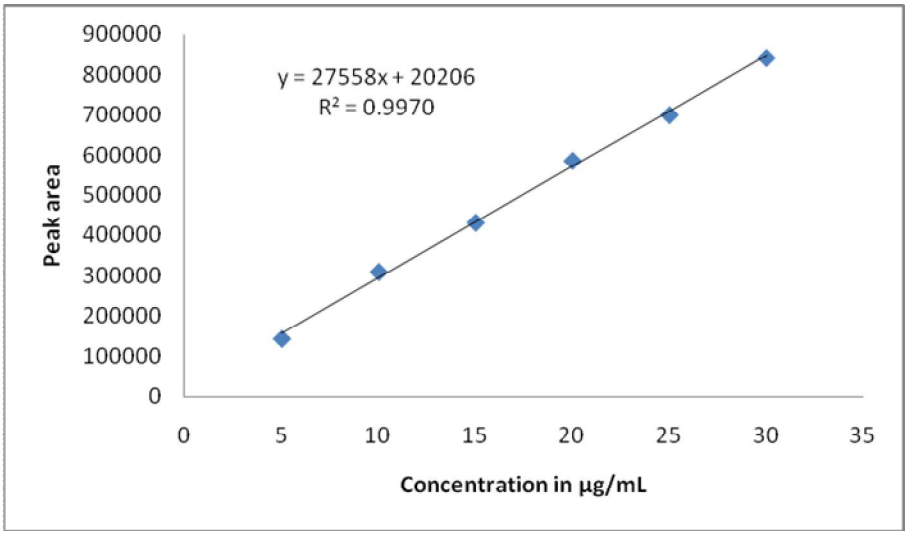


Figure 4.2.3: Calibration curve of TNV

Table 4.2.1: Optical and regression characteristics (n=3)

Parameter	TNV
Linearity range (µg/mL)	5-30
Linearity equation	y = 27558x + 20206
LOD (µg/mL)	0.055
LOQ (µg/mL)	0.166
Correlation coefficient (r)	0.9970

4.2.2.2.4 Accuracy (% Recovery)

The accuracy study was carried out by the standard addition method. The percent recoveries were found in the range of 97.66-100.89 %, which indicated accuracy of the method.

Table 4.2.2: Results of recovery study (n=3)

Amount Taken (µg/mL)	Amount added (µg/mL)	Amount found (µg/mL)	Recovery ± S.D, %	% RSD
10	0	9.76	97.66 ± 1.54	1.54
10	5	14.76	98.42 ± 1.79	1.79
10	10	20.17	100.89 ± 0.84	0.84
10	15	25.06	100.24 ± 1.19	1.19

4.2.2.2.5 Precision

The % RSD for repeatability of TNV was found to be 1.08. The value of % RSD for intra-day precision was found to be in the range of 0.78 - 1.00% and inter-day precision was found to be in the range of 1.06 - 1.22 %, which indicated that the method was precise.

Table 4.2.3: Results of repeatability (n=6)

Drug	TNV Peak area
1	309824.0
2	315686.0
3	308876.0
4	306549.0
5	307652.0
6	312484.0
Mean	310178.5
SD	3377.17
% RSD	1.08

Table 4.2.4: Results of Intra-day and Inter-day precision (n=3)

TNV (µg/mL)	Intra-day precision		Inter-day precision	
	Mean peak area ± SD	**% RSD**	**Mean peak area ± SD**	**% RSD**
10	309824.0 ± 2415.28	0.78	304587.4 ± 3245.17	1.06
20	585770.0 ± 5871.26	1.00	589472.1 ± 7187.33	1.22
30	842658.0 ± 6857.84	0.81	842764.2 ± 9564.21	1.13

4.2.2.2.6 Limit of detection and limit of quantification

The Limit of detection (LOD) for TNV was found to be 0.055 µg/mL. while the Limit of quantification (LOQ) was 0.166 µg/mL.

4.2.2.2.7 Robustness

The method was found to be robust as the results were not significantly affected by slight variation in extraction time, composition of mobile phase, and wavelength and flow rate of the mobile phase.

4.2.2.2.8 System-Suitability Test

The % RSD of system-suitability test parameters was found satisfactory. The results are listed in Table 4.2.5

Table 4.2.5: System suitability test parameters (n = 6)

No.	Retention time, Min.	Tailing factor	Theoretical plates
1	2.95	1.42	9193.01
2	2.95	1.40	9157.22
3	2.95	1.40	9078.1
4	2.93	1.41	9121.66
5	2.91	1.42	9044.24
6	2.90	1.42	9156.0
Mean	2.93	1.41	9125.038
SD	0.022	0.0098	55.42
% RSD	0.76	0.69	0.60

4.2.2.3 Analysis of Tablet Dosage Form

The proposed RP-HPLC method was successfully applied for determination of TNV from tablet dosage form. The percentage of TNV was found to be satisfactory; which was Comparable with the corresponding label claim.

Table 4.2.6: Analysis results of tablet dosage form (n=3)

Formulation	Labelled amount (mg)	Amount found (mg)	Assay ± SD, %
TENOF®	300	296.46	98.82 ± 1.61

4.2.2.4 RESULTS OF DEGRADATION STUDY

Forced degradation study of TNV was carried out under various stress conditions and resultant chromatograms are depicted in Figure 4.2.2.

4.2.2.4.1 Effect of Acid, Alkaline and Neutral Hydrolysis

TNV was found to undergo 31.32 and 90.00 % decomposition under acidic and alkaline stress conditions respectively with a degradation product at retention time of about 2.12 min and 2.10 min (Figure. 4.2.2 (a, b) respectively. Under neutral degradation condition, 78.32% degradation was observed with a degradation product at retention time of 2.07 min.(Figure 4.2.2 (c)). Hence, TNV was found to be highly degradable in basic and neutral condition, and moderately degradable in acidic condition.

4.2.2.4.2 Effect of Oxidation

In oxidation stress condition, almost 17.64 % of TNV was degraded and degradation peak appeared in chromatogram at 2.10 min retention time. (Figure 4.2.2 (d)),

4.2.2.4.3 Effect of Heat

Under dry thermal stress condition, TNV was degraded about 32.16% and degradation peak appeared in chromatogram at 2.07 min retention time. (Figure 4.2.2 (e)).

4.2.2.4.4 Effect of light

When TNV in solution state was exposed to sun light; and TNV in powder state was exposed to UV light, no degradation was observed, respectively (Figure 4.2.2 (f,g)).

The samples exposed to acidic, alkaline, neutral, oxidative, thermal and photolytic conditions were colorless. In Photolytic stability, TNV was stable showing no degradation. All degradates were resolved from TNV peak and the percentage degradation for each condition indicated that there was no interference from degradates in determination of the TNV in tablet dosage form. Thus, the proposed, method was found to be "Stability Indicating".

Table 4.2.7: Results of stress degradation study

Stress conditions/duration	% Degradation
Acidic/0.1N HCl / 72 h	31.32
Alkaline/ 0.1N NaOH / 72 h	90.00
Neutral/water / 72 h	78.32
Oxidative/ 3% H_20_2 / 72 h	17.64
Photolysis/ Sun light / 48 h	0.00
Photolysis/ UV light / 48 h	0.00
Thermal 60°C / 72 h	32.16

4.2.3 CONCLUSION:

An isocratic stability indicating reverse phase liquid chromatographic method has been developed and validated for the estimation of TNV in tablet dosage form, the method was found to be specific as there was no interference of any co-eluting impurities after stress degradation study. The proposed method was found to be simple, accurate, precise, sensitive and robust. Hence, it can be used successfully for the routine analysis of TNV in pharmaceutical dosage forms, and for analysis of stability samples obtained during accelerated stability study

CHAPTER 5

RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF LOPINAVIR AND RITONAVIR IN BULK AND TABLET DOSAGE FORM

5. RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF LOPINAVIR AND RITONAVIR IN BULK AND TABLET DOSAGE FORM

5.1 EXPERIMENTAL:

5.1.1 Instrumentation

- A Perkin Elmer (USA) HPLC system (series 200) equipped with Perkin Elmer series 200 pump system having back pressure 5000psi, manual injector of 20 µL loop capacity, UV-Visible detector and Brownlee C_{18} column (250 mm x 4.6 mm i.d., 5µm)
- BP211D, Sartorious Gottingen AG (Germany), analytical balance
- An ultra-sonic cleaner (TEC-4, Roop Telesonic Ultrasonix)
- A Shimadzu model 1800 double beam UV/Vis. spectrophotometer with a pair of 10 mm matched quartz cells
- pH meter (*Testronix 35 420 A (ORION)*

5.1.2 Reagents and Materials

- Lopinavir (LPV) and Ritonavir (RTV) were kindly gifted by Emcure Pharmaceuticals Ltd., Pune, India.
- Acetonotrile, methanol and water HPLC grade (Rankem, RFCL Ltd., New Delhi).
- Tablets (LOPIMUNE®, Cipla Ltd., Mumbai) containing Lopinavir (200 mg) and Ritonavir (50 mg) were purchased from local market.
- Nylon membrane filter 0.45 µm (Gelman laboratory, Mumbai, India)
- Ammonium acetate crystalline pure(E. Merck, Mumbai, India)
- Orthophosphoric acid HPLC grade (Spectrochem Pvt. Ltd., Mumbai)

5.1.3 Chromatographic Condition

The chromatographic separation was achieved on Brownlee C_{18} column. The HPLC system was operated isocratically, at 25°C column oven temperature, using mobile phase acetonitrile : 10mM ammonium acetate buffer (pH 4.5 ± 0.05 adjusted with orthphosphoric acid) : methanol (40:30:30 v/v/v), at a flow rate of 1.0 mL/min. The mobile phase was filtered through nylon 0.45 µm

membrane filter and was degassed before use. The determination was carried out at 210 nm wavelength by UV-Visible detector. The injection volume was 20 µL and total run time was 15 min. The analysis was performed at 25 ± 2 ^{0}C temperatures.

5.1.4 Preparation of the mobile phase

The mobile phase was prepared by mixing 40 mL acetonitrile, 30 mL 10mM ammonium acetate buffer (pH 4.5 ± 0.05 adjusted with orthphosphoric acid) and 30 mL methanol previously filtered through 0.45 µm nylon membrane filter. The mobile phase was degassed for 15 minutes by sonicating the solution before use.

5.1.5 Preparation of Standard Solution

Accurately weighed LPV (25 mg) and RTV (25 mg) were transferred to a 25 mL volumetric flask, dissolved in and diluted to the mark with methanol to obtain a standard stock solution (1 mg/mL LPV and RTV).

5.1.5A Preparation of working standard solution of LPV (10 µg/mL)

Accurately weighed LPV (25 mg) was transferred to a 25 mL volumetric flask, dissolved in and diluted to the mark with methanol to obtain a standard stock solution (1 mg/mL LPV). The solution (0.1 mL) was transferred in a 10 mL volumetric flask and diluted to the mark with mobile phase.

5.1.5B Preparation of working standard solution of RTV (10 µg/mL)

Accurately weighed RTV (25 mg) was transferred to a 25 mL volumetric flask, dissolved in and diluted to the mark with methanol to obtain a standard stock solution (1 mg/mL RTV). The solution (0.1 mL) was transferred in a 10 mL volumetric flask and diluted to the mark with mobile phase.

5.1.6 Selection of Wavelength for Determination

The working standard solution of LPV (10 µg/mL) and RTV (10 µg/mL) were scanned in the range of 200 - 400 nm using methanol as blank and overlain spectra was obtained. Both the drugs showed significant absorbance at 210 nm which was selected for the determination.

5.1.7 Analysis of Tablet Dosage Form

Twenty tablets were weighed and average weight was calculated. The tablets were powdered; a quantity of powder equivalent to 10mg LPV and 2.5 mg RTV was accurately weighed and transferred to a volumetric flask of 10 mL capacity. Methanol (6 mL) was transferred to volumetric flask and sonicated for 20 mins. The flask was shaken and volume was made up to the mark with methanol to obtain the sample stock solution (1000 µg/mL LPV and 250 µg/mL RTV). The solution was filtered through 0.45 µm membrane filter. Aliquot (1 mL) was taken and transferred to 10 mL volumetric flask and diluted up to the mark with methanol to give a solution containing 100 µg/mL LPV and 25 µg/mL RTV. The solution (2 mL) was transferred to 10 mL volumetric flask and diluted up to the mark with methanol to give a solution containing 20 µg/mL LPV and 5 µg/mL RTV. An aliquot (20 µL) was injected and the chromatogram was recorded. The peak area was noted and the amount of LPV and RTV were calculated from the regression equations LPV and RTV.

5.1.8 METHOD VALIDATION

As per the ICH guideline Q2 (R1), the method validation parameters like specificity, linearity, accuracy, precision, limit of detection, limit of quantitation and robustness were studied.

5.1.8.1 Solution Stability

Sample solutions were kept at 25 ± 2°C (24 hours) and 2 - 8^0C (3 days), respectively. Assay percentages of both the drugs at initial time period were compared with these two time periods. The change in the assay percentage was calculated. The difference between assay results should not be more than 2 % for formulation, and 0.5 % for API.

5.1.8.2 Specificity

Specificity of an analytical method is its ability to measure the analyte accurately and specifically in presence of component that may be expected to be present in the sample matrix. Chromatograms of standard and sample solutions of LPV and RTV were compared in order to provide an indication of specificity of the method.

5.1.8.3 Linearity (Calibration Curve)

Standard solutions (0.05, 0.1, 0.15, 0.2, 0.25, 0.3 and 0.35 mL equivalent to 5.0, 10.0, 15.0, 20.0, 25.0, 30.0 and 35 µg/mL of LPV and RTV) were transferred in a series of 10 mL volumetric flasks and diluted to the mark with methanol. An aliquot (20 µL) of each solution was injected under the operating chromatographic conditions as described earlier. Chromatograms were recorded. Methanol (20 µL) blank was also injected under the same conditions and chromatogram of methanol was recorded for the correction of the response of methanol in the chromatograms containing responses of LPV and RTV. Calibration curves were constructed by plotting peak areas versus concentrations, and the regression equations were calculated. Each response was average of three determinations.

5.1.8.4 Accuracy (% Recovery)

Accuracy of the method was determined by calculating percentage recovery of LPV and RTV by the standard addition method. Known amount of standard solutions of LPV (0, 5, 10 and 15 µg/mL) and RTV (0, 5, 10 and 15 µg/mL) were added to a pre-analyzed sample solution of LPV (10 µg/mL) and RTV (10 µg/mL). Each solution (20 µL) was injected in triplicate and the percentage recovery was calculated by measuring the peak areas and fitting these values into the regression equations of the calibration curves.

5.1.8.5 Precision

Repeatability was checked by repeatedly (n = 6) injecting the solution containing LPV (10 µg/mL) and RTV (10 µg/mL) and recording the chromatograms.

Intra-day and inter-day precisions of the developed method was determined by measuring the corresponding responses 3 times on the same day and on 3 different days over a period of 1 week for 3 different concentration of LPV (10.0, 20.0 and 30.0 µg/mL) and RTV (10.0, 20.0 and 30.0 µg/mL). The results were reported in terms of relative standard deviation.

5.1.8.6 Limit of Detection and Limit of Quantification

Limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the standard deviation of response (σ) and slope (S) of the calibration curve.

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

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

5.1.8.7 Robustness

The robustness was studied by analyzing the samples of LPV and RTV by deliberate variation in the method parameters. The change in the response of LPV and RTV was noted.

Robustness of the method was studied by changing the extraction time of LPV and RTV from tablet dosage form by ± 2 min, composition of mobile phase by ± 2% of organic solvents, wavelength by ± 2 nm, flow rate by ± 0.2 mL/min and column oven temperature by ± 2ºC. The changes in the response of LPV and RTV were noted and compared with the original one.

5.1.8.8 System-Suitability Test

System suitability tests were used to verify that the resolution and repeatability of the system were adequate for the analysis intended. The parameters used in this test were retention time, tailing factor and theoretical plates of chromatographic peaks of LPV and RTV as RSD of peak areas for replicate injections.

5.2 RESULTS AND DISCUSSION:

5.2.1 Selection of Column and Mobile Phase

As per the published literature and knowledge of the molecule, reverse phase liquid chromatography (RP-HPLC) is suitable for analysis of LPV and RTV. In case of RP-HPLC various columns are available, but as the main aim of the method was to resolve the peaks of LPV and RTV, C_{18} column (250 mm x 4.6 mm i.d., 5 µm particle size) was preferred over the other columns. Resolution is the most important criteria for the method, it is imperative to achieve good resolution among the compounds. As per the value of pKa and solubility of compounds various composition of mobile phase were tried. The buffers of various pH ranges (4 to 6) were tried. The best resolution was obtained with ammonium acetate buffer with pH 4.5 ± 0.05.

The chromatographic conditions were optimized which can separate LPV and RTV with good resolution. Mobile phase consisting of acetonitrile : 10mM ammonium acetate buffer (pH 4.5 ± 0.05 adjusted with orthphosphoric acid) : methanol (40:30:30 v/v/v) at a flow rate of 1.0 mL/min, was found to be satisfactory to obtain well-resolved peaks with better reproducibility and repeatability for LPV and RTV.

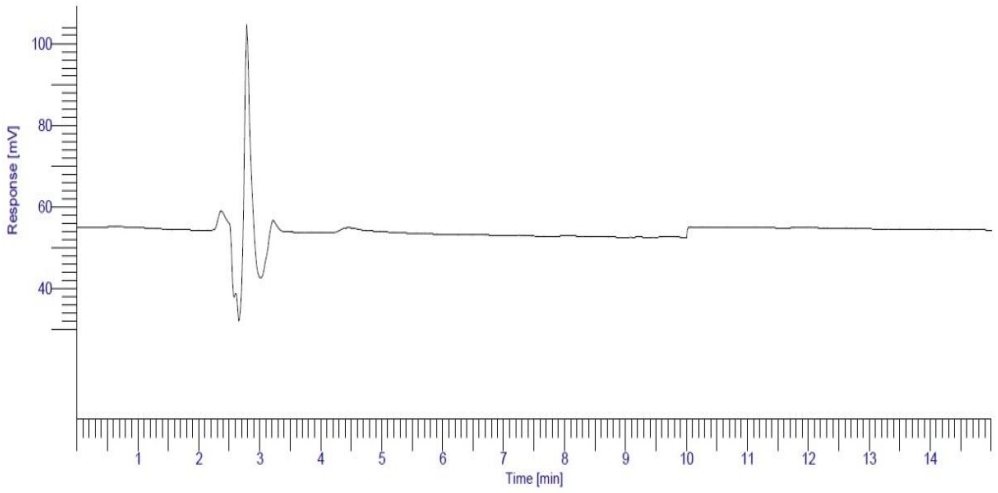


Figure 5.1: HPLC chromatogram of methanol blank at 210 nm

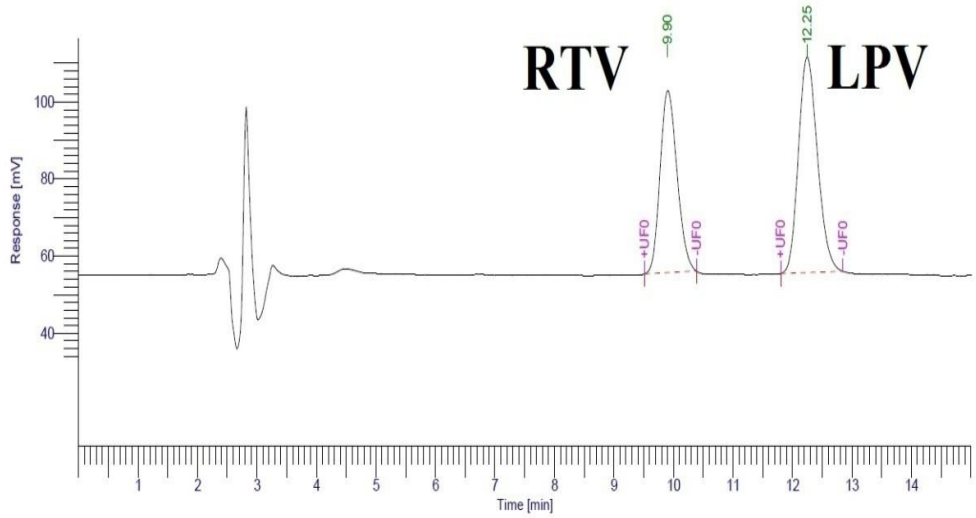


Figure 5.2: HPLC chromatogram of RTV (35 µg/mL) and LPV (35 µg/mL) standard with retention time of 9.90 and 12.25 min. respectively at 210nm

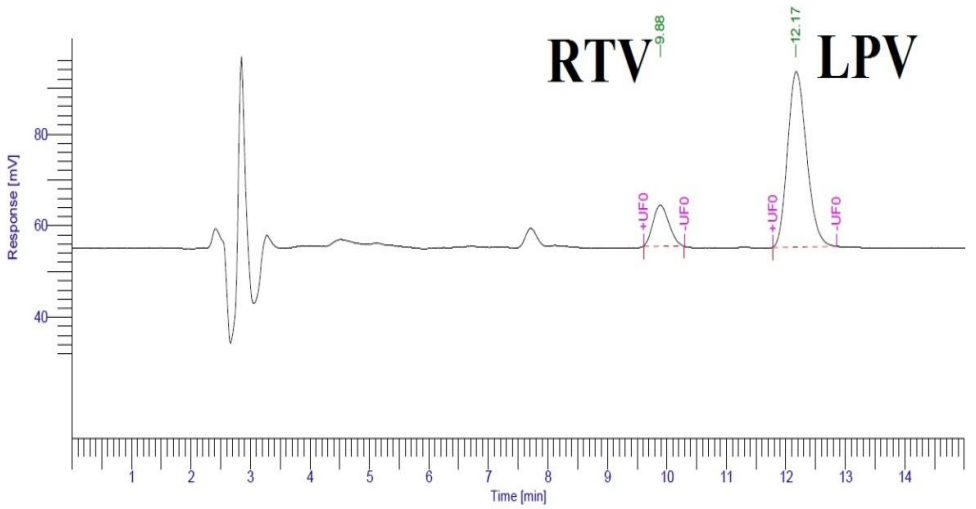


Figure 5.3: HPLC chromatogram of RTV (5 µg/mL) and LPV (20 µg/mL) tablet sample with retention time of 9.88 and 12.17 min. respectively at 210nm

5.2.2 METHOD VALIDATION

5.2.2.1 Solution Stability

The change in assay results after storage at 25°C (24 hours) and 2 - 8°C (3 days) was evaluated. It was found that the difference in assay results was not

more than 2 % for formulation, and 0.5% for API, indicating stability of LPV and RTV solution.

5.2.2.2 Specificity

The specificity of the method was determined by analyzing chromatogram of standard and sample solution. It was revealed that there was no interference from excipients or impurity was found in determination of LPV and RTV present in tablet which indicated that the proposed method was specific.

5.2.2.3 Linearity

Linear correlation was obtained between peak area and concentration of LPV and RTV in the range of 5-35 µg/ml., the linearity of the calibration curves were validated by the value of correlation coefficient of the regression (r), the regression analysis of the calibration curves is listed in Table 5.1

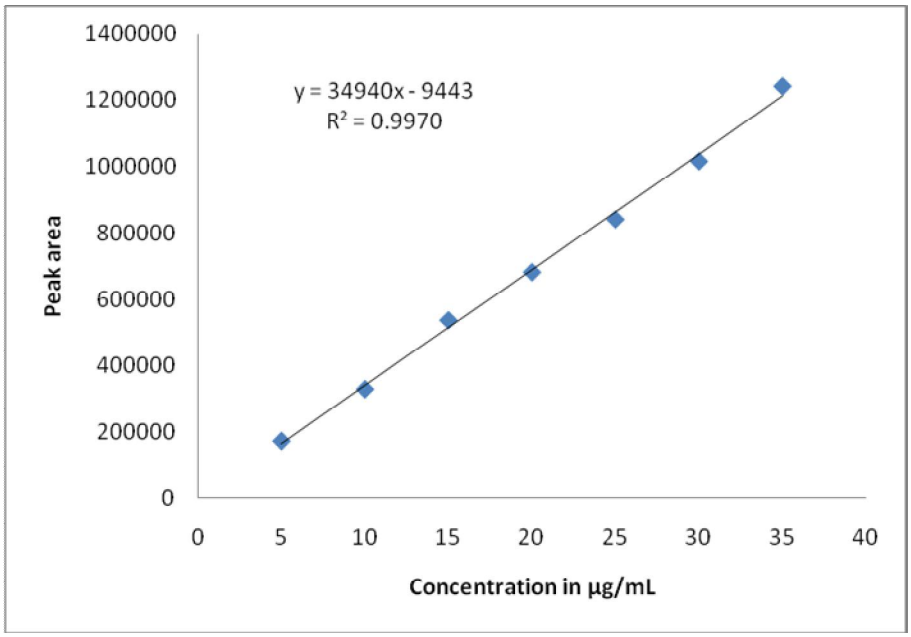


Figure 5.4: Calibration curve of LPV

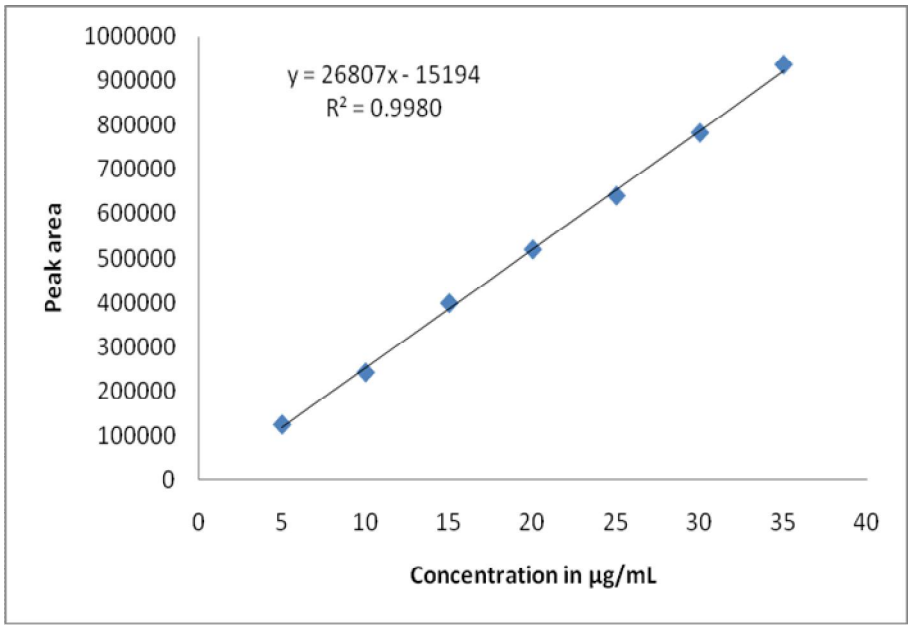


Figure 5.5: Calibration curve of RTV

Table 5.1: Optical and regression characteristics (n=3)

Parameter	LPV	RTV
Linearity range (µg/mL)	5-35	5-35
Linearity equation	y = 34940x - 9443	y = 26807x – 15194
LOD (µg/mL)	0.285	0.138
LOQ (µg/mL)	0.863	0.418
Correlation coefficient(r)	0.9970	0.9980

5.2.2.4 Accuracy (% Recovery)

The accuracy study was carried out by the standard addition method. The percent recoveries were found in the range of 97.31-100.41 % and 98.29-101.54 % for LPV and RTV respectively, which indicated accuracy of the method.

Table 5.2: Results of recovery study (n=3)

DRUG	Amount taken (µg/mL)	Amount added (µg/mL)	Amount found (µg/mL)	Recovery ± SD, %	% RSD
LPV	10	0	9.82	98.24 ± 1.21	1.21
	10	5	15.06	100.41 ± 1.39	1.39
	10	10	19.94	99.71 ± 0.97	0.97
	10	15	24.32	97.31 ± 1.14	1.14
RTV	10	0	9.82	98.29 ± 0.89	0.89
	10	5	14.75	98.34 ± 1.46	1.46
	10	10	19.91	99.58 ± 1.66	1.66
	10	15	25.38	101.54 ± 0.79	0.79

5.2.2.5 Precision

The % RSD for repeatability of LPV and RTV were found to be 1.84 and 1.62 respectively.

Table 5.3 Results of repeatability (n=6)

Drug	LPV Peak area	RTV Peak area
1	328802.3	242595.5
2	325673.2	239547.3
3	330984.4	247658.3
4	322154.8	245162
5	319468.5	241358.9
6	336042.6	236653.2
Mean	327187.6	242162.5
SD	6043.85	3930.03
% RSD	1.84	1.62

The value of % RSD for intra-day precision was found to be in the range of 0.71 - 0.91% and 0.69 - 0.91% while inter-day precision was found to be in the range of 1.01 - 1.28 % and 1.18 - 1.42% for LPV and RTV respectively, which indicated that the method was precise.

Table 5.4 Results of Intra-day and Inter-day precision (n=3)

Drug	**Con (µg/mL)**	**Intra-day precision**		**Inter-day precision**	
		Mean peak area ± SD	**% RSD**	**Mean peak area ± SD**	**% RSD**
LPV	10	328802.31 ± 2865.34	0.87	326977.13 ± 4187.32	1.28
	20	682953.09 ± 4876.45	0.71	684132.15 ± 6973.25	1.01
	30	1018343.77 ± 9248.77	0.91	1016594.12 ± 12568.36	1.23
RTV	10	242595.53 ± 1680.56	0.69	243865.45 ± 3467.41	1.42
	20	519441.60 ± 4769.74	0.91	518238.35 ± 6152.34	1.18
	30	783323.13 ± 6846.35	0.87	785182.91 ± 9587.73	1.22

5.2.2.6 Limit of detection and limit of quantification

The Limit of detection (LOD) was found to be 0.138 and 0.285 µg/mL while the Limit of quantification (LOQ) was 0.418 and 0.863 µg/mL for RTV and LPV respectively.

5.2.2.7 Robustness

The method was found to be robust as the results were not significantly affected by slight variation in extraction time, composition of mobile phase, and wavelength and flow rate of the mobile phase.

5.2.2.8 System-Suitability Test

The % RSD of system-suitability test parameters was found satisfactory. Resolution between the peaks of LPV and RTV was found to be 4.095. The results are listed in Table 5.5

Table 5.5: System suitability test parameters for LPV (n = 6)

No.	Retention time, Min.	Tailing factor	Theoretical plates
1	12.25	1.18	6546.5
2	12.17	1.18	6571.24
3	12.25	1.20	6523.26
4	12.20	1.18	6581.35
5	12.25	1.19	6540.58
6	12.22	1.17	6590.2
Mean	12.22	1.18	6558.85
SD	0.033	0.010	26.06
% RSD	0.27	0.87	0.39

Table 5.6: System suitability test parameters for RTV (n = 6)

No.	Retention time, Min.	Tailing factor	Theoretical plates
1	9.90	1.20	5334.77
2	9.88	1.20	5371.4
3	9.90	1.22	5289.72
4	10.12	1.21	5319.51
5	9.85	1.20	5327.86
6	10.12	1.21	5346.63
Mean	9.96	1.206	5331.64
SD	0.124	0.008	27.32
% RSD	1.24	0.67	0.51

5.2.3 Analysis of Tablet Dosage Form

The proposed RP-HPLC method was successfully applied for determination of LPV and RTV from combined tablet dosage form. The percentage of LPV and RTV were found to be satisfactory; which was comparable with the corresponding label claim.

Table 5.7: Analysis results of tablet dosage form (n=3)

Formulation	Drug	Labeled amount (mg)	Amount found (mg)	Assay ± SD, %
LOPIMUNE®	LPV	200	197.98	98.99 ± 1.22
	RTV	50	49.83	99.67 ± 1.25

5.3 CONCLUSION:

An isocratic reverse phase high performance liquid chromatographic method has been developed and validated for the determination of LPV and RTV from tablet dosage form. The method was found to be specific as there was no interference of any excipients and impurities. The proposed method was found to be simple, accurate, precise, sensitive and robust. Hence, it can be used successfully for the routine analysis of LPV and RTV in their pharmaceutical dosage forms.

CHAPTER 6

DETERMINATION OF ANTIRETROVIRAL AGENTS IN BULK AND TABLET DOSAGE FORM USING HPTLC METHOD

6. DETERMINATION OF ANTIRETROVIRAL AGENTS IN BULK AND DOSAGE FORM USING HPTLC METHOD

6.1 DETERMINATION OF EFAVIRENZ IN BULK AND TABLET DOSAGE FORM

6.1.1 EXPERIMENTAL:

6.1.1.1 Instrumentation:

- A Camag HPTLC with Linomat V auto spotter and Camag Scanner-III
- Camag flat bottom and twin trough developing chamber (10 cm x 10 cm)
- HPTLC plates used were silica gel with fluorescent indicator 254 nm, layer thickness (0.2 mm) 10 cm x 10 cm aluminium (E-Merck-KgaA).
- UV cabinet with dual wavelength UV lamp
- Ultrasonic bath (TEC-4, Roop Telesonic Ultrasonix)

6.1.1.2 Chemicals and materials:

- Efavirenz (EFV) was kindly gifted by Aurobindo Pharmaceuticals Ltd., Hyderabad, India.
- Ethyl acetate AR grade (Spectrochem Pvt. Ltd., Mumbai, India)
- Toluene and methanol HPLC grade (Rankem, RFCL Ltd., New Delhi)
- Tablets (ESTIVA 600®, Genix Pharma) containing efavirenz (600 mg) were purchased from local market.

6.1.1.3 Chromatographic conditions:

6.1.1.3.1 Pre-treatment of HPTLC plates

HPTLC plate was placed in twin-trough glass chamber containing methanol as mobile phase. Methanol was allowed to run up to the upper edge of plate (ascending method). The Plate was removed and allowed to dry in oven at 60^0C for 5 min. For the actual experiment the plate was allowed to come to room temperature and used immediately.

6.1.1.3.2 Chromatographic separation

The chromatographic separation was achieved on HPTLC plates using mobile phase ethyl acetate : toluene (6:4 v/v). EFV reference standard solution was

prepared using methanol as solvent. From the prepared standard solution, appropriate volume of aliquots were applied to silica gel 60 F_{254} HPTLC plates (10 cm x 10 cm) as spot bands of 6mm using LINOMAT V. Mobile phase components were mixed prior to use and the development chamber was left for saturation with mobile phase vapors for 10 min before each run. Development of the plate was carried out by the ascending technique to a migration distance of 7 cm. Then the plates were allowed to dry. All the analysis was carried out in a laboratory with temperature control (25 ± 2^0C). Densitometry scanning was done in absorbance mode at 254 nm using a deuterium lamp. The slit dimensions were set at 6 mm x 0.30 mm, the scanning speed of 10 mm/s, and the data resolution at 100 µm/step. Single wavelength detection was performed since the main components were only analyzed.

6.1.1.4 Preparation of the Mobile phase

The mobile phase was prepared by mixing 6.0 mL ethyl acetate with 4.0 mL toluene. The mobile phase was transferred into a twin-trough chamber covered with lid and allowed to stand for 30 min before use.

6.1.1.5 Preparation of Standard stock solution of EFV:

EFV (100 mg) was accurately weighed and transferred into 100 mL volumetric flask ,and dissolved in methanol. The volume was made up to the mark with methanol. Aliquot was further 4 times diluted with methanol to get the final concentration of 250 µg/mL EFV which was used for calibration purpose. Aliquots (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 µL) were applied to the HPTLC plate as bands of 6 mm.

6.1.1.6 Selection of Wavelength for Determination

The standard solution of EFV was scanned in the range of 200 - 400 nm against reagent blank. Maximum absorbance for the compound was observed at 254 nm which was selected for the determination.

6.1.1.7. Analysis of Tablet Dosage Form:

Twenty tablets, each containing 600 mg of EFV, were weighed and their average weight was calculated. The tablets were finely powdered and powder equivalent to 100 mg EFV was accurately weighed and transferred in to 100 mL volumetric flask. Methanol (60 mL) was added to it and shaken for 10 minutes. The volume was made up to the mark with methanol. The solution was sonicated for 30min, filtered through the whatman no.41 filter paper. Aliquot was further 4 times diluted with methanol to get the final concentration of 250 µg/mL EFV. An aliquot (2 µL equivalent to 500 ng/spot EFV) was applied to the HPTLC plate. The plate was developed and analyzed as described under 6.1.1.3.2. The chromatogram was recorded. The peak area was noted and amount of EFV was calculated from the regression equation.

6.1.1.8 METHOD VALIDATION

As per ICH guidelines Q2 (R1), the method validation parameters studied were solution stability, specificity, linearity, accuracy, precision, limit of detection, limit of quantitation and robustness.

6.1.1.8.1 Solution Stability

Sample solutions were kept at 25^0C (24 hours) and 2 - 8^0C (3 days), respectively. Assay of initial time period was compared with these two time periods. The falls in the assay values were evaluated. The difference between assays should not be more than 2 % for formulation, and 0.5% for API.

6.1.1.8.2 Specificity

Specificity of an analytical method is its ability to measure the analyte accurately and specifically in the presence of component that may be expected to be present in the sample matrix. Chromatograms of standard and sample solution of EFV were compared, and peak purity spectra at three different levels i.e., peak start (S), peak apex (M) and peak end (E) of a spot were recorded in order to provide an indication of specificity of the method.

6.1.1.8.3 Linearity (Calibration curve)

Standard stock solution containing 1000 µg/mL EFV was prepared in methanol and 4 times diluted. Aliquots (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 µL) were applied to the HPTLC plate to deliver 125, 250, 375, 500, 625 and 750 ng of EFV per spot. The plate was developed and analyzed as described under 6.1.1.3.2. The chromatograms were recorded and the peak areas were noted. Calibration curve was constructed by plotting peak area versus concentration, and the regression equation was calculated. Each response was average of three determinations.

6.1.1.8.4 Accuracy (% Recovery)

The accuracy of the method was determined by calculating recovery of EFV by the standard addition method. Known amount of standard solutions of EFV (equivalent to 0, 125, 250 and 375 ng/spot) were applied to the sample spot of EFV (250 ng/spot) on the plate. Each solution was applied in triplicate. The plate was developed and analyzed as described under 6.1.1.3.2. The percentage recovery was calculated by measuring the peak areas and fitting these values into the regression equation of the calibration curve.

6.1.1.8.5 Precision

The repeatability of measurement of peak area was checked by repeatedly (n = 6) measuring area of one band of EFV (500 ng/spot), while repeatability of sample application was checked by repeatedly (n = 6) measuring area of six bands having same concentration of EFV (500 ng/spot) applied on the same plate without changing the position of plate. The intra-day and inter-day precisions of the proposed method was determined by measuring the corresponding responses 3 times on the same day and on 3 different days over a period of 1 week for 3 different concentrations of EFV (250, 500 and 750 ng/spot). The results were reported in terms of relative standard deviation.

6.1.1.8.6 Limit of Detection and Limit of Quantification:

Limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the standard deviation of response (σ) and slope (S) of the calibration curve.

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

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

6.1.1.8.7 Robustness

The robustness was studied by analyzing the samples of EFV by deliberate variation in the method parameters. The change in the response of EFV was noted. Robustness of the method was studied by changing the extraction time of EFV from tablet dosage form by ± 2 min, composition of mobile phase by ± 2% of organic solvent, development distance by ± 1 cm, wavelength by ± 2 nm and temperature by ± 2^0C. The changes in the response of EFV were noted and compared with the original one.

6.1.2 RESULTS AND DISCUSSION:

6.1.2.1 Selection of mobile phase

Resolution is the most important criteria for the method, it is imperative to achieve good resolution among the compounds. As per the value of pKa and solubility of compound various composition of mobile phase were tried.

The chromatographic conditions were optimized with mobile phase consisting of ethyl acetate : toluene (6:4 v/v), which was found satisfactory to obtain sharp, well defined EFV peak with better reproducibility and repeatability (Figure 6.1.1).

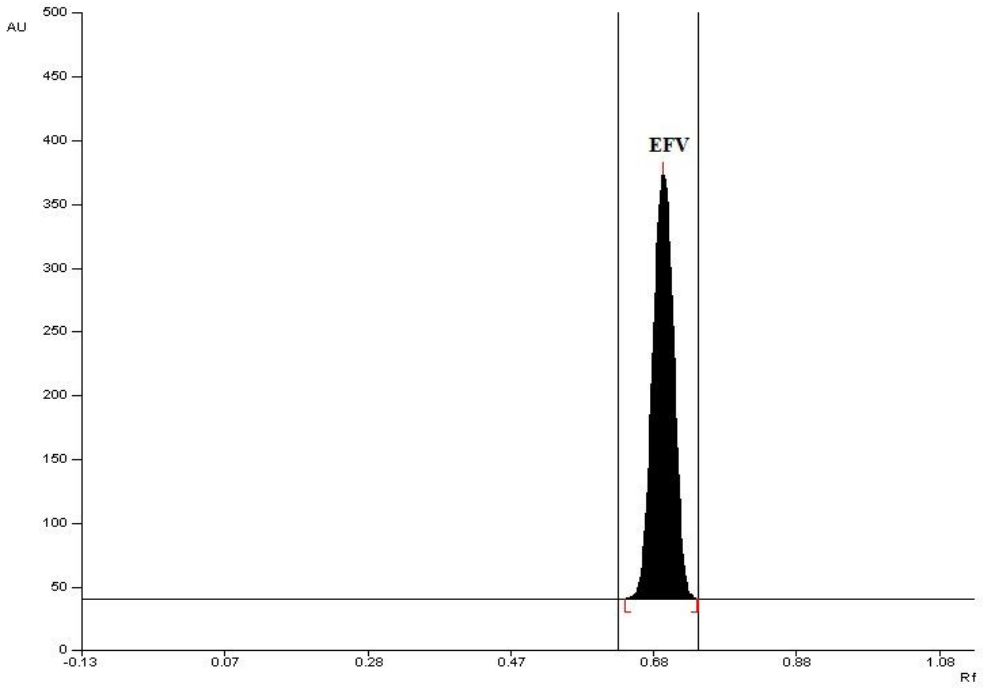


Figure 6.1.1(a): HPTLC chromatogram of EFV (750 ng/spot) standard with corresponding R_f at 254 nm

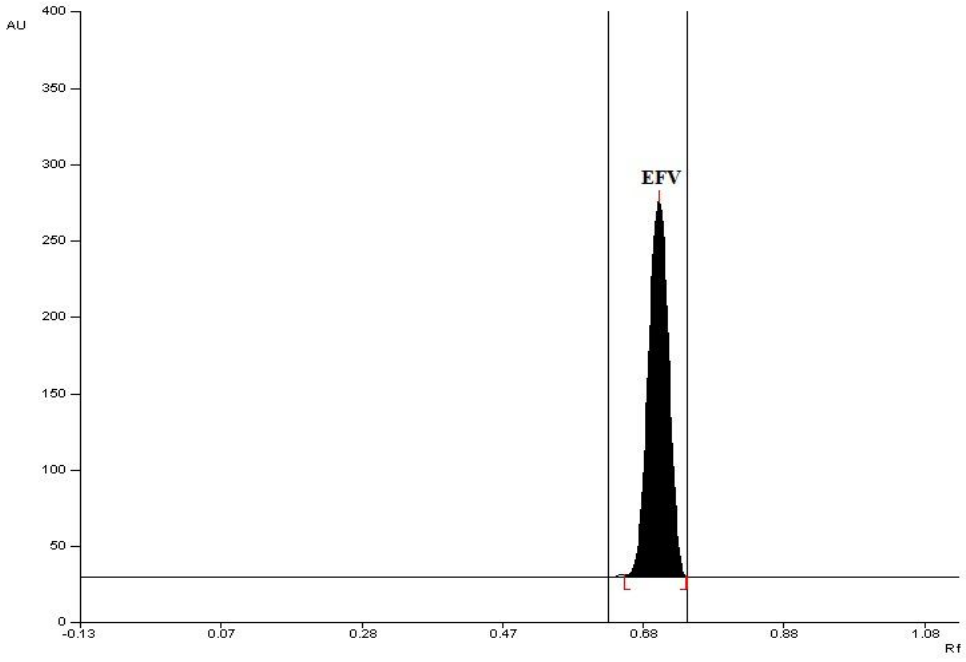


Figure 6.1.1(b): HPTLC chromatogram of EFV (500 ng/spot) tablet sample with corresponding R_f at 254 nm

6.1.2.2 METHOD VALIDATION

6.1.2.2.1 Solution Stability

The change in assay results after storage at 25°C (24 hours) and 2-8°C (3 days) was evaluated. It was found that the difference in assay results was not more than 2 % for formulation, and 0.5% for API, indicating stability of EFV solution.

6.1.2.2.2 Specificity

The proposed method was found to be specific as no interference of excipients or impurities was found in separation and determination of the peak purity of EFV, as r(S, M) = 0.9999 and r(M, E) = 0.9998 (Figure 6.1.2), and good correlation (r = 0.9999 and 0.9998) was obtained between standard and sample spectra of EFV, respectively. The peak purity and correlation > 0.99 indicated that the method is specific.

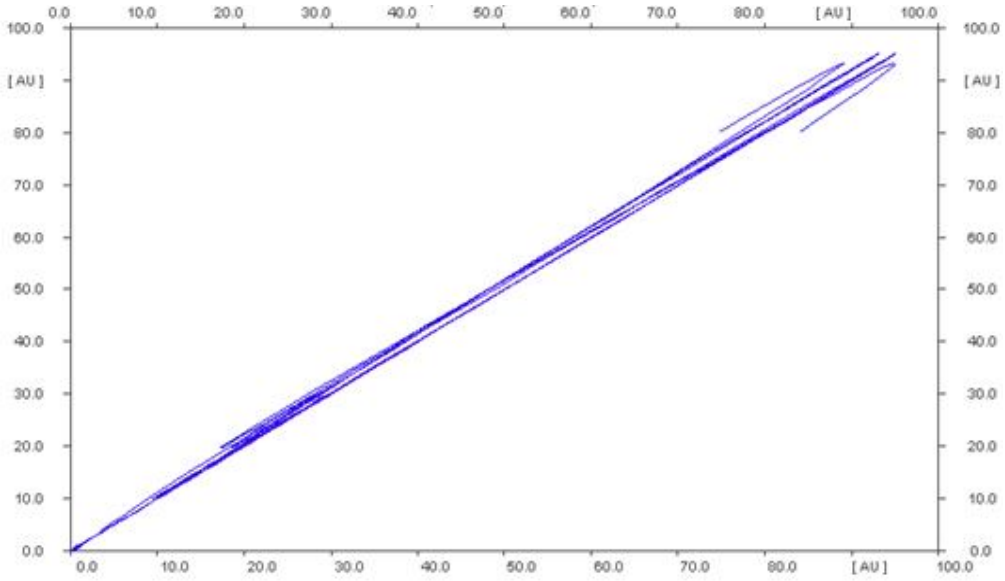


Figure 6.1.2: Peak purity spectra of EFV from tablet dosage form

6.1.2.2.3 Linearity

Linear correlation was obtained between peak area and concentration of EFV in the range of 125- 750 ng/spot. The linearity of the calibration curve was validated by the value of correlation coefficients of the regression (r). The optical and regression characteristics are listed in Table 6.1.1.

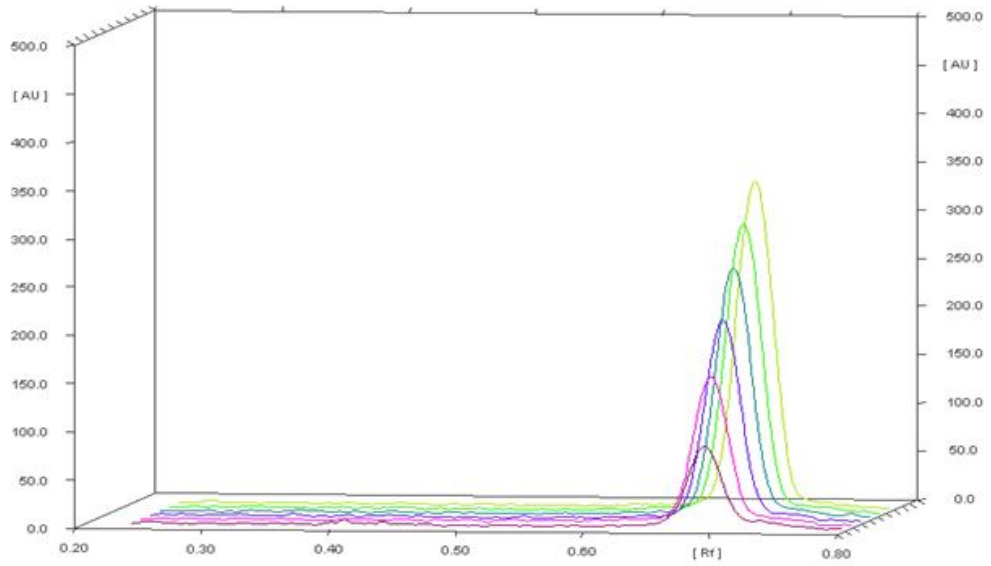


Figure 6.1.3: 3D Chromatogram showing peaks of EFV standards in different concentrations

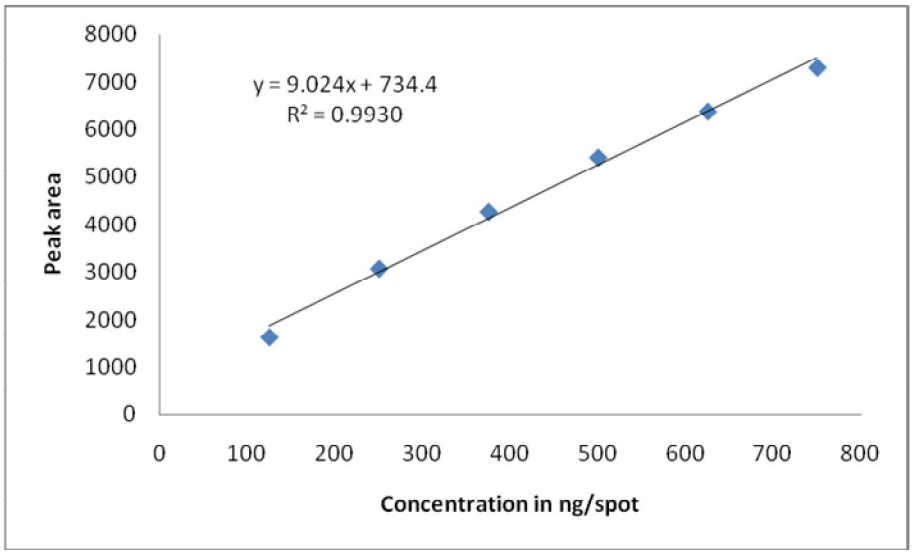


Figure 6.1.4: Calibration curve of EFV

Table 6.1.1: Optical and regression characteristics (n=3)

Parameter	EFV
Linearity range (ng/spot)	125-750
Linearity equation	y = 9.024x + 734.4
LOD (ng/spot)	8.36
LOQ (ng/spot)	25.33
Correlation coefficient (r)	0.9930

6.1.2.2.4 Accuracy (% Recovery)

Accuracy study was carried out by the standard addition method. The percent recovery was found in the range of 98.20 – 101.29 % for EFV, which indicated accuracy of the method.

Table 6.1.2: Results of recovery study (n=3)

Drug	Amount taken (ng/spot)	Amount added (ng/spot)	Amount found (ng/spot)	Recovery ± SD, %	% RSD
EFV	250	0	245.52	98.20 ± 1.43	1.43
	250	125	379.86	101.29 ± 0.69	0.69
	250	250	496.64	99.32 ± 0.86	0.86
	250	375	616.92	98.70 ± 1.64	1.64

6.1.2.2.5 Precision

The % RSD of the repeatability of measurement of peak area was found to be 0.77; while of the repeatability of sample application was found to be 1.80 for EFV. The % RSD for intra-day precision was found to be in the range of 0.65 - 0.92 %; while inter-day precision was found to be in the range of 0.90 - 1.24 % for EFV, which indicated that the method was precise.

Table 6.1.3: Results of repeatability (n=6)

Drug	EFV	
	Measurement of peak area	Sample application
1	4275.0	4321.2
2	4234.6	4286.7
3	4284.2	4354.3
4	4221.9	4408.4
5	4312.3	4186.4
6	4264.6	4257.9
Mean	4265.43	4302.48
SD	33.12	77.41
% RSD	0.77	1.80

Table 6.1.4: Results of Intra-day and Inter-day precision (n=3)

Drug	Concen-tration (ng/spot)	Intra-day precision		Inter-day precision	
		Mean peak area ± SD	% RSD	Mean peak area ± SD	% RSD
EFV	250	3077.0 ± 27.38	0.89	3122.4 ± 28.41	0.91
	500	5418.0 ± 35.21	0.65	5346.6 ± 48.11	0.90
	750	7310.2 ± 67.56	0.92	7245.4 ± 89.96	1.24

6.1.2.2.6 Limit of detection and limit of quantification

The Limit of detection (LOD) was found to be 8.36 ng/spot while the Limit of quantification (LOQ) was found to be 25.33 ng/spot for EFV.

6.1.2.2.7 Robustness

The method was found to be robust as the results were not significantly affected by slight variation in extraction time, composition of mobile phase, development distance, wavelength and temperature.

6.1.2.2.8 Analysis of Tablet Dosage Form

The proposed HPTLC method was successfully applied for determination of EFV from tablet dosage form. The percentage of EFV was found to be satisfactory, which was comparable with the corresponding label claim.

Table 6.1.5: Analysis results of tablet dosage form (n=3)

Formulation	Drug	Labelled amount (mg)	Amount found (mg)	Assay ± SD, %
ESTIVA 600®	EFV	600	594.66	99.11 ± 1.70

6.1.3 CONCLUSION:

A high performance thin layer chromatographic method has been developed and validated for the determination of EFV from tablet dosage form. The method was found to be specific as there was no interference of excipients and impurity. The proposed method was found to be simple, accurate, precise, sensitive and robust. Hence, it can be used successfully for the routine analysis of EFV in pharmaceutical dosage forms.

6.2 DETERMINATION OF TENOFOVIR DISOPROXIL FUMARATE IN BULK AND TABLET DOSAGE FORM

6.2.1 EXPERIMENTAL:

6.2.1.1 Instrumentation:

Same as described under 6.1.1.1.

6.2.1.2 Chemicals and materials:

- Tenofovir disoproxil fumarate (TNV) was kindly gifted by Aurobindo Pharmaceuticals Ltd., Hyderabad, India.
- Ethyl acetate AR grade (Spectrochem Pvt. Ltd., Mumbai, India)
- Formic acid AR grade (Astron Chemicals, Ahmedabad)
- Methanol HPLC grade (Rankem, RFCL Ltd., New Delhi)
- Tablets (TENOF®, Genix Pharma) containing tenofovir disoproxil fumarate (300 mg) were purchased from local market.

6.2.1.3 Chromatographic conditions:

6.2.1.3.1 Pre-treatment of HPTLC plates

Same as described under 6.1.1.3.1

6.2.1.3.2 Chromatographic separation

The chromatographic separation was achieved on HPTLC plates using ethyl acetate : methanol : formic acid (7:2.5:0.5 v/v/v). TNV reference standard solution was prepared using methanol as solvent. From the prepared standard solution, appropriate volume of aliquots were applied to silica gel 60 F_{254} HPTLC plates (10 cm x 10 cm) as spot bands of 6mm using LINOMAT V. Mobile phase components were mixed prior to use and the development chamber was left for saturation with mobile phase vapors for 10min before each run. Development of the plate was carried out by the ascending technique to a migration distance of 7 cm. Then the plates were dried. All the analysis were carried out in a laboratory with temperature control (25 ± 2^0C). Densitometry scanning was done in absorbance mode at 266 nm using a deuterium lamp. The slit dimensions were set at 6 mm x 0.30 mm, the scanning speed of 10 mm/s, and the data resolution at 100 µm/step. Single

wavelength detection was performed since the main components were only analyzed.

6.2.1.4 Preparation of the Mobile phase

The mobile phase was prepared by mixing 7.0 mL ethyl acetate, 2.5 mL methanol with 0.5 mL formic acid. The mobile phase was transferred into a twin-trough chamber covered with lid and allowed to stand for 30 min before use.

6.2.1.5 Preparation of Standard stock solution of TNV

TNV (100 mg) was accurately weighed and transferred into100 mL volumetric flask, and dissolved in methanol. The volume was made up to the mark with methanol. Aliquot was further 4 times diluted with methanol to get the final concentration of 250 µg/mL TNV which was used for calibration purpose. Aliquots (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 µL) were applied to the HPTLC plates as bands of 6 mm.

6.2.1.6 Selection of Wavelength for Determination

The standard solution of TNV was scanned in the range of 200 - 400 nm against reagent blank. The drug showed significant absorbance at 266nm which was selected for analysis.

6.2.1.7 Analysis of Tablet Dosage Form

Twenty tablets, each containing 300 mg of TNV, were weighed and their average weight was calculated. The tablets were finely powdered and powder equivalent to 100 mg TNV was accurately weighed and transferred in to 100 mL volumetric flask. Methanol (60 mL) was added to it and shaken for 10 minutes. The volume was made up to the mark with methanol. The solution was sonicated for 30 min, filtered through the whatman no.41 filter paper. Aliquot was further 4 times diluted with methanol to get the final concentration of 250 µg/mL TNV. An aliquot (2 µL equivalent to 500 ng/spot TNV) was applied to the HPTLC plate. The plate was developed and analyzed as described under 6.2.1.3.2. The chromatogram was recorded. The peak area was noted and amount of TNV calculated from the regression equation.

6.2.1.8 METHOD VALIDATION

As per ICH guidelines Q2(R1), the method validation parameters studied were solution stability, specificity, linearity, accuracy, precision, limit of detection, limit of quantitation and robustness.

6.2.1.8.1 Solution Stability

Sample solutions were kept at 25^0C (24 hours) and 2 - 8^0C (3 days), respectively. Assay of initial time period was compared with these two time periods. The falls in the assay values were evaluated. The difference between assays should not be more than 2 % for formulation, and 0.5% for API.

6.2.1.8.2 Specificity

Specificity of an analytical method is its ability to measure the analyte accurately and specifically in the presence of component that may be expected to be present in the sample matrix. Chromatograms of standard and sample solution of TNV were compared, and peak purity spectra at three different levels i.e., peak start (S), peak apex (M) and peak end (E) of a spot were recorded in order to provide an indication of specificity of the method.

6.2.1.8.3 Linearity (Calibration curve)

Standard stock solution containing 1000 µg/mL TNV was prepared in methanol and 4 times diluted. Aliquots (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 µL) were applied to the HPTLC plate to deliver 125, 250, 375, 500, 625 and 750 ng of TNV per spot. The plate was developed and analyzed as described under 6.2.1.3.2. The chromatograms were recorded and the peak areas were noted. Calibration curve was constructed by plotting peak area versus concentration, and the regression equation was calculated. Each response was average of three determinations.

6.2.1.8.4 Accuracy (% Recovery)

The accuracy of the method was determined by calculating recovery of TNV by the standard addition method. Known amount of standard solutions of TNV (equivalent to 0, 125, 250 and 375 ng/spot) were applied to the sample spot of TNV (250 ng/spot) on the plate. Each solution was applied in triplicate. The

plate was developed and analyzed as described under 6.2.1.3.2. The percentage recovery was calculated by measuring the peak areas and fitting these values into the regression equation of the calibration curve.

6.2.1.8.5 Precision

The repeatability of measurement of peak area was checked by repeatedly (n = 6) measuring area of one band of TNV (500 ng/spot), while repeatability of sample application was checked by repeatedly (n = 6) measuring area of six bands having same concentration of TNV (500 ng/spot) applied on the same plate without changing the position of plate. The intra-day and inter-day precisions of the proposed method was determined by measuring the corresponding responses 3 times on the same day and on 3 different days over a period of 1 week for 3 different concentrations of TNV (250, 500 and 750 ng/spot). The results were reported in terms of relative standard deviation.

6.2.1.8.6 Limit of Detection and Limit of Quantification

Limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the standard deviation of response (σ) and slope (S) of the calibration curve.

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

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

6.2.1.8.7 Robustness

The robustness was studied by analyzing the samples of TNV by deliberate variation in the method parameters. The change in the response of TNV was noted. Robustness of the method was studied by changing the extraction time of TNV from tablet dosage form by ± 2 min, composition of mobile phase by ± 2% of organic solvent, development distance by ± 1 cm, wavelength by ± 2 nm and temperature by ± 2^0C. The changes in the response of TNV were noted and compared with the original one.

6.2.2 RESULTS AND DISCUSSION:

6.2.2.1 Selection of mobile phase

Resolution is the most important criteria for the method, it is imperative to achieve good resolution among the compounds. As per the value of Ka and solubility of compound various composition of mobile phase were tried.

The chromatographic conditions were optimized with mobile phase consisting of ethyl acetate : methanol : formic acid (7:2.5:0.5 v/v/v), which was found satisfactory to obtain sharp, well-defined TNV peak with better reproducibility and repeatability (Figure 6.2.1).

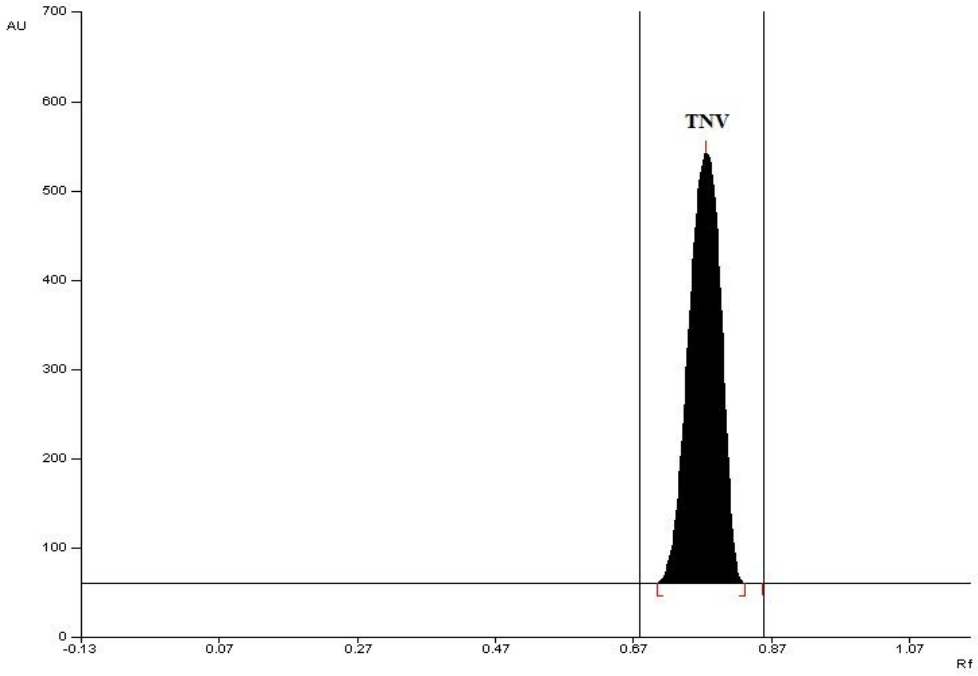


Figure 6.2.1(a): HPTLC chromatogram of TNV (750 ng/spot) standard with corresponding R_f at 266 nm

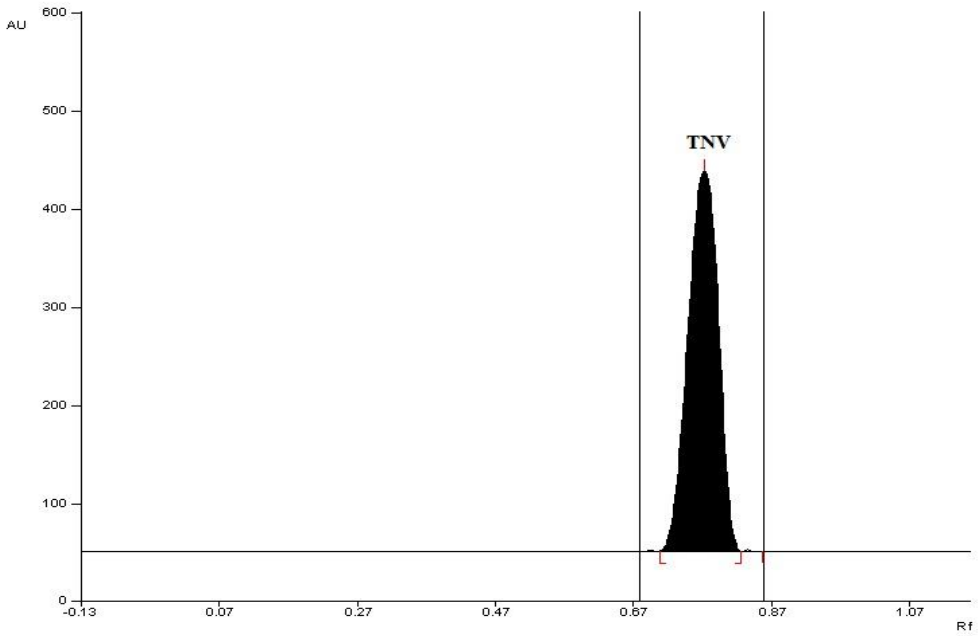


Figure 6.2.1(b): HPTLC chromatogram of TNV (500 ng/spot) tablet sample with corresponding R_f at 266 nm

6.2.2.2 METHOD VALIDATION

6.2.2.2.1 Solution Stability

The change in assay results after storage at 25°C (24 hours) and 2 - 8°C (3 days) was evaluated. It was found that the difference in assay results was not more than 2 % for formulation, and 0.5% for API, indicating stability of TNV solution.

6.2.2.2.2 Specificity

The proposed method was found to be specific as no interference of excipients or impurities was found in separation and determination of the peak purity of TNV, as r(S, M) = 0.9999 and r(M, E) = 0.9998 (Figure 6.1.2), and good correlation (r = 0.9999 and 0.9998) was obtained between standard and sample spectra of TNV, respectively. The peak purity and correlation > 0.99 indicated the method specificity.

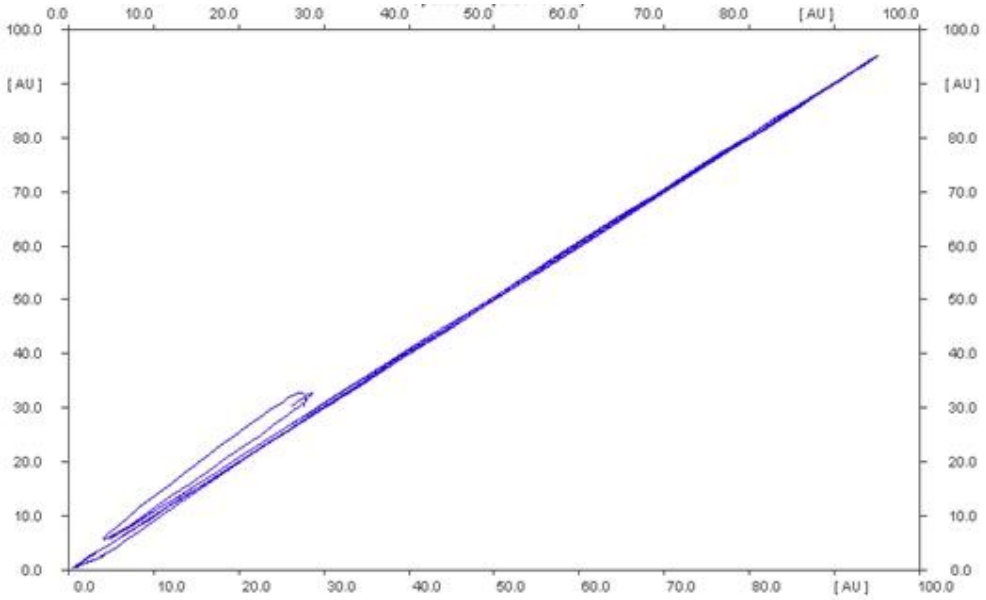


Figure 6.2.2: Peak purity spectra of TNV from tablet dosage form

6.2.2.2.3 Linearity

Linear correlation was obtained between peak area and concentration of TNV in the range of 125- 750 ng/spot. The linearity of the calibration curve was validated by the value of correlation coefficients of the regression (r). The optical and regression characteristics are listed in Table 6.2.1.

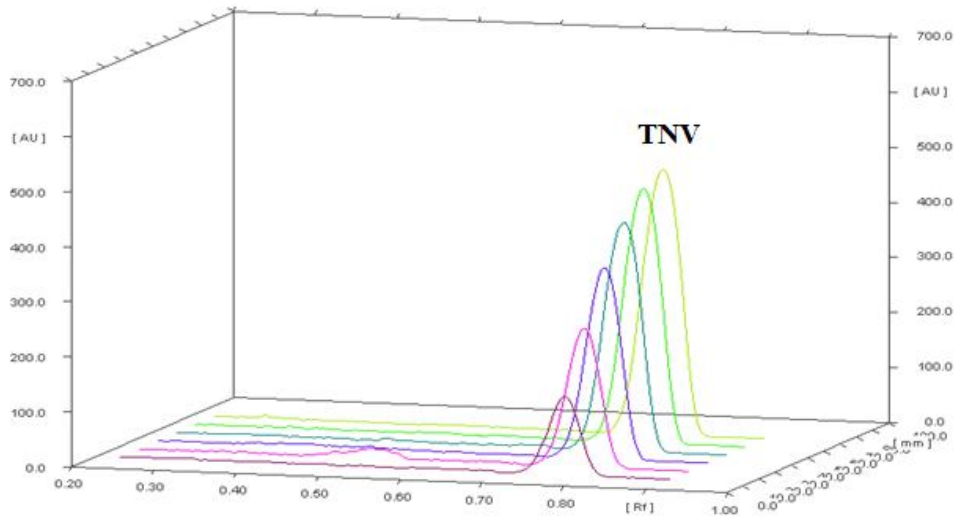


Fig 6.2.3: 3D Chromatogram showing peaks of TNV standards in different concentrations

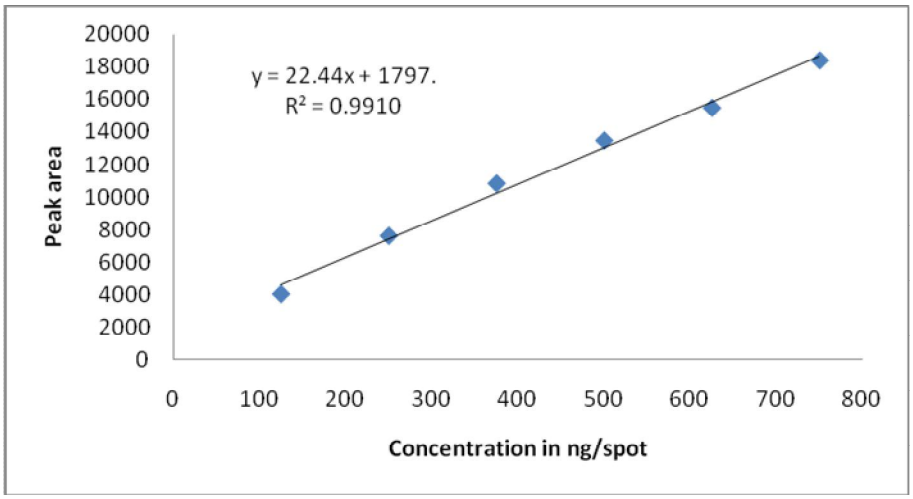


Fig. 6.2.4: Calibration curve of TNV

Table 6.2.1: Optical and regression characteristics (n=3)

Parameter	TNV
Linearity range (ng/spot)	125-750
Linearity equation	y = 22.44x + 1797.
LOD (ng/spot)	7.90
LOQ (ng/spot)	23.93
Correlation coefficient (r)	0.9910

6.2.2.2.4 Accuracy (% Recovery)

Accuracy study was carried out by the standard addition method. The percent recovery was found in the range of 98.75 – 101.34 % for TNV, which indicated accuracy of the method.

Table 6.2.2: Results of recovery study (n=3)

Drug	Amount taken (ng/spot)	Amount added (ng/spot)	Amount found (ng/spot)	Recovery± SD, %	% RSD
TNV	250	0	246.87	98.75 ± 0.87	0.87
	250	125	371.73	99.13 ± 1.65	1.65
	250	250	506.70	101.34 ± 1.39	1.39
	250	375	617.87	98.86 ± 0.94	0.94

6.2.2.2.5 Precision

The % RSD of the repeatability of measurement of peak area was found to be 0.41; while of the repeatability of sample application was found to be 0.70 for TNV. The % RSD for intra-day precision was found to be in the range of 0.71-1.48 %; while inter-day precision was found to be in the range of 0.93 - 1.53% for TNV, which indicated that the method was precise.

Table 6.2.3: Results of repeatability (n=6)

Drug	TNV	
	Measurement of peak area	Sample application
1	13455.1	13417.5
2	13489.6	13387.2
3	13375.4	13564.1
4	13532.7	13402.6
5	13479.8	13365.3
6	13511.4	13582.4
Mean	13474	13453.18
SD	55.13	94.77
% RSD	0.41	0.70

Table 6.2.4: Results of Intra-day and Inter-day precision (n=3)

Drug	Concen-tration (ng/spot)	Intra-day precision		Inter-day precision	
		Mean peak area ± SD	% RSD	Mean peak area ± SD	% RSD
TNV	250	7587.0 ± 54.11	0.71	7616.4 ± 71.46	0.93
	500	13455.1 ± 175.87	1.30	13564.2 ± 195.38	1.44
	750	18383.0 ± 272.63	1.48	18422.4 ± 283.15	1.53

6.2.2.2.6 Limit of detection and limit of quantification

The Limit of detection (LOD) was found to be 7.90 ng/spot while the Limit of quantification (LOQ) was found to be 23.93 ng/spot for TNV.

6.2.2.2.7 Robustness

The method was found to be robust as the results were not significantly affected by slight variation in extraction time, composition of mobile phase, development distance, wavelength and temperature.

6.2.2.2.8 Analysis of Tablet Dosage Form

The proposed HPTLC method was successfully applied for determination of TNV from tablet dosage form. The percentage of TNV was found to be satisfactory, which was comparable with the corresponding label claim.

Table 6.2.5: Analysis results of tablet dosage form (n=3)

Formulation	Drug	Labelled amount (mg)	Amount found (mg)	Assay ± SD, %
TENOF®	TNV	300	296.70	98.90 ± 1.47

6.2.3 CONCLUSION:

A high performance thin layer chromatographic method has been developed and validated for the determination of TNV from tablet dosage form. The method was found to be specific as there was no interference of excipients and impurity. The proposed method was found to be simple, accurate, precise, sensitive and robust. Hence, it can be used successfully for the routine analysis of TNV in pharmaceutical dosage forms.

6.3 DETERMINATION OF LOPINAVIR AND RITONAVIR IN BULK AND THEIR COMBINED TABLET DOSAGE FORM

6.3.1 EXPERIMENTAL:

6.3.1.1 Instrumentation:

Same as described under 6.1.1.1

6.3.1.2 Chemicals and materials:

- Lopinavir (LPV) and Ritonavir (RTV) were kindly gifted by Emcure Pharmaceuticals Ltd., Pune, India.
- Chloroform HPLC grade (Rankem, RFCL Ltd., New Delhi)
- 1,4-dioxane GC grade (Spectrochem Pvt. Ltd., Mumbai)
- Methanol HPLC grade (Rankem, RFCL Ltd., New Delhi)
- Tablets (LOPIMUNE®, CiplaLtd) containing Lopinavir (200 mg) and Ritonavir (50 mg) were purchased from local market.

6.3.1.3 Chromatographic conditions:

6.3.1.3.1 Pre-treatment of HPTLC plates

Same as described under 6.1.1.3.1

6.3.1.3.2 Chromatographic separation

The chromatographic separation was achieved on HPTLC plates using chloroform: 1,4- dioxane (7:3 v/v). LPV and RTV reference standard solution was prepared using methanol as solvent. From the prepared standard solution, appropriate volume of aliquots were applied to silica gel 60 F_{254} HPTLC plates (10 cm x 10 cm) as spot bands of 6mm using LINOMAT V. Mobile phase components were mixed prior to use and the development chamber was left for saturation with mobile phase vapors for 10min before each run. Development of the plate was carried out by the ascending technique to a migration distance of 7 cm. Then the plates were dried on a hot plate .All the analysis were carried out in a laboratory with temperature control (25 ± 2^0C). Densitometry scanning was done in absorbance mode at 210 nm using a deuterium lamp. The slit dimensions were set at 6 mm x 0.30 mm, the scanning speed of 10 mm/s, and the data resolution at 100 µm/step. Single

wavelength detection was performed since the main components were only analyzed.

6.3.1.4 Preparation of the Mobile phase

The mobile phase was prepared by mixing 7.0 mL chloroform with 3.0 mL 1,4-dioxane. The mobile phase was transferred into a twin-trough chamber covered with lid and allowed to stand for 30 min before use.

6.3.1.5 Preparation of Standard stock solution of LPV and RTV

LPV (200 mg) and RTV (50 mg) were accurately weighed and transferred into100 mL volumetric flask ,and dissolved in methanol. The volume was made up to the mark with methanol. The resulting stock solution was further 10 times diluted with methanol to get the final concentration of 200 µg/mL LPV and 50 µg/mL RTV which was used for calibration purpose of both the drugs. Aliquots (0.8, 1.6, 2.4, 3.2, 4.0 and 4.8 µL) were applied to the HPTLC plates as bands of 6 mm.

6.3.1.6 Selection of Wavelength for Determination

The standard solutions of LPV and RTV were scanned in the range of 200-400 nm against reagent blank. Both the drugs showed significant absorbance at 210 nm which was selected for analysis.

6.3.1.7 Analysis of tablet dosage form:

For analysis of tablet dosage form, twenty tablets, each containing 200 mg of LPV and 50mg RTV, were weighed and their average weight was calculated. The tablets were finely powdered and powder equivalent to 200 mg LPV and 50mg RTV was accurately weighed and transferred in to 100 mL volumetric flask. Methanol (60 mL) was added to it and shaken for 10 minutes. The volume was made up to the mark with methanol. The solution was sonicated for 30 min, filtered through the whatman no.41 filter paper. Aliquot was further 10 times diluted with methanol to get the final concentration of 200 µg/mL LPV and 50 µg/mL RTV. An aliquot (3 µL equivalent to 600 ng/spot LPV and 150 ng/spot RTV) was applied to the HPTLC plate. The plate was developed and analyzed as described under 6.3.1.3.2. The chromatogram was recorded. The

peak areas of both the drugs were noted and amount of LPV and RTV were calculated from the regression equation of the respective drug.

6.3.1.8 METHOD VALIDATION

As per ICH guidelines Q2 (R1), the method validation parameters studied were solution stability, specificity, linearity, accuracy, precision, limit of detection, limit of quantitation and robustness.

6.3.1.8.1 Solution Stability

Sample solutions were kept at 25^0C (24 hours) and 2 - 8^0C (3 days), respectively. Assays of both the drugs at initial time period were compared with these two time periods. The falls in the assay values were evaluated. The difference between assays should not be more than 2 % for formulation, and 0.5% for API.

6.3.1.8.2 Specificity

Specificity of an analytical method is its ability to measure the analyte accurately and specifically in the presence of component that may be expected to be present in the sample matrix. Chromatograms of standard and sample solutions of LPV and RTV were compared, and peak purity spectra at three different levels i.e., peak start (S), peak apex (M) and peak end (E) of a spot were recorded in order to provide an indication of specificity of the method.

6.3.1.8.3 Linearity (Calibration curve)

Standard stock solution containing 2000 µg/mL LPV and 500 µg/mL RTV was prepared in methanol and 10 times diluted. Aliquots (0.8, 1.6, 2.4, 3.2, 4.0 and 4.8 µL) were applied to the HPTLC plate to deliver 160, 320, 480, 640, 800 and 960 ng of LPV per spot and 40, 80, 120, 160, 200 and 240 ng of RTV per spot. The plate was developed and analyzed as described under 6.3.1.3.2. The chromatograms were recorded and the peak areas were noted. Calibration curve was constructed by plotting peak area versus concentration, and the regression equation was calculated. Each response was average of three determinations.

6.3.1.8.4 Accuracy (% Recovery)

The accuracy of the method was determined by calculating recovery of LPV and RTV by the standard addition method. Known amount of standard solutions of LPV (equivalent to 0, 160, 320 and 480 ng/spot) and RTV (equivalent to 0, 40, 80 and 120 ng/spot) were applied to the sample spot of LPV (320 ng/spot) and RTV (80 ng/spot) respectively on the plate. Each solution was applied in triplicate. The plate was developed and analyzed as described under 6.3.1.3.2. The percentage recovery was calculated by measuring the peak areas and fitting these values into the regression equations of the calibration curves.

6.3.1.8.5 Precision

The repeatability of measurement of peak area was checked by repeatedly (n = 6) measuring area of one band of LPV (800 ng/spot) and RTV (200 ng/spot), while repeatability of sample application was checked by repeatedly (n = 6) measuring area of six bands having same concentration of LPV (800 ng/spot) and RTV (200 ng/spot) applied on the same plate without changing the position of plate. The intra-day and inter-day precisions of the proposed method was determined by measuring the corresponding responses 3 times on the same day and on 3 different days over a period of 1 week for 3 different concentrations of LPV (320, 640 and 960 ng/spot) and RTV (80, 160 and 240 ng/spot). The results were reported in terms of relative standard deviation.

6.3.1.8.6 Limit of Detection and Limit of Quantification:

Limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the standard deviation of response (σ) and slope (S) of the calibration curve.

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

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

6.3.1.8.7 Robustness

The robustness was studied by analyzing the samples of LPV and RTV by deliberate variation in the method parameters. The change in the response of LPV and RTV was noted. Robustness of the method was studied by changing the extraction time of LPV and RTV from tablet dosage form by ± 2 min, composition of mobile phase by ± 2% of organic solvent, development distance by ± 1 cm, wavelength by ± 2 nm and temperature by ± 2^0C. The changes in the response of LPV and RTV were noted and compared with the original one.

6.3.2 RESULTS AND DISCUSSION:

6.3.2.1 Selection of mobile phase

Resolution is the most important criteria for the method, it is imperative to achieve good resolution among the compounds. As per the value of Ka and solubility of compound various composition of mobile phase were tried.

The chromatographic conditions were optimized with mobile phase consisting of chloroform : 1,4 - dioxane (7:3 v/v), which was found satisfactory to obtain sharp, well defined LPV and RTV peaks with better reproducibility and repeatability (Figure 6.3.1).

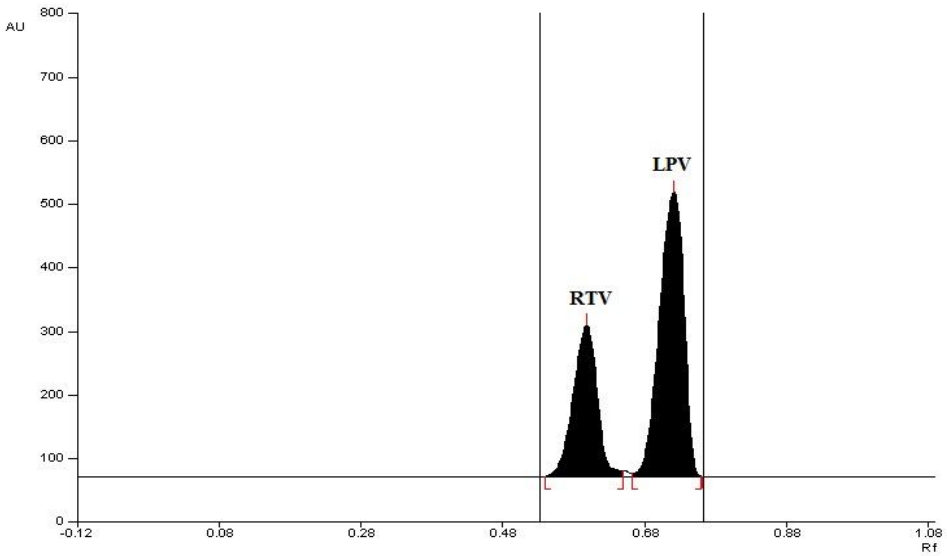


Figure 6.3.1(a): HPTLC chromatogram of RTV (240 ng/spot) and LPV (960 ng/spot) standard with corresponding R_f at 210 nm

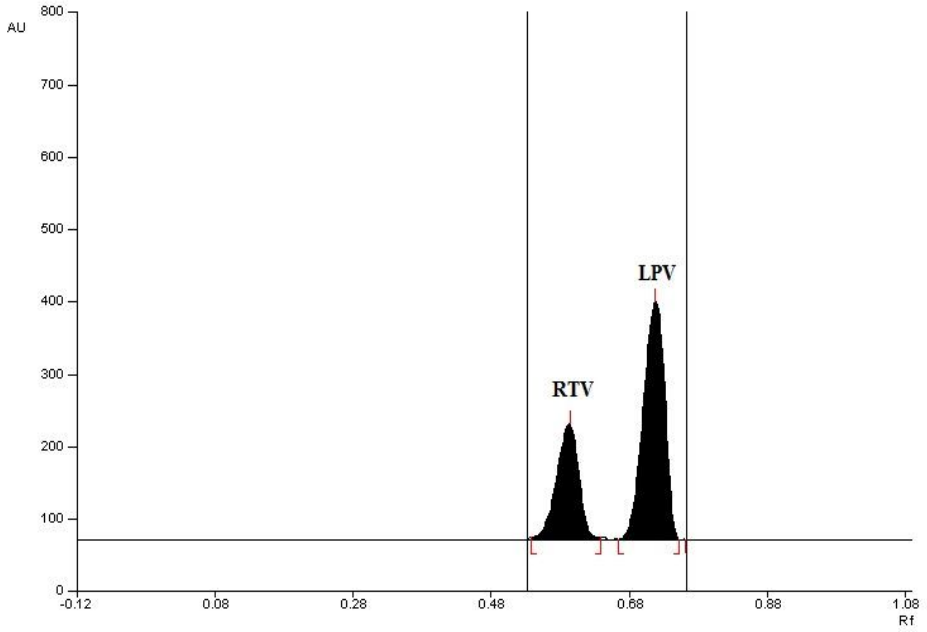


Figure 6.3.1(b): HPTLC chromatogram of RTV (150 ng/spot) and LPV (600 ng/spot) tablet sample with corresponding R_f at 210 nm

6.3.2.2 METHOD VALIDATION

6.3.2.2.1 Solution Stability

The change in assay results after storage at 25°C (24 hours) and 2 - 8°C (3 days) was evaluated. It was found that the difference in assay results was not more than 2 % for formulation, and 0.5% for API, indicating stability of LPV and RTV solution.

6.3.2.2.2 Specificity

The proposed method was found to be specific as no interference of excipients or impurities was found in separation and determination of the peak purity as r(S, M) = 0.9999 and r(M, E) = 0.9998 for LPV; r(S, M) = 0.9999 and r(M, E) = 0.9998 for RTV (Figure 6.1.2(a) and (b)), and good correlation (r = 0.9999 and 0.9998) was obtained between standard and sample spectra of LPV and RTV, respectively. The peak purity and correlation > 0.99 indicated the method specificity.

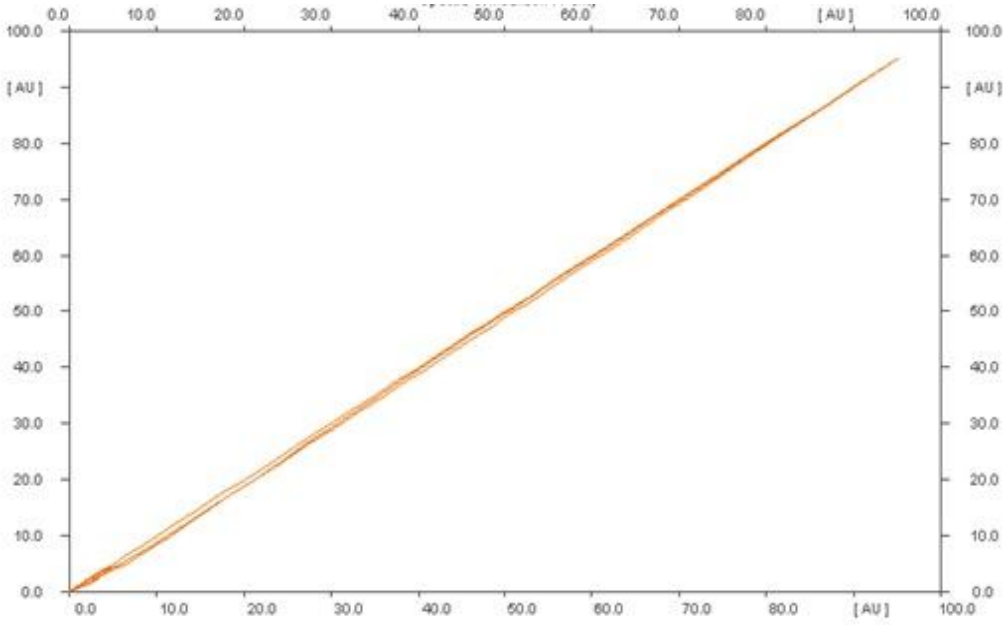


Figure 6.3.2(a): Peak purity spectra of LPV from tablet dosage form

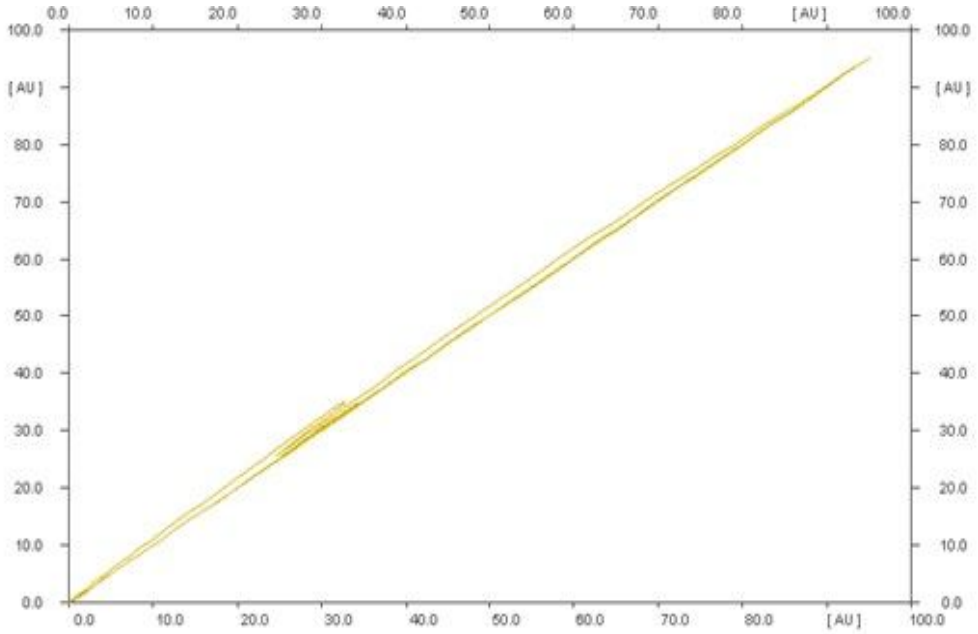


Figure 6.3.2(b): Peak purity spectra of RTV from tablet dosage form

6.3.2.2.3 Linearity

Linear correlation was obtained between peak area and concentration of LPV and RTV in the range of 160-960 ng/spot & 40-240 ng/spot, respectively. The linearity of the calibration curve was validated by the value of correlation coefficients of the regression (r). The optical and regression characteristics are listed in Table 6.3.1.

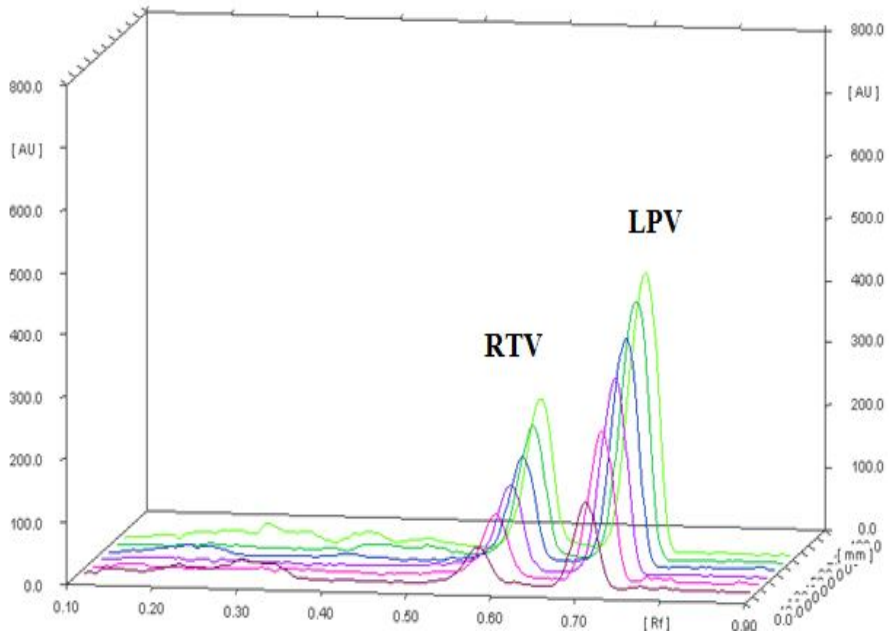


Figure 6.3.3: 3D Chromatogram showing peaks of RTV and LPV standards in different concentrations

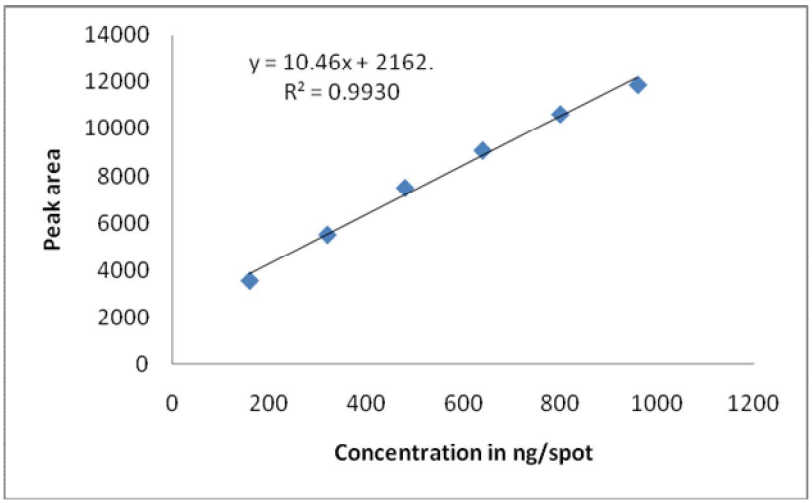


Figure 6.3.4: Calibration curve of LPV

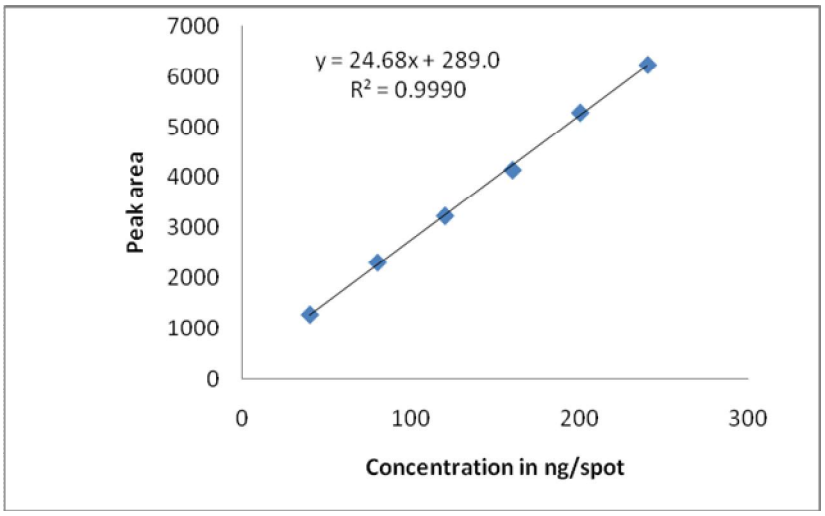


Figure 6.3.5: Calibration curve of RTV

Table 6.3.1: Optical and regression characteristics (n=3)

Parameter	LPV	RTV
Linearity range (ng/spot)	160-960	40-240
Linearity equation	y = 10.46x + 2162.0	y = 24.68x + 289.0
LOD (ng/spot)	9.56	6.82
LOQ (ng/spot)	28.96	20.66
Correlation coefficient (r)	0.9930	0.9990

6.3.2.2.4 Accuracy (% Recovery)

Accuracy study was carried out by the standard addition method. The percent recovery was found in the range of 97.83-99.58 % and 97.69-101.28 % for LPV and RTV, respectively, which indicated accuracy of the method.

Table 6.3.2: Results of recovery study (n=3)

Drug	Amount taken (ng/spot)	Amount added (ng/spot)	Amount found (ng/spot)	Recovery ± SD, %	% RSD
LPV	320	0	316.28	98.84 ± 0.84	0.84
	320	160	473.13	98.57 ± 0.37	0.37
	320	320	626.11	97.83 ± 1.74	1.74
	320	480	796.64	99.58 ± 1.32	1.32
RTV	80	0	81.02	101.28 ± 1.27	1.27
	80	40	119.11	99.26 ± 0.63	0.63
	80	80	156.3	97.69 ± 0.82	0.82
	80	120	198.68	99.34 ± 0.94	0.94

6.3.2.2.5 Precision

The % RSD of the repeatability of measurement of peak area was found to be 0.90 and 0.98; while of the repeatability of sample application was found to be 1.76 and 1.57 for LPV and RTV respectively. The % RSD for intra-day precision was found to be in the range of 0.21 - 1.06 % and 0.43 - 0.68 %; while inter-day precision was found to be in the range of 0.47 - 1.41 % and 0.74 - 1.82 % for LPV and RTV, respectively, which indicated that the method was precise.

Table 6.3.3: Results of repeatability (n=6)

Drug	LPV		RTV	
	Measurement of peak area	Sample application	Measurement of peak area	Sample application
1	10626.5	10564.54	5278.4	5234.36
2	10654.2	10156.87	5204.5	5376.95
3	10471.6	10434.77	5232.9	5145.75
4	10598.5	10226.45	5354.7	5172.67
5	10432.3	10587.36	5263.8	5182.92
6	10648.3	10257.24	5294.4	5227.76
Mean	10571.9	10371.21	5271.44	5223.4
SD	95.74	183.31	52.018	82.41
% RSD	0.90	1.76	0.98	1.57

Table 6.3.4: Results of Intra-day and Inter-day precision (n=3)

Drug	Concen-tration (ng/spot)	Intra-day precision		Inter-day precision	
		Mean peak area ± SD	% RSD	Mean peak area ± SD	% RSD
LPV	320	5502.41 ± 18.15	0.33	5567.64 ± 31.17	0.56
	640	9076.65 ± 19.06	0.21	8974.72 ± 42.18	0.47
	960	11878.12 ± 125.90	1.06	11842.41± 166.97	1.41
RTV	80	2308.54 ± 10.38	0.45	2278.45 ± 41.46	1.82
	160	4141.62 ± 28.29	0.68	4235.54 ± 40.66	0.96
	240	6227.71 ± 26.82	0.43	6158.35 ± 45.57	0.74

6.3.2.2.6 Limit of detection and limit of quantification

The Limit of detection (LOD) was found to be 9.56 ng/spot and 6.82 ng/spot while the Limit of quantification (LOQ) was found to be 28.96 ng/spot and 20.66 ng/spot for LPV and RTV, respectively.

6.3.2.2.7 Robustness

The method was found to be robust as the results were not significantly affected by slight variation in extraction time, composition of mobile phase, development distance, wavelength and temperature.

6.3.2.2.8 Analysis of Tablet Dosage Form

The proposed HPTLC method was successfully applied for determination of LPV and RTV from tablet dosage form. The percentage of LPV and RTV was found to be satisfactory, which was comparable with the corresponding label claim.

Table 6.3.5: Analysis results of tablet dosage form (n=3)

Formulation	Drug	Labelled amount (mg)	Amount found (mg)	Assay ± SD, %
LOPIMUNE®	LPV	200	198.20	99.10 ± 1.87
	RTV	50	49.36	98.72 ± 0.77

6.3.3 CONCLUSION:

A high performance thin layer chromatographic method has been developed and validated for the determination of LPV and RTV from tablet dosage form. The method was found to be specific as there was no interference of excipients and impurity. The proposed method was found to be simple, accurate, precise, sensitive and robust. Hence, it can be used successfully for the routine analysis of LPV and RTV in their combined pharmaceutical dosage forms.

CHAPTER 7

SPECTROPHOTOMETRIC DETERMINATION OF ANTIRETROVIRAL AGENTS IN TABLET DOSAGE FORM

7. SPECTROPHOTOMETRIC DETERMINATION OF ANTIRETRO VIRAL AGENTS IN TABLET DOSAGE FORM

7.1 DETERMINATION OF EFAVIRENZ IN TABLET DOSAGE FORM

7.1.1 EXPERIMENTAL:

7.1.1.1 Instrumentation

- A Shimadzu model 1800 double beam UV/Visile spectrophotometer with spectral width of 1 ± 0.2nm, wavelength accuracy of ± 0.1 nm and a pair of 10 mm matched quartz cells
- BP211D,Sartorious Gottingen AG (Germany), analytical balance
- An ultra-sonic cleaner (TEC-4, Roop Telesonic Ultrasonix)

7.1.1.2 Reagents and Materials

- Efavirenz (EFV) was kindly gifted by Aurobindo Pharmaceuticals Ltd., Hyderabad, India.
- Tablets (ESTIVA 600®, Genix Pharma) containing Efavirenz (600 mg) were purchased from local market.
- Methanol AR grade (SD Fine Chemicals Pvt. Ltd., Ahmedabad, India)
- Whatman filter paper no. 41
- Hydrochloric acid (36%) and sodium hydroxide AR grade (Finar Chemicals Pvt. Ltd, Ahmedabad, India)

7.1.1.3 Preparation of Standard Solution

Accurately weighed EFV (25 mg) was transferred to a 25 mL volumetric flask, dissolved in and diluted to the mark with methanol to obtain a standard stock solution (1 mg/mL). Aliquot (1 mL) was transferred to 10 mL volumetric flask and diluted up to the mark with methanol to obtain the working standard solution (100 µg/mL)

7.1.1.3A Preparation of hydrochloric acid (0.1N)

Accurately transferred 0.85 mL concentrated hydrochloric acid (36%) to 100mL volumetric flask and diluted up to the mark with distilled water.

7.1.1.3B Preparation of sodium hydroxide (0.1N)

Accurately weighed and transferred 0.4 gm sodium hydroxide to 100 mL volumetric flask, dissolved in 60 mL distilled water and diluted up to the mark with distilled water.

7.1.1.4 Selection of Wavelength for Determination

Aliquot (1 mL) was taken from EFV stock solution (100 µg/mL) in duplicate 10 ml volumetric flasks, and volumes were made up with 0.1N HCl and 0.1N NaOH respectively to prepare standard solutions containing 10 µg/ml EFV in 0.1N HCl and 0.1N NaOH. The above solutions were scanned in the UV range of 200 nm to 400 nm to obtain difference spectra by keeping acidic form (i.e. EFV in 0.1N HCl) in reference cell and basic form (i.e. EFV in 0.1N NaOH) in sample cell using 0.1N HCl (in reference cell) and 0.1N NaOH (in sample cell) as blank. The maximum absorbance was observed at 267.50 nm which was selected for analysis.

7.1.1.5 Analysis of Tablet Dosage Form

Twenty tablets were weighed and average weight was calculated. The tablets were powdered, a quantity of powder equivalent to 25 mg EFV was weighed and transferred to a 25 mL volumetric flask containing 15 mL methanol, and sonicated for 15 minutes. The flask was allowed to stand at room temperature for 5 min and the volume was made up to the mark with methanol to obtain the sample stock solution (1 mg/mL). An aliquot (1 mL) from the stock solution was transferred to 10 mL volumetric flask and diluted up to the mark with methanol to obtain the working standard solution (100 µg/mL). The solution was filtered through whatman filter paper no. 41. An aliquot (1.5 mL) was taken in duplicate 10 mL volumetric flasks, and volumes were made up to the mark with 0.1N HCl

and 0.1N NaOH respectively to prepare tablet test solutions containing 15 µg/mL EFV in 0.1N HCl and 0.1N NaOH. The above solutions were scanned in the UV range of 200 nm to 400 nm to obtain difference spectra by keeping acidic form (i.e. EFV in 0.1N HCl) in reference cell and basic form (i.e. EFV in 0.1N NaOH) in sample cell using 0.1N HCl (in reference cell) and 0.1N NaOH (in sample cell) as blank. The difference absorbance of the sample solution was noted at 267.50 nm which was put in regression equation to calculate the amount of EFV.

7.1.1.6 METHOD VALIDATION

As per ICH guidelines Q2 (R1), the method validation parameters studied were solution stability, specificity, linearity, accuracy, precision, limit of detection, limit of quantitation and robustness.

7.1.1.6.1 Solution Stability

Sample solutions were kept at 25 ± 2°C (24 hours) and 2 - 8^0C (3 days), respectively. Assay percentage of initial time period was compared with these two time periods. The change in the assay percentage was calculated. The difference between assay results should not be more than 2 % for formulation, and 0.5% for API.

7.1.1.6.2 Specificity

Specificity of an analytical method is its ability to measure the analyte accurately and specifically in the presence of component that may be expected to be present in the sample matrix. Difference spectra of standard and sample solutions of EFV in 0.1N HCl (in reference cell) and 0.1N NaOH (in sample cell) were compared in order to provide an indication of specificity of the method.

7.1.1.6.3 Linearity (Calibration Curve)

Standard solutions (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mL equivalent to 5, 10, 15, 20, 25 and 30 µg/mL) were transferred in a series of duplicate 10 mL volumetric flasks and volumes were made up with 0.1N HCl and 0.1N NaOH respectively to

prepare series of standard solutions containing 5 - 30 µg/mL EFV in 0.1N HCl and 0.1N NaOH. All the above solutions were scanned in the UV range of 200 nm to 400 nm to obtain their difference spectra by keeping acidic form (i.e. EFV in 0.1N HCl) in reference cell and basic form (i.e. EFV in 0.1N NaOH) in sample cell. Difference absorbance at 267.50 nm was noted for each solution. Calibration curve was constructed by plotting difference absorbance versus concentrations, and the regression equation was calculated. Each response was average of three determinations.

7.1.1.6.4 Accuracy (% Recovery)

Accuracy of the method was determined by calculating recovery of EFV by the standard addition method. Known amount of standard solutions of EFV (0, 5, 10 and 15 µg/mL) were added to a pre-analysed sample solution of EFV (10 µg/mL). The percentage recovery was calculated by measuring the difference absorbances and fitting these values into the regression equation of the calibration curve. Each response was average of three determinations.

7.1.1.6.5 Precision

The repeatability of EFV was checked by repeatedly (n = 6) measuring difference absorbances of EFV solution (10 µg/mL).

The intra-day and inter-day precisions of the proposed method was determined by measuring the corresponding responses 3 times on the same day and on 3 different days over a period of 1 week for 3 different concentrations of EFV (10, 20 and 30 µg/mL). The results were reported in terms of relative standard deviation.

7.1.1.6.6 Limit of Detection and Limit of Quantification

Limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the standard deviation of response (σ) and slope (S) of the calibration curve.

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

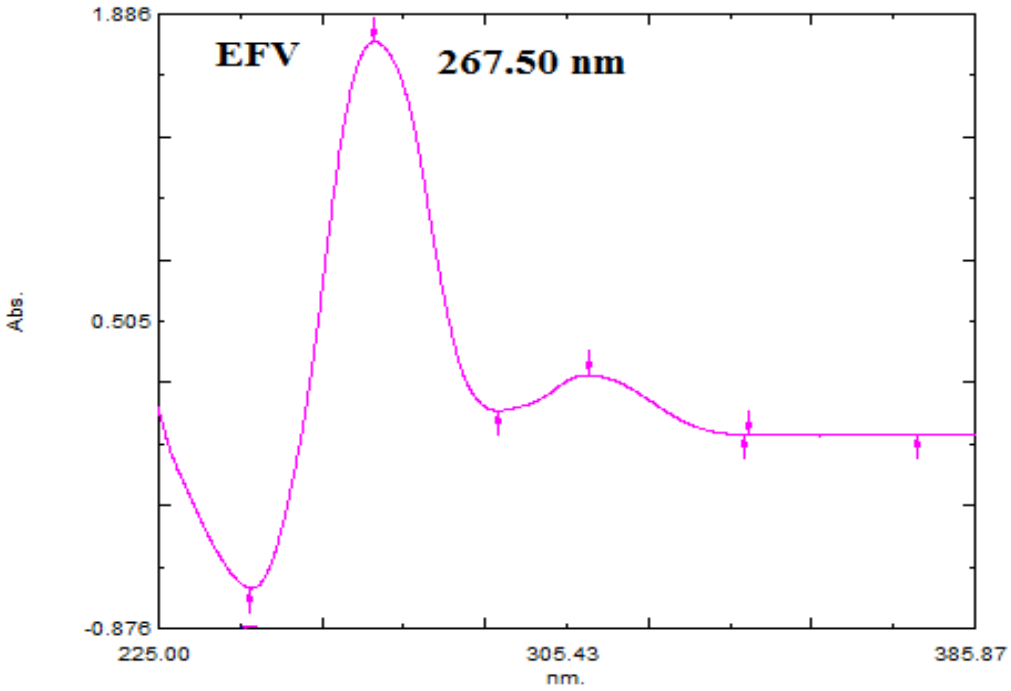
$$LOQ = 10 \times \sigma/S$$

7.1.1.6.7 Robustness

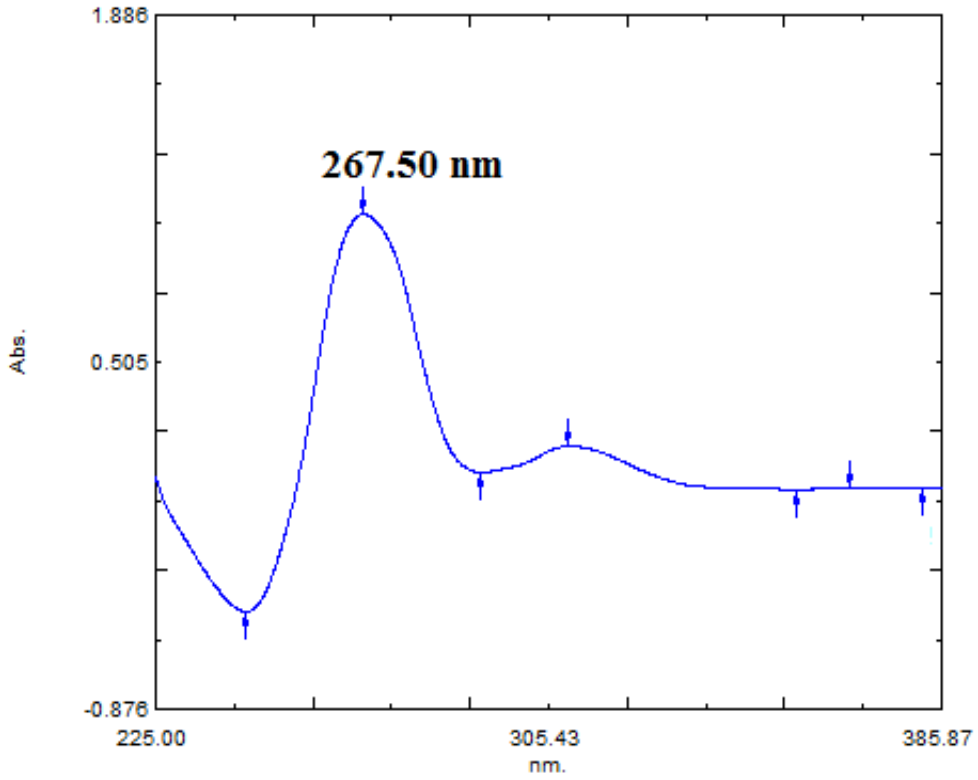
Robustness was studied by analyzing the samples of EFV by deliberate variation in the method parameters. The change in the response of EFV was noted. Robustness of the method was studied by changing the extraction time of EFV from tablet dosage form by ± 2 min, wavelength by ±1 nm and temperature by 25 ± 2^0C. The changes in the response of EFV were noted and compared with the original one.

7.1.2 RESULTS AND DISCUSSION:

The satisfactory results were obtained with 0.1N HCl (in reference cell) and 0.1 NaOH (in sample cell). The wavelength of maximum absorbance of EFV in 0.1N HCl (reference) and 0.1N NaOH (sample) was found to be at 267.50 nm (Figure 7.1.1).



(a)



(b)

Figure 7.1.1: Difference spectra of EFV in 0.1 N HCl(reference) and 0.1N NaOH (sample) from (a) standard (25 µg/mL) and (b) tablet dosage form (15 µg/mL)

7.1.2.1 METHOD VALIDATION

7.1.2.1.1 Solution Stability

The change in assay results after storage at 25^{0}C (24 hours) and 2 - 8^{0}C (3 days) was evaluated. It was found that the difference in assay results was not more than 2 % for formulation, and 0.5% for API, indicating stability of EFV solution.

7.1.2.1.2 Specificity

The analytical method was found to be specific as no interference of excipients and impurities was found in determination of the EFV

7.1.2.1.3 Linearity

The Beer's law was obeyed. Linear correlation was obtained between difference absorbance and concentration of EFV in the range of 5-30 µg/mL. The linearity of the calibration curve was validated by the value of correlation coefficient of the regression (r). The optical and regression characteristics are listed in Table 7.1.1.

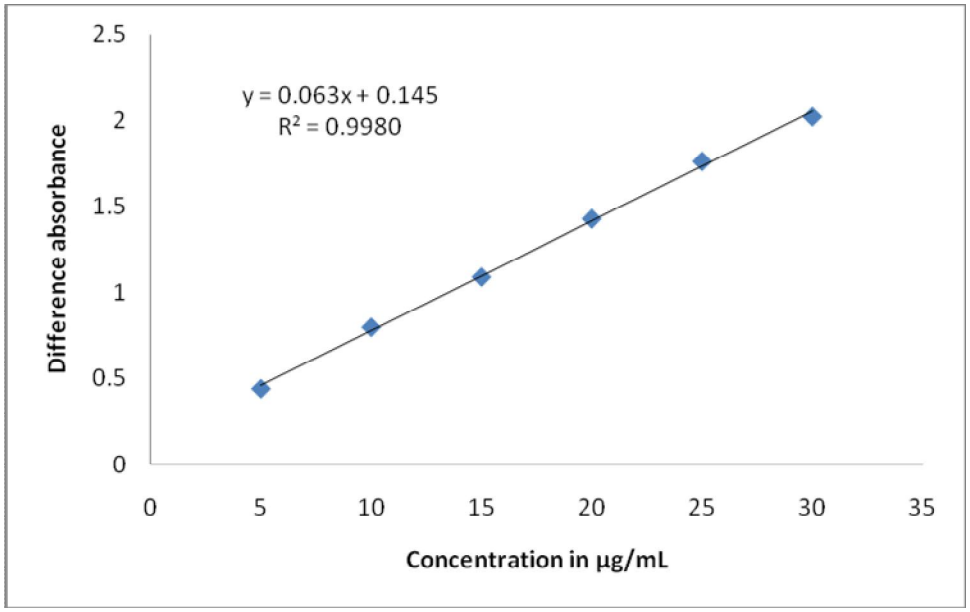


Figure 7.1.2: Calibration curve of EFV

Table 7.1.1: Optical and regression characteristics (n=3)

Parameter	EFV
Linearity range (µg/mL)	5-30
Linearity equation	y = 0.063x + 0.145
LOD (µg/mL)	0.352
LOQ (µg/mL)	1.066
Correlation coefficient (r)	0.998

7.1.2.1.4 Accuracy (% Recovery)

The accuracy study was carried out by the standard addition method. The percent recoveries were found in the range of 98.64 – 102.05 %, which indicated accuracy of the method.

Table 7.1.2: Results of recovery study (n=3)

Amount taken (µg/mL)	Amount added (µg/mL)	Amount found (µg/mL)	Recovery ± S.D, %	% RSD
10	0	10.20	102.05 ± 1.31	1.28
10	5	14.89	99.30 ± 1.16	1.16
10	10	20.27	98.64 ± 0.58	0.58
10	15	25.11	100.47 ± 0.83	0.83

7.1.2.1.5 Precision

The % RSD for repeatability of EFV was found to be 0.95. The value of % RSD for intra-day precision was found to be in the range of 0.93 – 1.06% and inter-day precision was found to be in the range of 1.19 - 1.31%, which indicated that the method was precise.

Table 7.1.3: Results of repeatability (n=6)

Drug	EFV Diff. absorbance
1	0.800
2	0.812
3	0.805
4	0.807
5	0.795
6	0.816
Mean	0.805
SD	0.0076
% RSD	0.95

Table 7.1.4: Results of Intra-day and Inter-day precision (n=3)

EFV (µg/mL)	Intra-day precision		Inter-day precision	
	Mean diff. absorbance ± SD	% RSD	Mean diff. absorbance ± SD	% RSD
10	0.800 ± 0.0076	0.95	0.816 ± 0.0097	1.19
20	1.428 ± 0.0152	1.06	1.419 ± 0.0186	1.31
30	2.022 ± 0.0189	0.93	2.057 ± 0.0262	1.27

7.1.2.1.6 Limit of detection and limit of quantification

The limit of detection (LOD) for EFV was found to be 0.352 µg/mL, while the limit of quantification (LOQ) was 1.066 µg/mL.

7.1.2.1.7 Robustness

The method was found to be robust as the results were not significantly affected by slight variation in extraction time, wavelength and temperature.

7.1.2.2 Analysis of Tablet Dosage Form

The proposed UV spectrophotometric method was successfully applied for determination of EFV in tablet dosage form. The percentage of EFV was found to be satisfactory, which was comparable with the corresponding label claim.

Table 7.1.5: Analysis results of tablet dosage form (n=3)

Formulation	Labelled amount (mg)	Amount found (mg)	Assay ± SD, %
ESTIVA 600®	600	604.92	100.82 ± 1.31

7.1.3 CONCLUSION:

A UV spectrophotometric method has been developed and validated for the determination of EFV in tablet dosage form. The method was found to be specific as there was no interference of any excipients and impurities. The proposed method was found to be simple, accurate, precise and robust. Hence, it can be used successfully for the routine analysis of EFV in pharmaceutical dosage forms.

7.2 DETERMINATION OF TENOFOVIR DISOPROXIL FUMARATE IN TABLET DOSAGE FORM

7.2.1 EXPERIMENTAL:

7.2.1.1 Instrumentation

Same as described under 7.1.1.1

7.2.1.2 Reagents and Materials

- Tenofovir disoproxil fumarate (TNV) was kindly gifted by Aurobindo Pharmaceuticals Ltd., Hyderabad, India.
- Tablets (TENOF®, Genix Pharma) containing tenofovir disoproxil fumarate (300 mg) were purchased from local market.
- Methanol AR grade (SD Fine Chemicals Pvt. Ltd., Ahmedabad, India)
- Whatman filter paper no. 41
- Hydrochloric acid (36%) AR grade (Finar Chemicals Pvt. Ltd, Ahmedabad, India)

7.2.1.3 Preparation of standard solution

Accurately weighed TNV (25 mg) was transferred to a 25 mL volumetric flask, dissolved in and diluted to the mark with methanol to obtain a standard stock solution (1 mg/mL).

7.2.1.3A Preparation of hydrochloric acid (0.1N)

Same as described under 7.1.1.3A

7.2.1.3B Preparation of working standard solution (10 µg/mL)

Standard solution (0.1 mL) was transferred in a 10 mL volumetric flask and diluted to the mark with 0.1N HCl.

7.2.1.4 Selection of Wavelength for Determination

TNV working standard solution (10 µg/mL) was scanned in the spectrum mode between 200 and 400 nm, using 0.1N HCl as a blank. The wavelength of

maximum absorbance (λ max) was observed at 258.50 nm. Then 1st derivative spectrum of TNV was calculated by UV Probe 2.31 software which shown peak maxima at 247.06 nm and peak minima at 273.45 nm. Both these wavelengths were selected for analysis.

7.2.1.5 Analysis of Tablet Dosage Form

Twenty tablets were weighed and average weight was calculated. The tablets were powdered, a quantity of powder equivalent to 25 mg TNV was weighed and transferred to a 25 mL volumetric flask containing 15 mL methanol, and sonicated for 15 minutes. The flask was allowed to stand at room temperature for 5 min and the volume was made up to the mark with methanol to obtain the sample stock solution (1 mg/mL). The solution was filtered through whatman filter paper no. 41. An aliquot (0.2 mL) was transferred to a 10 mL volumetric flask and diluted to the mark with 0.1N HCl to obtain a sample solution (20 µg/mL). The spectrum was recorded and 1st derivative spectrum was calculated. The absorbances were noted at 247.06 nm as well as 273.45 nm. The amplitude difference of the absorbances between these two wavelengths was calculated and amount of TNV present per tablet was calculated from the regression equation.

7.2.1.6 METHOD VALIDATION

As per ICH guidelines Q2 (R1), the method validation parameters studied were solution stability, specificity, linearity, accuracy, precision, limit of detection, limit of quantitation and robustness.

7.2.1.6.1 Solution Stability

Sample solutions were kept at 25 ± 2°C (24 hours) and 2 - 8^0C (3 days), respectively. Assay percentage of initial time period was compared with these two time periods. The change in the assay percentage was calculated. The difference between assay results should not be more than 2 % for formulation, and 0.5% for API.

7.2.1.6.2 Specificity

Specificity of an analytical method is its ability to measure the analyte accurately and specifically in the presence of component that may be expected to be present in the sample matrix. First derivative spectra of standard and sample solutions of TNV in 0.1N HCl were compared in order to provide an indication of specificity of the method.

7.2.1.6.3 Linearity (Calibration Curve)

Standard solutions (0.05, 0.1, 0.15, 0.2, 0.25 and 0.30 mL equivalent to 5, 10, 15, 20, 25 and 30 µg/mL) were transferred in a series of 10 mL volumetric flasks, and diluted to the mark with 0.1N HCl. The absorbances of each solution were measured at 247.06 nm as well as 273.45 nm and amplitude difference was calculated for each solution. Calibration curve was constructed by plotting amplitude difference versus concentrations, and the regression equation was calculated. Each response was average of three determinations.

7.2.1.6.4 Accuracy (% Recovery)

Accuracy of the method was determined by calculating recovery of TNV by the standard addition method. Known amount of standard solutions of TNV (0, 5, 10 and 15 µg/mL) were added to a pre-analysed sample solution of TNV (10 µg/mL). The percentage recovery was calculated by measuring the amplitude differences and fitting these values into the regression equation of the calibration curve. Each response was average of three determinations.

7.2.1.6.5 Precision

The repeatability of TNV was checked by repeatedly (n = 6) measuring amplitude differences of TNV solution (10 µg/mL). The intra-day and inter-day precisions of the proposed method was determined by measuring the corresponding responses 3 times on the same day and on 3 different days over a period of 1 week for 3 different concentrations of TNV (10, 20 and 30 µg/mL). The results were reported in terms of relative standard deviation.

7.2.1.6.6 Limit of Detection and Limit of Quantification

Limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the standard deviation of response (σ) and slope (S) of the calibration curve.

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

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

7.2.1.6.7 Robustness

Robustness was studied by analyzing the samples of TNV by deliberate variation in the method parameters. The change in the response of TNV was noted. Robustness of the method was studied by changing the extraction time of TNV from tablet dosage form by ±2 min, wavelength by ± 1 nm and temperature by 25 ± 2 ^{0}C. The changes in the response of TNV were noted and compared with the original one.

7.2.2 RESULTS AND DISCUSSION:

The satisfactory results were obtained with 0.1N HCl. The wavelength of maximum absorbance of TNV in 0.1N HCl was found to be at 258.50 nm (Figure 7.2.1).

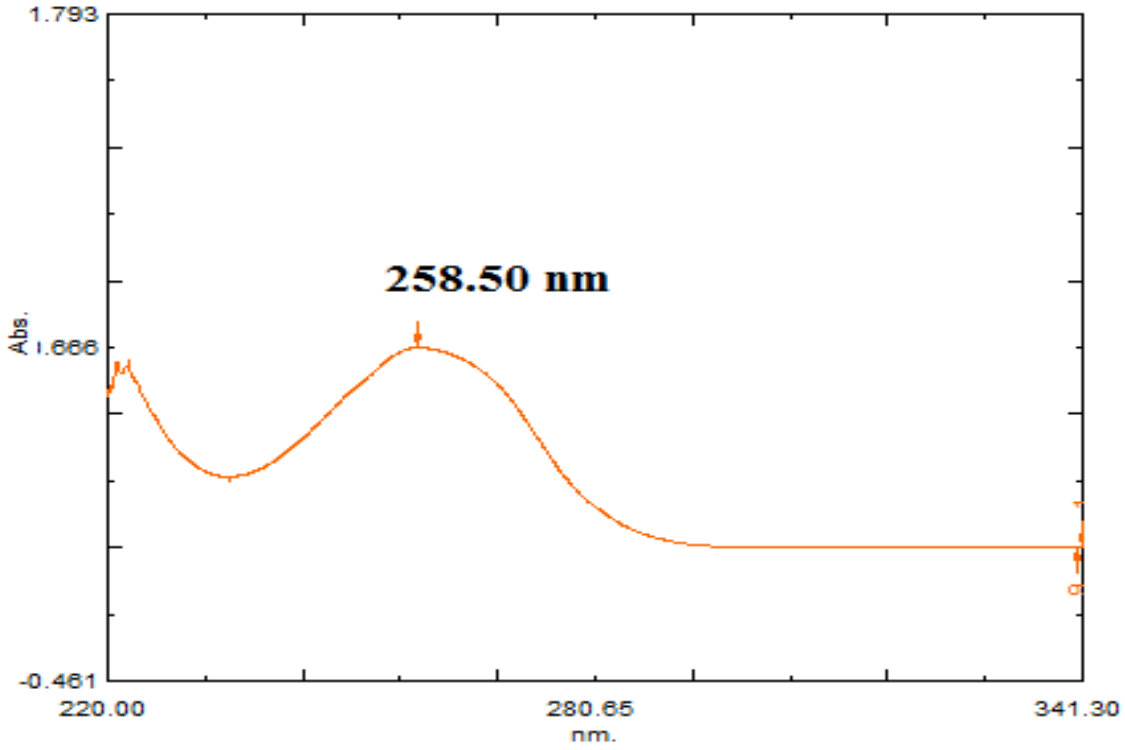
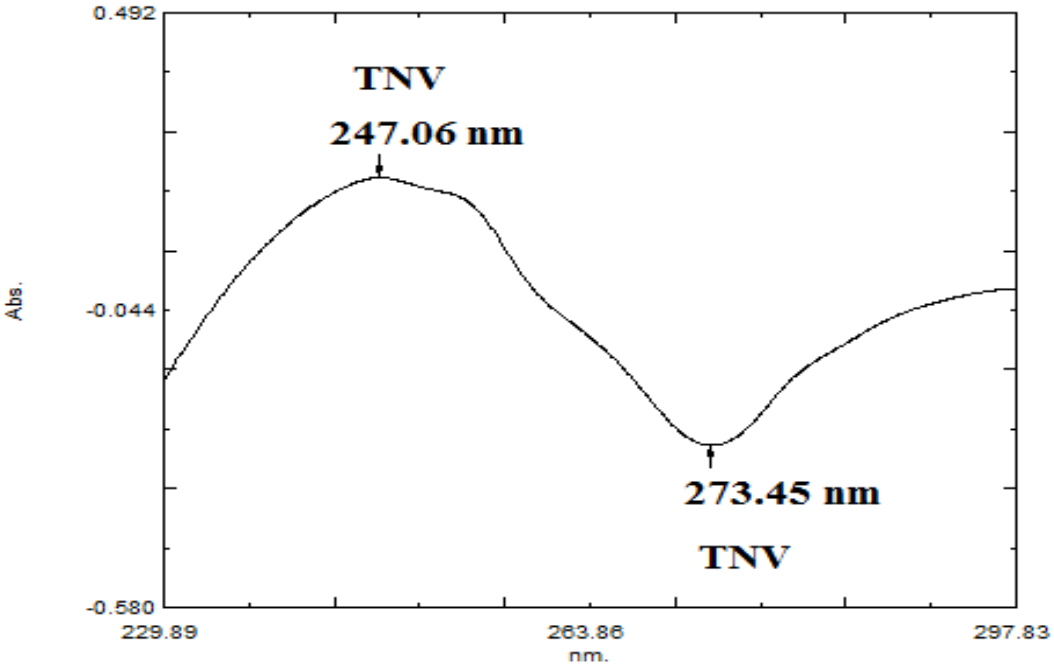
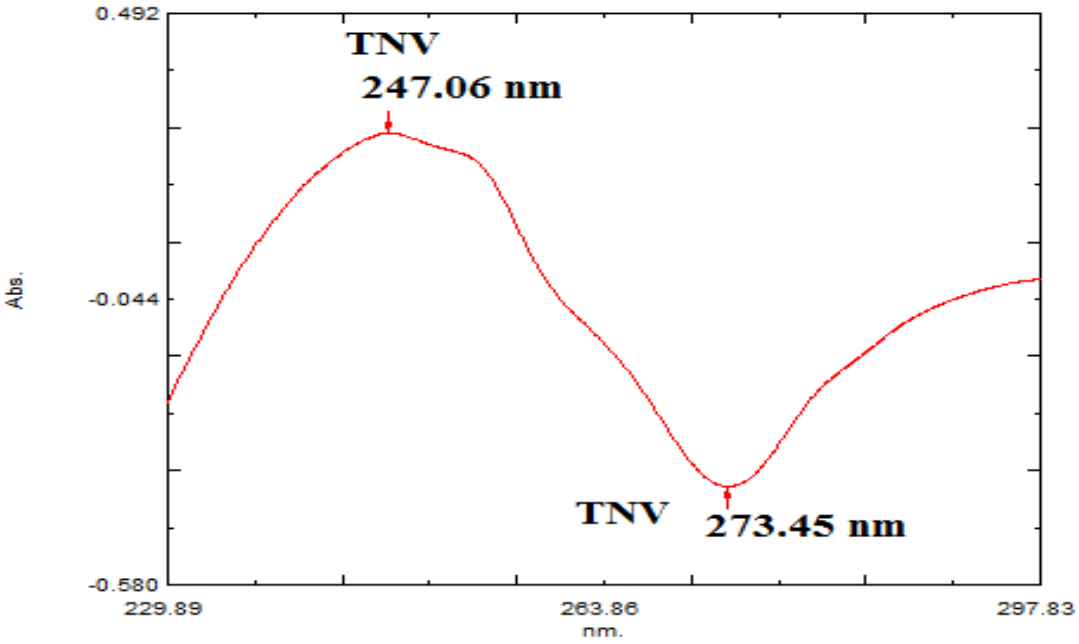


Figure 7.2.1: UV spectrums of TNV in 0.1N HCl.

In 1st derivative spectrums of TNV in 0.1N HCl, peak maxima and peak minima were obtained at 247.06 nm and 273.45 nm respectively (Figure 7.2.2).



(a)



(b)

Figure 7.2.2: Spectra of TNV from (a) standard (15 µg/mL) and (b) tablet dosage form (20 µg/mL)

7.2.2.1 METHOD VALIDATION

7.2.2.1.1 Solution Stability

The change in assay results after storage at 25^{0}C (24 hours) and 2 - 8^{0}C (3 days) was evaluated. It was found that the difference in assay results was not more than 2 % for formulation, and 0.5% for API, indicating stability of TNV solution.

7.2.2.1.2 Specificity

The analytical method was found to be specific as no interference of excipients and impurities was found in determination of the TNV

7.2.2.1.3 Linearity

The Beer's law was obeyed. Linear correlation was obtained between amplitude difference and concentration of TNV in the range of 5-30 µg/mL. The linearity of the calibration curve was validated by the value of correlation coefficient of the regression (r). The optical and regression characteristics are listed in Table 7.2.1.

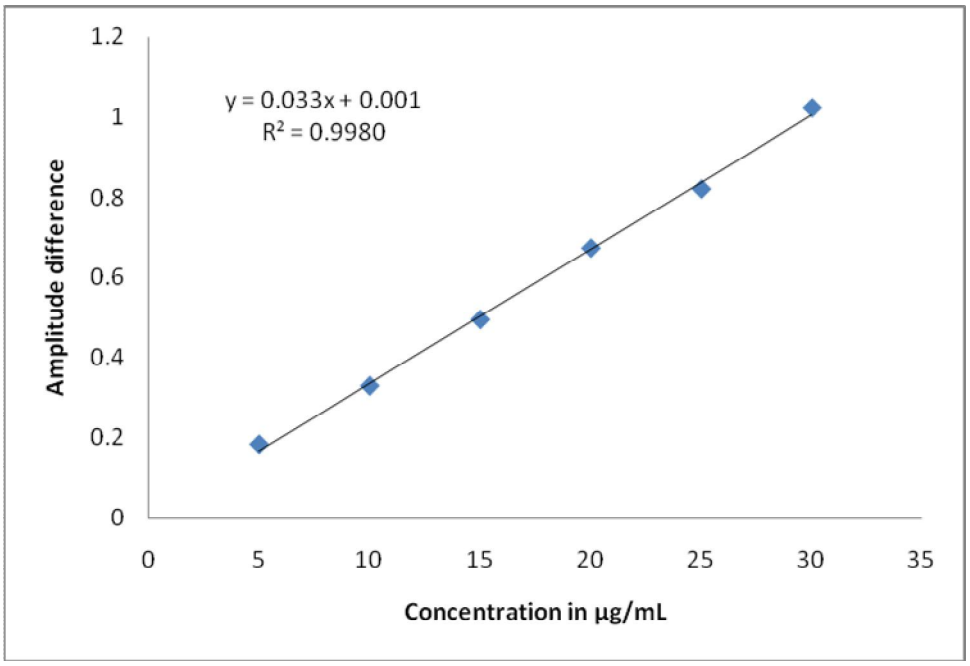


Figure 7.2.3: Calibration curve of TNV

Table 7.2.1: Optical and regression characteristics (n=3)

Parameter	TNV
Linearity range (µg/mL)	5-30
Linearity equation	y = 0.033x + 0.001
LOD (µg/mL)	0.269
LOQ (µg/mL)	0.815
Correlation coefficient (r)	0.9980

7.2.2.1.4 Accuracy (% Recovery)

The accuracy study was carried out by the standard addition method. The percent recoveries were found in the range of 97.68 - 98.79 %, which indicated accuracy of the method.

Table 7.2.2: Results of recovery study (n=3)

Amount taken (µg/mL)	Amount added (µg/mL)	Amount found (µg/mL)	Recovery ± S.D, %	% RSD
10	0	9.84	98.41 ± 1.06	1.06
10	5	14.65	97.68 ± 0.87	0.87
10	10	19.63	98.17 ± 1.26	1.26
10	15	24.69	98.79 ± 0.79	0.79

7.2.2.1.5 Precision

The % RSD for repeatability of TNV was found to be 1.105. The value of % RSD for intra-day precision was found to be in the range of 0.35 - 0.85% and inter-day precision was found to be in the range of 0.95 - 1.59%, which indicated that the method was precise.

Table 7.2.3 Results of repeatability (n=6)

Drug	TNV Amplitude diff.
1	0.328
2	0.321
3	0.323
4	0.325
5	0.331
6	0.327
Mean	0.325
SD	0.0036
% RSD	1.105

Table 7.2.4 Results of Intra-day and Inter-day precision (n=3)

TNV (µg/mL)	Intra-day precision		Inter-day precision	
	Mean amplitude diff. ± SD	% RSD	Mean amplitude diff. ± SD	% RSD
10	0.328 ± 0.0028	0.85	0.327 ± 0.0052	1.59
20	0.672 ± 0.0024	0.35	0.672 ± 0.0065	0.96
30	1.021 ± 0.0059	0.57	1.023 ± 0.0098	0.95

7.2.2.1.6 Limit of detection and limit of quantification

The limit of detection (LOD) for TNV was found to be 0.269 µg/mL, while the limit of quantification (LOQ) was 0.815 µg/mL.

7.2.2.1.7 Robustness

The method was found to be robust as the results were not significantly affected by slight variation in extraction time, wavelength and temperature.

7.2.2.2 Analysis of Tablet Dosage Form

The proposed UV spectrophotometric method was successfully applied for determination of TNV in tablet dosage form. The percentage of TNV was found to be satisfactory, which was comparable with the corresponding label claim.

Table 7.2.5: Analysis results of tablet dosage form (n=3)

Formulation	Labelled amount (mg)	Amount found (mg)	Assay ± SD, %
TENOF®	300	294.75	98.25 ± 0.42

7.2.3 CONCLUSION:

A UV spectrophotometric method has been developed and validated for the determination of TNV in tablet dosage form. The method was found to be specific as there was no interference of any excipients and impurities. The proposed method was found to be simple, accurate, precise and robust. Hence, it can be used successfully for the routine analysis of TNV in pharmaceutical dosage forms.

7.3 DETERMINATION OF RITONAVIR IN TABLET DOSAGE FORM

7.3.1 EXPERIMENTAL:

7.3.1.1 Instrumentation

Same as described under 7.1.1.1

7.3.1.2 Reagents and Materials

- Ritonavir (RTV) was kindly gifted by Emcure Pharmaceuticals Ltd., Pune, India.
- Tablets (VIRITON®, Ranbaxy, Mumbai) containing Ritonavir (100 mg) were purchased from local market.
- Methanol AR grade (SD Fine Chemicals Pvt. Ltd., Ahmedabad, India)
- Whatman filter paper no. 41
- Hydrochloric acid (36%) and sodium hydroxide AR grade (Finar Chemicals Pvt. Ltd, Ahmedabad, India)

7.3.1.3 Preparation of standard solution

Accurately weighed RTV (25 mg) was transferred to a 25 mL volumetric flask, dissolved in and diluted to the mark with methanol to obtain a standard stock solution (1 mg/mL). Aliquot (1 mL) from the stock solution was transferred to 10 mL volumetric flask and diluted up to the mark with methanol to obtain the working standard solution (100 µg/mL)

7.3.1.3A Preparation of hydrochloric acid (0.1N)

Same as described under 7.1.1.3A

7.3.1.3B Preparation of sodium hydroxide (0.1N)

Same as described under 7.1.1.3B

7.3.1.4 Selection of Wavelength for Determination

Aliquot (1 mL) was taken from RTV stock solution (100 µg/mL) in duplicate 10 ml volumetric flasks and volumes were made up with 0.1N HCl and 0.1N NaOH respectively to prepare standard solutions containing 10 µg/ml RTV in 0.1N HCl and 0.1N NaOH. The above solutions were scanned in the UV range of 200 nm to 400 nm to obtain difference spectra by keeping acidic form (i.e. RTV in 0.1N HCl) in sample cell and basic form (i.e. RTV in 0.1N NaOH) in reference cell using 0.1N HCl (in sample cell) and 0.1N NaOH (in reference cell) as blank. The maximum absorbance was observed at 256.80 nm which was selected for analysis.

7.3.1.5 Analysis of Tablet Dosage Form

Twenty tablets were weighed and average weight was calculated. The tablets were finely powdered. A quantity of powder equivalent to 25 mg RTV was accurately weighed and transferred to a 25 mL volumetric flask containing 15 mL methanol, and sonicated for 15 minutes. The flask was allowed to stand at room temperature for 5 min and the volume was made up to the mark with methanol to obtain the sample stock solution (1 mg/mL). An aliquot (1 mL) from the stock solution was transferred to 10 mL volumetric flask and diluted up to the mark with methanol to obtain 100 µg/mL RVT. The solution was filtered through whatman filter paper no. 41. An aliquot (2 mL) was taken in duplicate 10 mL volumetric flasks, and diluted up to the mark with 0.1N HCl and 0.1N NaOH respectively to prepare tablet test solutions containing 20 µg/mL RTV in 0.1N HCl and 0.1N NaOH. The above solutions were scanned in the UV range of 200 nm to 400 nm to obtain difference spectra by keeping acidic form (i.e. RTV in 0.1N HCl) in sample cell and basic form (i.e. RTV in 0.1N NaOH) in reference cell using 0.1N HCl (in sample cell) and 0.1N NaOH (in reference cell) as blank. The difference absorbance of the sample solution was noted at 256.80 nm which was put in regression equation to calculate the amount of RTV.

7.3.1.6 METHOD VALIDATION

As per ICH guidelines Q2 (R1), the method validation parameters studied were solution stability, specificity, linearity, accuracy, precision, limit of detection, limit of quantitation and robustness.

7.3.1.6.1 Solution Stability

Sample solutions were kept at 25 ± 2°C (24 hours) and 2 - 8^0C (3 days), respectively. Assay percentage of initial time period was compared with these two time periods. The change in the assay percentage was calculated. The difference between assay results should not be more than 2 % for formulation, and 0.5% for API.

7.3.1.6.2 Specificity

Specificity of an analytical method is its ability to measure the analyte accurately and specifically in the presence of component that may be expected to be present in the sample matrix. Difference spectra of standard and sample solutions of RTV in 0.1N HCl (in sample cell) and 0.1N NaOH (in reference cell) were compared in order to provide an indication of specificity of the method.

7.3.1.6.3 Linearity (Calibration Curve)

Standard solutions (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mL equivalent to 5, 10, 15, 20, 25 and 30 µg/mL) were transferred in a series of duplicate 10 mL volumetric flasks and volumes were made up with 0.1N HCl and 0.1N NaOH respectively to prepare series of standard solutions containing 5- 30 µg/mL RTV (standard) in 0.1N HCl and 0.1N NaOH. All the above solutions were scanned in the UV range of 200 nm to 400 nm to obtain their difference spectra by keeping acidic form (i.e. RTV in 0.1N HCl) in sample cell and basic form (i.e. RTV in 0.1N NaOH) in reference cell. Difference absorbance at 256.80 nm was noted for each solution. Calibration curve was constructed by plotting difference absorbance versus concentrations, and the regression equation was calculated. Each response was average of three determinations.

7.3.1.6.4 Accuracy (% Recovery)

Accuracy of the method was determined by calculating recovery of RTV by the standard addition method. Known amount of standard solutions of RTV (0, 5, 10 and 15 µg/mL) were added to a pre-analyzed sample solution of RTV (10 µg/mL). The percentage recovery was calculated by measuring the difference absorbances and fitting these values into the regression equation of the calibration curve. Each response was average of three determinations.

7.3.1.6.5 Precision

The repeatability of RTV was checked by repeatedly (n = 6) measuring difference absorbances of RTV solution (20 µg/mL).

The intra-day and inter-day precisions of the proposed method was determined by measuring the corresponding responses 3 times on the same day and on 3 different days over a period of 1 week for 3 different concentrations of RTV (10, 20 and 30 µg/mL). The results were reported in terms of relative standard deviation.

7.3.1.6.6 Limit of Detection and Limit of Quantification

Limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the standard deviation of response (σ) and slope (S) of the calibration curve.

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

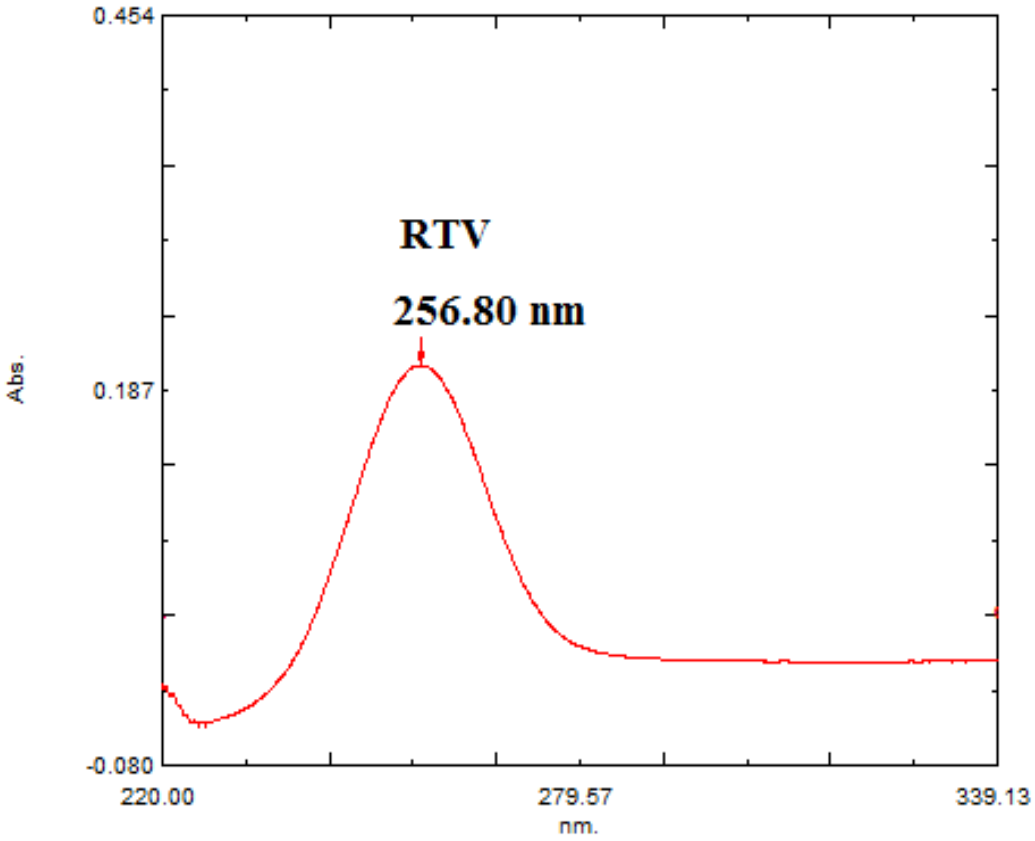
$$LOQ = 10 \times \sigma/S$$

7.3.1.6.7 Robustness

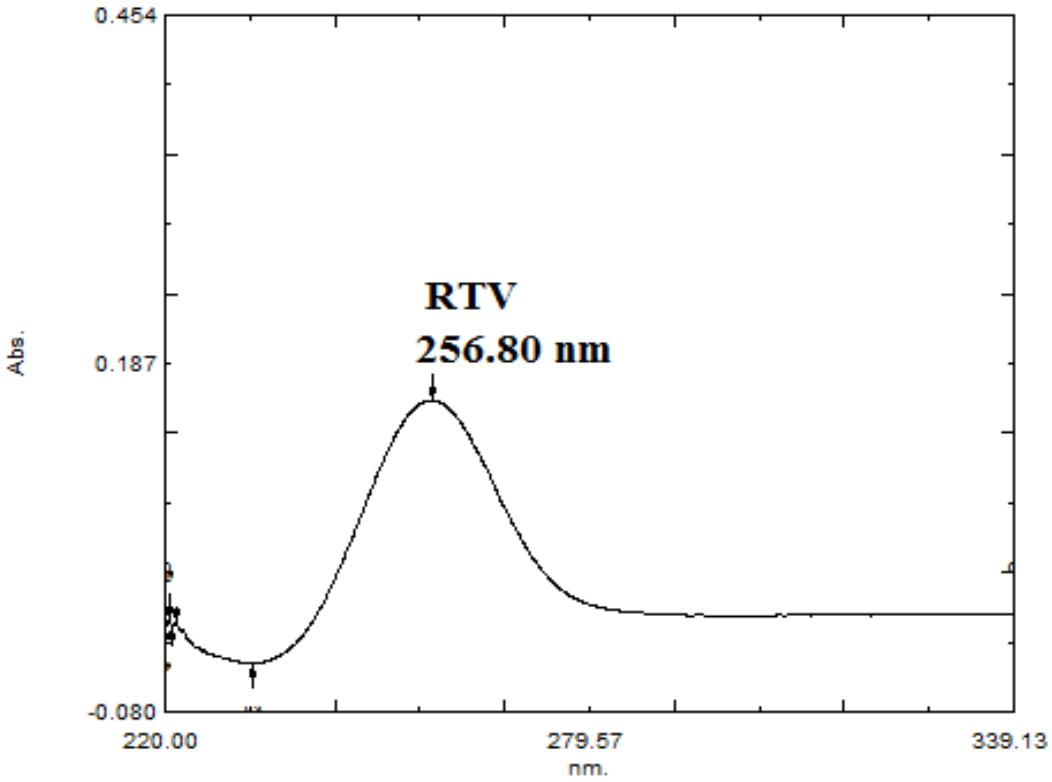
Robustness was studied by analyzing the samples of RTV by deliberate variation in the method parameters. The change in the response of RTV was noted. Robustness of the method was studied by changing the extraction time of RTV from tablet dosage form by ± 2 min, wavelength by ±1 nm and temperature by 25 ± 2 ^{0}C. The changes in the response of RTV were noted and compared with the original one.

7.3.2 RESULTS AND DISCUSSION:

The satisfactory results were obtained with 0.1N HCl (in sample cell) and 0.1 NaOH(in reference cell). The wavelength of maximum absorbance of RTV in 0.1N HCl (sample) and 0.1N NaOH (reference) was found to be at 256.80 nm (Figure 7.3.1).



(a)



(b)

Figure 7.3.1: Difference spectra of RTV in 0.1 N HCl (sample) and 0.1N NaOH (reference) from (a) standard (25 µg/mL) and (b) tablet dosage form (20 µg/mL)

7.3.2.1 METHOD VALIDATION

7.3.2.1.1 Solution Stability

The change in assay results after storage at 25^0C (24 hours) and 2 - 8^0C (3 days) was evaluated. It was found that the difference in assay results was not more than 2 % for formulation, and 0.5% for API, indicating stability of RTV solution.

7.3.2.1.2 Specificity

The analytical method was found to be specific as no interference of excipients and impurities was found in determination of the RTV

7.3.2.1.3 Linearity

The Beer's law was obeyed. Linear correlation was obtained between difference absorbance and concentration of RTV in the range of 5-30 µg/mL. The linearity of the calibration curve was validated by the value of correlation coefficient of the regression (r). The optical and regression characteristics are listed in Table 7.3.1.

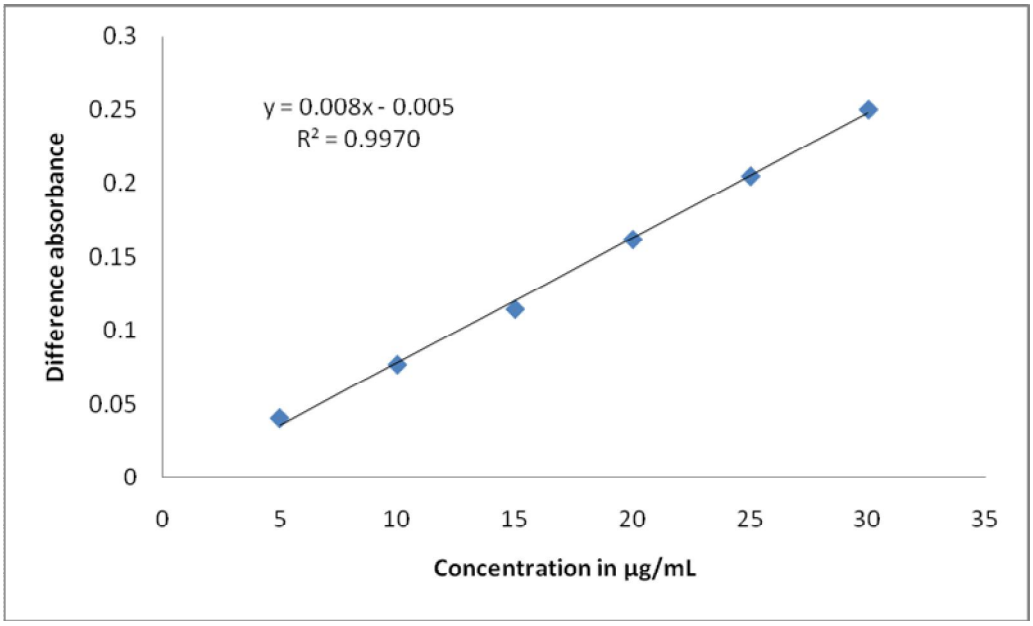


Figure 7.3.2: Calibration curve of RTV

Table 7.3.1: Optical and regression characteristics (n=3)

Parameter	RTV
Linearity range (µg/mL)	5-30
Linearity equation	y = 0.008x – 0.005
LOD (µg/mL)	0.627
LOQ (µg/mL)	1.90
Correlation coefficient (r)	0.9970

7.3.2.1.4 Accuracy (% Recovery)

The accuracy study was carried out by the standard addition method. The percent recoveries were found in the range of 97.26 – 101.85 %, which indicated accuracy of the method.

Table 7.3.2: Results of recovery study (n=3)

Amount taken (µg/mL)	Amount added (µg/mL)	Amount found (µg/mL)	Recovery ± S.D, %	% RSD
10	0	9.72	97.26 ± 1.21	1.24
10	5	14.71	98.09 ± 0.82	0.83
10	10	20.35	101.85 ± 1.27	1.24
10	15	24.70	98.37 ± 1.39	1.41

7.3.2.1.5 Precision

The % RSD for repeatability of RTV was found to be 1.74. The value of % RSD for intra-day precision was found to be in the range of 0.90 – 1.31% and inter-day precision was found to be in the range of 1.54 - 1.89 %, which indicated that the method was precise.

Table 7.3.3: Results of repeatability (n=6)

Drug	RTV Diff. absorbance
1	0.162
2	0.166
3	0.160
4	0.164
5	0.168
6	0.165
Mean	0.164
SD	0.0028
% RSD	1.74

Table 7.3.4: Results of Intra-day and Inter-day precision (n=3)

RTV (µg/mL)	Intra-day precision		Inter-day precision	
	Mean diff. absorbance ± SD	% RSD	Mean diff. absorbance ± SD	% RSD
10	0.076 ± 0.0010	1.31	0.079 ± 0.0015	1.89
20	0.166 ± 0.0015	0.90	0.162 ± 0.0025	1.54
30	0.248 ± 0.0023	0.92	0.253 ± 0.0046	1.81

7.3.2.1.6 Limit of detection and limit of quantification

The limit of detection (LOD) for RTV was found to be 0.627 µg/mL, while the limit of quantification (LOQ) was 1.90 µg/mL.

7.3.2.1.7 Robustness

The method was found to be robust as the results were not significantly affected by slight variation in extraction time, wavelength and temperature.

7.3.2.2 Analysis of Tablet Dosage Form

The proposed UV spectrophotometric method was successfully applied for determination of RTV in tablet dosage form. The percentage of RTV was found to be satisfactory, which was comparable with the corresponding label claim.

Table 7.3.5: Analysis results of tablet dosage form (n=3)

Formulation	Labelled amount (mg)	Amount found (mg)	Assay ± SD, %
VIRITON®	100	98.39	98.39 ± 0.97

7.3.3 CONCLUSION:

A UV spectrophotometric method has been developed and validated for the determination of RTV in tablet dosage form. The method was found to be specific as there was no interference of any excipients and impurities. The proposed method was found to be simple, accurate, precise and robust. Hence, it can be used successfully for the routine analysis of RTV in pharmaceutical dosage forms.

CHAPTER 8

DETERMINATION OF ANTIRETROVIRAL AGENTS IN HUMAN PLASMA

8. DETERMINATION OF ANTIRETROVIRAL AGENTS IN HUMAN PLASMA

8.1 RP-HPLC METHOD FOR DETERMINATION OF EFAVIRENZ IN HUMAN PLASMA.

8.1.1 EXPERIMENTAL

8.1.1.1 Instrumentation:

High Performance Liquid Chromatography (HPLC)

Model : Perkin Elmer series 200

Make : Perkin Elmer, USA

Column : C_{18} , Brownlee

Particle size : 5 µm.

Length : 250 mm

Diameter : 4.6 mm

Pump : Perkin Elmer series 200 pump, back pressure 5000 psi.

Injector : Rhenodyne valve

Injection volume : 20 µL

Detector : UV-visible detector model: Perkin Elmer series 200

8.1.1.2 Chemicals and materials:

- Efavirenz was kindly gifted by Aurobindo Pharmaceuticals, Ltd Hyderabad.
- Acetonitrile and water HPLC grade (Rankem, RFCL Ltd., New Delhi)
- Ammonium acetate crystalline pure (E. Merck, Mumbai, India)
- Nylon membrane filter 0.45 µm (Gelman laboratory, Mumbai, India)

8.1.1.3 Chromatographic conditions

Brownlee C_{18} column (250 mm x 4.6 mm i.d., 5 µm) was used at ambient temperature. The mobile phase comprised of acetonitrile : 10mM ammonium acetate buffer (pH 6.5 ± 0.05) (80:20 v/v). The mobile phase was filtered through 0.45 µm nylon membrane filter and was degassed before use. The elution was monitored at 254 nm. The injection volume was 20 µL.

8.1.1.4 Preparation of mobile phase

The mobile phase was prepared by mixing 80 mL acetonitrile and 20 mL 10mM ammonium acetate buffer (pH 6.5 ± 0.05) previously filtered through 0.45 µm nylon membrane filter. The mobile phase was degassed for 15 minutes by sonicating the solution before use.

8.1.1.5 Preparation of EFV standard stock solution.

Accurately weighed EFV (25mg) was transferred to 25 mL volumetric flask and dissolved and diluted up to the mark with acetonitrile to obtain a standard solution having concentration of EFV (1000 µg/mL).

8.1.1.6 Extraction of sample (EFV)

Drug/metabolite free plasma samples with fixed aliquots of EFV (50 µL) and volunteer plasma sample (950 µL) was taken in glass centrifuge tubes and mixed with 1.0mL acetonitrile for protein precipitation of plasma as well as 0.1 mL saturated sodium chloride solution. The samples were vortexed for 1.0 min., and then precipitated proteins were separated by centrifugation at 15000 rpm for 15 min. Supernant from the tube was collected and filtered through 0.45 µm nylon membrane filter before injection and then injected into the HPLC system.

8.1.1.7 Selection of Wavelength for Determination

The standard solutions of EFV were scanned in the range of 200 – 400 nm against reagent blank. Maximum absorbance for EFV was observed at 254 nm which was selected for analysis.

8.1.1.8 METHOD VALIDATION:

8.1.1.8.1 Calibration Curve (Linearity)

Accurately measured standard stock solutions of EFV (0.1, 0.2, 0.4, 0.8, 1.2, 1.6, 2.0 and 2.4 mL) were transferred in a series of 10 mL volumetric flasks and diluted with acetonitrile. The above solutions (50 µL) were spiked with 950 µL of drug free plasma to obtain EFV concentration of 500, 1000, 2000, 4000, 6000, 8000, 10000 and 12000 ng/mL.

8.1.1.8.2 Accuracy

Accuracy of the measurement of EFV in plasma was determined by standard addition method at four different concentration levels of EFV (6000, 8000, 10000 and 12000 ng/mL). The working solution (4000 ng/mL) was transferred to four different glass tubes. To each tube, 950 µL of drug/metabolite free plasma was added. Further 2000, 4000, 6000 and 8000 ng/mL of solutions were serially added into these tubes. These samples were extracted as described above in the extraction procedure.

8.1.1.8.3 Recovery

EFV recoveries (relative and absolute) from human plasma were determined by spiking drug-free plasma (five replicates for each standard) with known amounts of the drug to achieve EFV concentrations of 500, 4000, 8000 and 12000 ng/mL. The spiked samples were processed and analysed with the developed procedure. The relative (analytical) recovery was calculated by comparing the concentrations obtained from the drug-supplemented plasma with actual added amounts. The absolute (extraction) recovery was obtained by comparing the observed peak areas obtained from the processed standard samples to direct injections of standard aqueous solutions prepared at concentrations which represented 100% recovery.

8.1.1.8.4 Precision

8.1.1.8.4.1 Method Precision (repeatability)

Method precision experiment was performed by preparing the working solution of EFV (4000 ng/mL) for seven times and analysed as per described under recovery.

8.1.1.8.4.2 Inter-day and intra-day precision

The intra and inter day precisions of EFV assay in human plasma were assessed from replicate samples spiked at four different concentrations (500, 4000, 8000 and 12000 ng/mL). The selection of concentrations for analysis was made according to definition of precision at low, medium and high concentrations of the linear range. The precision of the method is expressed

as the relative standard deviation (% RSD) of the mean estimated concentrations.

8.1.1.8.5 Limit of detection and limit of quantification

The limit of detection and the limit of quantification of the drug were calculated using the following equations.

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

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

Where σ is the standard deviation of the peak areas of the drug and S is the slope for the corresponding calibration curve.

8.1.1.8.6 Stability

The effect of frozen storage on EFV stability in human plasma was assessed through storing of EFV plasma samples at -70^0C over a period of 1 month. EFV plasma samples were analysed immediately after preparation and at selected time intervals after storage over the study period. Stability was defined as <10% loss of initial drug concentration. Moreover, stability in heparinized plasma samples through five freeze and thaw cycles (-70^0C ± 5^0C to room temperature) has been confirmed. Samples, after thawing, were allowed to stand on the bench top, under room lighting till 2 hr had elapsed since their removal from the freezer. On the other hand , on-machine stability of the processed samples was evaluated by injecting the quality control samples, stored at room temperature in the HPLC, immediately after preparation and then at selected time intervals for 24 hr.

8.1.1.8.7 Specificity

The specificity of the assay for the analyte in the presence of endogenous substances in the matrix was assessed by comparing the response obtained from the concentration used to produce the calibration plots with response obtained from plasma of different persons.

8.1.2 RESULTS AND DISCUSSION

8.1.2.1 Chromatography

The mobile phase acetonitrile : 10mM ammonium acetate buffer (pH 6.5 ± 0.05) (80:20 v/v) at the flow rate of 1.0 mL/min achieved optimum separation of efavirenz without interferences from endogenous components in the plasma. Figure 8.1.1, 8.1.2 and 8.1.3 represent typical chromatograms of drug-free human plasma and drug-free human plasma spiked with efavirenz (12000 ng/mL and 10000 ng/mL) respectively. As illustrated in these chromatograms, efavirenz eluted at retention time of approximately 4.42 min. The peak of interest was clearly resolved with no interfering peaks from endogenous components in the plasma within the time frame where efavirenz was eluted.

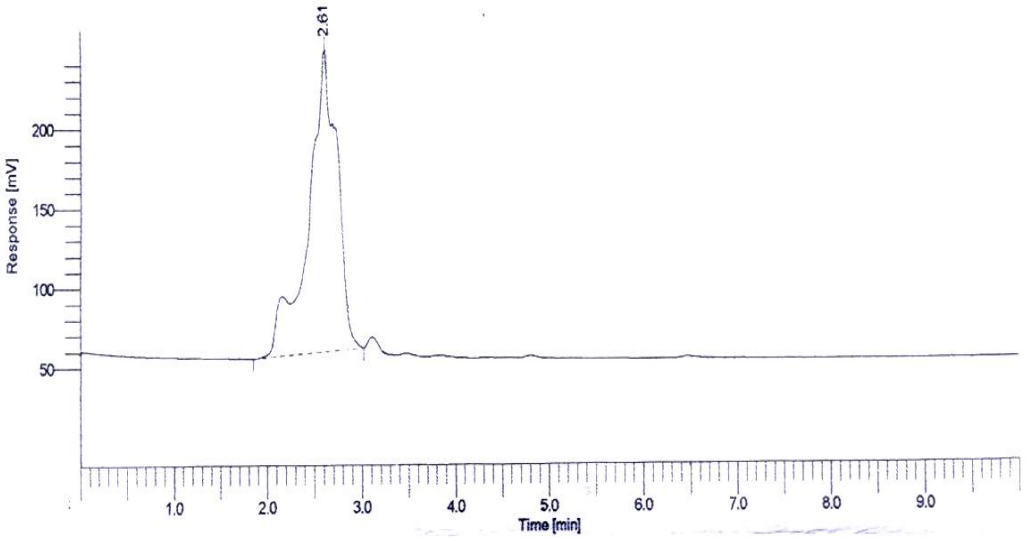


Figure 8.1.1: Chromatogram of drug-free human plasma.

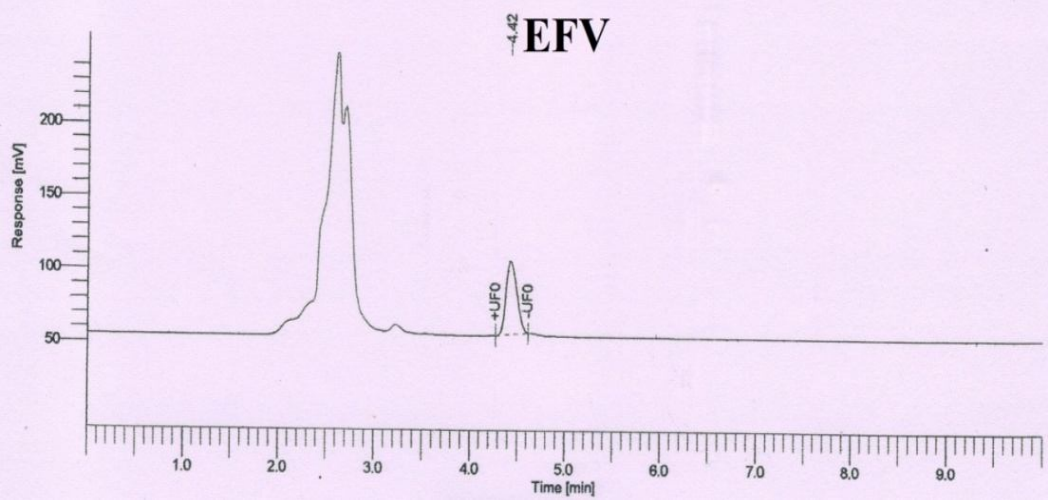


Figure 8.1.2: Chromatogram of drug-free human plasma spiked with EFV (12000 ng/mL).

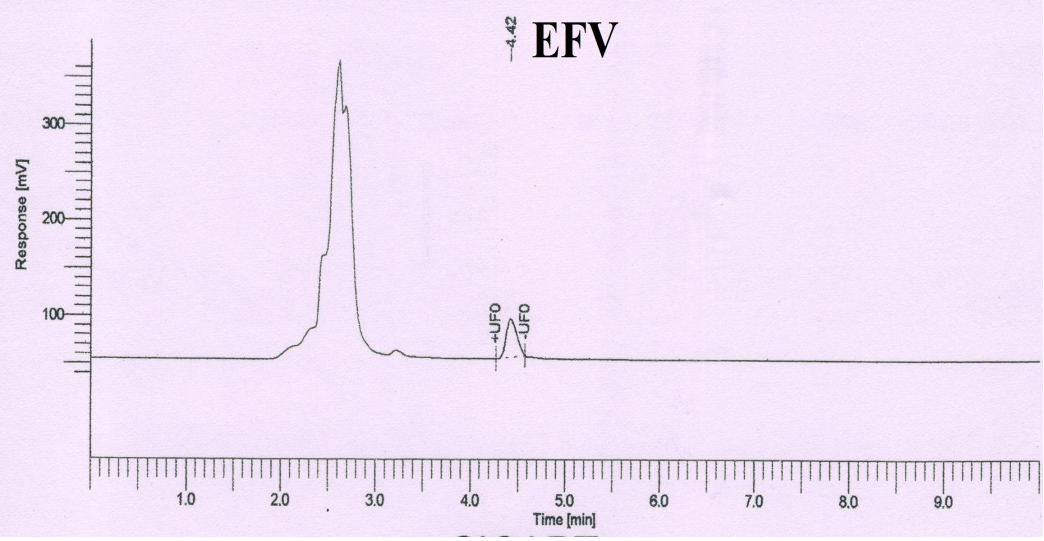


Figure 8.1.3: Chromatogram of drug-free human plasma spiked with EFV (10000 ng/mL).

8.1.2.2 VALIDATION OF THE METHOD

8.1.2.2.1 Linearity

Linear correlation was obtained between peak areas and concentrations of EFV in range of 500 - 12000 ng/mL.

The linearity of the calibration graphs was validated by the high value of correlation coefficients of the regression (Table 8.1.1).The linearity of the calibration graphs was validated by the high value of correlation coefficients of the regression (figure 8.1.4).

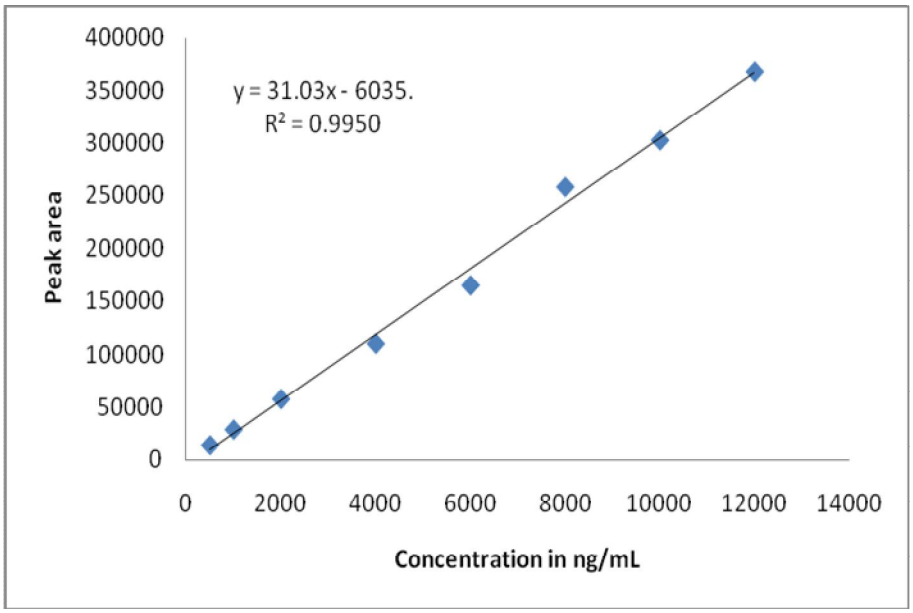


Figure 8.1.4: Calibration curve of EFV

Table 8.1.1: Optical and regression characteristic

Parameter	EFV
Linearity range	500 - 12000 ng/mL
Regression equation (y)	y = 31.03x - 6035
Correlation coefficient (r)	0.9950

8.1.2.2.2 Accuracy:

The recovery experiments were carried out as in the text. The percent recoveries obtained were 86.11 to 92.57% for EFV. The results of recovery study are given in table 8.1.2

Table 8.1.2: Data of recovery study (n = 5)

Compound	Amount taken (ng/mL)	Amount added (ng/mL)	Amount recovered (ng/mL)	Accuracy ± SD%
EFV	4000	2000	5460.00	91.00 ± 11.76
	4000	4000	6888.80	86.11 ±10.42
	4000	6000	9257.00	92.57 ± 8.35
	4000	8000	10718.40	89.32 ± 6.87

8.1.2.2.3 Recovery:

The recovery experiments were carried out as in the text. The absolute and relative mean recovery found to be 83.75% and 85.00%, respectively (Table 8.3.3).

Table 8.1.3: Absolute and Relative Recoveries of EFV from human plasma

Nominal concentration (ng/mL)	Absolute recovery (mean ± SD%) (n = 5)	Relative recovery (mean ± SD%) (n = 5)
500	82.65 ± 11.2	83.34 ± 10.6
4000	84.89 ± 7.52	85.79 ±5.28
8000	86.34 ± 8.28	86.21 ± 3.88
12000	81.14 ± 9.71	84.68 ± 6.74

8.1.2.2.4 Precision

8.1.2.2.4.1 Method precision (repeatability)

Relative standard deviation of all the parameters was less than 15% (table 8.1.4), which indicated that the proposed method was repeatable.

Table 8.1.4: Method Precision data

EFV (4000 ng/mL)	Retention time (min.)	Peak area	Tailing factor
1	4.42	57424.8	1.42
2	4.40	53634.2	1.40
3	4.41	59276.6	1.41
4	4.39	54766.0	1.41
5	4.42	52865.9	1.42
6	4.42	57145.6	1.40
7	4.40	56358.5	1.42
Mean	4.40	55924.5	1.411
SD	0.012	2277.03	0.009
% RSD	0.27	4.07	0.63

8.1.2.2.4.2 Intra-day and inter-day precision:

The intraday precision (% RSD) ranged from 3.89 to 5.94% while the interday precision (% RSD) ranged from 5.05 to 7.90% (Table 8.1.5). The intra- and inter-day precision data showed that acceptable precision was obtained over the entire assay range.

Table 8.1.5: Intra and Inter- day precision of EFV in human plasma.

Nominal concentration. (ng/mL)	**Observed concentration. (ng/mL)**	**(% RSD)**
Intra-day (n = 7)		
500	502.72 ± 23.72	4.72
4000	4005.36 ± 155.80	3.89
8000	7989.54 ± 329.96	4.13
12000	12011.65 ± 713.49	5.94
Inter-day (n = 7)		
500	497.34 ± 31.23	6.28
4000	3988.45 ± 201.41	5.05
8000	7982.54 ± 530.83	6.65
12000	12008.58 ± 948.67	7.90

8.1.2.2.5 Limit of detection and limit of quantification:

The limit of detection (LOD) of EFV was determined to be 119.82 ng/mL, whereas, quantitative limit (LOQ) was 382.43 ng/mL.

8.1.2.2.6 Stability study:

The stability results showed that efavirenz was stable for at least 4 weeks when kept frozen at -70 ^{0}C. Thus, analysis up to 4 weeks storage confirmed adequate EFV stability at this temperature. Moreover, the results of freeze/thaw stability indicate that EFV was stable in plasma for at least five freeze/thaw cycles (Table 8.1.6).

Table 8.1.6: Summary of stability testing of EFV in human plasma.

Stability	Nominal concentration (ng/mL)	Observed concentration. (ng/mL)	Precision (% RSD)
Freeze/thaw (n = 5)	500	486.22	4.87
	4000	3878.43	3.67
	8000	7539.54	5.22
	12000	11529.96	7.35
Long term (n = 5)	500	465.87	8.12
	4000	3773.63	6.42
	8000	7458.42	4.31
	12000	11464.26	5.64

8.1.3 CONCLUSION

The developed method was found to be simple, accurate, precise and repeatable. It can be applied for the determination of concentration of EFV in the human plasma for bioavailability and bioequivalence study.

8.2 RP-HPLC METHOD FOR DETERMINATION OF TENOFOVIR DISOPROXIL FUMARATE IN HUMAN PLASMA.

8.2.1 EXPERIMENTAL

8.2.1.1 Instrumentation

Same as described under 8.1.1.1

8.2.1.2 Chemicals and materials:

- Tenofovir disoproxil fumarate was kindly gifted by Aurobindo Pharmaceuticals, Ltd Hyderabad.
- Acetonitrile and water HPLC grade (Rankem, RFCL Ltd., New Delhi)
- Ammonium acetate crystalline pure (E. Merck, Mumbai, India)
- Nylon membrane filter 0.45 µm (Gelman laboratory, Mumbai, India)

8.2.1.3 Chromatographic conditions

Brownlee C_{18} column (250 mm x 4.6 mm i.d., 5 µm) was used at ambient temperature. The mobile phase comprised of acetonitrile : 10 mM ammonium acetate buffer (pH 6.5 ± 0.05) (60:40 v/v). The mobile phase was filtered through 0.45 µm nylon membrane filter and was degassed before use. The elution was monitered at 254 nm. The injection volume was 20 µL.

8.2.1.4 Preparation of mobile phase

The mobile phase was prepared by mixing 60 mL acetonitrile and 40 mL 10mM ammonium acetate buffer (pH 6.5 ± 0.05) previously filtered through 0.45 µm nylon membrane filter. The mobile phase was degassed for 15 minutes by sonicating the solution before use.

8.2.1.5 Preparation of TNV standard stock solution.

Accurately weighed TNV (25 mg) was transferred to 25 mL volumetric flask and dissolved and diluted up to the mark with acetonitrile to obtain a standard solution having concentration of TNV (1000 µg/mL).

8.2.1.6 Extraction of sample (TNV)

Drug/metabolite free plasma samples with fixed aliquots of TNV (50 µL) and volunteer plasma sample (950 µL) was taken in glass centrifuge tubes and mixed with 1.0mL acetonitrile for protein precipitation of plasma as well as 0.1 mL saturated sodium chloride solution. The samples were vortexed for 1.0 min., and then precipitated proteins were separated by centrifugation at 15000 rpm for 15 min. Supernant from the tube was collected and filtered through 0.45 µm nylon membrane filter before injection and then injected into the HPLC system.

8.2.1.7 Selection of Wavelength for Determination

The standard solutions of TNV were scanned in the range of 200 – 400 nm against reagent blank. Significant absorbance for TNV was observed at 254 nm which was selected for analysis.

8.2.1.8 METHOD VALIDATION

8.2.1.8.1 Calibration Curve (Linearity)

Accurately measured standard stock solutions of TNV (0.01, 0.02**,** 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mL) were transferred in a series of 10 mL volumetric flasks and diluted with acetonitrile. The above solutions (50 µL) were spiked with 950 µL of drug free plasma to obtain TNV concentration of 50, 100, 500, 1000, 2000, 3000, 4000 and 5000 ng/mL.

8.2.1.8.2 Accuracy

Accuracy of the measurement of TNV in plasma was determined by standard addition method at four different concentration levels of TNV (1500, 2000, 2500 and 3000, ng/mL). The working solution (1000 ng/mL) was transferred to four different glass tubes. To each tube, 950 µL of drug/metabolite free plasma was added. Further 500, 1000, 1500 and 2000 ng/mL of solutions were serially added into these tubes. These samples were extracted as described above in the extraction procedure.

8.2.1.8.3 Recovery

TNV recoveries (relative and absolute) from human plasma were determined by spiking drug-free plasma (five replicates for each standard) with known amounts of the drug to achieve TNV concentrations of 100, 500, 3000 and 5000 ng/mL. The spiked samples were processed and analysed with the developed procedure. The relative (analytical) recovery was calculated by comparing the concentrations obtained from the drug-supplemented plasma with actual added amounts. The absolute (extraction) recovery was obtained by comparing the observed peak areas obtained from the processed standard samples to direct injections of standard aqueous solutions prepared at concentrations which represented 100% recovery.

8.2.1.8.4 Precision

8.2.1.8.4.1 Method Precision (repeatability)

Method precision experiment was performed by preparing the working solution of TNV (1000 ng/mL) for seven times and analysed as per described under recovery.

8.2.1.8.4.2 Inter day and intraday precision

The intra and inter day precisions of TNV assay in human plasma were assessed from replicate samples spiked at four different concentrations (100, 500, 3000 and 5000 ng/mL). The selection of concentrations for analysis was made according to definition of precision at low, medium and high concentrations of the linear range.The precision of the method is expressed as the relative standard deviation (% RSD) of the mean estimated concentrations.

8.2.1.8.5 Limit of detection and limit of quantification:

The limit of detection and the limit of quantification of the drug were calculated using the following equations.

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

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

Where σ is the standard deviation of the peak areas of the drug and S is the slope for the corresponding calibration curve.

8.2.1.8.6 Stability

The effect of frozen storage on TNV stability in human plasma was assessed through storing of TNV plasma samples at -70^{0}C over a period of 1 month. TNV plasma samples were analysed immediately after preparation and at selected time intervals after storage over the study period. Stability was defined as <10% loss of initial drug concentration. Moreover, stability in heparinized plasma samples through five freeze and thaw cycles (-70^{0}C ± 5^{0}C to room temperature) has been confirmed. Samples, after thawing , were allowed to stand on the bench top, under room lighting till 2 hr had elapsed since their removal from the freezer. On the other hand , on-machine stability of the processed samples was evaluated by injecting the quality control samples, stored at room temperature in the HPLC, immediately after preparation and then at selected time intervals for 24 hr.

8.2.1.8.7 Specificity

The specificity of the assay for the analyte in the presence of endogenous substances in the matrix was assessed by comparing the response obtained from the concentration used to produce the calibration plots with response obtained from plasma of different patients.

8.2.2 RESULTS AND DISCUSSION

8.2.2.1 Chromatography

The mobile phase acetonitrile : 10mM ammonium acetate buffer (pH 6.5 ± 0.05) (60:40 v/v) at the flow rate of 1.0 mL/min achieved optimum separation of TNV without interferences from endogenous components in the plasma. Figure 8.2.1, 8.2.2 and 8.2.3 represent typical chromatograms of drug-free human plasma and drug-free human plasma spiked with TNV (5000 ng/mL and 3000 ng/mL) respectively. As illustrated in these chromatograms, TNV eluted at retention time of approximately 3.38 min. The peak of interest was clearly resolved with no interfering peaks from endogenous components in the plasma within the time frame where TNV was eluted.

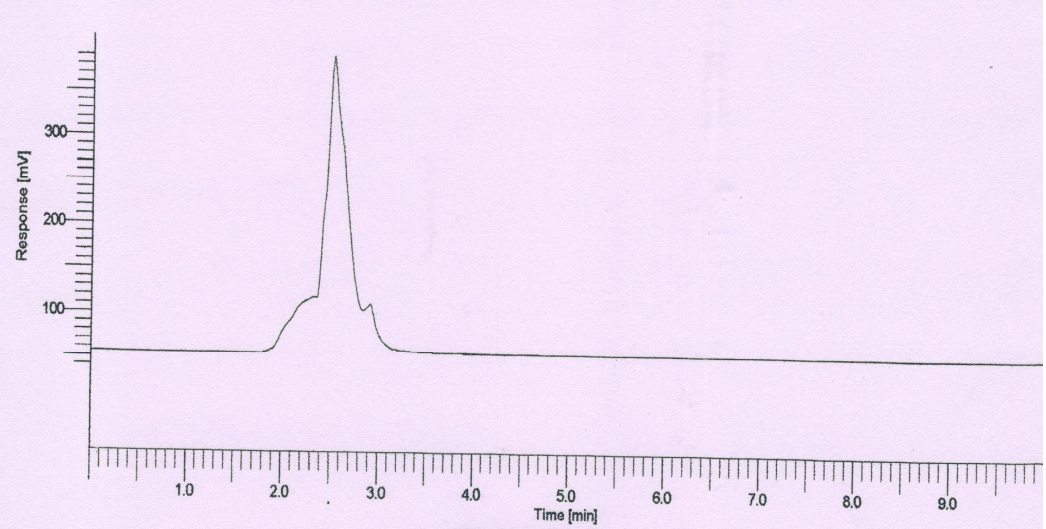


Figure 8.2.1: Chromatogram of drug-free human plasma.

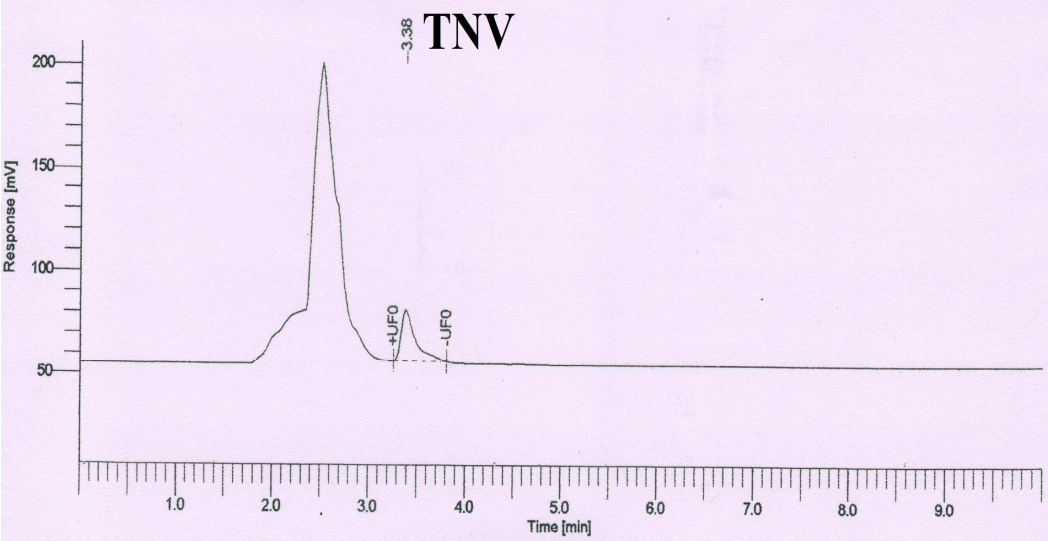


Figure 8.2.2: Chromatogram of drug-free human plasma spiked with TNV (5000 ng/mL).

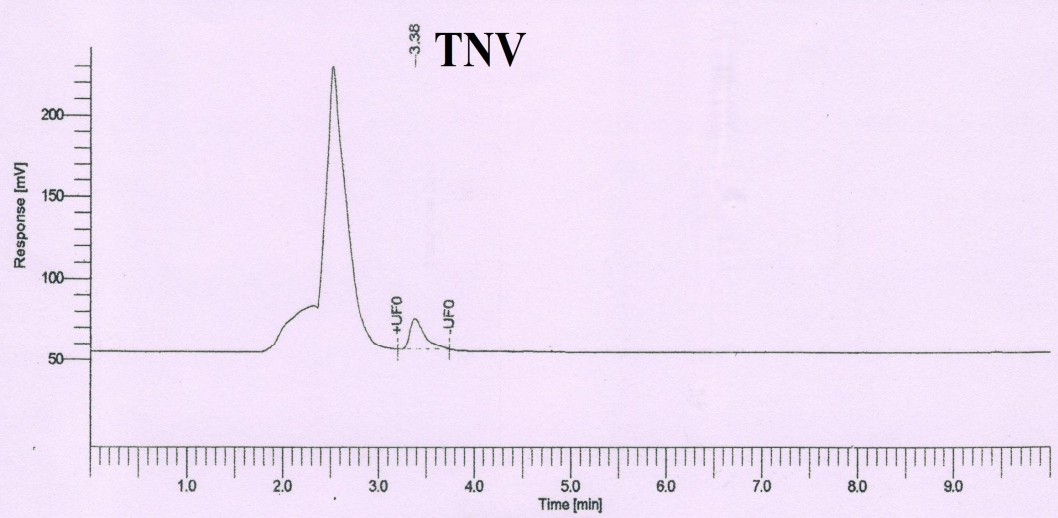


Figure 8.2.3: Chromatogram of drug-free human plasma spiked with TNV (3000 ng/mL).

8.2.2.2 VALIDATION OF THE METHOD

8.2.2.2.1 Linearity

Linear correlation was obtained between peak areas and concentrations of TNV in range of 50 - 5000 ng/mL. The linearity of the calibration graphs was

validated by the high value of correlation coefficients of the regression (Table 8.2.1).The linearity of the calibration graphs was validated by the high value of correlation coefficients of the regression (figure 8.2.4).

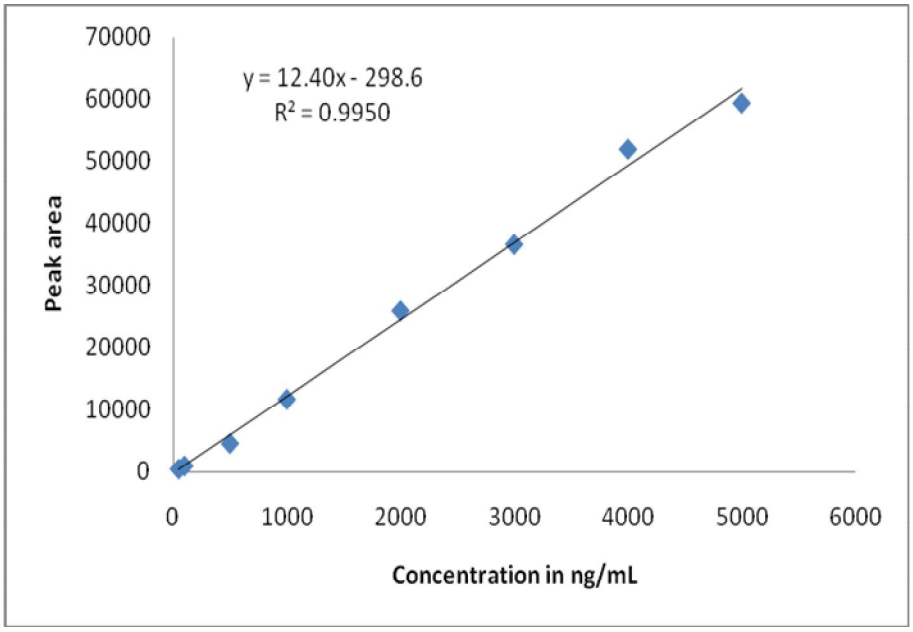


Figure 8.2.4: Calibration curve of TNV

Table 8.2.1: Optical and regression characteristic

Parameter	TNV
Linearity range	50 - 5000 ng/mL
Regression equation (y)	y = 12.40x – 298.6
Correlation coefficient (r)	0.9950

8.2.2.2.2 Accuracy

The recovery experiments were carried out as in the text. The percent recoveries obtained were 86.06 to 91.51% for TNV. The results of recovery study are given in table 8.2.2

Table 8.2.2: Data of recovery study (n = 5)

Compound	Amount taken (ng/mL)	Amount added (ng/mL)	Amount found (ng/mL)	Accuracy ± SD%
TNV	1000	500	1320.00	88.00 ± 3.24
	1000	1000	1830.20	91.51 ± 11.55
	1000	1500	2151.50	86.06 ± 4.87
	1000	2000	2722.80	90.76 ± 7.42

8.2.2.2.3 Recovery:

The recovery experiments were carried out as in the text. The absolute and relative mean recovery found to be 84.37% and 84.26%, respectively (Table 8.2.3).

Table 8.2.3: Absolute and Relative Recoveries of TNV from human plasma.

Nominal concentration (ng/mL)	Absolute recovery (mean ± SD%) (n = 5)	Relative recovery (mean ± SD%) (n = 5)
100	83.35 ± 10.1	82.21 ± 8.95
500	82.71 ± 6.55	81.47 ± 6.32
3000	85.24 ± 7.21	87.66 ± 5.57
5000	86.18 ± 5.61	85.72 ± 3.28

8.2.2.2.4 Precision

8.2.2.2.4.1 Method precision (repeatability)

Relative standard deviation of all the parameters was less than 15% (table 8.2.4), which indicated that the proposed method was repeatable.

Table 8.2.4: Method Precision data

TNV (1000 ng/mL)	Retention time (min.)	Peak area	Tailing factor
1	3.38	11756.34	1.69
2	3.36	9588.48	1.69
3	3.38	12164.21	1.68
4	3.38	11232.45	1.67
5	3.28	10975.54	1.69
6	3.22	13667.91	1.68
7	3.38	11824.38	1.68
Mean	3.34	11601.33	1.68
SD	0.064	1241.01	0.0075
% RSD	1.92	10.69	0.45

8.2.2.2.4.2 Intra day and inter day precision:

The intraday precision (% RSD) ranged from 6.44 to 8.10% while the interday precision (% RSD) ranged from 8.57 to 10.60% (Table 8.2.5). The intra- and inter-day precision data showed that acceptable precision was obtained over the entire assay range.

Table 8.2.5: Intra and Inter- day precision of TNV in human plasma.

Nominal concentration. (ng/mL)	Observed concentration. (ng/mL)	(% RSD)
Intra-day (n = 7)		
100	102.40 ± 6.81	6.65
500	489.55 ± 39.65	8.10
3000	2976.49 ± 210.43	7.07
5000	5024.72 ± 323.59	6.44
Inter-day (n =7)		
100	97.23 ± 8.33	8.57
500	508.67 ± 53.91	10.60
3000	3018.39 ± 298.21	9.88
5000	4922.86 ± 441.08	8.96

8.2.2.2.5 Limit of detection and limit of quantification

The limit of detection (LOD) of TNV was determined to be 14.86 ng/mL, whereas, quantitative limit (LOQ) was 46.78 ng/mL.

8.2.2.2.6 Stability study:

The stability results showed that TNV was stable for at least 3 weeks when kept frozen at -70 ^{0}C. Thus, analysis up to 3 weeks storage confirmed adequate TNV stability at this temperature. Moreover, the results of freeze/thaw stability indicate that TNV was stable in plasma for at least five freeze/thaw cycles (Table 8.2.6).

Table 8.2.6: Summary of stability testing of TNV in human plasma.

Stability	Nominal concentration (ng/mL)	Observed concentration. (ng/mL)	Precision (% RSD)
Freeze/thaw (n = 5)	100	92.68	5.51
	500	482.71	7.35
	3000	2868.65	3.96
	5000	4814.52	8.64
Long term (n = 5)	100	91.12	4.61
	500	491.08	9.86
	3000	2911.76	7.74
	5000	4708.31	5.63

8.2.3 CONCLUSION

The developed method was found to be simple, accurate, precise and repeatable. It can be applied for the determination of concentration of TNV in the human plasma for bioavailability and bioequivalence studies.

8.3 RP-HPLC METHOD FOR DETERMINATION OF LOPINAVIR AND RITONAVIR IN HUMAN PLASMA.

8.3.1 EXPERIMENTAL

8.3.1.1 Instrumentation

Same as described under 8.1.1.1

8.3.1.2 Chemicals and materials:

- Lopinavir and Ritonavir were kindly gifted by Emcure pharmaceuticals ltd., Pune
- Acetonitrile and water HPLC grade (Rankem, RFCL Ltd., New Delhi)
- Ammonium acetate crystalline pure (E. Merck, Mumbai, India)
- Orthophosphoric acid HPLC grade (Spectrochem Pvt. Ltd., Mumbai)
- Nylon membrane filter 0.45 µm (Gelman laboratory, Mumbai, India)

8.3.1.3 Chromatographic conditions

Brownlee C_{18} column (250mm x 4.6mm i.d., 5 µm) was used at ambient temperature. The mobile phase comprised of acetonitrile : 10 mM ammonium acetate buffer (pH 4.5 ± 0.05 adjusted with orthphosphoric acid) : methanol (40:30:30 v/v/v). The mobile phase was filtered through 0.45 µm nylon membrane filter and was degassed before use. The elution was monitered at 210 nm. The injection volume was 20 µL.

8.3.1.4 Preparation of mobile phase

The mobile phase was prepared by mixing 40 mL acetonitrile, 30 mL 10mM ammonium acetate buffer (pH 4.5 ± 0.05 adjusted with orthphosphoric acid) and 30 mL methanol previously filtered through 0.45 µm nylon membrane filter. The mobile phase was degassed for 15 minutes by sonicating the solution before use.

8.3.1.5 Preparation of LPV and RTV standard stock solution.

Accurately weighed LPV and RTV (25 mg each) were transferred to same 25 mL volumetric flask and dissolved and diluted up to the mark with acetonitrile

to obtain a standard solution having concentration of LPV and RTV (1000 µg/mL).

8.3.1.6 Extraction of sample (LPV and RTV)

Drug/metabolite free plasma samples with fixed aliquots of LPV and RTV (50 µL) and volunteer plasma sample (950 µL) was taken in glass centrifuge tubes and mixed with 1.0 mL acetonitrile for protein precipitation of plasma as well as 0.1 mL saturated sodium chloride solution. The samples were vortexed for 1.0 min., and then precipitated proteins were separated by centrifugation at 15000 rpm for 15 min. Supernant from the tube was collected and filtered through 0.45 µm nylon membrane filter before injection and then injected into the HPLC system.

8.3.1.7 Selection of Wavelength for Determination

The standard solutions of LPV and RTV were scanned in the range of 200 - 400 nm against reagent blank. Both the drugs showed significant absorbance at 210 nm which was selected for analysis.

8.3.1.8 METHOD VALIDATION

8.3.1.8.1 Calibration Curve (Linearity)

Accurately measured standard stock solution containing LPV and RTV (0.06, 0.2, 0.4, 0.8, 1.2, 2.0, 2.4 and 3.2 mL) were transferred in a series of 10 mL volumetric flasks and diluted with acetonitrile. The above solutions (50 µL) were spiked with 950 µL of drug free plasma to obtain LPV and RTV concentration of 300, 1000, 2000, 4000, 6000, 10000, 12000 and 16000 ng/mL.

8.3.1.8.2 Accuracy

Accuracy of the measurement of LPV and RTV in plasma was determined by standard addition method at four different concentration levels of LPV and RTV (6000, 8000, 10000 and 12000 ng/mL). The working solution (4000 ng/mL) was transferred to four different glass tubes. To each tube, 950 µL of drug/metabolite free plasma was added. Further 2000, 4000, 6000 and 8000

ng/mL of solutions were serially added into these tubes. These samples were extracted as described above in the extraction procedure.

8.3.1.8.3 Recovery

LPV and RTV recovery (relative and absolute) from human plasma were determined by spiking drug-free plasma (five replicates for each standard) with known amounts of the drug to achieve TNV concentrations of 300, 2000, 6000 and 12000 ng/mL. The spiked samples were processed and analysed with the developed procedure. The relative (analytical) recovery was calculated by comparing the concentrations obtained from the drug-supplemented plasma with actual added amounts. The absolute (extraction) recovery was obtained by comparing the observed peak areas obtained from the processed standard samples to direct injections of standard aqueous solutions prepared at concentrations which represented 100% recovery.

8.3.1.8.4 Precision

8.3.1.8.4.1 Method Precision (repeatability)

Method precision experiment was performed by preparing the working solution of LPV and RTV (4000 ng/mL) for seven times and analysed as per described under recovery.

8.3.1.8.4.2 Inter day and intra day precision

The intra and inter day precisions of LPV and RTV assay in human plasma were assessed from replicate samples spiked at four different concentrations (300, 2000, 6000 and 12000 ng/mL). The selection of concentrations for analysis was made according to definition of precision at low, medium and high concentrations of the linear range. The precision of the method is expressed as the relative standard deviation (% RSD) of the mean estimated concentrations.

8.3.1.8.5 Limit of detection and limit of quantification

The limit of detection and the limit of quantification of the drug were calculated using the following equations.

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

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

Where σ is the standard deviation of the peak areas of the drug and S is the slope for the corresponding calibration curve.

8.3.1.8.6 Stability

The effect of frozen storage on LPV and RTV stability in human plasma was assessed through storing of LPV and RTV plasma samples at -70^{0}C over a period of 1 month. LPV and RTV plasma samples were analysed immediately after preparation and at selected time intervals after storage over the study period. Stability was defined as <10% loss of initial drug concentration. Moreover, stability in heparinized plasma samples through five freeze and thaw cycles (-70^{0}C ± 5^{0}C to room temperature) has been confirmed. Samples, after thawing, were allowed to stand on the bench top, under room lighting till 2 hr had elapsed since their removal from the freezer. On the other hand , on-machine stability of the processed samples was evaluated by injecting the quality control samples, stored at room temperature in the HPLC, immediately after preparation and then at selected time intervals for 24 hr.

8.3.1.8.7 Specificity

The specificity of the assay for the analyte in the presence of endogenous substances in the matrix was assessed by comparing the response obtained from the concentration used to produce the calibration plots with response obtained from plasma of different patients.

8.3.2 RESULTS AND DISCUSSION

8.3.2.1 Chromatography

The mobile phase acetonitrile : 10mM ammonium acetate buffer (pH 4.5 ± 0.05 adjusted with orthphosphoric acid) : methanol (40:30:30 v/v/v) at the flow rate of 1.0 mL/min achieved optimum separation of LPV and RTV without interferences from endogenous components in the plasma. Figure 8.3.1, 8.3.2 and 8.3.3 represent typical chromatograms of drug-free human plasma and drug-free human plasma spiked with LPV and RTV (16000 ng/mL and 12000 ng/mL) respectively. As illustrated in these chromatograms, LPV and RTV eluted at retention times of approximately 12.58 min and 10.30 min respectively. The peaks of interest were clearly resolved with no interfering peaks from endogenous components in the plasma within the time frame where LPV and RTV were eluted.

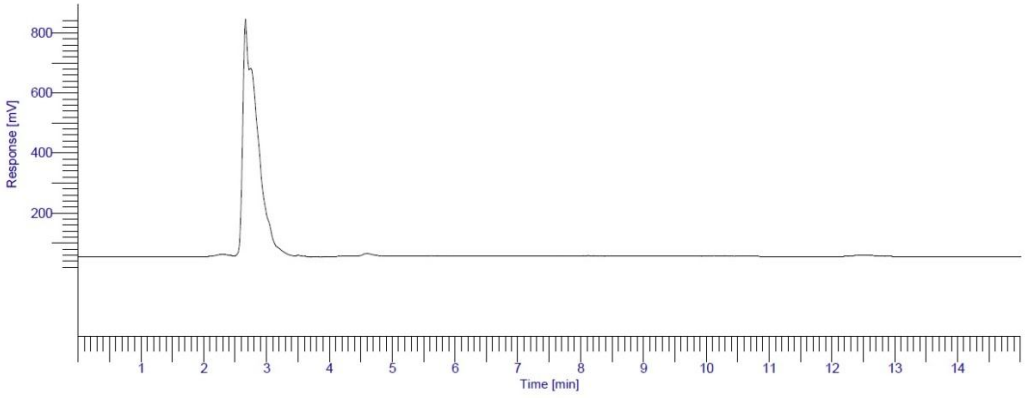
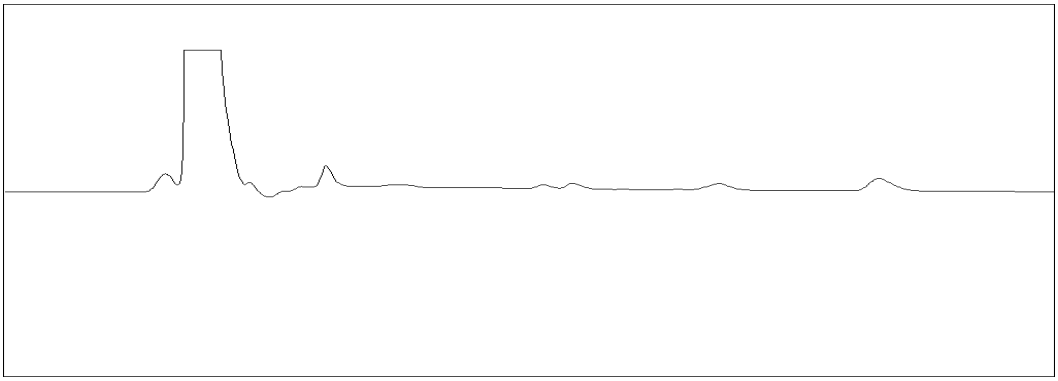


Figure 8.3.1: Chromatogram of drug-free human plasma.



Enlargement of Figure 8.3.1

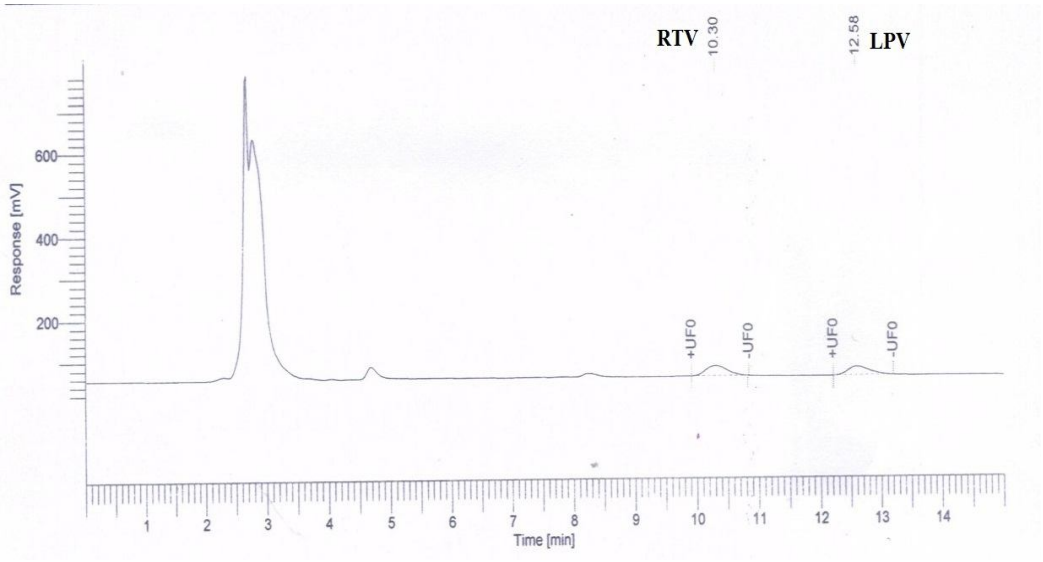
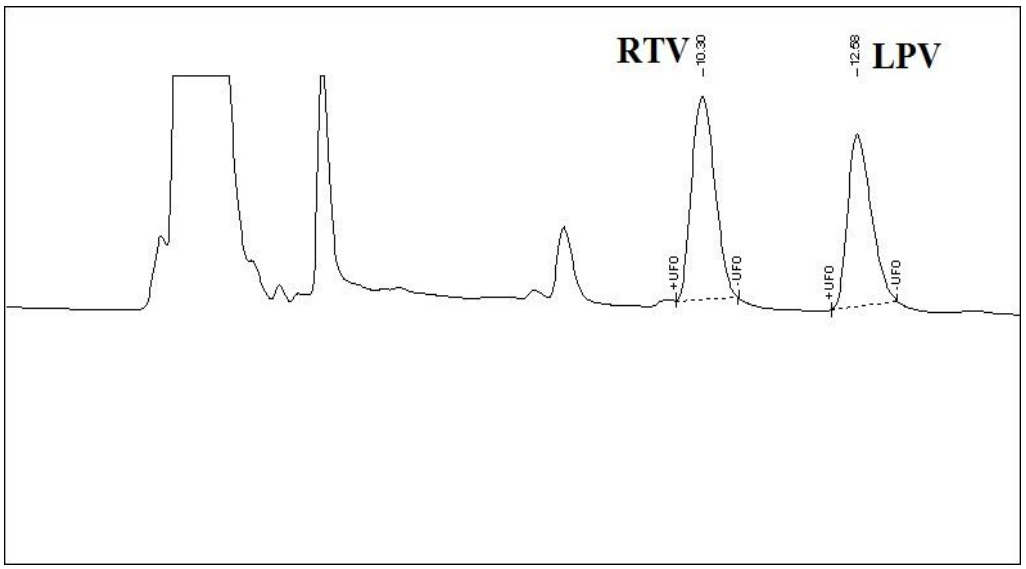


Figure 8.3.2: Chromatogram of drug-free human plasma spiked with RTV and LPV (16000 ng/mL).



Enlargement of Figure 8.3.2

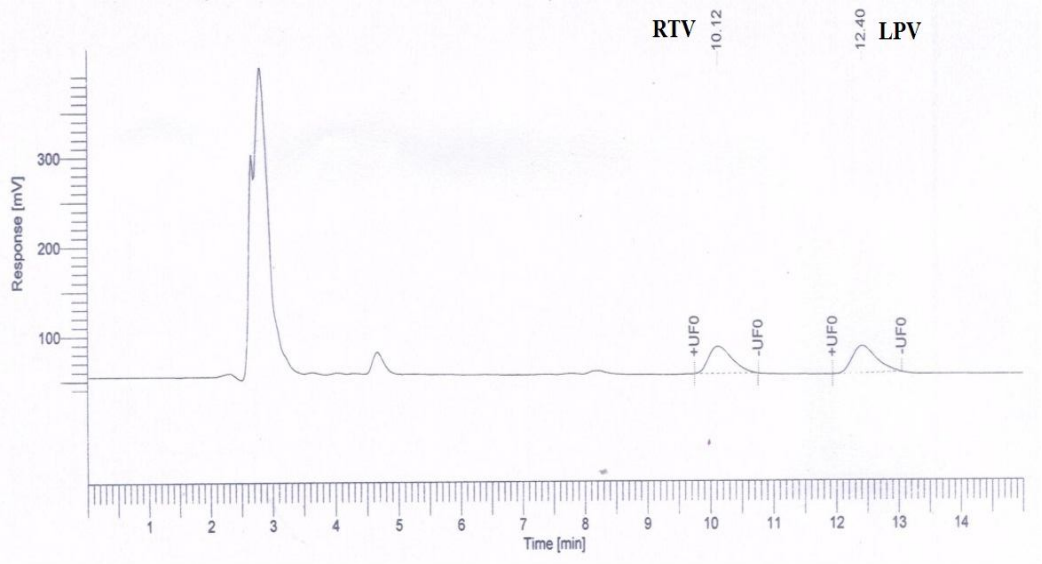
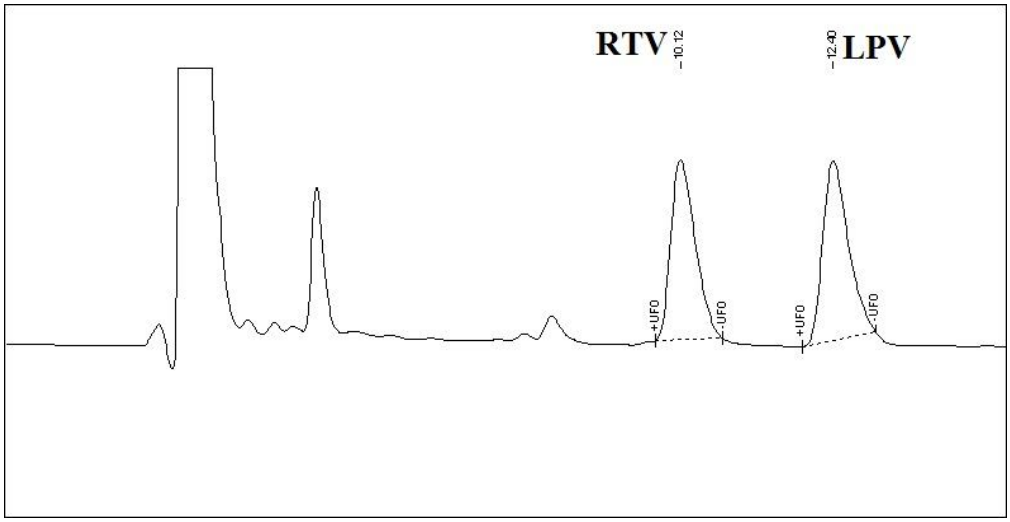


Figure 8.3.3: Chromatogram of drug-free human plasma spiked with RTV and LPV (12000 ng/mL).



Enlargement of Figure 8.3.3

8.3.2.2 VALIDATION OF THE METHOD

8.3.2.2.1 Linearity

Linear correlation was obtained between peak areas and concentrations of LPV and RTV in range of 300 - 16000 ng/mL. The linearity of the calibration graphs were validated by the high value of correlation coefficients of the

regression (Table 8.3.1).The linearity of the calibration graphs were validated by the high value of correlation coefficients of the regression (figure 8.3.4 and 8.3.5).

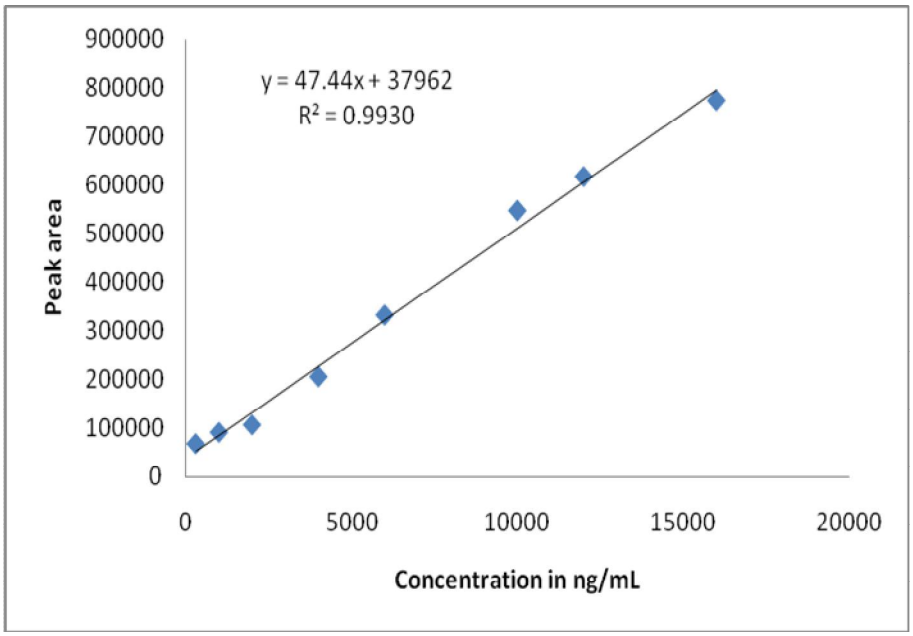


Figure 8.3.4: Calibration curve of RTV

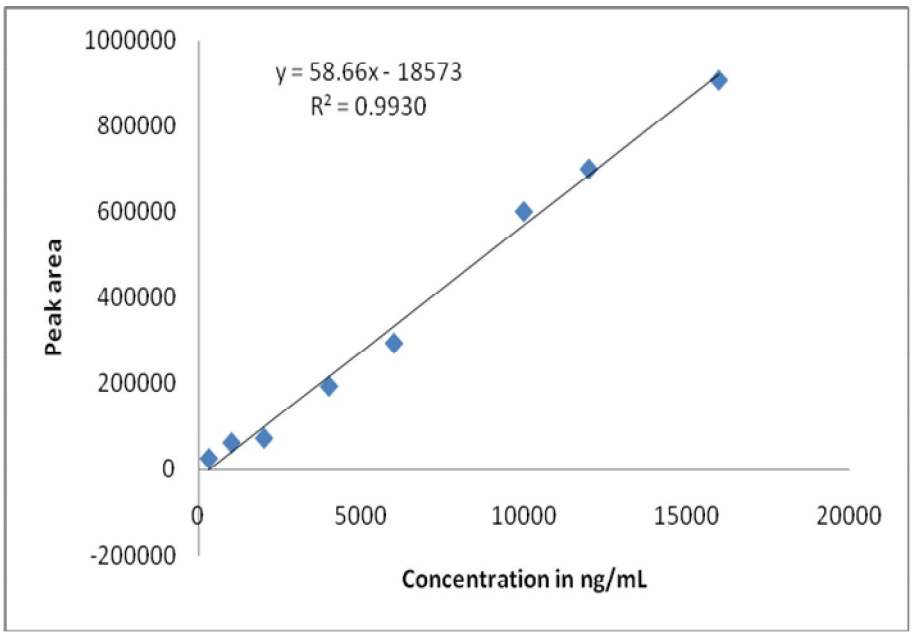


Figure 8.3.5: Calibration curve of LPV

Table 8.3.1: Optical and regression characteristics

Parameter	RTV	LPV
Linearity range	300 - 16000 ng/mL	300 - 16000 ng/mL
Regression equation (y)	y = 47.44x + 37962	y = 58.66x - 18573
Correlation coefficient (r)	0.9930	0.9930

8.3.2.2.2 Accuracy

The recovery experiments were carried out as in the text. The percent recoveries obtained were 86.38 to 93.24% for RTV and 88.56 to 92.34% for LPV. The results of recovery study are given in Table 8.3.2.

Table 8.3.2: Data of recovery study (n = 5)

Compound	Amount taken (ng/mL)	Amount added (ng/mL)	Amount recovered (ng/mL)	Accuracy ± SD%
RTV	4000	2000	5268.00	87.80 ± 6.30
	4000	4000	7172.00	89.65 ± 10.89
	4000	6000	9324.00	93.24 ± 7.71
	4000	8000	10365.60	86. 38 ± 10.53
LPV	4000	2000	5540.40	92.34 ± 8.28
	4000	4000	7084.80	88.56± 12.48
	4000	6000	9164.00	91.64± 7.23
	4000	8000	10491.60	87.43 ± 5.76

8.3.2.2.3 Recovery

The recovery experiments were carried out as in the text. The absolute and relative mean recovery found to be 86.44% and 85.81% for RTV as well as 86.68% and 85.77% for LPV respectively (Table 8.3.3 and 8.3.4).

Table 8.3.3: Absolute and Relative Recoveries of RTV from human plasma.

Nominal concentration (ng/mL)	Absolute recovery (mean ± SD%) (n = 5)	Relative recovery (mean ± SD%) (n = 5)
300	85.12 ± 8.83	84.28 ± 10.7
2000	87.35 ± 5.43	86.82 ± 7.24
6000	84.56 ± 9.57	88.42 ± 4.98
12000	88.74 ± 11.64	83.74 ± 2.74

Table 8.3.4: Absolute and Relative Recoveries of LPV from human plasma.

Nominal concentration (ng/mL)	Absolute recovery (mean ± SD%) (n = 5)	Relative recovery (mean ± SD%) (n = 5)
300	89.67 ± 11.4	85.32 ± 6.29
2000	85.78 ± 9.54	83.37 ± 8.21
6000	87.94 ± 7.58	89.13 ± 11.42
12000	83.34 ± 5.51	85.28 ± 4.46

8.3.2.2.4 Precision

8.3.2.2.4.1 Method precision (repeatability)

Relative standard deviation of all the parameters was less than 15% (Table 8.3.5 and 8.3.6), which indicated that the proposed method was repeatable.

Table 8.3.5: Method Precision data for RTV

RTV (4000 ng/mL)	Retention time (min.)	Peak area	Tailing factor
1	10.30	205254.4	1.20
2	9.96	179423.7	1.22
3	10.38	224376.3	1.20
4	10.35	202532.1	1.19
5	10.37	187654.3	1.20
6	10.38	218765.2	1.21
7	10.32	233542.9	1.20
Mean	10.29	207364.1	1.20
SD	0.15	19586.01	0.0095
% RSD	1.46	9.44	0.79

Table 8.3.6: Method Precision data for LPV

LPV (4000 ng/mL)	Retention time (min.)	Peak area	Tailing factor
1	12.58	193992.6	1.18
2	12.22	168575.2	1.17
3	12.34	224563.3	1.19
4	12.52	209874.8	1.18
5	12.55	198734.3	1.18
6	12.55	236458.7	1.19
7	12.92	207545.6	1.20
Mean	12.525	205677.8	1.184
SD	0.22	21933.93	0.0097
% RSD	1.74	10.66	0.82

8.3.2.2.4.2 Intraday and inter day precision:

The intra-day precision (% RSD) ranged from 6.73 to 9.86% and 6.57 to 10.76% for RTV and LPV respectively while the inter-day precision (% RSD) ranged from 9.40 to 12.26% and 9.27 to 12.07% for RTV and LPV respectively (Table 8.3.7 and 8.3.8). The intra- and inter-day precision data showed that acceptable precision was obtained over the entire assay range.

Table 8.3.7: Intra and Inter- day precision of RTV in human plasma.

Nominal concentration. (ng/mL)	**Observed concentration. (ng/mL)**	**(% RSD)**
Intra-day (n = 7)		
300	304.71± 30.04	9.86
2000	1983.37 ± 133.48	6.73
6000	6022.45 ± 445.66	7.40
12000	11968.26± 1072.35	8.96
Inter-day (n = 7)		
300	289.23± 32.33	11.18
2000	2004.34± 188.40	9.40
6000	5813.74 ± 572.07	9.84
12000	12014.38 ± 1472.96	12.26

Table 8.3.8: Intra- and Inter- day precision of LPV in human plasma.

Nominal concentration. (ng/mL)	**Observed concentration. (ng/mL)**	**(% RSD)**
Intra-day (n = 7)		
300	294.22± 21.27	7.23
2000	2003.56 ± 215.58	10.76
6000	5888.71 ± 495.82	8.42
12000	11976.34± 786.84	6.57
Inter-day (n = 7)		
300	287.36± 29.79	10.37
2000	1989.58± 240.14	12.07
6000	6011.26 ± 626.97	10.43
12000	11983.48 ± 1110.86	9.27

8.3.2.2.5 Limit of detection and limit of quantification

The limit of detection (LOD) of RTV and LPV were found to be 67.86 ng/mL and 86.80 ng/mL, whereas, quantitative limit (LOQ) were 193.75 ng/mL and 258.47 ng/mL respectively.

8.3.2.2.6 Stability study

The stability results showed that LPV and RTV were stable for at least 4 weeks when kept frozen at -70 ^{0}C. Thus, analysis up to 4 weeks storage confirmed adequate LPV and RTV stability at this temperature. Moreover, the results of freeze/thaw stability indicate that LPV and RTV were stable in plasma for at least five freeze/thaw cycles (Table 8.3.9 and 8.3.10).

Table 8.3.9: Summary of stability testing of RTV in human plasma.

Stability	Nominal concentration (ng/mL)	Observed concentration. (ng/mL)	Precision (% RSD)
Freeze/thaw (n = 5)	300	296.34	8.57
	2000	1981.66	6.90
	6000	5934.32	9.82
	12000	11575.49	3.45
Long term (n = 5)	300	276.12	7.53
	2000	1964.56	5.35
	6000	5781.42	10.63
	12000	11384.58	8.46

Table 8.3.10: Summary of stability testing of LPV in human plasma.

Stability	Nominal concentration (ng/mL)	Observed concentration. (ng/mL)	Precision (% RSD)
Freeze/thaw (n = 5)	300	282.23	6.72
	2000	1957.38	9.45
	6000	5768.58	7.43
	12000	11354.39	10.36
Long term (n = 5)	300	271.34	8.30
	2000	1872.48	10.52
	6000	5739.45	6.37
	12000	11458.76	5.23

8.3.3 CONCLUSION

The developed method was found to be simple, accurate, precise and repeatable. It can be applied for the determination of concentration of lopinavir and ritonavir in the human plasma for bioavailability and bioequivalence studies.

CHAPTER 9

STATISTICAL COMPARISION OF THE DEVELOPED METHODS

9. STATISTICAL COMPARISION OF THE DEVELOPED METHODS

9.1. DETERMINATION OF TENOFOVIR DISOPROXIL FUMARATE IN TABLET DOSAGE FORM

Table 9.1: Comparision of HPLC, HPTLC and UV method for determination of TNV

Parameters	HPLC	HPTLC	UV
TNV ± SD, % (n=3)	98.82 ± 1.61	98.90 ± 1.47	98.25 ± 0.42
Tabulated *F*- Value	5.591		
Calculated *F*- Value	0.334		

The assay results for TNV in tablet dosage form, obtained using HPLC, HPTLC and UV spectrophotometric methods were compared statistically by applying the *F*-test. The calculated *F*- value (0.334) for TNV is less than the tabulated *F*- value (5.591) at the 95% confidence interval. Therefore no significant difference was found in the content of TNV determined by the proposed HPLC, HPTLC and UV spectrophotometric methods.

$$F_{calculated} < F_{tabulated}$$

9.2. DETERMINATION OF EFAVIRENZ IN TABLET DOSAGE FORM

Table 9.2: Comparision of HPLC, HPTLC and UV method for determination of EFV

Parameters	HPLC	HPTLC	UV
EFV ± SD, % (n=3)	99.65 ± 1.72	99.11 ± 1.70	100.82 ± 1.31
Tabulated *F*- Value	5.591		
Calculated *F*- Value	0.814		

The assay results for EFV in tablet dosage form, obtained using HPLC, HPTLC and UV spectrophotometric methods were compared statistically by applying the *F*-test. The calculated *F*- value (0.814) for EFV is less than the tabulated *F*- value (5.591) at the 95% confidence interval. Therefore no significant difference was found in the content of EFV determined by the proposed HPLC, HPTLC and UV spectrophotometric methods.

$$F_{calculated} < F_{tabulated}$$

9.3. SIMULTANEOUS DETERMINATION OF LOPINAVIR AND RITONAVIR IN TABLET DOSAGE FORM

Table 9.3: Comparision of HPLC and HPTLC method for determination of LPV and RTV

Parameters	**LPV**		**RTV**	
	HPLC	**HPTLC**	**HPLC**	**HPTLC**
Drug ± SD, % (n=3)	98.99 ± 1.21	99.10 ± 1.87	99.67 ± 1.25	98.72 ± 0.77
Tabulated *t*- Value	4.30		4.30	
Calculated *t*- Value	0.088		0.976	

The assay results for LPV and RTV in tablet dosage form, obtained using HPLC and HPTLC methods were compared statistically by applying the two tail paired *t*-test. The calculated *t*- value for LPV (0.088) and RTV (0.976) is less than the tabulated *t*- value (4.30) at the 95% confidence interval. Therefore no significant difference was found in the content of LPV and RTV determined by the proposed HPLC and HPTLC methods.

$$t_{calculated} < t_{tabulated}$$

CHAPTER 10

SUMMARY

10. SUMMARY

- Anti retroviral agents and their tablet dosage forms which are available in the market, i.e., Efavirenz tablet, Tenofovir disoproxil fumarate tablet, Lopinavir and Ritonavir combined tablets as well as Ritonavir tablet were selected for the study.
- Various methods including spectrophotometric, RP-HPLC, HPTLC etc. were reviewed for the estimation of Efavirenz, Tenofovir disoproxil fumarate, Lopinavir and Ritonavir in alone as well as combined dosage form.
- Stability indicating RP-HPLC method was developed for the estimation of Efavirenz. Forced degradation study was carried out under acidic, alkaline, neutral, oxidative, thermal and photolytic conditions. The degraded products were well resolved from Efavirenz, indicating the method can also be useful for the estimation of Efavirenz in presence of degraded product. The developed RP- HPLC method was applied and optimized for the estimation of Efavirenz in human plasma.
- Stability indicating RP-HPLC method was developed for the estimation of Tenofovir disoproxil fumarate. Forced degradation study was carried out under acidic, alkaline, neutral, oxidative, thermal and photolytic conditions. The degraded products were well resolved from Tenofovir disoproxil fumarate, indicating the method can also be useful for the estimation of Tenofovir disoproxil fumarate in presence of degraded product. The developed RP- HPLC method was applied and optimized for the estimation of Tenofovir disoproxil fumarate in human plasma.
- RP-HPLC method was developed for the estimation of Lopinavir and Ritonavir in their combined tablet dosage form. The developed RP-HPLC method was applied and optimized for the estimation of Lopinavir and Ritonavir in human plasma.
- HPTLC method was developed for the estimation of Tenofovir disoproxil fumarate and Efavirenz in tablet dosage form.
- HPTLC method was developed for the estimation of Lopinavir and Ritonavir in their combined tablet dosage form.

- First derivative UV spectrophotometric method was developed for the estimation of Tenofovir disoproxil fumarate in tablet dosage form.
- Difference spectrometric method was developed for the estimation of Efavirenz and Ritonavir in tablet dosage form.
- The developed HPLC, HPTLC and UV spectrophotometric methods were validated as per ICH guideline for accuracy, linearity, precision, robustness, limit of detection and limit of quantification.
- The optimized bioanalytical RP-HPLC methods (for the analysis of antiretroviral agents in human plasma) were validated as per USFDA guideline for accuracy, linearity, precision and stability.
- The developed HPLC, HPTLC and UV spectrophotometric methods were compared statistically by applying the *F*-test and *t*-test. The calculated *F*-value and *t*-value were found to be less than the tabulated *F*-value and *t*-value indicated no significant difference in the content of antiretroviral agents determined by the proposed methods.

CHAPTER 11

PUBLICATIONS AND PRESENTATIONS

11. PUBLICATIONS AND PRESENTATIONS

RESEARCH PAPERS PUBLISHED

1. Mardia R. B., Suhagia B. N., Pasha T. Y., Chauhan S. P. and Solanki S. D., Development and validation of HPTLC method for simultaneous analysis of lopinavir and ritonavir in their combined tablet dosage form, *International journal for pharmaceutical research scholars* **2012**, 1(1), 39 - 44.

2. Mardia R. B., Suhagia B. N., Pasha T. Y., Chauhan S. P. and Solanki S. D., Development and validation of HPTLC method for estimation of tenofovir disoproxil fumarate in tablet dosage form, *Journal of pharmaceutical science and bioscientific research* **2012,** 2(2), 73-76.

RESEARCH PAPERS PRESENTED

Following research papers presented in the National level conference **"Advances in Chromatography"** held at Ganpat University, Kherva, Mehsana, Gujarat, India on 9th and 10th April 2012.

1. Development and validation of RP-HPLC method for simultaneous analysis of lopinavir and ritonavir in their combined tablet dosage form.
2. Development and validation of RP-HPLC method for estimation of tenofovir disoproxil fumarate in tablet dosage form.
3. Development and validation of normal phase HPTLC method for simultaneous analysis of lopinavir and ritonavir in their combined tablet dosage form.
4. Development and validation of normal phase HPTLC method for estimation of tenofovir disoproxil fumarate in tablet dosage form.