"DEVELOPMENT AND VALIDATION OF SPCTROPHOTOMETRIC, HPTLC AND RP-HPLC METHODS FOR SIMULTANEOUS ESTIMATION OF ATORVASTATIN CALCIUM AND TELMISARTAN IN THEIR COMBINED DOSAGE FORMS"

A THESIS SUBMITTED TO

NIRMA UNIVERSITY

In partial fulfillment of the requirements for the degree of

Master of Pharmacy

in

Pharmaceutical Analysis

BY

BALVANT D. PATEL (08MPH302.), B. Pharm.

GUIDE

Dr. Hardik G. Bhatt, M. Pharm., Ph. D.

DEPARTMENT OF PHARMACEUTICAL ANALYSIS

INSTITUTE OF PHARMACY

NIRMA UNIVERSITY

SARKHEJ-GANDHINAGAR HIGHWAY

AHMEDABAD-382481

GUJARAT, INDIA

APRIL 2010

CERTIFICATE

This is to certify that Mr. BALVANT D. PATEL has prepared his thesis entitled "DEVELOPMENT AND VALIDATION OF SPCTROPHOTOMETRIC, HPTLC AND RP-HPLC METHODS FOR SIMULTANEOUS ESTIMATION OF ATORVASTATIN CALCIUM AND TELMISARTAN IN THEIR COMBINED DOSAGE FORMS", in partial fulfillment for the award of M. Pharm. degree of the Nirma University, under my guidance. He has carried out the work at the Department of Pharmaceutical Analysis, Nirma University.

Guide:

Dr. Hardik G. Bhatt
M. Pharm., Ph.D.,
Assistant Professor,
Department of Pharmaceutical Analysis,
Institute of Pharmacy,
Nirma University.

Forwarded Through:

Dr. Priti J. Mehta
Head,
Dept. of Pharmaceutical Analysis,
Institute of Pharmacy,
Nirma University,
Ahmedabad.

Dr. Manjunath Ghate
I/c Director,
Institute of Pharmacy,
Nirma University,
Ahmedabad.

Date: 21/4/2010

DECLARATION

I declare that the thesis entitled "DEVELOPMENT AND VALIDATION OF SPCTROPHOTOMETRIC, HPTLC AND RP-HPLC METHODS FOR SIMULTANEOUS ESTIMATION OF ATORVASTATIN CALCIUM AND TELMISARTAN IN THEIR COMBINED DOSAGE FORMS" has been prepared by me under the guidance of Dr. Hardik G. Bhatt, Assistant professor, Department of Pharmaceutical Analysis, Nirma University. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

BALVANT D. PATEL (08MPH302)

Department of Pharmaceutical Analysis,
Institute of Pharmacy,
Nirma University,
Sarkhej - Gandhinagar Highway,
Ahmedabad-382481.
Gujarat, India.

Date: 20/04/2010

ACKNOWLEDGEMENT

First I thank Almighty God, for it is He who began this work in me and carried it to completion. It is He who has blest me with the people whose names I feel privileged to mention here.

It gives me immense pleasure today when I take an opportunity to acknowledge all those personalities who contributed directly or indirectly to my project. This research would not have been possible without the whole hearted encouragement, guidance, support, and cooperation of my beloved family, teachers, friends, well wishers and relatives. Probably I would have never achieved this without their support and blessings. With profound appreciation, I acknowledge to one and all.

I am indebted infinitely to love, care and trust being showered on me by my ***Family*** *without their consistent prayers, affectionate blessings, selfless care and endless confidence in me, I would have never come to the stage of writing this acknowledge.*

I wish to express my sincere thanks, with a deep sense of gratitude, to my respected guide ***Dr. Hardik G. Bhatt,*** *Assistant Professor, Dept. of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University for initiating and suggesting the theme of work, for his valuable guidance, supervision, creative suggestions and meticulous attention, sustained interest, immense guidance, dedicated support he has bestowed upon me for the timely completion of this work. I am extremely indebted to him for his motivational inspiration, kind expertise during the writing*

up of my thesis and the scientific attitude he has nurtured in me which will definitely stand in all my future endeavours.

I am extremely grateful to **Dr. Priti Mehta** *and* **Nrupesh sir**, *Dept., of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University for their continous encouragement and everlasting support throughout the course of this dissertation work.*

I am grateful to **Dr. Manjunath Ghate,** *I/C Director, Head of Dept. of Pharmaceutics, Institute of Pharmacy, Nirma University for providing all necessary help and facility for my work and also for his constant support and encouragement.*

I am also thankful to **Mr. Deepak Khatri and Mr. Omkar** *for constantly helping out in my work and solving my queries.*

I acknowledge my colleagues **Modh, Yogesh, Shaggy, Oza, Beravala, Pandya, Deesha, Janki and Modi** *for their amicable support and help.*

I am also thankful to my juniors **Mikul, Pankti, Shalav** *and all of the others for helping me as and when required.*

I owe special thanks to **Nitinbhai** *for helping me in maximum utilization of computer lab. I also wish to acknowledge* **Satejbhai, Shreyasbhai, Rajubhai and shaileshbhai, Bipinbhai, Vikrambhai, Surindarbhai** *for providing me all the materials required in my work.*

Date: 20104/2010

Balvant D. Patel

ABBREVIATIONS

CHEMICALS

ATR	Atorvastatin calcium
TEL	Telmisartan
HCl	Hydrochloric acid
TEA	Triethyl Amine
KH_2PO_4	Potassium Di-hydrogen Phosphate
NH_3	Ammonia
ACN	Acetronitrile

SYMBOLS

°C	Degree centigrade
r^2	Correlation coefficient
L	Liter
M	Molar
N	Normal
µL	Micro liter
mL	Milliliter
µm	Micro meter
mg	Milligram
µg	Microgram
cm	Centimeter
<	Less than
>	Greater than
%	Percentage
Min	Minute
i.d.	Internal diameter
v/v	Volume by volume
w/w	Weight by weight
Kpa	Kilo Pascal
mm	millimeter

RSD	Relative standard deviation

OTHERS

IP	Indian pharmacopoeia
UV	Ultra violet
HPTLC	High Performance Thin Layer chromatography
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
RP-HPLC	Reverse phase high Performance Liquid Chromatography
MS	Mass Spectroscopy
LOD	Limit of Detection
LOQ	Limit of Quantification
ICH	International Conference on Harmonization
IUPAC	International Union of Pure and Applied Chemistry
RSD	Relative Standard Deviation
EP	European Pharmacopoeia
IP	Indian Pharmacopoeia
BP	British Pharmacopoeia
USP	United States Pharmacopoeia
UV	Ultraviolet
IR	Infrared
ARB	Angiotensin receptor blocker
CVD	Cardio vascular disease

CHAPTER 1

INTRODUCTION

CONTENTS

1.1 INTRODUCTION TO HYPERTENSION

Hypertension is a chronic medical condition in which the blood pressure is elevated. It is also referred to as **high blood pressure** or shortened to **HT**, **HTN** or **HPN**. The word "hypertension", by itself, normally refers to systemic, arterial hypertension. [1]

Hypertension can be classified as either **essential** (primary) or **secondary**. Essential or primary hypertension means that no medical cause can be found to explain the raised blood pressure and represents about 90-95% of hypertension cases. [2-5] Secondary hypertension indicates that the high blood pressure is a result of (*i.e.*, secondary to) another condition, such as kidney disease or tumours (adrenal adenoma or pheochromocytoma).

Persistent hypertension is one of the risk factors for strokes, heart attacks, heart failure and arterial aneurysm, and is a leading cause of chronic renal failure.[6] Even moderate elevation of arterial blood pressure leads to shortened life expectancy. At severely high pressures, defined as mean arterial pressures 50% or more above average, a person can expect to live no more than a few years unless appropriately treated. [7] Beginning at a systolic pressure (which is peak pressure in the arteries, which occurs near the end of the cardiac cycle when the ventricles are contracting) of 115 mmHg and diastolic pressure (which is minimum pressure in the arteries, which occurs near the beginning of the cardiac cycle when the ventricles are filled with blood) of 75 mmHg (commonly written as 115/75 mmHg), cardiovascular disease (CVD) risk doubles for each increment of 20/10 mmHg. [8]

- **Classification**

Table 1.Classification of hypertension

Classification	**Systolic pressure**		**Diastolic pressure**	
	mmHg	**kPa**	**mmHg**	**kPa**
Normal	90–119	12–15.9	60–79	8.0–10.5
Prehypertension	120–139	16.0–18.5	80–89	10.7–11.9
Stage 1	140–159	18.7–21.2	90–99	12.0–13.2
Stage 2	≥160	≥21.3	≥100	≥13.3
Isolated systolic hypertension	≥140	≥18.7	<90	<12.0

Signs and symptoms

Accelerated hypertension is associated with

- Headache
- Somnolence
- Confusion
- Visual disturbances,
- Vomiting

Chronic hypertension is associated with

- Centripetal obesity
- Abdominal striate
- Diabetes
- Glucocorticoid excess
- Cushing's syndrome

- Hyperthyroidism,
- Hypothyroidism

1.1.1 PREVENTION AND TREATMENT[8]

The process of managing hypertension according the guidelines of the British Hypertension Society suggest that non-pharmacological options should be explored in all patients who are hypertensive or pre-hypertensive. These measures include;

- Weight reduction and regular aerobic exercise [9] (e.g., walking)
- Reducing dietary sugar intake.
- Reducing sodium (salt) in the diet may be effective: It decreases blood pressure in about 33% of people (see above). Many people use a salt substitute to reduce their salt intake. [10]

- Increase in dietary potassium, which offsets the effect of sodium has been shown to be highly effective in reducing blood pressure.[11]
- Discontinuing tobacco use and alcohol consumption has been shown to lower blood pressure. The exact mechanisms are not fully understood, but blood pressure (especially systolic) always transiently increases following alcohol or nicotine consumption. Besides, abstention from cigarette smoking is important for people with hypertension because it reduces the risk of many dangerous outcomes of hypertension, such as stroke and heart attack. Note that coffee drinking (caffeine ingestion) also increases blood pressure transiently but does *not* produce chronic hypertension.
- Reducing stress, for example with relaxation therapy, such as meditation and other mindbody relaxation techniques. [12]

Medication

Commonly used drugs include the typical groups of: [13]

- ACE inhibitors such as captopril, enalapril, fosinopril (Monopril), lisinopri (Zestril), quinapril, ramipril.
- Angiotensin II receptor antagonists may be used where ACE inhibitors are not tolerated: eg, telmisartan, irbesartan, losartan, valsartan, candesartan, olmesartan.
- Calcium channel blockers such as nifedipine (Adalat)[14] amlodipine (Norvasc), diltiazem, verapamil.
- Diuretics: eg, bendroflumethiazide, chlorthalidone, hydrochlorothiazide
- Additional diuretics such a furosemideor low-dosages of spironolactone
- Alpha blockers such as prazosin, or terazosin. Doxazosin has been shown to increase risk of heart failure, and to be less effective than a simple diuretic.[15]
- Beta blockers such as atenolol, labetalol, metoprolol, propranolol.[16]
- Direct renin inhibitors such as aliskiren[17]

1.2 INTRODUCTION TO ATHEROSCLEROSIS

Atherosclerosis (also known as **arteriosclerotic vascular disease** or **ASVD**) is the condition in which an artery wall thickens as the result of a build-up of fatty materials such as cholesterol. It is a syndrome affecting arterial blood vessels, a chronic inflammatory response in the walls of arteries, in large part due to the accumulation of macrophage white blood cells and promoted by Low-density lipoproteins (plasma proteins that carry cholesterol and triglycerides) without adequate removal of fats and cholesterol from the macrophages by functional high density lipoproteins (HDL). It is commonly referred to as a hardening or furring of the arteries. It is caused by the formation of multiple plaques within the arteries. [18]

Atherosclerosis, though typically asymptomatic for decades, eventually produces two main problems: First, the atheromatous plaques, though long compensated for by artery enlargement, eventually lead to plaque ruptures and clots inside the artery lumen over the ruptures. The clots heal and usually shrink but leave behind stenosis (narrowing) of the artery

(both locally and in smaller downstream branches), or worse, complete closure, and, therefore, an insufficient blood supply to the tissues and organ it feeds. Second, if the compensating artery enlargement process is excessive, then a net aneurysm results.

These complications of advanced atherosclerosis are chronic, slowly progressive and cumulative. Most commonly, soft plaque suddenly ruptures, causing the formation of a thrombus that will rapidly slow or stop blood flow, leading to death of the tissues fed by the artery in approximately 5 minutes. This catastrophic event is called an infraction. One of the most common recognized scenarios is called coronary thrombosis of a coronary artery, causing myocardial infarction (a heart attack). Even worse is the same process in an artery to the brain, commonly called stroke. Another common scenario in very advanced disease is claudication from insufficient blood supply to the legs, typically due to a combination of both stenosis and aneurysmal segments narrowed with clots. Since atherosclerosis is a body-wide process, similar events occur also in the arteries to the brain, intestines, kidneys, legs, etc.

Causes

Atherosclerosis develops from low-density lipoprotein molecules (LDL) becoming oxidized by free radicals, particularly reactive oxygen species (ROS). When oxidized LDL comes in contact with an artery wall, a series of reactions occur to repair the damage to the artery wall caused by oxidized LDL. The LDL molecule is globular shaped with a hollow core to carry cholesterol throughout the body. Cholesterol can move in the bloodstream only by being transported by lipoproteins.

The body's immune system responds to the damage to the artery wall caused by oxidized LDL by sending specialized white blood cells (macrophages and T-lymphocytes) to absorb the oxidized-LDL forming specialized foam cells. Unfortunately, these white blood cells are not able to process the oxidized-LDL, and ultimately grow then rupture, depositing a greater amount of oxidized cholesterol into the artery wall. This triggers more white blood cells, continuing the cycle.

Eventually, the artery becomes inflamed. The cholesterol plaque causes the muscle cells to enlarge and form a hard cover over the affected area. This hard cover is what causes a narrowing of the artery, reduces the blood flow and increases blood pressure.

Some researchers believe that atherosclerosis may be caused by an infection of the vascular smooth muscle cells. Chickens, for example, develop atherosclerosis when infected with the Marek's disease herpesvirus.[19] Herpesvirus infection of arterial smooth muscle cells has been shown to cause cholesteryl ester (CE) accumulation.[20] Cholesteryl ester accumulation is associated with atherosclerosis.

Sign and Symptoms[21-22]

Atherosclerosis typically begins in early adolescence, and is usually found in most major arteries, yet is asymptomatic and not detected by most diagnostic methods during life. Atheroma in arm, or more often in leg arteries, which produces decreased blood flow is called Peripheral Artery Occlusive Disease (PAOD).

Cardiac stress testing, traditionally the most commonly performed non-invasive testing method for blood flow limitations, in general, detects only lumen narrowing of ~75% or greater, although some physicians claim that nuclear stress methods can detect as little as 50%.

Modificable risk factors for artheromatous disease

- Raised low density lipoprotein
- Reduced high density lipoprotein[23]
- Hypertension
- Diabetes mellitus[23]
- Cigarette smoking
- Obesity
- Physical inactivity
- Raised C-reactive protein[23-24]

- Raised coagulation factors
- Raised homocysteine
- Raised lipoprotein(a)

Prevention and Treatment

If atherosclerosis leads to symptoms, some symptoms such as angina pectoris can be treated. Non-pharmaceutical means are usually the first method of treatment, such as cessation of smoking and practicing regular exercise. If these methods do not work, medicines are usually the next step in treating cardiovascular diseases, and, with improvements, have increasingly become the most effective method over the long term. However, medicines are criticized for their expense, patented control and occasional undesired effects.

Primary and secondary prevention [25]

Lipid lowering drugs

- Statins: HMG COA reductase inhibitors
- Fibrates
- Bile acid-binding resins.
- Nicotinic acid

STATINS[25]

- It is the HMG COA reductase inhibitors it inhibit denovo synthesis of cholesterol in the liver. This increases expression of LDL receptors on heaptocytes, increasing LDL uptake from plasma and makedly lowering circulating LDL and total cholesterol.

- Secondary prevention of myocardial infarction and stroke in patients who have symptomatic atherosclerotic disease (e.g. angina, transientischaemic attacks, following acute myocardial infarction or stroke)

- Atorvastatin lowers serum cholesterol in patients with homozygous familial hypercholesterolaemia
- In severe drug-resistant dyslipidemia
- e.g simvastatin, lovastatin and pravastatin

FIBRATES[25]

- It activates PPARα (Peroxisome Proliferator Adenosine receptors) decrease VLDL production, increase activity of lipoprotein lipase, increasing activity of lipoprotein lipase, increasing breckdown of chylomicra and VLDL in muscle adipose disease
- Mixed dyslipidemia
- Patient with low HDL and high risk of atheromatous diseases
- Combined with other lipId lowering drugs in patients with severe treatment-resistant dyslipidemia

BILE ACID BINDING RESINS[25]

- Sequester bile acids in the intenstine. This reduces the absorption of exogenous cholesterol and incrases the metabolism of endogenous cholesterol and increase the metabolism of endogenous cholesterol into bile acids.
- As an addition to a statin when response has been inadequate, e.g. in patients with heterozygous familial hypercholesterolaemia.

  For hypercholesterolaemia when a statin is contraindicated.
- e.g. colystyramine

NICOTINIC ACID[25]

It is a vitamin that has been used in gram quantities as a lipid-lowering agent. Acipimox is a derivative of nicotinic acid that is used in lower dose and may have less markd adverse effects. These drugs inhibit hepatic triglyceride production and VLDL secretion, which leads indirectly to a modest reduction in LDL and increase in HDL.

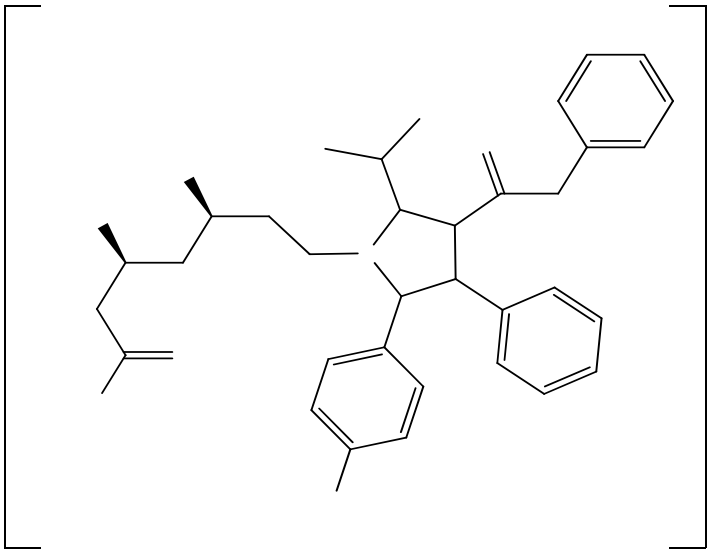
Adverse effects[25]

- Gastrointestinal disturbance,
- Increased plasma concentrations of liver enzymes
- Insomnia
- Rash
- Myositis
- Acute renal failure

1.3 INTRODUCTION TO DRUGS

1.3.1 Drug Profile for Atorvastatin calcium

1. Drug class:[26] Lipid lowering drug
2. Category:[26] HMG-CoA reductase inhibitor
3. CAS number: [26]134523-03-8
4. Chemical name:[26] (3*R*,5*R*)-7-[2-(4-fluorophenyl)- 3-phenyl-4-(phenylcarbamoyl)-5-(propan-2-yl)- 1*H*-pyrrol-1-yl]- 3,5- dihydroxyheptanoic acid calcium salt
5. Molecular formula:[26] $2(C_{33}H_{34}FN_2O_5)\cdot Ca$
6. Molecular weight:[26] 1155.36 g/mol
7. Structural formula: [26]



8. Dose**:**[26] The usually effective dose is 10, 20, 40 or 80 mg for oral administration once daily. Tablets are white, elliptical, and film coated.

9. Physiological properties:[26]

- Solubility: slightly soluble in water (insoluble in aqueous solutions of pH 4 or below); soluble in methanol

- Appearance: white to off-white crystalline powder
- Assay: 98.0% - 101.0%
- Stability: Stable under ordinary conditions.

10. Pharmacology[27]

As with other statins, Atorvastatin is a competitive inhibitor of HMG-CoA reductase. Unlike most others, however, it is a completely synthetic compound. HMG-CoA reductase catalyzes the reduction of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to mevalonate, which is the rate-limiting step in hepatic cholesterol biosynthesis. Inhibition of the enzyme decreases *de novo* cholesterol synthesis, increasing expression of low-density lipoprotein receptors (LDL receptors) on hepatocytes. This increases LDL uptake by the hepatocytes, decreasing the amount of LDL-cholesterol in the blood. Like other statins, atorvastatin also reduces blood levels of triglycerides and slightly increases levels of HDL-cholesterol.

11. Pharmacokinetics[27]

- **Absorption**: Atorvastatin has rapid oral absorption with an approximate time to maximum plasma concentration (Tmax) of 1–2 hours. The absolute bioavailability of atorvastatin is approximately 14%, however, the systemic availability for HMG-CoA reductase activity is approximately 30%. Atorvastatin undergoes high intestinal clearance and first-pass metabolism, which is the main cause for the low systemic availability. Food has been shown to reduce the rate and extent of Atorvastatin absorption. Administration of atorvastatin with food produces a 25% reduction in Cmax (rate of absorption) and a 9% reduction in AUC (extent of absorption). However, food does not affect the plasma LDL-C lowering efficacy of Atorvastatin. Evening Atorvastatin dose administration is known to reduce the Cmax (rate of absorption) and AUC (extent of absorption) by 30% each. However, time of administration does not affect the plasma LDL-C lowering efficacy of Atorvastatin.

- **Protein binding:** Atorvastatin is highly protein bound (≥98%).
- **Mechanism of action:** The primary proposed mechanism of atorvastatin metabolism is through cytochrome P450 3A4 hydroxylation to form active ortho- and parahydroxylated metabolites, as well as various beta-oxidation metabolites. The ortho- and parahydroxylated metabolites are responsible for 70% of systemic HMG-CoA reductase activity. The ortho-hydroxy metabolite undergoes further metabolism via glucuronidation. As a substrate for the CYP3A4 isozyme it has shown susceptibility to inhibitors and inducers of CYP 3A4 to produce increased or decreased plasma concentrations, respectively. This interaction was tested in vitro with concurrent administration of erythromycin, a known CYP 3A4 isozyme inhibitor, which resulted in increased plasma concentrations of atorvastatin. Atorvastatin is also an inhibitor of cytochrome 3A4.
- **Excretion:** It is primarily eliminated via hepatic biliary excretion with less than 2% of atorvastatin recovered in the urine. Bile elimination follows hepatic and/or extra-hepatic metabolism. There does not appear to be any entero-hepatic recirculation. Atorvastatin has an approximate elimination half-life of 14 hours. Noteworthy, the HMG-CoA reductase inhibitory activity appears to have a half-life of 20–30 hours, which is thought to be due to the active metabolites. Atorvastatin is also a substrate of the intestinal P-glycoprotein efflux transporter, which pumps the drug back into the intestinal lumen during drug absorption.

12. Drug and food interactions[27]

- Co-administration of Atorvastatin with one of CYP3A4 inhibitors like itraconazole, telithromycin, and voriconazole, may increase serum concentrations of atorvastatin, which may lead to adverse reactions. This is less likely to happen with other CYP3A4 inhibitors like diltiazem, erythromycin, fluconazole, ketoconazole, clarithromycin, cyclosporine, protease inhibitors, verapamil. And only rarely with other CYP3A4 inhibitors like amiodarone, and aprepitat.often bosentan, fosphenytoin, and phenytoin which are CYP3A4 inducers can decrease the plasma concentrations of atorvastatin. But only rarely barbiturates carbamazepine, efavirenz, nevirapine, oxcarbazepine,

rifampin, and rifamycin which are CYP3A4 inducers can decrease the plasma concentrations of atorvastatin. Oral contraceptives increased AUC values for norethindrone and ethinyl estradiol, these increases should be considered when selecting an oral contraceptive for a woman taking atorvastatin.

13. Adverse effects[28]

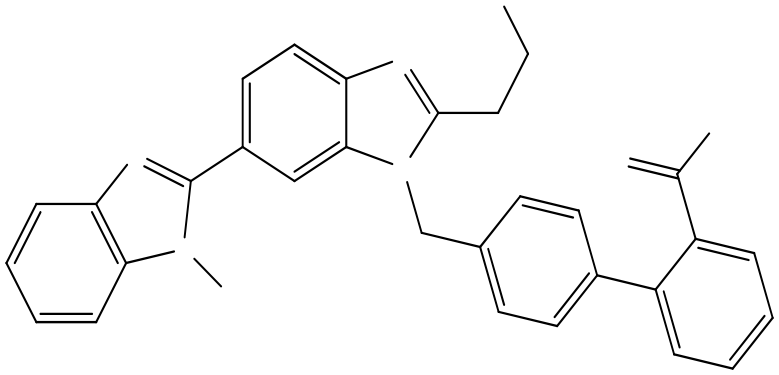
- Weakness
- Insomnia and dizziness
- Chest pain and peripheral edema
- Rash
- Abdominal pain, constipation, diarrhea, dyspepsia, flatulence, nausea.
- Urinary tract infection
- Arthralgia, myalgia, back pain, arthritis
- Sinusitis, pharyngitis, bronchitis, rhinitis
- Infection, flu-like syndrome, allergic reaction.

Table 2.Marketed formulations of Atorvastatin calcium

Dosage form	Brand Name	Name of manufacturer
Tablets	TG TOR 10MG TAB	Uni Chem. laboratories
	TG TOR 20MG TAB	Uni Chem. laboratories
	TG TOR 5MG TAB	Uni Chem. laboratories
	TG TOR PLUS TAB	Nicholas Piramal India ltd
	TGGOAL TAB	Nicholas Piramal India ltd

1.3.2 Drug Profile for Telmisartan:

1. Drug class: Antihypertensive
2. Category: Angiotensin II receptor antagonist
3. CAS number[28]: 144701-48-4
4. Chemical name[28]: 2-(4-{[4-methyl-6-(1-methyl-1*H*-1,3-benzodiazol-2-yl)-2-propyl-1*H*-1,3-benzodiazol-1-yl]methyl}phenyl)benzoic acid
5. Molecular formula[28]: $C_{33}H_{30}N_4O_2$
6. Molecular weight[28]: 514.617 g/mol
7. Structural formula:



8. Physicochemical properties:[29]
    a. Description and Solubility: white to off-white, odorless crystalline powder
    b. It is freely soluble in methanol; It is practically insoluble in water or an aqueous solution in the pH range of 3 to 9, and sparingly soluble in a strong acid, with the exception of hydrochloric acid in which it is insoluble. Telmisartan is soluble in a strong base.
    c. Melting point[30]: 261°C – 264°C

9. Dose: The usually effective dose is 40 mg once daily. Some patients may already benefit at a daily dose of 20 mg. In cases where the target blood pressure is not achieved, Telmisartan dose can be increased to a maximum of 80 mg once daily.

10. Storage condition[21]: Store at 25°C (77°F); excursions permitted to 15°-30°C (59°-86°F.

11. Mechanism of action: [31] Telmisartan is an Angiotensin Receptor Blocker (ARB) that shows high affinity for the angiotensin II type 1 (AT_1) receptors, has a long duration of action, and has the longest half-life of any ARB. In addition to blocking the Renin-Angiotensin System (RAS), telmisartan acts as a selective modulator of Peroxisome proliferator-activated receptor gamma (PPAR-γ), a central regulator of insulin and glucose metabolism. It is believed that telmisartan's dual mode of action may provide protective benefits against the vascular and renal damage caused by diabetes and cardiovascular disease (CVD). Telmisartan has binding affinity 3000 times with AT-2 receptor than AT-1 receptor.

12. Pharmacokinetics:[32] Following oral administration, peak concentrations (Cmax) of Telmisartan are reached in 0.5-1 hour after dosing. Food slightly reduces the bioavailability of Telmisartan, with a reduction in the area under the plasma concentration-time curve (AUC) of about 6% with the 40 mg tablet and about 20% after a 160 mg dose. The absolute bioavailability of telmisartan is dose dependent. At 40 and 160 mg the bioavailability was 42% and 58%, respectively. The pharmacokinetics of orally administered telmisartan is nonlinear over the dose range 20-160 mg, with greater than proportional increases of plasma concentrations (Cmax and AUC) with increasing doses.
    a. **Absorption:** Absolute bioavailability depends on dosage. Food slightly decreases the bioavailability (a decrease of about 6% is seen when the 40-mg dose is administered with food).

b. **Protein binding:** ≥99.5%

c. **Biotransformation:** Minimally metabolized by conjugation to form a pharmacologically inactive acylglucuronide; the glucuronide of the parent compound is the only metabolite that has been identified in human plasma and urine. The cytochrome P450 isoenzymes are not involved in the metabolism of telmisartan.

d. **Half life:** 24 hours

13. Side effects: most common telmisartan side effects include:

- Upper respiratory infection, such as the common cold or flu -- up to 7 percent of people
- Back pain -- up to 3 percent of people
- Diarrhea -- up to 3 percent of people
- Inflammation of the sinuses (sinusitis) -- up to 3 percent

Some of these possible rare side effects of telmisartan include, but are not limited to:

- Insomnia
- Impotence
- Constipation
- Dry mouth
- Gout
- Diabetes
- Depression
- Middle ear infection (otitis media)
- Asthma
- Vision problems
- Serious breakdown of muscle (known medically as rhabdomyolysis).

14. Interactions**:**

Drug –drug interaction

- ✓ Diuretics, such as torsemide, furosemide , hydrochlorothiazide, and others
- ✓ Potassium supplements
- ✓ Potassium-sparing diuretics, such as spironolactone , triamterene, and amiloride
- ✓ Digoxin
- ✓ Warfarin

Table 3.Marketed formulations of Telmisartan

Dosage form	Brand Name	Name of manufacturer
Tablets	TELI 20MG TAB	CADILA COSMETICS DIVISION
	TELAST 20MG TAB	INTAS PHARMACEUTICALS LTD
	TELISTA RM 2.5MG TAB	LUPIN LABORATORIES LTD
	TELCON 40MG TAB	CONCERTINA PHARMA PRIVATE LTD
	TELMA 20MG TAB	GLENMARK PHARMACEUTICALS LTD
	TELEACT 40MG TAB	RANBAXY LABORATORIES LTD (RAN)
	TELEACT 80MG TAB	RANBAXY LABORATORIES LTD
	TELDAY 20MG TAB	TORRENT PHARMACEUTICAL LTD

1.3 RATIONAL FOR COMBINATION

Evidence for Combination Therapy

There is a strong rationale for combining an angiotensin receptor blocker (ARB) with a statin from the preclinical studies. Statins may enhance the effect of an ARB to improve vascular remodeling in a mouse vascular injury model. They demonstrated that a combination of Telmisartan and Atorvastatin acted synergistically to attenuate neointimal formation at doses that were without effect when administered alone and were devoid of any effects on BP or cholesterol levels. It has also reported a synergistic antioxidative effect of Atorvastatin and Telmisartan against LDL oxidation in a small group (n=21) of hypercholesterolemic and hypertensive men.[33] Statins have been demonstrated to directly downregulate AT_1 receptor expression in isolated vascular smooth muscle cells.[33-34] This mechanism may explain some of the cholesterol-independent beneficial effects of statins. A statin and an ARB could produce synergistic effects against CVDs.

However, in which patients after myocardial infarction[35] were treated with either Telmisartan, revealed that additional treatment with a statin resulted in a marked reduction of death and subsequent myocardial infarction. One ongoing trial is testing the hypothesis that reducing cholesterol (with statins) and BP (with ACE inhibitors plus calcium channel blockers vs ß-blocker plus a diuretic) may have a CVD preventive effect, regardless of cholesterol diagnosis.

Who Would Benefit From Combined Therapy of ARBs and Statins?

Accumulating evidence suggests that preventive medications are a future trend in a growing population in whom longevity is increasing. Thus, one can envisage that combination therapy of an ARB and a statin would find utilization in people with cardiovascular risk factors and provocatively, also in people without symptomatic disease but who are >55 years old, as age also becomes a risk factor for CVDs. Patients presenting with 2 or more linked risk factors for CVD would benefit from the combination of cholesterol lowering and antihypertensive drug therapy (i.e. patients with hypertension, moderate cholesterol levels, the metabolic syndrome, type 2 diabetes, and a previous cardiovascular event such as stroke or myocardial infarction).

The Metabolic Syndrome[36]

Metabolic syndrome arises from a number of causes, including predetermined genetic factors such as insulin resistance, as well as acquired or lifestyle characteristics such as obesity, physical inactivity, and high-carbohydrate diets (>60% total calories). Dyslipidemia, which includes hypercholesterolemia and hypertriglyceridemia, is associated with hypertension and central obesity, all of which, together with insulin resistance, constitute the metabolic syndrome. The components of the metabolic syndrome are associated with insulin resistance, disturbances of coagulation and fibrinolysis, endothelial dysfunction, and elevated markers of subclinical inflammation. This syndrome is presumably a powerful determinant of diabetes and CVD. The end product of the metabolic syndrome is atherosclerosis leading to CVD, myocardial infarction, stroke, peripheral arterial disease, and endothelial dysfunction. The prevalence of the metabolic syndrome is increasing worldwide, with an estimated 20% to 25% of American adults being affected. The statins be first-line therapy for patients with high LDL levels, but this group also recognized the need for combination therapy to treat total lipid profiles. They also recommended aggressive treatment of any hypertension present.[37]

Type 2 Diabetes

Type 2 diabetes increases the risk for hypertension and associated CVDs. The worldwide prevalence of type 2 diabetes has exploded in recent years.[38] The frequent association of diabetes with dyslipidemia, hypertension, and endothelial and metabolic abnormalities also aggravates the underlying vascular disease process in patients who possess these comorbid conditions. Diabetes is a predictor of atherosclerosis, and this is the main cause of morbidity and mortality in this patients.[39] Insulin induces AT_1 receptor over expression. Clinical trials have shown that ARBs effectively reduce cardiovascular end points in hypertensive diabetics and preserve renal function in diabetics with nephropathy. The American Diabetes Association recommends the use of statins to treat dyslipidemia in patients with type 2 diabetes, as well as the use of antihypertensives to a target BP goal of 130/80 mm Hg. Therefore, a combination of an ARB and a statin could have beneficial effects in type 2 diabetes.

Heart Failure[40]

Recent studies have demonstrated a beneficial effect of ARBs in heart failure. There is also evidence that statins are beneficial in heart failure and further ongoing trials are examining the effects of statins[41] in heart failure. It has demonstrated direct effects of statins on the myocardium. Therefore, patients at risk from heart failure would probably be ideal candidates for combination therapy with an ARB and a statin.

Table 4.Marketed formulations for combination of Atorvastatin calcium (10 mg) and Telmisartan (40mg)

Formulation	Brand Name	Name of Manufacturer
Tablet	Telsartan™-ATR	DR.REDDY'S
Tablet	Arbitel-AV	MICRO LABS LIMITED
Tablet	TELDAY AV	TORRENT PHARMACEUTICALS LTD.
Tablet	TELEACT ST 40	RANBAXY LAB. LTD.

CHAPTER 2

REVIEW OF LITERATURE

2.1 REVIEW OF LITERATURE

Table 5.Reported Methods for Determination of Atorvastatin calcium

SR. NO.	TITLE	MATRIX	DESCRIPTION	REF. NO.
1	Development and validation of the reverse-phase high-performance liquid chromatographic method simultaneous determination of Atorvastatin calcium and Amlodipine besylate in tablet dosage forms.	Tablets	**Method: HPLC** **Column:** phenomenex Luna C-185 µm column having 250 × 4.6 mm i.d in isocratic mode, **Mobile phase:** methanol: acetonitrile: 50 mM KH_2PO_4 (20:50:30; pH 3.5) **pH:** 3.5 **wavelength for measurement** 240nm **Flow rate:** 1 ml/min **Beer's law limit:** 5-100 µg/ml RT for atorvastatin calcium 7.6 min RT for amlodipine besylate 3.2 min	42
2	RP-HPLC Method for the Determination of Atorvastatin calcium and Nicotinic acid in Combined Tablet Dosage Form	Tablets	**Method: HPLC** **Column:** phenomenex Luna C-185 µm column having 250 × 4.6 mm i.d **Mobile phase:** 0.02 M potassium dihydrogen phosphate: methanol: acetonitrile (20:40:40,) **pH:** 4 **wavelength for measurement**	43

			240nm **Flow rate:** 1 ml/min **Beer's law limit:** atorvastatin calcium-0.08-20 µg/ml a0.1-20 µg/ml for RT for atorvastatin calcium 3.6min RT for nicotinic acid - 2.4 min, respectively.	

SR. NO.	TITLE	MATRIX	DESCRIPTION	REF. NO.
3	Simultaneous RP-HPLC Estimation of Atorvastatin Calcium and Fenofibrate	Tablets	**Method**: HPLC **Stationary phase**: Luna C18 column **Mobile phase:** methanol:acetate buffer (82:18 v/v) **pH:** 3.7 **Flow rate:** 1.5 ml/min **Detection:** UV detector (248 nm) **Beer's law limit:** Atorvastatin calcium :1-5 µg/ml Fenofibrate : 16-80 µg/ml **RT time for** Atorvastatin calcium: 3.02+0.1	44

SR. NO.	TITLE	MATRIX	DESCRIPTION	REF. NO.
4	An HPLC method for the determination of Atorvastatin and its	Bulk drug samples and pharmaceutical	**Method:** HPLC **Stationary phase**:	45

	impurities in bulk drug and tablets	dosage forms	Luna C18 column **Mobile phase:** acetonitrile-ammonium acetate buffer pH 4-tetrahydrofuran (THF) **Detection:** UV detector (248 nm)	
6	RP-HPLC Estimation of Aspirin and Atorvastatin Calcium in Combined Dosage Forms	Dosage forms	**Method:** HPLC **Stationary phase**: Luna C18 column **Mobile phase:** methnol:water RT for aspirin: 1.98 RT for atorvastatin calcium: 7.99 min **Beer's law limit:** aspirin and Atorvastatin calcium were in the range of 4-20 µg/ml. **Detection:** 242 nm	46

SR. NO.	**TITLE**	**MATRIX**	**DESCRIPTION**	**REF. NO.**
7	Application of UV-Spectrophotometry and RP-HPLC for Simultaneous Determination of Atorvastatin Calcium and Ezetimibe in Pharmaceutical Dosage Form	Bulk drug samples	**Method: UV** UV-spectrophotometric determination of two drugs, using simultaneous equation method. It involves absorbance measurement at 232.5 nm (λmax of Ezetimibe) and 246.0 nm (λmax of Atorvastatin calcium) in methanol **Beer's limit**: 5 – 25 µg.mL^{-1} **Method**:HPLC **Stationary phase**:	47

			Luna C18 column **Beer's limit:** 8-22 µg.mL-1	
8	Stability-indicating high performance liquid chromatographic determination of atorvastatin calcium in pharmaceutical dosage form	Pharmaceutical dosage forms**.**	**Method:** HPLC **Stationary phase**: **reversed-phase C18 column (250 x 4.6 mm)** **Mobile phase:** Methanol: Acetonitrile: Phosphate Buffer solution in the ratio (45:45:10). **Flow rate : 1ml/min** **RT** for atorvastatin calcium: 6.98 min **Beer's law limit:** Atorvastatin calcium were in the range of 4-20 µg/ml. Detection : 246 nm	48
9	Simultaneous HPTLC analysis of Atorvastatin calcium, ramipril, and aspirin in a capsule dosage form	Pharmaceutical dosage forms**.**	**Method:** HPTLC **Mobile phase:** methanol-benzene-ethyl acetate-glacial acetic acid 0.36:5.6:4.0:0.04 (*v* / *v*) R_F values were approximately 0.38, 0.06, and 0.86 for atorvastatin calcium, ramipril, and aspirin, respectively **Limits of detection (LOD**) : 4.88 ng for atorvastatin calcium, 2.91 ng for ramipril, and 18.63 ng for aspirin	49

SR. NO.	TITLE	MATRIX	DESCRIPTION	REF. NO.
10	A simple and sensitive HPTLC method for the determination of content uniformity of Atorvastatin calcium tablets	Pharmaceutical dosage forms	**Method:** HPTLC **Stationary phase:** silica gel 60 F_{254} **Mobile phase:** benzene: methanol, (7:3 v/v). **Beer's law limit** (200-600 ng/spots Detection: 281 nm	50
11	Development and validation of a HPTLC method for the simultaneous estimation of Atorvastatin calcium and ezetimibe	Pharmaceutical dosage forms	**Method:** HPTLC **Mobile phase:** of chloroform: benzene: methanol: acetic acid (6.0:3.0:1.0:0.1 v/v/v/v) **Beer's law limit** 0.8 and 4.0 ng/spot **Detection**: UV detection (250 nm)	51
12	Validated Method Development for Estimation of Atorvastatin Calcium and Fenofibrate in Fixed Dose Combination by HPTLC	Pharmaceutical dosage forms	**Method:** HPTLC **Stationary phase:** silica gel 60 F_{254} **Mobile phase:** chloroform–methanol (8:2 v/v) Rf values 0.29 and 0.77 for atorvastatin calcium and fenofibrate, **Beer's law limit** 200-1000 ng/band **Detection**: UV detection (285 nm)	52

13	Estimation of Atorvastatin Calcium and Fenofibrate in Tablets by Derivative Spectrophotometry and Liquid Chromatography	Pharmaceutical dosage forms	**Method: U.V and** HPLC ATO and FEN in combined preparations (tablets) were quantitated using the second-derivative responses at 245.64 nm for ATO and 289.56 nm for FEN in spectra of their solution in methanol **Stationary phase:** **Hypersil ODS-C18 column** **Mobile phase:** **mobile phase methanol–water (90 :10, v/v),** **pH;5.5** RT values 1.95 min for atorvastatin calcium and 5.55min for fenofibrate, **Detection**: UV detection (285 nm)	53

Table 6.IP reported method for Atorvastatin calcium

1.	HPLC	Tablets	**Stationary phase**: stainless steel column 25 cm x 4.6 mm, packed with octadecylsilane bonded to porous silica (5 µm). mobile phase: A. a mixture of 92.5 volumes of acetonitrile and 7.5 volumes tetrahydrofuran, B. a mixture of 58 volumes of a buffer solution prepared by dissolving 5.75 g of ammonium dihydrogen orthophosphate in 1000 ml of water and 42 volumes of mobile phase A, C. a mixture of 20 volumes of the	54

			buffer solution, 20 volumes of mobile phase A and 60 volumes of methanol, spectrophotometer set at 246 nm, **flow rate**:1.8 ml per minute,	

7. Reported Methods for Determination of Telmisartan

1.	UV-Vis Spectrophotometric method for estimation of Telmisartan	Pharmaceutical dosage forms	Telmisartan exists in two different forms in acidic and basic mediums that differ in their UV spectra. Difference spectrum, obtained by keeping telmisartan in 0.01 N NaOH in reference cell and telmisartan in 0.01 N HNO_3 in sample cell, showed two characteristic peaks at 295 nm and 327 nm with positive and negative absorbance respectively. Difference of absorbance between these two maxima was calculated to find out the amplitude, which was plotted against concentration. **Linearity and range:**2-12 µg/ml	55
2.	Colorimetry method for estimation of Telmisartan in Urine & Plasma	Pharmaceutical formulations, Urine & Plasma	Formation of colored complex of Telmisartan and Congo red in pH 2.5 HCL-NaAc Buffer solution Detection : 593 nm	56
3.	Colorimetry method for estimation of Telmisartan in tablets	Tablets	Formation of colored chloroform extractable ion pair complexes with Bromocresol green (BCG) Detection: 440 nm	57
4.	Fluorimetric method for estimation of	Bulk drug, Pharmaceutical	Reaction of Telmisartan with 1 M NaOH solution, which shows	58

	Telmisartan	formulations & Plasma	strong native fluorescence λ_{ex} = 230 nm λ_{em} =365 nm	
5.	HPLC method for estimation of Telmisartan	Tablets	**Method** : HPLC **Stationary phase** :Hypersil C18 BDS (25×0.46 cm) **Mobile phase:** acetonitrile : Methanol (60:40 v/v) **Flow rate**: 1.2 ml/min **Detection**:245nm **Retention time:** 2.72 min **Linearity and range:** 1-10 µg/ml	59
6.	HPLC method for estimation of Telmisartan	Tablets	**Method** : HPLC **Stationary phase** :Hypersil C18 BDS (25×0.46 cm) **Mobile phase**: acetonitrile : 0.05M KH_2PO_4 pH 3.0 (60:40, v/v) **Flow rate**: 1.0 ml/min **Detection** :271nm **Retention time:** 5.19 min **Linearity and range:** 4.1-20.48µg/ml	60
7.	HPLC method for estimation of Telmisartan	Tablets	**Stationary phase** :Genesis C18(25×0.46 cm, I.D.,10 µm) **Mobile phase**: 0.01M KH_2PO_4 pH 3.4 : Methanol : acetonitrile (15:15:70 v/v/v) **Flow rate**: 1.0 ml/min **Detection**:210nm **Retention time:** 4.98 min	61

			Linearity and range: 28-52 µg/ml **LOQ:** 1.5 µg/ml **LOD:** 0.5 µg/ml	
8.	LC-MS method for estimation of Telmisartan	Human plasma	**Stationary phase** : Venusil XBP-C8 column Mobile phase: acetonitrile– 10 mM ammonium acetate– formic acid *m*/*z* 513.0–469.4 **Linearity and range:** 1–600 ng/ml	62
9.	HPLC- Fluorimetric method for estimation of Telmisartan	Urine	**Stationary phase:** Novapak C18 column (3.9×150 mm, 4 µm). **Mobile phase**: acetonitrile – phosphate buffer (pH 6.0, 5 mM) (45:55, v/v	63
11.	LC method for estimation of Telmisartan	Tablets	C-18 column Mobile phase: Buffer (pH 3.0): acetonitrile (55:45 v/v). Buffer contains 0.1M sodium perchlorate monohydrate in double distilled water. Flow rate: 2.8 ml/min **Linearity and range:** 20-400 µg/ml	64

12.	HPTLC method for estimation of Telmisartan	Tablets	Precoated silica gel 60F254 Mobile phase: chloroform : methanol: toluene) (2:5:5; v/v/v) **Detection** : 272nm **Retention factor:** 0.33 **Linearity and range:** 250–500 ng /spot **LOQ:** 190 ng/spot **LOD:** 75 ng/spot	65

CHAPTER 3

AIM OF PRESENT WORK

Quantitative analysis of any drug is an important tool in an industry, it is important to determine that the raw material, intermediate products as well as final products meet its specification and are of required quality. The numbers of drugs and drug formulation introduced into market have been increasing at an alarming rate. These drugs or formulation may be either new entities or partial structural modification of existing ones or novel dosage forms.

Multi component dosage forms are to be effective due to their combined mode of action in the body. The development of assay procedures for such dosage forms poses considerable challenges to analytical chemist owing to complexity of these dosage forms as they contain multiple drug entities and a variety of drug excipients. The estimation of the individual drugs in these multicomponent dosage forms becomes difficult due to cumbersome extraction or isolation procedures.

The literature review revealed that Chromatographic and Spectroscopic methods are reported for the determination of Atorvastatin calcium and Telmisartan in its individual dosage forms. So this combination was selected for development of spctrophotometric, HPTLC and RP-HPLC methods. There are no records of methods reported for simultaneous determination of Atorvastatin calcium and Telmisartan in their combined dosage forms. So, it was thought of interest to develop a spetrophotometric, HPTLC, RP-HPLC method for determination of both drugs in their combined dosage forms.

- ✓ Prime objective of work was to develop a specific, simple, rapid, sensitive and reproducible Spctrophotometric, HPTLC and RP-HPLC methods for quantitative estimation of Atorvastatin calcium and Telmisartan in their combined dosage form.

CHAPTER 4
INTRODUCTION TO METHOD OF ANALYSIS AND METHOD VALIDATION

Contents

4.1 U.V METHOD FOR ANALYSIS OF DRUG COMPONENTS

Ultraviolet-visible spectroscopy[66] or ultraviolet-visible spectrophotometry (UV-Vis or UV/Vis) involves the spectroscopy of photons in the UV-visible region. This means it uses light in the visible and adjacent (near ultraviolet (UV) and near infrared (NIR)) ranges. The absorption in the visible ranges directly affects the color of the chemicals involved. In this region of the electromagnetic spectrum, molecules undergo electronic transitions. This technique is complementary to fluorescence spectroscopy, in that fluorescence deals with transitions from the excited state to the ground state, while absorption measures transitions from the ground state to the excited state.

There are many methods available for the simultaneous UV estimation of the drug combinations like

(a)Assay as a single-component sample

(b) Assay using absorbance corrected for interference

(c) Simultaneous equation method

(d) Absorbance ratio method

(e) Geometric correction method

(f) Orthogonal polynomial method

(g) Difference Spectrophotometry

(h) Derivative Spectrophotometry

(i) Least square approximation

(j) Dual Wavelength Spectrophotometry

In simultaneous Spectrophotometric determination[66] the absorbance are additive, provided there is no reaction between the two solutes. We may write

$A\lambda_1 = \lambda_1 A_1 + \lambda_1 A_2$

$A\lambda 2 = \lambda_2 A_1 + \lambda_2 A_2$

Where A1 and A2 are the measured absorbance at the wavelengths λ_1 and λ_2; the subscripts 1 and 2 refer to the two different substances, and the subscripts λ_1 and λ_2 refer to the different wavelengths. The wavelengths are selected to coincide with the absorption maxima of the two solutes. Substance 1 absorbs strongly at wavelength λ_1 and weakly at λ_2, and substance 2 absorbs strongly at λ_2 and weakly at λ_1. Now

A = Єcl where Є is the molar absorption coefficient (molar absorptivity) at any particular wavelength, c is the concentration (mol-1) and l is the thidkeness, or length, of the absorbing solution (cm). if we set l = 1 cm then

$A\lambda_1 = \lambda_1 Є_1 c_1 + \lambda_1 Є_2 c_1$

$A\lambda_2 = \lambda_2 Є_1 c_1 + \lambda_2 Є_2 c_2$

Solution of these simultaneous equations gives

$$C_1 = \frac{\lambda_2 Є_2 A\lambda_1 - \lambda_1 Є_2 A\lambda_2}{\lambda_1 Є_1 \lambda_2 Є_2 - \lambda_1 Є_2 \lambda_2 Є_1}$$

$$C_2 = \frac{\lambda_1 Є_1 A\lambda_2 - \lambda_2 Є_1 A\lambda_1}{\lambda_1 Є_1 \lambda_2 Є_2 - \lambda_1 Є_2 \lambda_2 Є_1}$$

The values of the molar absorption coefficients Є1 and Є2 can be deduced from measurements of the absorbances of pure solutions of substances 1 and 2. by measuring the absorbances of the mixture at wavelengths λ1 and λ2, the concentrations of the two components can be calculated.

4.2 HPTLC METHOD FOR ANALYSIS OF DRUG COMPONENTS

4.2.1 Introduction to HPTLC

HPTLC added a new dimension to chromatography as it was demonstrated that precision could be improved ten-fold, analysis time could be reduced by a similar factor, less mobile phase was required, and the development distances on the layers could be reduced. The technique could now be made fully instrumental to give accuracy comparable with HPLC.[67]

For multi-component samples fractions of interest from an HPLC separation can be collected and subsequent re-chromatography of these on HPTLC can give a ‘‘fine tuned’’ separation of the components of the fractions.[68-69] HPTLC has been

successfully hyphenated with high performance liquid chromatography (HPLC), mass spectroscopy (MS), Fourier transform infra-red (FTIR), and Raman spectroscopy, to give far more detailed analytical data on separated compounds.[70] Even the UV/visible diode array technique has been utilized in HPTLC to determine peak purity or the presence of unresolved analytes.

HPTLC uses the same type of silica gel 60 layers with a thickness of 0.20-0.25 mm. However the particle size is much smaller, typically ranging from 4-8 μm, with optimum 5-6 μm.

Table 8.Comparison of HPTLC and TLC

PARAMETERS	HPTLC	TLC
Particle size	5-6 μm	10-12 μm
Pore diameter	60 Å	60-100 Å
Layer thickness	0.20-0.25 mm	0.20-0.25 mm
Number of samples can be applied per plate	Up to 75	UP to 16
Spot size recommended	~1 mm	2-5 mm
Spot loading	50-200 nl	1-5 μl
Band size recommended	5-10 mm	10-15 mm
Band loading	1-4 μl	5-10 μl
Sensitivity limit	Upper pg	ng
Normal development time	2-30 minutes	15-120 minutes

- **Mechanism of HPTLC separation**
  - Adsorption
  - Partition
  - Ion-exchange

4.2.2 Steps involved in HPTLC [71-72]

- Selection of chromatographic layer
- Sample and standard preparation
- Layer pre-washing and pre-conditioning
- Application of sample and standard
- Chromatographic development
- Detection of spots

- Scanning and Documentation of chromatic plate

Sample Application

The solvent used to apply the sample to the TLC plate can have a decisive influence on the spot size. The least polar single solvent or mixture of solvents in which the analyte are completely soluble or completely extracted from the sample matrix can be used.

Even for basic qualitative TLC, there are advantages in band over spot application. Although the spot can be easily applied manually using a glass capillary, the application of sample as a band usually require more dexterity and is more accurately accomplished with semi- or fully automated equipment. The Linomat 5 offers semi-automatic sample application for qualitative and quantitative analyses as well as for preparative separations

Table 9.Advantages of Band Application over Spot Application

Advantage of Band over Spot Application	Application of Spot over Band Application
Better resolution of analytes-near origin	Can require less automation
More even distribution of sample	Can be very inexpensive
Less error on choice of scanner slit width	Application usually less time consuming
Greater accuracy – lower % standard deviation	
More flexibility in sample loading	

Solvent Selection

Selectivity of separation is greatly influenced by the choice of solvent or solvent mixture.

- Trial and error
- Experience and Literature
- 3 - 4 component mobile phase should be avoided
- Multi component mobile phase once used not recommended for further use
- Twin trough chambers are used only 10 -15 ml of mobile phase is required
- Components of mobile phase should be mixed introduced into the twin - trough chamber

Development chambers

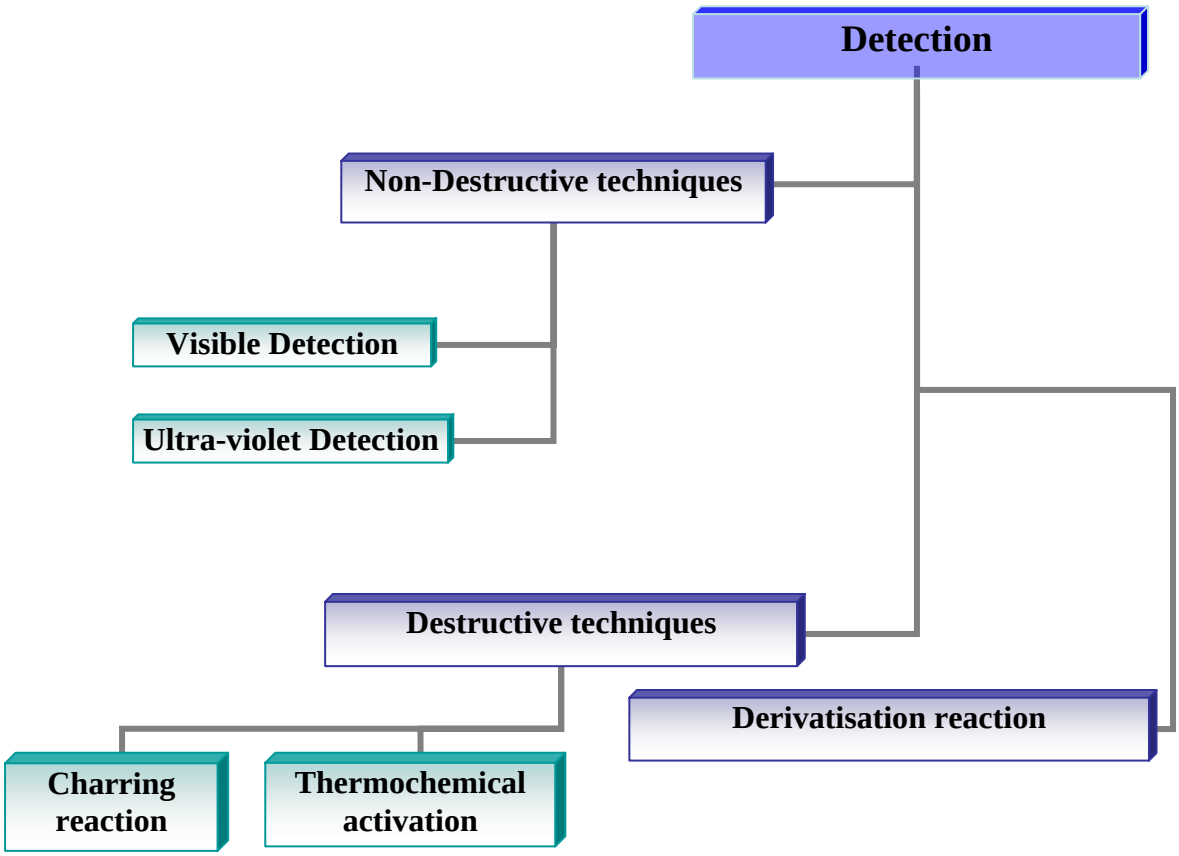
There are a variety of different types of chambers, each designed with particular features to control to the greater or lesser extent the parameters of chromatogram development reproducibility.

As solvent vapour saturation, sorbent vapour adsorbed, solvent vapour "demixing" and solvent front and edge effects on the chromatographic layer can have a bearing on separation achieved, it is important to eliminate unwanted effects and to utilize those features that will improve resolution.

The types of HPTLC chambers are

- Nu-chamber
- Ns-chamber
- Twin-through chamber
- Su-chamber
- Ss- chamber
- Horizontal chamber
- Automatic development chamber (ADC)
- Vario chambers

Detection



Quantification

1) Densitometry

Densitometry is a means of measuring the concentration of the chromatographic zones on the developed HPTLC layer without damaging the separated substance.

There are three possible scanning modes, single beam, single wavelength, double beam using a beam splitter and dual wavelength and double beam combined into a single beam. The single beam format is most popular, as the beam of electromagnetic radiation hits the chromatographic layer, some passes into and through the layer whilst the remainder is reflected back from the surface. Reflectance occurs due to the opaqueness of the layer. This reflected radiation is measured by the photomultiplier unit or photoelectric cell in the instrument.

The spectrodensitometric scanner scan separate tracks and wavelength produces vast amount data. These data includes peak heights and areas, and position of zones (start, middle and end) for every resolved component on every chromatographic track on the HPTLC plate. A baseline adjustment is applied so that all peaks can be accurately integrated ready for possible quantification.

2) Video imaging and Densitometry

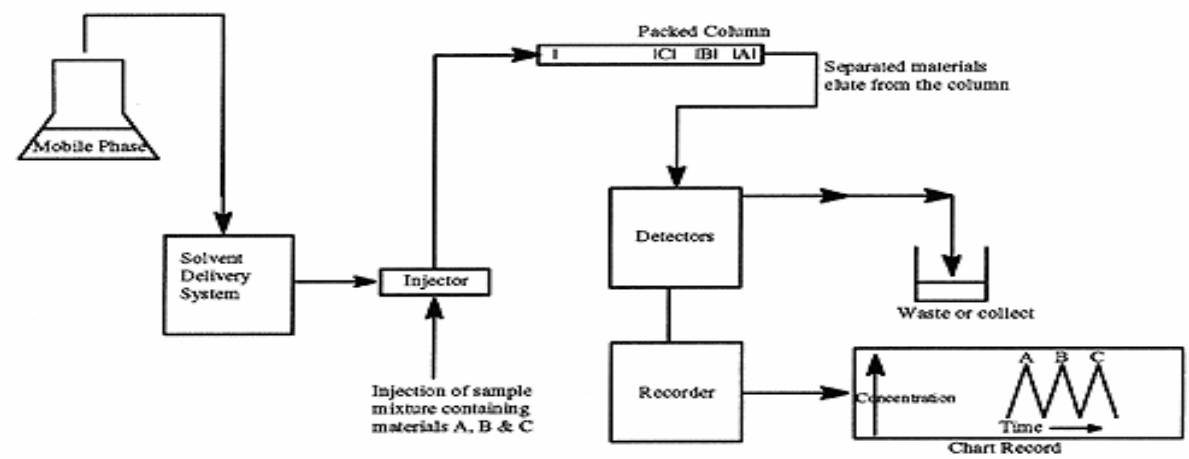
The developed chromatogram is illuminated from above with visible, 254 nm (UV) or 366 nm (UV) light, depending on the radiation required to visualize the analytes. Illumination from below the plate can often improve the brightness of the image. With the plate suitably lit, an image acquisition device, usually CCD (charged coupled device) camera with zoom attachment is positioned vertically above. The CCD camera transmits a digital signal to a computer and video printer.

4.3 RP-HPLC METHOD FOR ANALYSIS OF DRUG COMPONENTS

4.3.1 Mechanism:[73]

HPLC instrument consist of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector. Compounds are separated by injecting a plug of the sample mixture onto the column.

4.3.2 Schematic diagram:[74]



4.3.3 Mobile phase[74-75]

- Methanol CH_3OH
- Acetonitrile CH_3CN
- Water H_2O
- Buffer
- Tetrahydrofuran (THF)

4.3.4 Solvent degassing[74-75]

- Sparging (online degassing)
- Heat (offline degassing)
- Vaccum (offline degassing)
- Sonication (offline degassing)

4.3.5 Solvent delivery systems / Pump[74-75]

- **Types of the pumps:**

1. Syringe pump (screw driven)
2. Reciprocating pump
3. Pneumatic pump:

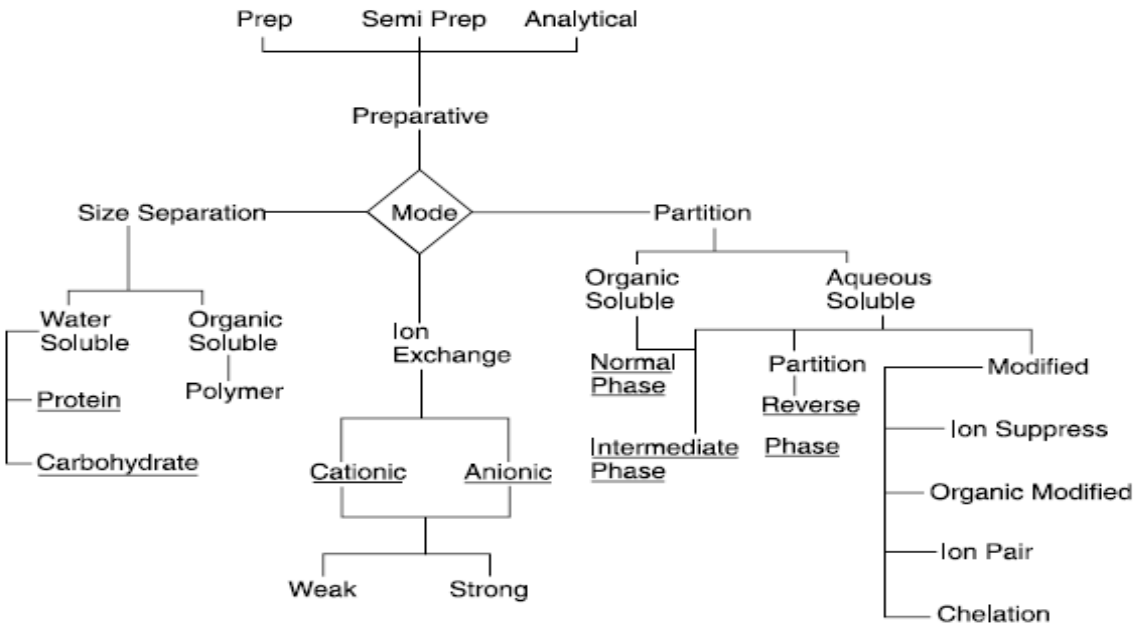
Table 10.Types of pump

Types of pump	Advantage	Disadvantage
Syringe Position displacement	1) Pulse free delivery at high pressure. 2) FR independent of viscosity of MP.	Limited solvent capacity. Change in solvent is inconvenient.
Reciprocating pump	1) FR independent of viscosity of MP. 2) Suitable for continuous operation.	Detection of noise due to pulsating out flow.
Pneumatic pump	1) Rugged 2) Inexpensive 3) easy to operate 4) Pulse free	FR dependent of viscosity of MP. Change in solvent is inconvenient. Gradient operation is difficult.

- **Desired Pump characteristics**:
  - Pressure in PSI : 3000-5000
  - FR in ml/min : 0.5-2 (analytical)
    : 0-10 (preparative)
  - Accuracy : ±5%
  - Reproducibility : ±1 %
  - Solvent storage : 200-500 ml
  - Puse free delivery of mobile phase : essential

4.3.6 HPLC columns[74-75]

- It is the heart of the HPLC.
- Stable and high performance column is essential requisite for rugged, reproducible method.



- **Column selection**[74-75]

- Separation Column: Usually a stainless steel tubing with i.d. of 2-6 mm (analytical separations).
- Larger columns (e.g. 30-200 mm i.d.): preparative separations in the range of 100 mg to 1 Kg can be performed.

A fine grained chromatographic material (e.g. silica gel or RP-18), serves as stationary phase with particle size of 5-10 μm (analytical separations) and 10-50 μm (preparative separations). To overcome the flow resistance of stationary phase,mobile phase must placed under a relatively high pressure up to 100 bar.

- **Stationary phase**[74-75]

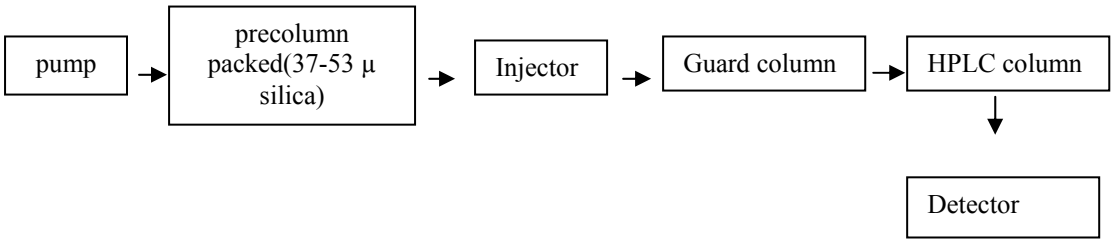
Table 11.Type of group in stationary phase

Group	Type	Particle diameter in µ
Amino	Normal	5 & 10
Nitrile	Normal	5 & 10
Amine & nitrile	Normal	40
Octyl	Reverse phase	3,4,5 & 10
Octadecyl	Reverse phase	4,5,10 & 40

4.3.7 Precolumn[74-75]

Some of operating conditions which cause acute dissolution of silica are such as pH>3 or <2, temperature >50°C, concentrated buffer and ion pair reagents. This dissolution leads to column bed subsidence resulting in formation of void at top of the column which can lead to broadening of peak and increase pressure. Precolumn fitted between pump and injection valve ensures that the mobile phase is fully saturated with silicates ions prior to the sample injection. Thus the use of precolumn shall reduce adverse effects of low or high ph mobile phase. It extends life of column.

.



4.3.8 Column parameter affecting the detection[76]

- **Internal diameter**

The internal diameter (ID) of an HPLC column is an important parameter that influences the detection sensitivity and separation selectivity in gradient elution. It also determines the quantity of analyte that can be loaded onto the column. Larger columns are usually seen in industrial applications, such as the purification of a drug product for later use. Low-ID columns have improved sensitivity and lower solvent consumption at the expense of loading capacity.

- Larger ID columns (over 10 mm) are used to purify usable amounts of material because of their large loading capacity.
- Analytical scale columns (4.6 mm) have been the most common type of columns, though smaller columns are rapidly gaining in popularity. They are used in traditional quantitative analysis of samples and often use a UV-Vis absorbance detector.
- Narrow-bore columns (1-2 mm) are used for applications when more sensitivity is desired either with special UV-vis detectors, fluorescence detection or with other detection methods like liquid chromatography-mass spectrometry
- Capillary columns (under 0.3 mm) are used almost exclusively with alternative detection means such as mass spectrometry. They are usually made from fused silica capillaries, rather than the stainless steel tubing that larger columns employ.

- **Particle size**

Most traditional HPLC is performed with the stationary phase attached to the outside of small spherical silica particles (very small beads). These particles come in a variety of sizes with 5 μm beads being the most common. Smaller particles generally provide more surface area and better separations, but the pressure required for optimum linear velocity increases by the inverse of the particle diameter squared.

This means that changing to particles that are half as big, keeping the size of the column the same, will double the performance, but increase the required pressure by a

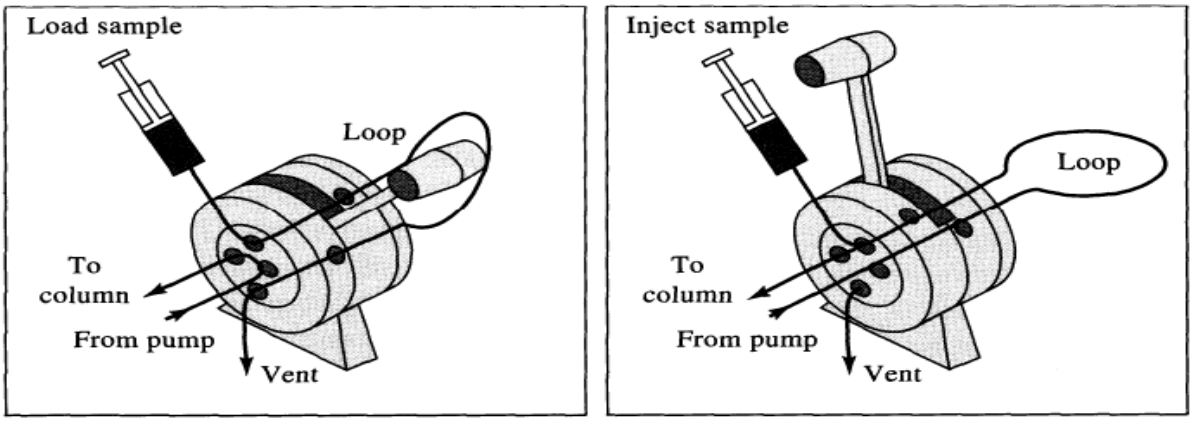
factor of four. Larger particles are used in preparative HPLC (column diameters 5 cm up to >30 cm) and for non-HPLC applications such as solid-phase extraction.

- **Pore size**

Many stationary phases are porous to provide greater surface area. Small pores provide greater surface area while larger pore size has better kinetics, especially for larger analytes. For example, a protein which is only slightly smaller than a pore might enter the pore but does not easily leave once inside.

4.3.8 Sample Injection System:

Injection systems, includes manual injector, standard auto sampler, high-performance auto sampler, high-performance auto sampler SL plus, micro well-plate auto sampler, preparative auto sampler and dual-loop auto sampler as well as the thermostat.



4.3.9 Guard column:

The purpose of the guard column is to protect expensive of analytical column by removing partical garbage and strongly irreversible retained sample component which decrease the life time of analytical column.

4.3.10 Detectors

- **Two types of detectors:**

Bulk property detectors: They are based on some bulk properties of eluent, such as RI and are not suitable for gradient elution and are usually lees sensitive than solute property.

1) **Solute property detectors:** Performed by measuring some types of physical or chemical property that is specific to solute only. So can be used with gradient elution.

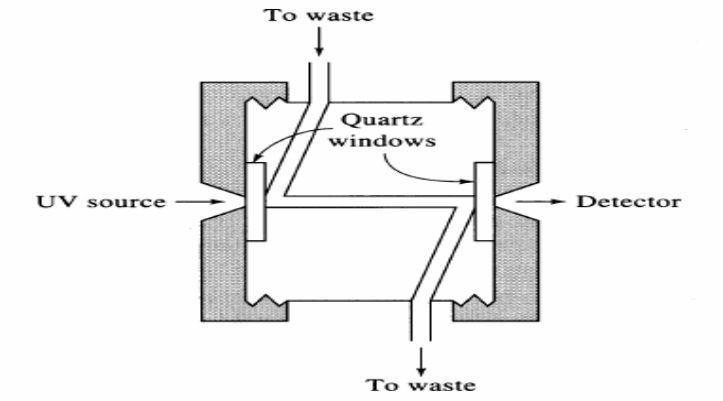


Figure: schematic diagram of UV VIS detector

- UV VIS Detector
- Variable wave length detector
- Photo diode array
- Refractive index
- Evaporating light scattering detector
- Chiral detector
- Conductivity detector
- Polarimeter for enantiomer
- Viscometer for polymers

- Radioisotope for flow detector

4.4 VALIDATION OF ANALYTICAL METHODS

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice.

Validation is an act of proving that any procedure, process, equipment, material, activity or system performs as expected under given set of conditions and also give the required accuracy, precision, sensitivity, ruggedness etc. When extended to an analytical procedure, depending upon the application, it means that a method works reproducibly, when carried out by some different persons, in same or different laboratories, using different reagents, different equipments, etc.

Advantage of analytical method validation

The biggest advantage of method validation is that it builds a degree of confidence, not only for the developer but also to the user. Although the validation exercise may appear costly and time consuming, it results are inexpensive and eliminates frustrating repetitions, leads to better time management in the end. Minor change in the conditions such as reagents supplier or grade, analytical setup are unavoidable due to obvious reasons but the method validation absorbs the shock of such conditions and pays for more than invested on the process.

The objective of the analytical procedure should be clearly understood since this will govern the validation characteristics that are evaluated. Typical validation characteristics that may be considered are listed below:

1. Accuracy
2. Precision
3. Linearity
4. Range
5. Limit of Detection (LOD) and Limit of Quantification (LOQ)
6. Specificity
7. Selectivity

8. Robustness

4.3.1 Accuracy

The accuracy of an analytical method is the extent to which test results generated by the method and the true value agree. As per ICH guideline, accuracy is defined as "the closeness of agreement between the value that is adopted, either as a conventional, true or accepted reference value, and the value found". Accuracy is a measure of exactness of the analytical method.

Accuracy can be measured by several methods:

The true value can be obtained from an established reference method. In this approach assumes that the uncertainty of the reference method is known.

Accuracy can be assessed by analyzing a sample with known concentration, for example, a certified reference material, and comparing measured value with the true value as supplied with the material.

Recovery is found from the following formula [77]

$$\%\,\text{Recovery} = \frac{N(\sum xy) - (\sum x) * (\sum y)}{N(\sum x^2) - (\sum y^2)}$$

Where N = Number of observations

x = Amount of standard drug added

y = Amount of drug added

or

% Recovery = ($C_{fortified} - C_{unfortified}$) x 100 / $C_{std\ added}$ (QAAC)

Where,

$C_{fortified}$ is concentration of drug from matrix with standard addition of drug

$C_{unfortified}$ is concentration of drug without addition

$C_{std\ added}$ is standard added drug in solution of drug

4.3.2 Precision

The precision of a method is the extent to which the individual test results of multiple injections of a series of standards agree. As per ICH guideline, precision of a method is "express the closeness of agreement (degree of scatter) between a series of

successive measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions".[78-79]

The measured standard deviation can be subdivided into 3 categories:

- Repeatability
- Intermediate precision
- Reproducibility

Repeatability gives the degree of precision obtained when the method is replicated in the same laboratory within short intervals and in the same conditions. Reproducibility represents precision obtained under variations in conditions of assays such as different analyst, equipment and reagents, laboratory and days.

The precision of an analytical method is usually expressed as the standard deviation (SD) or relative standard deviation (RSD).

The standard deviation is calculated from the following formula

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

Where,

Xi = Individual measurement in the set

X = Arithmetic mean of the set

N = Number of replicates taken in the set

$$RSD = \frac{SD}{X}$$

%RSD or coefficient of variance (CV) is expressed as

$$\% RSD = CV = \frac{SD}{X} * 100$$

4.3.3 Linearity

The linearity of an analytical method is its ability to elicit test results that are directly proportional to the concentration of analytes in samples within a given range or proportional by means of well-defined mathematical transformations. Linearity may

be demonstrated directly on the test substance (by dilution of a standard stock solution) and/or by using separate weighings of synthetic mixtures of the test product components, using the proposed procedure. [80]

The relationship between the sample concentration and its signal is first order type. This line, known as the calibration line, is expressed by an estimated first order equation.

y = mx + c

where, y = measured signal

x = concentration of sample

c = intercept

m = slope of line

4.3.4 Range

The range of an analytical method is the interval between the upper and lower levels (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity. The range is normally expressed in the same units as the test results (e.g., percentage, parts per million) obtained by the analytical method. [80]

4.3.5 Specificity and Selectivity

Specificity of an analytical method is the ability to measure accurately and specifically the analyte in presence of components that may be expected to be present in the sample matrix.[80]

Specificity is expressed as degree of bias of test results obtained by analysis of sample containing added impurities, degradation product, related chemical compounds or placebo ingredients when compared with test results from samples without added samples. Bias may be expressed as difference in assay results between two group samples. Thus specificity is a measure of the degree of interference in the analysis of complex sample mixture.

Selectivity is ability of analytical method to differentiate various substances in the sample.[80] One basic difference in selectivity and specificity is that, selectivity is restricted to qualitative detection of the components from the sample matrix whereas specificity means quantitative measurement of one or more analytes. Selectivity generally applies to a separative method whereas specificity is applicable to a non-

separative method. The titration methods are good examples of specificity and chromatographic methods are both selective and specific.

The selectivity is an essential requirement for all types of methods used in identification i.e. spectroscopic, chromatographic or chemical. On the other hand, the selectivity is an essential for non chromatographic assay methods and for stability indicating assays whether chromatographic or non chromatographic.[81]

Procedure for establishment of selectivity of a method

1. Analyze sample and reference materials by the candidate and other independent methods.
2. Assess the ability of the methods to confirm free identity analyte and their ability to measure the analyte in solution from the interference present
3. Choose the most appropriate method
4. Analyze sample containing various suspected interference in the presence of the analyte of present
5. Examine the effect of interferences and whether further development is required.

4.3.6 Sensitivity

Sensitivity of the method expressed in terms of sandell's sensitivity. Sandell's sensitivity refers to the number of milligrams of the drug determined converted to the colored product, which in a column solution of cross section 1 cm^2 shows an absorbance of 0.001 (expressed as log cm^2, 0.001 absorbance $unit^{-1}$).

$$S = N \frac{M}{\varepsilon}$$

Where, M = Molecular weight,

ε = Molar absorptivity of colored species

N = Number of atoms in molecule

4.3.7 Limit of detection (LOD) and Limit of Quantification

Limit of detection (LOD)

It is a quantitative parameter. LOD is the lowest concentration of the analyte in sample that can be detected, but not necessarily quantities precisely and accurately.[81] It is expressed in terms of concentration units. Limit of Detection values are always specific for a particular set of experimental conditions.

Limit of Detection by definition encompasses

1. Instrumental detection limit (IDL) is the lowest limit that the instrument can detect and is based on the samples that have not gone any sample preparative steps.
2. Method detection limit (MDL) is similar to IDL but is based on samples that have gone through entire sequence of sample preparation prior to analysis.

In chromatography, the detection limit is the injected amount that results in a peak with a height at least two or three times as high as the baseline noise level. Besides this signal/noise method, the ICH describes three more methods:

1. Visual inspection: The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.
2. Standard deviation of the response based on the standard deviation of the blank: Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.
3. Standard deviation of the response based on the slope of the calibration curve: A specific calibration curve is studied using samples containing an analyte in the range of the limit of detection. The residual standard deviation of a regression line, or the standard deviation of y-intercepts of regression lines, may be used as the standard deviation.

Limit of Quantification (LOQ)

It is the lowest concentration of analyte in a sample that may be measured in a sample matrix such as impurities in bulk drug substances and degradation products in finished

pharmaceuticals. The value of LOQ is almost 3-10 times higher than LOD. The LOQ also varies with the type of method employed and nature of samples.[82]

Table 10. Approaches for Determining the LOD and LOQ

APPROACH	LIMIT OF DETECTION	LIMIT OF QUANTIFICATION
Signal-to-noise	3:1 or 2:1	10:01
Standard deviation of the response and the slope (S)	3.3 x σ/S	10 x σ/S

S = The slope of calibration curve

σ = The standard deviation of the response

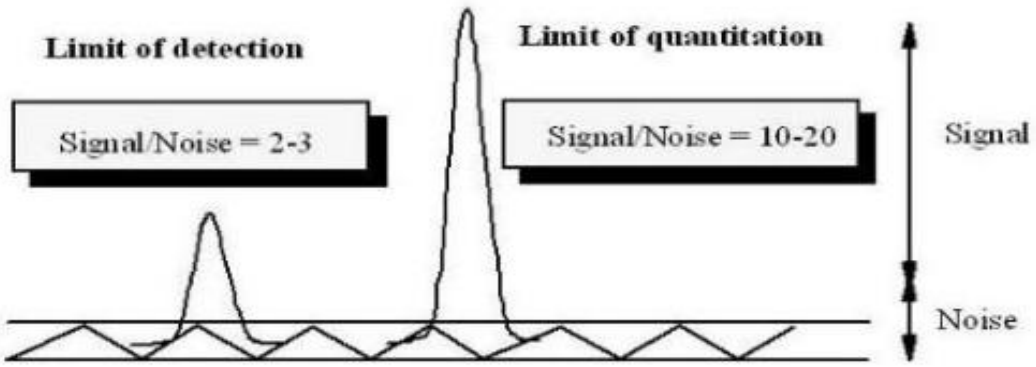
The slope S may be estimated from the calibration curve of the analyte. The estimation of σ may be carried out in a variety of ways, for example:

1. **Based on the standard deviation of the blank**

   Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

2. **Based on calibration curve**

   A specific calibration curve should be studied using samples containing an analyte in the range of detection limit. The residual standard deviation of the regression line or the standard deviation of y-intercepts of regression line may be used as the standard deviation.



4.3.8 Ruggedness

Ruggedness is the degree of reproducibility of test results obtained by the analysis of the same sample under a variety of normal test conditions such as different laboratories, different analysts, different instruments and different batches or brands of reagents, different elapsed assay times and different assay temperature etc.[82]

4.3.9 Robustness

Robustness is the measurement of capability of analytical method to remain unaffected by small but deliberate deviation in the method parameters. Robustness testing is normally restricted to methods that are to be used repetitively in the same laboratory. It means that the method repeatable when intentional variations such as changes in concentration, use of different analyte, wavelength of detection, use of different dilutions, change of column of the same type, small changes in the mobile phase etc are introduced in the method.

CHAPTER 5

EXPERIMENTAL WORK

Contents

5.1 IDENTIFICATION OF DRUGS:

Drug samples were received from pharmaceutical companies; these samples were subjected to identification. Identification of these drugs was carried out by following methods:

5.1.1 Determination of Melting Point:

Melting point of both the drug samples were determined by capillary method and obtained results as under:

Table 13.Melting point of drugs

Drugs	Standard	Obtained
Atorvastatin calcium	176°C -178°C	179°C -181°C
Telmisartan	261°C – 264°C	260°C – 262°C

5.1.2 UV spectra of Atorvastatin calcium and Telmisartan

UV-spectrum of Atorvastatin calcium (10µg/ml) and Telmisartan (10µg/ml) in methanol was taken. Atorvastatin calcium was found to show absorption maxima at 246 nm. Telmisartan was found to show absorption maxima at 296nm.

Figure 1.UV spectrum of Atorvastatin calcium (10µg/ml) in methanol

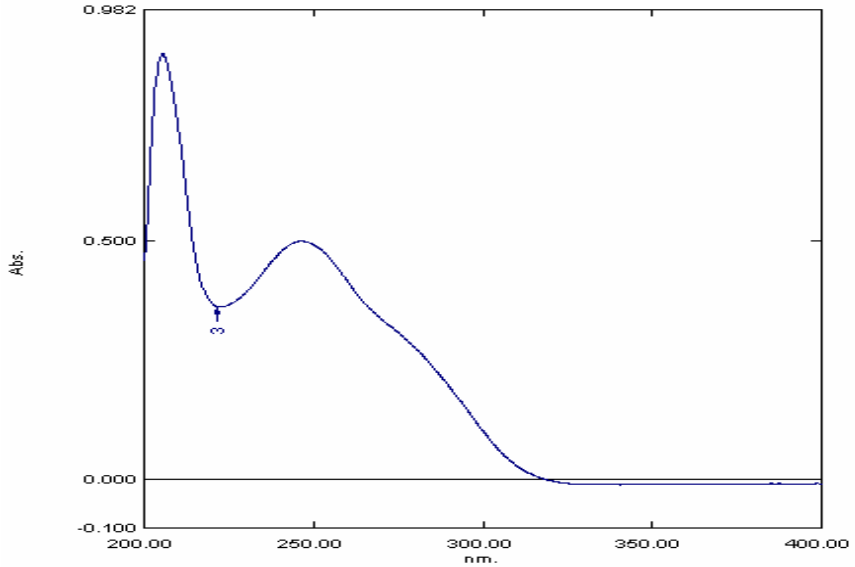


Figure 2.UV spectrum of Telmisartan (10 µg/ml) in methanol

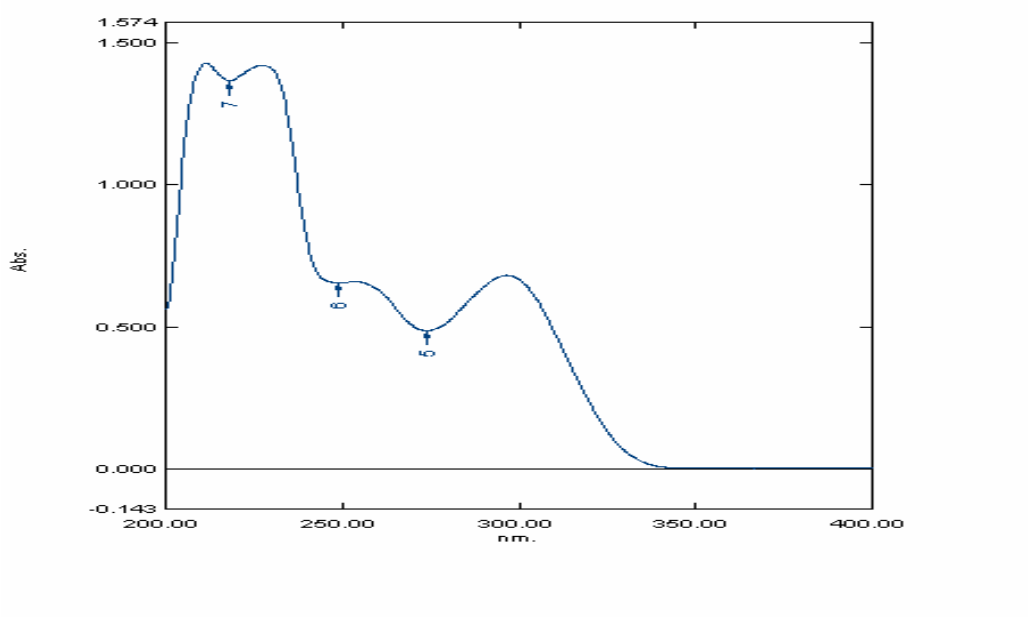


Table 14.Wavelength Maxima for Telmisartan and Atorvastatin calcium

DRUG NAME	REPORTED PEAK (NM)	PEAK OBTAINED (NM)
Atorvastatin calcium	246	246
Telmisartan	296	296,250,230

5.1.3 Raman spectra of Atorvastatin calcium and Telmisartan

Raman spectrum of **Atorvastatin calcium and Telmisartan** was taken for the identification of the drugs.

Figure 3.Recorded Raman Spectrum of Atorvastatin calcium

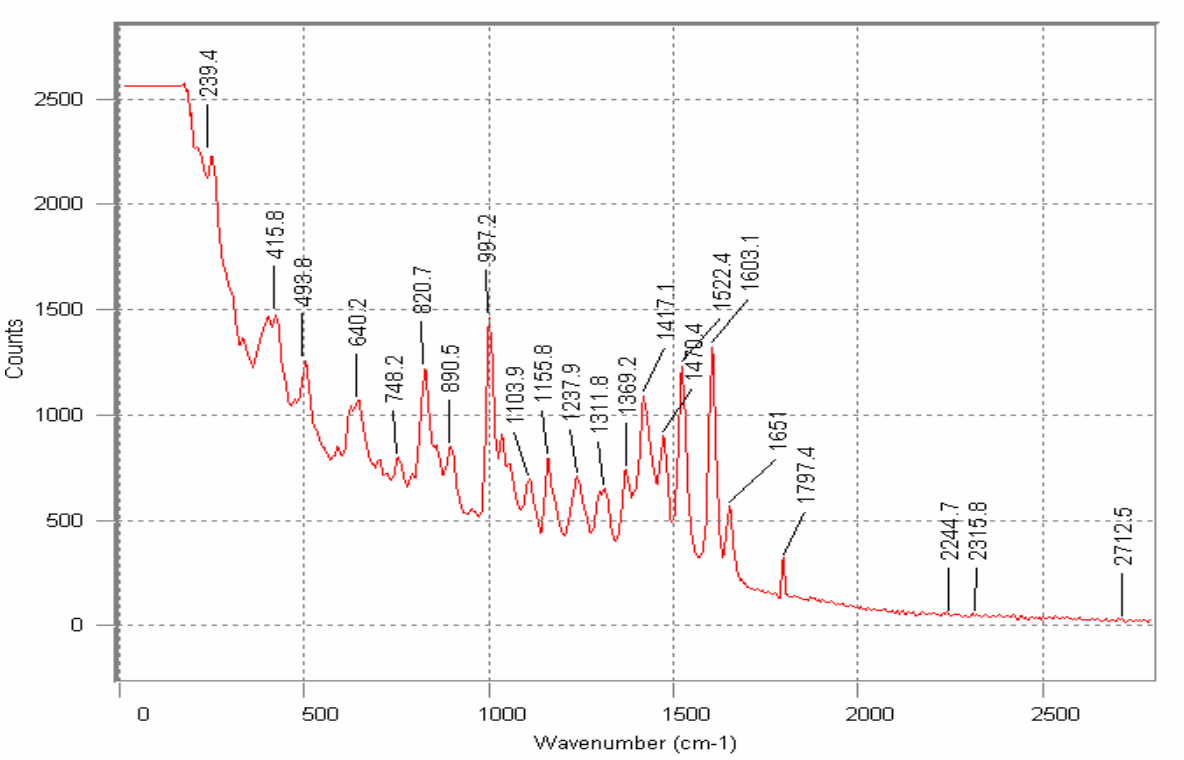


Table 15.Specification of Raman spectrum of Atorvastatin calcium

SPECIFICATION OF RAMAN PEAK FOR ATORVASTATIN CALCIUM	**RECORDED WAVE NUMBER (CM^{-1})**
C-F stretch	820.7
p-substituted benzene	748.2
Mono substituted benzene	997.2
Secondary amine	890.5
Benzene derivatives	1522.4
C=O stretch	1603.1

Figure 4.Recorded Raman Spectrum of Telmisartan

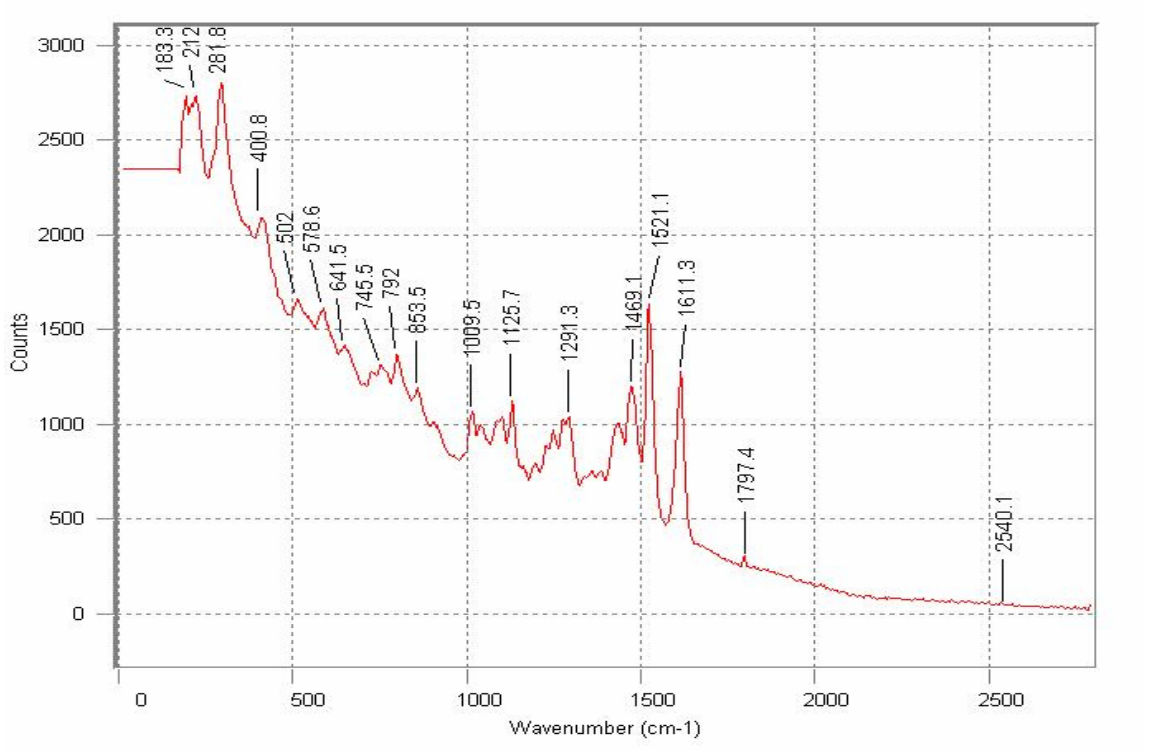


Table 16.Specification of Raman spectrum of Telmisartan

SPECIFICATION OF RAMAN PEAK FOR TELMISARTAN	**RECORDED WAVE NUMBER (CM^{-1})**
C-C skeletal stretch(n-alkanes)	853.5
Mono substituted benzene	1009.5
Secondary amine	853.5
Ring stretch doublet (benzene)	1521.1
Symmetric C=O stretch	1611.3

5.2 DEVELOPMENT AND VALIDATION OF SPECTROPHOTOMETRIC METHOD FOR SIMULTANEOUS ESTIMATION OF ATORVASTATIN CALCIUM AND TELMISARTAN IN THEIR COMBINED DOSAGE FORMS

5.2.1 Abstract

A simple, accurate and precise Spectrophotometric method was developed for the simultaneous estimation of Atorvastatin calcium (ATR) and Telmisartan (TEL). The estimation of ATR and TEL was carried out by simultaneous equation method. The λmax of Atorvastatin calcium is 246 nm and λmax of Telmisartan is 296 nm. Both drugs show absorbance at λmax of each other. So Atorvastatin calcium was estimated at 246 nm and 296 nm, as well as Telmisartan was estimated at this two Wavelength. The method was validated according to ICH guidelines and it was found to be accurate, precise and selective. Developed and validated method was applied for estimation of ATR and TEL in their combined dosage forms, procured from the local market.

5.2.2 Instrumentation

(1) UV-Visible Double-Beam spectrophotometer:

Matched quartz cell (1cm)

Model: UV-2450 Pc series

Manufacturer: Shimadzu Inc. Japan.

Wavelength range: 200.00 to 400.00 nm

(2) Analytical Balance:

Model: KEROY

Manufacturer: Keroy (balance) Pvt. Ltd. Varanasi, India

Weighing capacity: 100 gm

(3) Sonicator:

Model: TRANS-O-SONIC; D-compact

Capacity: 2 Lit.

5.2.3 Materials and Methods

5.2.3.1 Reagents and chemicals:

- Tablets containing Atorvastatin calcium (10 mg) and Telmisartan (40 mg).
    1. (Brand name: **Telsartan™-ATR;** Name of manufacturer: **DR.REDDY'S)**

2. (Brand name: **Arbitel-AV;** Name of manufacturer: **MICRO LABS LIMITED)**
3. (Brand name: **TELDAY AV;** Name of manufacturer: **TORRENT PHARMACEUTICALS LTD.)**
4. (Brand name: **TELEACT ST 40;** Name of manufacturer: **RANBAXY LAB. LTD.)**

- Tablets containing Atorvastatin calcium (10 mg) and Telmisartan (20 mg).
  1. (Brand name: **TELEACT ST 20;** Name of manufacturer: **RANBAXY LAB. LTD.)**

5.2.3.2 Preparation of Standard Stock Solution of Atorvastatin calcium and Telmisartan

UV analysis was done by using the standard stock solution of Atorvastatin calcium and Telmisartan. Aliquots were prepared by using this stock solution of Atorvastatin calcium and aliquots were prepared by using this stock solution of Telmisartan, for preparation of calibration curve.

5.2.3.3 Preparation of binary mixtures of Atorvastatin calcium and Telmisartan

Suitable aliquots of standard stock solution of Atorvastatin calcium and Telmisartan are mixed and diluted to volume with methanol to obtain different binary mixture solutions containing Atorvastatin calcium and Telmisartan in 1:1 ratio.

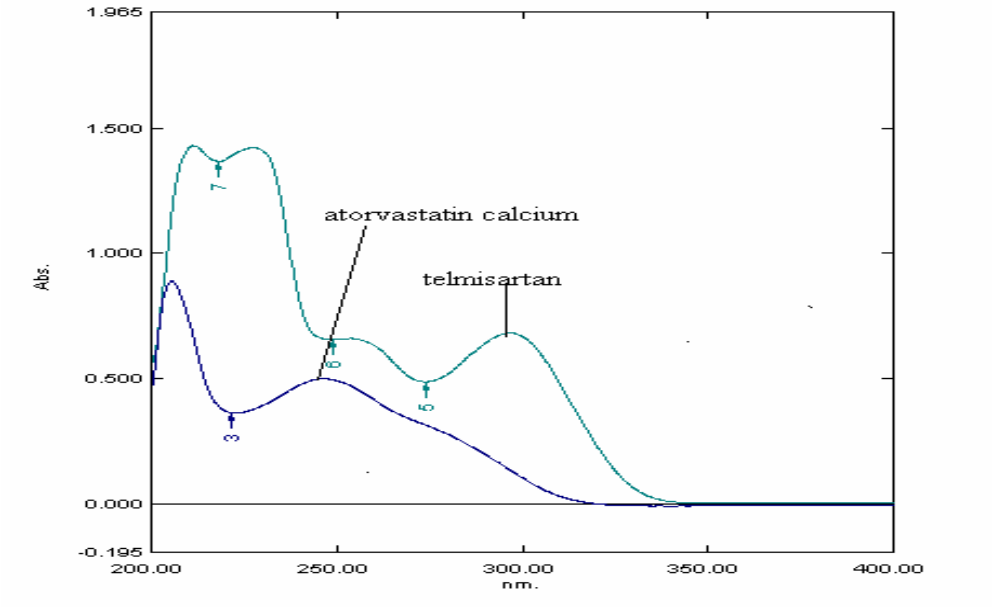
5.2.3.4 Wavelength selection and measurement

Solution of both drug were prepared and scanned in the U.V region separately. The wavelengths selected were 246nm and 296 nm for simultaneous estimation of both drugs.

The valus of absorbance were recorded at the selected wavelengths, and the absorptivity and molar absorptivity values for both drugs were determined for both drugs. Molar absorptivity values determined for Atorvastatin calcium at 246 nm and 296 nm were 58430.56 and 15849.46 cm^{-1} mol^{-1} lit^{-1} while respectively values for Telmisartan at 246 nm 296 nm were 38131.28 and 38009.01 cm^{-1} mol^{-1} lit^{-1} . Molecular weight of Atorvastatin calcium and Telmisartan is 1155.36 and 514.63 respectively.

Where A_1 and A_2 are values of absorbance of sample at 246 nm and 296 nm respectively, and c_1 and c_2 are concentrations of Atorvastatin calcium and Telmisartan in moles lit^{-1}. Overlay spectra of Atorvastatin calcium and Telmisartan is given in figure 3.

Figure 5.Overlain spectra of Atorvastatin calcium and Telmisartan



5.2.3.5 Method validation

1) Preparation of Linearity Curve

For estimation of Atorvastatin calcium, calibration curve (n=6) was plotted at 246 nm. For estimation of Telmisartan, calibration curve (n=6) was plotted between absorbance difference at 296 nm.

2) Precision

For Atorvastatin calcium, intraday precision was carried out by taking three different concentrations (2, 4, 6 µg/ml) repeated for 3 times in a day and interday precision was carried out by taking three different concentrations (2, 4, 6 µg/ml) repeated on three different days. For Telmisartan, intraday precision was carried out by taking three different concentrations (2, 4, 6 µg/ml) repeated for 3 times in a day and interday precision was carried out by taking three different concentrations (2, 4, 6 µg/ml) repeated on three different days.

3) LOD and LOQ

For this determination Calibration curve for both the drugs was repeated six times. The LOD & LOQ were measured by using mathematical equations given below.

LOD = 3.3 x σ/S

LOQ = 10 x σ/S

Where,

σ = Standard deviation of the intercept

S = Slope of calibration curve

4) Accuracy

4 µg/ml standard solution was taken for Atorvastatin calcium and 4 µg/ml standard solution for Telmisartan. After that accuracy of the method was determined by standard addition method at three different levels (80%, 100% and 120%).

5.2.2.6 Analysis of Tablet Samples

Total 20 tablets were weighed accurately and powdered. An amount equivalent to one tablet (containing 10 mg of Atorvastatin calcium and 40 mg of Telmisartan) was taken and dissolved in 100 ml methanol in 100 ml volumetric flask. The solution was sonicated for 15 minutes and then diluted with methanol to the mark. The solution was filtered by using Whatmann filter No.41. From this solution, 0.32 ml of sample solution was taken and diluted with 10 ml methanol to get the final solution containing 3.2 µg/ml concentration of Atorvastatin calcium and 12.8 µg/ml concentration of Telmisartan .

5.2.4 Result and discussion

5.2.4.1 Method validation

1) Preparation of Linearity Curve

Figure 6.Linear Curve for Atorvastatin calcium at 246 nm

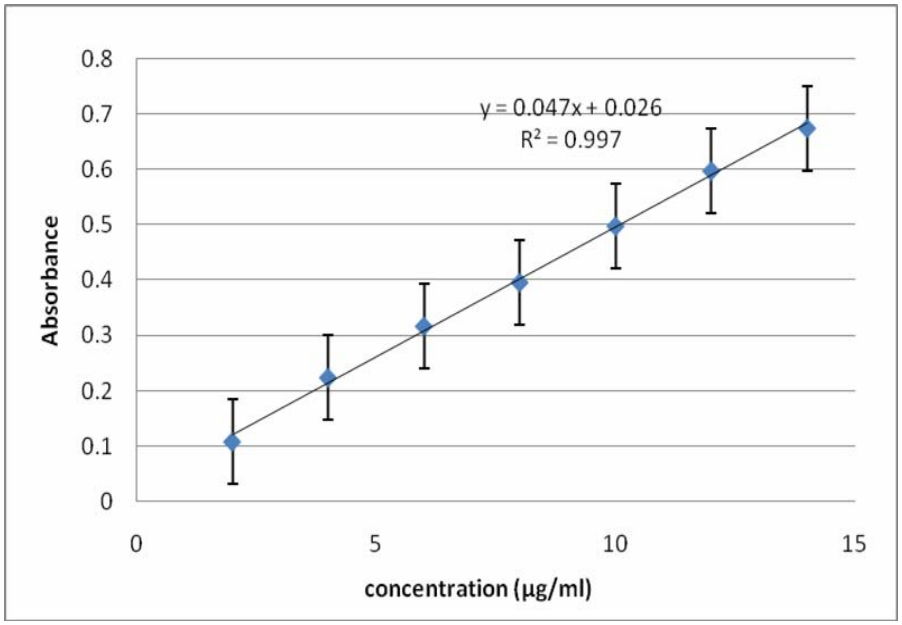


Table 17.Calibration curve data of Atorvastatin calcium at 246 nm

Conc(µg/ml)	Mean±S.D	%RSD
2	0.108±0.00083	0.77
4	0.224±0.0014	0.62
6	0.317±0.0010	0.31
8	0.396±0.00083	0.20
10	0.498±0.00109	0.21
12	0.598±0.0014	0.23
14	0.675±0.011	1.62

Figure 7.Linear Curve for Atorvastatin calcium at 296 nm

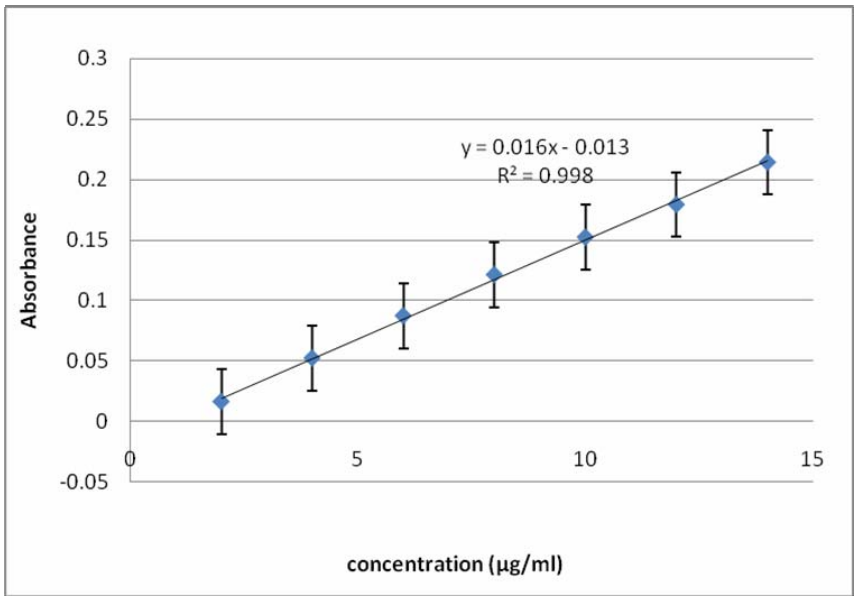


Table 18.Calibration curve data of Atorvastatin calcium at 296 nm

Conc.(µg/ml)	Mean±S.D	%RSD
2	0.016±0.00026	1.85
4	0.052±0.00041	0.80
6	0.087±0.00148	1.73
8	0.121±0.00208	1.71
10	0.152±0.00057	0.38
12	0.179±0.00246	1.49
14	0.214±0.00230	1.07

Figure 8.Linear Curve for Telmisartan at 246 nm

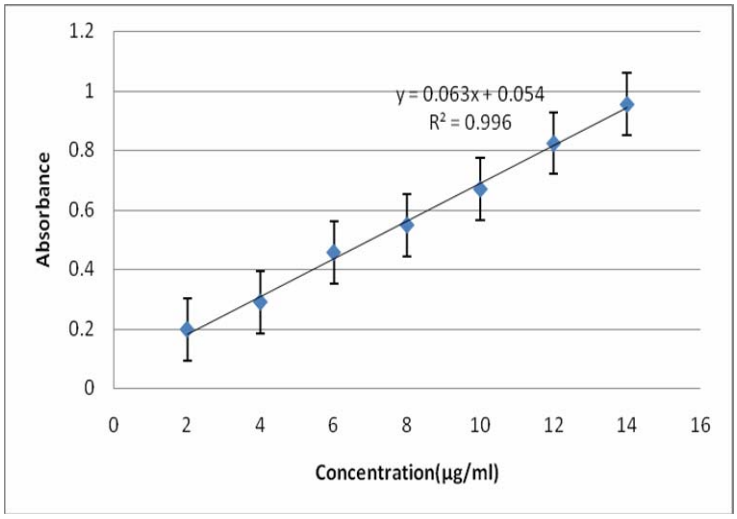


Table 19.Calibration curve data of Telmisartan at 246 nm

Conc.(µg/ml)	Mean±S.D*	%RSD
2	0.197±0.0030	1.50
4	0.289±0.0025	0.87
6	0.456±0.001	0.21
8	0.548±0.0015	0.27
10	0.669±0.0015	0.22
12	0.823±0.0014	0.18
14	0.954±0.0020	0.21

*indicates (n=3)

Figure 9.Linear Curve for Telmisartan at 296 nm

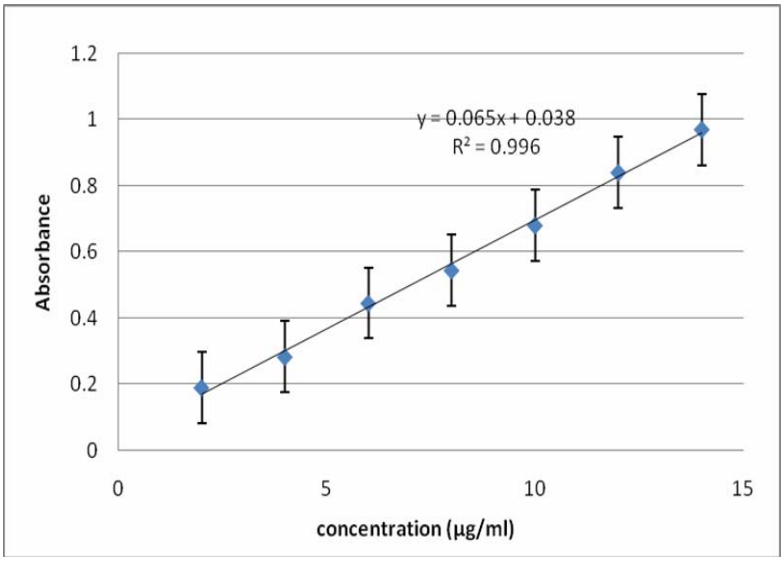


Table 20.Calibration curve data of Telmisartan at 296 nm

Conc.(µg/ml)	Mean±S.D*	%RSD
2	0.191±0.00041	0.21
4	0.284±0.0026	0.91
6	0.446±0.00148	0.33
8	0.545±0.00246	0.45
10	0.681±0.0050	0.73
12	0.841±0.00214	0.25
14	0.971±0.00148	0.15

*indicates (n=3)

Table 21.Calibration curve data for mixed std. solution of Atorvastatin calcium and Telmisartan at 246 nm and 296 nm

Conc.(µg/ml)	Mean±S.D (at 246 nm)	Mean±S.D (at 296nm)
2+2	0.298±0.0026	0.215±0.00015
4+4	0.540±0.0012	0.373±0.00045
6+6	0.740±0.0019	0.512±0.001
8+8	0.965±0.00045	0.674±0.0016
10+10	1.181±0.0087	0.823±0.0058
12+12	1.502±0.0048	1.052±0.0065
14+14	1.791±0.0014	1.254±0.0018

2) Precision

Intraday precision for both the drugs was done by analyzing three different concentrations (ng/ml) within linearity range, three times in a day (3*3 determinations).

Interday precision for both the drugs was done by analyzing three different concentrations (ng/ml) within linearity range, on three consecutive days.

Table 22.Intraday Precision for Atorvastatin calcium

Initial Conc.(µg/ml)	Conc. Obtained	Avg. ± S.D	%RSD
2	1.98	1.99±0.0208	1.04
	1.99		
	2.02		
4	4.01	4.00±0.0115	0.28
	3.99		
	4.02		
6	6.02	5.99±0.0251	0.41
	5.97		
	5.99		

Table 23.Interday Precision for Atorvastatin calcium

Initial Conc.(µg/ml)	Conc. Obtained	Avg. ± S.D	%RSD
2	1.95	1.95±0.0208	1.06
	1.94		
	1.98		
4	4.01	4.00±0.0116	0.28
	3.99		
	4.01		
6	5.97	5.99±0.0251	0.41
	6.02		
	5.99		

Table 24.Intraday Precision for Telmisartan

Initial Conc.(µg/ml)	Conc. Obtained	Avg. ± S.D	%RSD
2	1.97	1.98±0.036	1.82
	1.95		
	2.02		
4	4.01	4.01±0.030	0.74
	3.98		
	4.04		
6	6.01	5.96±0.040	0.67
	5.94		
	5.94		

Table 25.Interday Precision for Telmisartan

Initial Conc.(µg/ml)	Conc. Obtained	Avg. ± S.D	%RSD
2	1.96	1.96±0.01	0.51
	1.95		
	1.97		
4	4.00	3.98±0.037	0.95
	3.94		
	4.01		
6	6.00	5.97±0.030	0.51
	5.98		
	5.94		

3) Repeatability

Table 26.Repeatability for Atorvastatin calcium

Conc.(µg/ml)	Conc. obtained						Avg. ± S.D	%RSD
	1	2	3	4	5	6		
2	1.89	1.98	1.98	20.1	2.01	1.98	1.98±0.034	1.7
4	4.01	4.01	3.98	3.99	4.01	3.97	3.99±0.015	0.37
6	6.01	5.97	5.99	6.01	5.97	6.02	5.99±0.021	0.35

Table 27.Repeatability for Telmisartan

Conc(µg/ml)	Conc. obtained						Avg. ± S.D	%RSD
	1	2	3	4	5	6		
2	2.22	2.12	2.32	2.21	2.10	2.11	2.19±0.081	0.36
4	3.89	3.85	3.86	3.89	3.89	3.82	3.89 ±0.028	0.70
6	6.18	6.16	5.98	5.99	6.01	6.10	6.08 ±0.099	0.71

4) LOD and LOQ

For this determination Calibration curve for both the drugs was repeated six times. The LOD & LOQ were measured by using mathematical equations given below.

LOD = 3.3 x σ/S

LOQ = 10 x σ/S

Where,

σ = Standard deviation of the Intercept

S = Slope of calibration curve

5) Accuracy

Accuracy of the measurement of Atorvastatin calcium and Telmisartan was determined by standard addition method. Standard addition was done at three levels, 80%, 100% and 120% of a concentration in the linearity range.

Table 28.Accuracy observed for Atorvastatin calcium

Sample conc.(µg/ml)	Std addition (%)	conc after spiking(µg/ml)	Conc.recovered (µg/ml) Mean±S.D*	% Recovery Mean±S.D*
4	80%	7.2	7.18±0.017	99.54±0.058
4	100%	8	7.97±0.017	99.68±0.109
4	120%	8.8	8.74±0.005	99.54±0.005

*indicates n=3

Table 29.Accuracy observed for Telmisartan

sample conc.(µg/ml)	Std addition (%)	Conc. after spiking(µg/ml)	Conc. recovered (µg/ml) Mean±S.D*	% Recovery Mean±S.D*
4	80%	7.2	7.15±0.03	99.30±0.416
4	100%	8	7.96±0.015	99.54±0.190
4	120%	8.8	8.75±0.005	99.46±0.065

*indicates n=3

Table 30.Validation Parameters for Atorvastatin calcium

Parameters	Atorvastatin calcium	Telmisartan
Linearity range	2-14 µg/ml	2-14 µg/ml
Regression equation (at 246 nm)	Y = 0.047x + 0.0266	Y = 0.0163x – 0.0131
Correlation coefficient (r^2) (at 246 nm)	0.9970	0.9980
Regression equation (at 296 nm)	Y = 0.0634x + 0.0549	Y = 0.0659x + 0.0386
Correlation coefficient (r^2) (at 296 nm)	0.9960	0.9960
Intraday precision	0.28% - 1.04%	0.67% - 1.82%
Interday precision	0.28% - 1.06%	0.51% - 0.95%
Repeatability	0.35% -1.7%	0.36% - 0.71%
LOD (µg/ml)	0.26	0.09
LOQ (µg/ml)	0.80	0.29
Accuracy	99.54±0.058% to 99.68±0.109%	99.35±1.9 % to 99.96±1.76 %

5.2.4.2 Analysis of Tablet Samples.

The developed method was used to estimate Atorvastatin calcium and Telmisartan in the tablet dosage form. Five different brands of tablet formulations were procured from the market for analysis by the proposed method.

5.2.5 Conclusion

The proposed UV method was fast, accurate, precise and sensitive for determination of Atorvastatin calcium and Telmisartan in combined dosage forms. High recoveries show that the method is free from the interference from excipients used in the commercial pharmaceutical preparations. Hence, if can be successfully applied for routine estimation for Atorvastatin calcium and Telmisartan in quality control laboratories. The results of validation parameters are satisfactory level indicates the accuracy of proposed method for estimation of Atorvastatin calcium and Telmisartan.

5.3 DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR SIMULTANEOUS ESTIMATION OF ATORVASTATIN CALCIUM AND TELMISARTAN IN THEIR COMBINED DOSAGE FORMS

5.3.1 Abstract

A High Performance Thin Layer Chromatography method was developed for the simultaneous estimation of Atorvastatin and Telmisartan calcium in their combined dosage forms. The separation was achieved by TLC silica gel 60 F_{254} plates.

The method has been validated for linearity, accuracy, precision and migration rate of solvent front. Linearity for Atorvastatin calcium and Telmisartan were found in the range of 200-2000 ng/spot. The developed method was found to be precise, selective and rapid for simultaneous estimation of Atorvastatin calcium and Telmisartan in their combined dosage forms.

5.3.2 Instrumentation

1) HPTLC

- **Camag Applicator Linomat 5:**
  Semiautomatic application, band application by spray on technique (2 - 500µl)
- **Camag twin trough glass chamber:** (10 x 10 cm and 20 x 10 cm)
- **Camag TLC scanner 3:**
  Scanning speed up to 100mm/s, Spectral range 190 – 800nm
- **Camag UV cabinet with dual wavelength UV lamp:**
  Dual wavelength 254 / 366nm
- **Stationary Phase:** Pre- coated Silica gel on aluminum sheet G60 F_{254}
- **Camag 100µl Applicator syringe (Hamilton, Bonaduz, Schweiz)**
- **Data Resolution:** 100µm/step

2) Spectrophotometer

- **Model:** SHIMADZU 2450 double beam spectrophotometer, version 2.21
- **Slit width:** 1 nm

3) Analytical Balance

- **Model :** KEROY®
- **Manufacturer:** Keroy (Balance) Pvt. Ltd.

- **Capacity :** 0.0001 to 100 g

4) Sonicator

- **Model:** Trans-O-Sonic, D- compact
- **Capacity**: 2 L

5.3.3 Materials and methods

5.3.3.1 Reagents and chemicals

- Methanol (AR Grade, S.D. Fine chemicals Ltd., Mumbai, India)
- Chloroform (AR Grade, S.D. Fine chemicals Ltd., Mumbai, India)
- Tablets containing Atorvastatin calcium (10 mg) and Telmisartan (40 mg).
    1. (Brand name: **Telsartan™-ATR;** Name of manufacturer: **DR.REDDY'S)**
    2. (Brand name: **Arbitel-AV;** Name of manufacturer: **MICRO LABS LIMITED)**
    3. (Brand name: **TELDAY AV;** Name of manufacturer: **TORRENT PHARMACEUTICALS LTD.)**
    4. (Brand name: **TELEACT ST 40;** Name of manufacturer: **RANBAXY LAB. LTD.)**
- Tablets containing Atorvastatin calcium (10 mg) and Telmisartan (20 mg).
    1. (Brand name: **TELEACT ST 20;** Name of manufacturer: **RANBAXY LAB. LTD.)**
- API Atorvastatin calcium (gift sample from the Alembic Pharmaceutical Ltd.)
- API Telmisartan (gift sample from the Torrent Pharmaceutical Ltd.)

5.3.3.2 HPTLC Conditions

- **Slit dimension:** 4 x 0.45 mm (micro)
- **Band width:** 4 mm
- **Syringe capacity:** 100 µl

5.3.3.3 Preparation of Mobile Phase

A mixture of 8 ml of chloroform and 2 ml of methanol was mixed properly and it was used as a mobile phase.

5.3.3.4 Preparation of Standard Stock Solution of Atorvastatin calcium

100 mg Atorvastatin calcium was weighed accurately and dissolved in 100 ml methanol. 1 ml aliquots of the above solution were diluted to 10 ml with methanol to produce 100 µg/ml of Atorvastatin calcium in methanol.

5.3.3.5 Preparation of Standard Stock Solution of Telmisartan

100 mg Telmisartan was weighed accurately and dissolved in 100 ml methanol. 1 ml aliquots of the above solution were diluted to 10 ml with methanol to produce 100 µg/ml of Telmisartan in methanol.

5.3.3.6 HPTLC Analysis

1) Activation of Silica gel plate

Analysis was performed on 20 cm × 20 cm TLC silica gel 60 F_{254} plates (EM Science, Gibbstown, NJ, USA, an affiliate of Merck KGaA, Darmstadt, Germany). Layers were cleaned by predevelopment to the top with methanol and dried in a hot air oven at 60ºC for 20 minutes.

2) Spotting

Standard zones were applied to the layer as bands by Means of a Camag (Wilmington, NC, USA) Linomat V semi-automated spray-on applicator equipped with a 100-µl syringe and operated with the settings band length 4 mm, application rate 15 nl/s, distance between bands 4 mm, distance from the plate side edge 10 mm, and distance from the bottom of the plate 80 mm.

3) Development

Spotted plates were developed in saturated HPTLC twin-through chamber up to 80 mm solvent front. HPTLC twin-trough chamber is presaturated with mobile phase for 15 minutes with help of filter papers. After development, plates were removed immediately and dried in oven at 60ºC for 5 minutes.

4) Scanning of Plates

The developed plates were scanned with the help Camag TLC Scanner III with a deuterium and tungsten source, slit dimension was 4 × 0.45 mm, and a scanning rate of 10 mm s^{-1} using WINCATS-3 software.

5.3.3.7 Method validation

1) Preparation of Linearity Curve

For Atorvastatin and Telmisartan, (2.0-20.0 µl) volumes of the HPTLC standard solution (200-2000 ng/spot) were applied to the plate. Plates were developed and peak area of the spots was measured at 246 nm in the absorbance mode with Camag TLC scanner III. Calibration curve of peak area v/s concentration was plotted for the drug.

2) Precision

- **Intraday and Interday precision**

  Intraday and interday precision for the HPTLC developed were measured in terms of % RSD. The experiment was repeated three times in a day using three different concentration (3x3) for intraday precision and three different concentrations on three different days for interday precision.

- **Reproducibility**

  The reproducibility of the method was determined by using two different Analysts. The standard solution was spotted 7 times on the same plate and peak area was recorded. Reproducibility of the method was measured in terms of %RSD.

- **Repeatability**

  Peak area of standard solution prepared for calibration curve was scanned for seven times and % RSD was calculated.

3) LOD and LOQ

From the linearity curve equation, the standard deviation (SD) of the intercepts (response) was calculated. Then LOD and LOQ were measured by using mathematical expressions.

4) Accuracy

Accuracy of the method was determined by recovery study by standard addition method at three different levels (80%, 100% and 120%).

5) Flow Constant

The flow constant or velocity constant (K) is a measure of the migration rate of the solvent front. It is an important parameter for HPTLC and can be used to calculate development

times with different separation distances, provided that the sorbent, solvent system, chamber type and temperature remain constant. The flow constant is given by the following equation:

$$K = \frac{Z_F}{t}$$

K = Flow constant (mm^2/s)

Z_F = Distance between the solvent front and the solvent level [mm]

t = Development time [s].

5.3.3.8 Analysis of Tablets

Total 20 tablets (or chewable tablets) were weighed accurately and powdered. An amount of equivalent to one tablet (10 mg of Atorvastatin calcium and 40 mg Telmisartan) was dissolved in 100 ml methanol in 100 ml volumetric flask. The solution was sonicated for 15 minutes. The solution was filtered by using Whatmann filter No.41. Above solution containing 100 µg/ml of Atorvastatin and 400 µg/ml of Telmisartan. 4 µl of this solution was spotted on activated TLC plate.

5.3.4 RESULTS AND DISCUSSION:

Selection and optimization of solvent and mobile phase:

- Selection and optimization of a proper mobile phase is a challenging task in HPTLC method development. Several factors affects the selection of mobile phase such as polarity of the drugs, desired R_f values, practical problems such as diffusion of spots, tailing, proper peak shape after scanning.

5.3.4.1 Validation Parameters

1) Linearity

Linearity range of Atorvastatin calcium and Telmisartan were found to be 200-2000 ng/spot with correlation co-efficient 0.9980and 0.9970 respectively.

Table 33.Calibration data of Atorvastatin calcium by HPTLC with UV detection

Sr. No.	Concentration (µg/spot)	Peak Area		R_f
		Mean ± SD*	%RSD	
1	200	2990±25.23	0.84	0.22
2	500	5442±50.41	0.92	0.22
3	700	6887±12.47	0.18	0.22
4	1000	9039±18.85	0.20	0.22
5	1200	10400±69.96	0.67	0.22
6	1500	12339±68.52	0.55	0.22
7	2000	15850±28.52	0.17	0.22

*indicates (n=3)

Figure 10.Linearity curve for Atorvastatin calcium

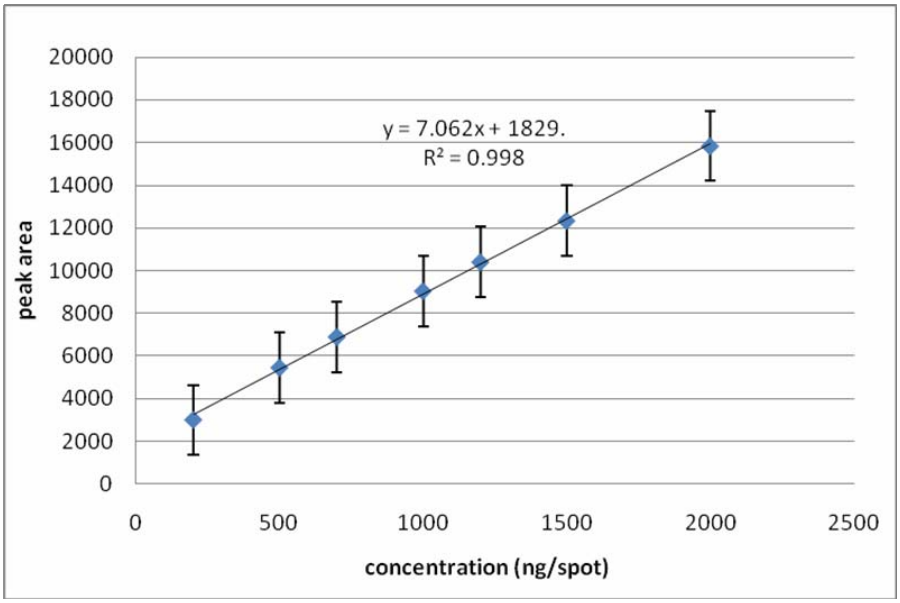


Figure 11.Linearity curve for Atorvastatin from winCATS software

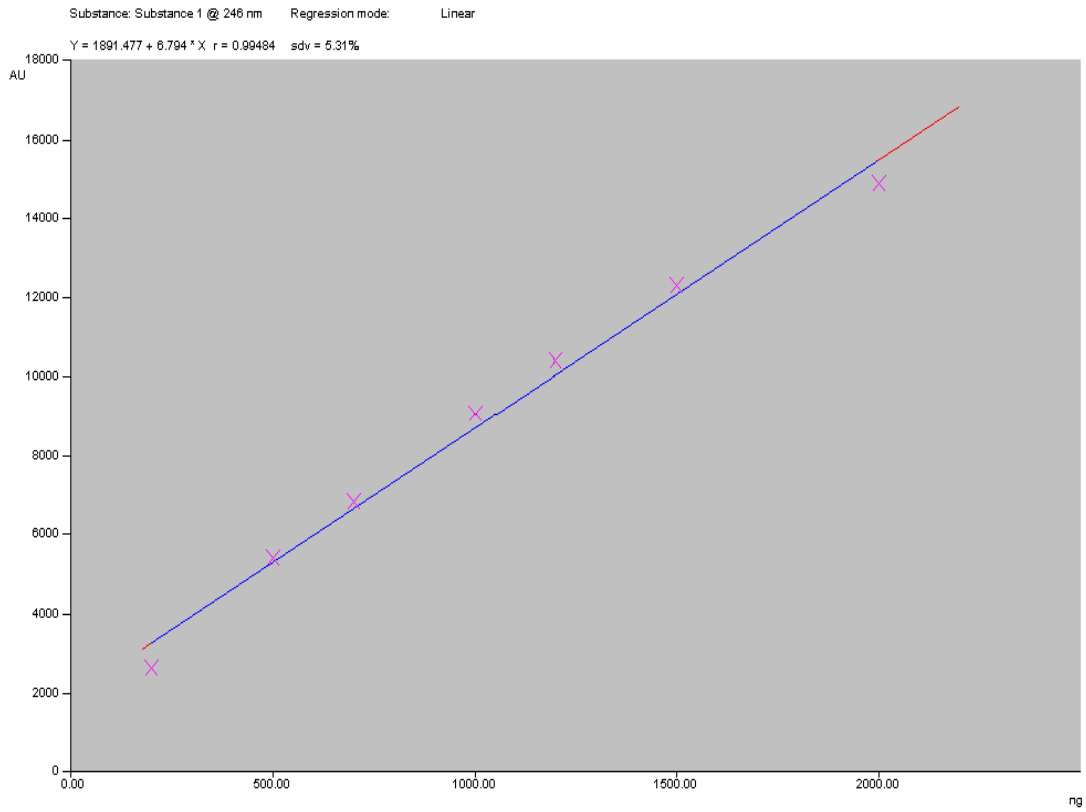


Table 34.Calibration data of Telmisartan by HPTLC with UV detection

Sr. No.	Concentration (µg/spot)	Peak Area		R_f
		Mean ± SD*	%RSD	
1	200	2890±20.95	0.72	0.57
2	500	5438±54.76	1.0	0.57
3	700	6530±12.65	0.19	0.57
4	1000	8239±10.78	0.13	0.57
5	1200	9725±60.67	0.62	0.57
6	1500	11450.2±53.84	0.46	0.57
7	2000	14820.5±20.99	0.14	0.57

*indicates (n=3)

Figure 12.Linearity curve for Telmisartan

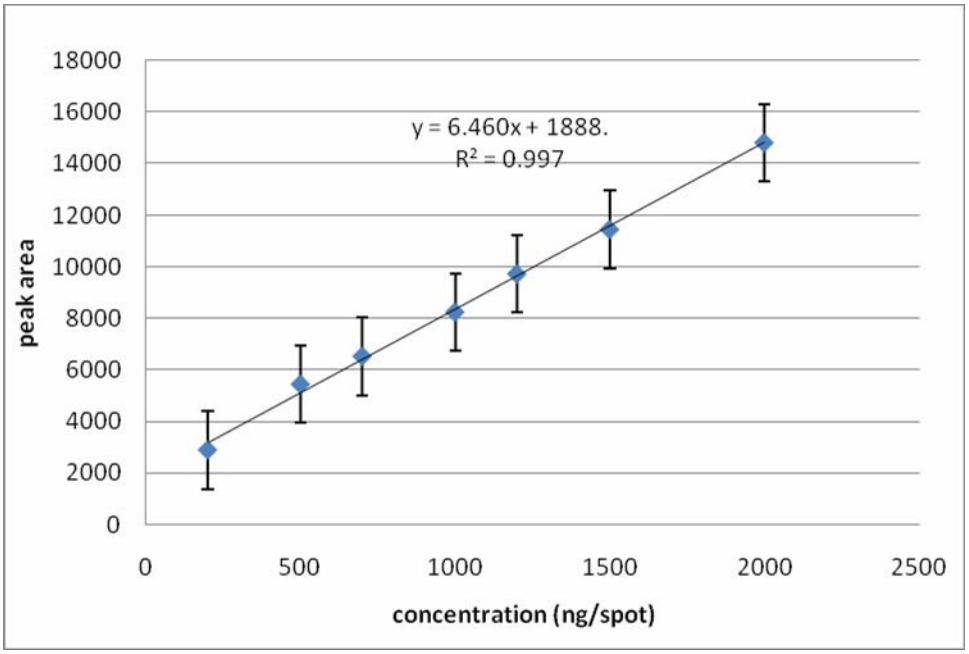


Figure 13.Linearity curve for Telmisartan from winCATS software

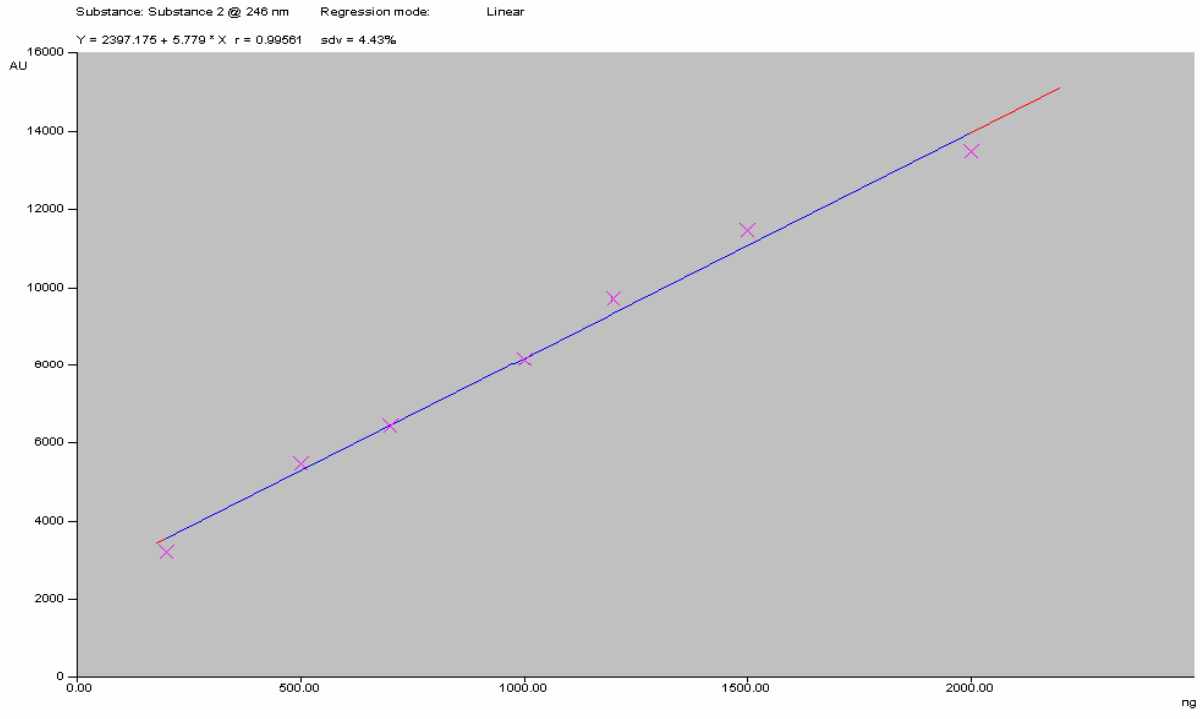


Figure 14.HPTLC chromatogram of Atorvastatin calcium (R_f = 0.22) and Telmisartan (R_f = 0.57) standard mixture.

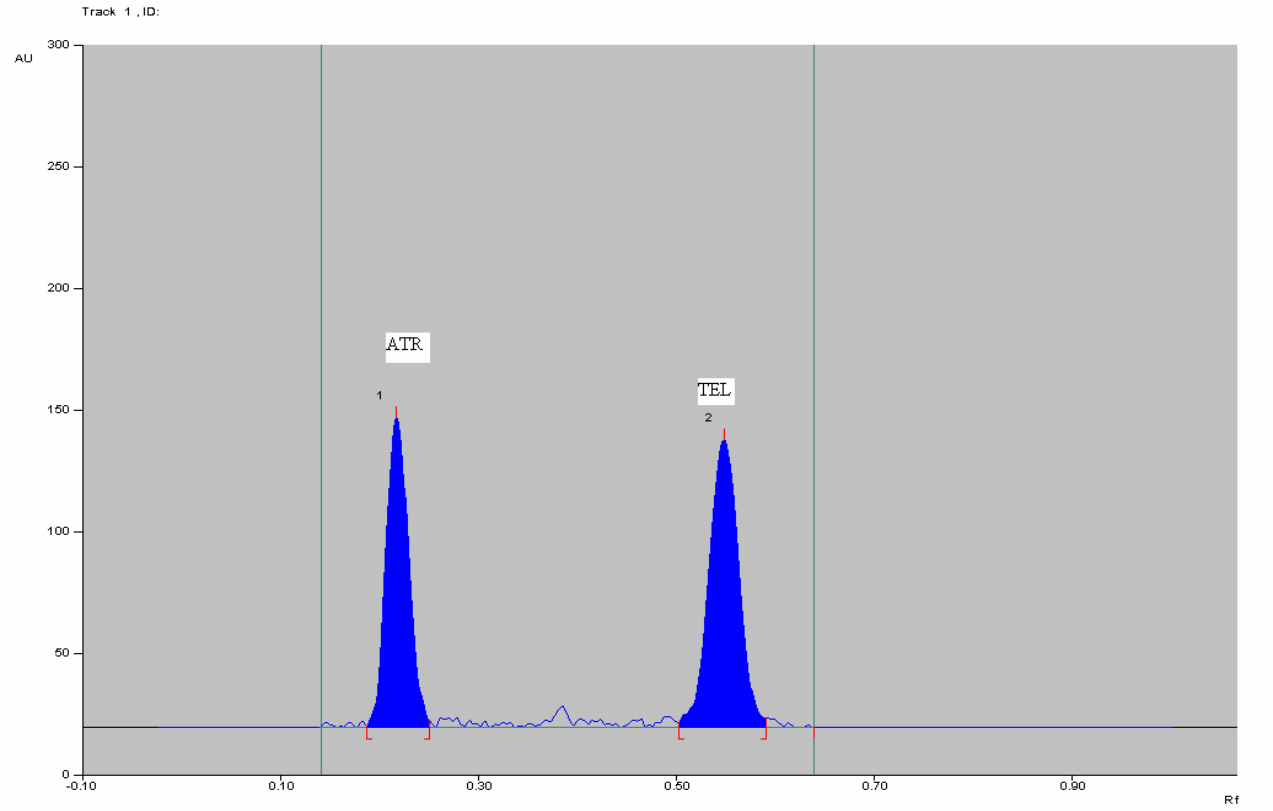
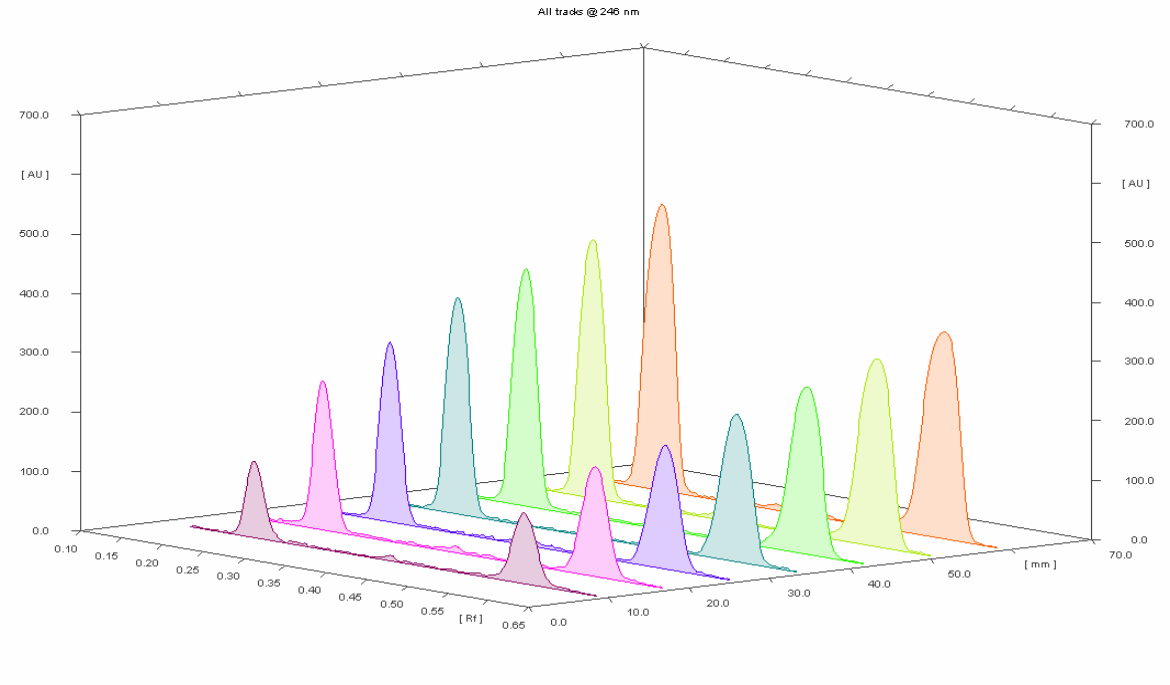


Figure 15.HPTLC chromatogram (3D view) for linearity of Atorvastatin calcium and Telmisartan



2) Repeatability

Table 35.Repeatability data of Atorvastatin calcium by HPTLC with UV detection

Time	Peak Area	Rf
1st	5442	0.22
2nd	5487	0.22
3rd	5489	0.21
4th	5875	0.22
5th	5574	0.21
6th	5478	0.22
7th	5547	0.22
Mean	5550	0.2174
S.D.	48.37	0.0048
%RSD	1.42	2.24

Table 36.Repeatability data of Telmisartan by HPTLC with UV detection

Time	Peak Area	Rf
1st	5438	0.57
2nd	5512	0.57
3rd	5418	0.57
4th	5487	0.57
5th	5519	0.57
6th	5481	0.57
7th	5389	0.58
Mean	5463	0.5714
S.D.	49.26	0.00378
%RSD	0.90	0.66

3) Intraday and Interday Precision:

Intraday precision for both the drugs was done by analyzing three different concentrations (ng/ml) within linearity range, three times in a day (3*3 determinations).

Interday precision for both the drugs was done by analyzing three different concentrations (ng/ml) within linearity range, on three consecutive days.

Table 37.Intraday precision data of Atorvastatin calcium by HPTLC with UV detection

Sr. No.	Concentration (ng/spot)	Peak Area		R_f
		Mean ± SD*	%RSD	
1	200	2934±39.31	1.33	0.23
2	500	5474±44.90	0.82	0.22
3	700	6782±32.78	0.48	0.22

*indicates (n=3)

Table 38.Interday precision data of Atorvastatin calcium by HPTLC with UV detection

Sr. No.	Concentration (ng/spot)	Peak Area		R_f
		Mean ± SD*	%RSD	
1	200	2910±32.78	1.12	0.23
2	500	5547±170.10	1.42	0.22
3	700	6864±51.72	0.80	0.22

*indicates (n=3)

Table 39.Intraday precision data of Telmisartan by HPTLC with UV detection

Sr. No.	Concentration (ng/spot)	Peak Area		R_f
		Mean ± SD*	%RSD	
1	200	2857±39.31	1.37	0.58
2	500	5475±40.24	0.73	0.57
3	700	6589±82.9	1.2	0.57

*indicates (n=3)

Table 40.Interday precision data of Telmisartan by HPTLC with UV detection

Sr. No.	Concentration (ng/spot)	Peak Area		R_f
		Mean ± SD*	%RSD	
1	200	2881±39.31	1.36	0.57
2	500	5386±38.24	0.70	0.58
3	700	6679±83.8	1.25	0.57

*indicates (n=3)

4) Accuracy

Accuracy of the measurement of Atorvastatin calcium and telmisartan was determined by standard addition method. Standard addition was done at three levels, 80%, 100% and 120% of a concentration in the linearity range.

Table 41.Accuracy data of Atorvastatin calcium by HPTLC with UV detection

Initial conc. (ng/spot) (A)	Std. Added (%) (ng/spot) (B)	Conc. After spiking (A + B)	Accuracy	
			Conc. Recovered Mean±S.D.*	%Recovery Mean±S.D*
500	80%	900	882.08±44.48	98.0±0.41
500	100%	1000	995.15±66.52	99.51±0.70
500	120%	1200	1097.56±89.18	99.77±0.28

*indicates (n=3)

Table 42.Accuracy data of telmisartan by HPTLC with UV detection

Initial conc. (ng/spot) (A)	Std. Added(%) (ng/spot) (B)	After spiking (A + B)	Accuracy	
			Conc. Recovered Mean±S.D.*	%Recovery Mean±S.D*
500	80%	900	907.08±129.63	100.7±0.35
500	100%	1000	983.51±44.82	98.35±0.41
500	120%	1200	1094.35±91.59	99.46±0.74

*indicates (n=3)

5) Specificity (peak purity)

Figure 16.Peak purity spectra of Atorvastatin calcium and Telmisartan

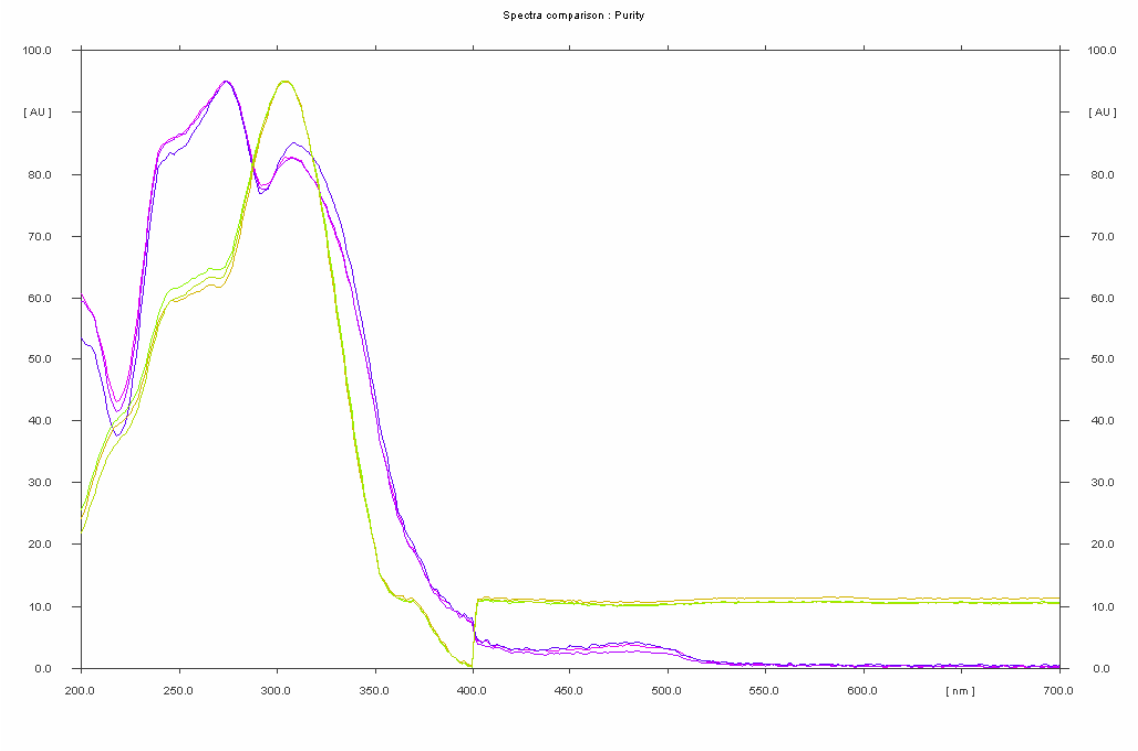


Table 43.Specificity data of Atorvastatin calcium and Telmisartan

Drugs	**Correlation coefficient**	**purity**
Atorvastatin calcium	0.999978	Pass
Telmisartan	0.999994	pass

6) Limit of detection

The minimum detectable concentration of Atorvastatin calcium was found to be **5.67 ng/spot.**

The minimum detectable concentration of Telmisartan was found to be **3.96 ng/spot**.

7) Limit of quantification

Limit of quantification for Atorvastatin calcium and Telmisartan was found as per the procedure given in section 7.5

The lowest quantifiable concentration of Atorvastatin calcium was found to be **17.21 ng/spot**

The lowest quantifiable concentration of Telmisartan was found to be **12.02 ng/spot**

Table 44.Summary of Validation parameters by HPTLC with UV detection

Sr. No	Parameters	Atorvastatin calcium	Telmisartan
1	Linearity range (ng/spot)	200-2000	200-2000
2	Regression equation	y = 7.062x + 1829.3	y = 6.46x + 1889
3	Correlation coefficient (r^2)	0.9980	0.9970
4	Intercept	1829	1889
5	Slope	7.0624	6.46
6	Precision Intraday % RSD (n = 3) Interday % RSD (n = 3) Repeatability of measurements % RSD	 0.87 1.11 1.42	 1.1 1.10 0.90
7	Limit of detection	5.67	3.96
8	Limit of quantification	17.21	12.02
9	Specificity	Specific	Specific
10	Recovery	98.0±0.41 % to 99.77±0.28 %	98.35±0.41 % to 98.35±0.41 %

5.3.4.2 Analysis of Tablets

The developed method was used to estimate Atorvastatin calcium and Telmisartan in the tablet dosage form. Five different brands of tablet formulations were procured from the market for analysis by the proposed method. The percentage of Atorvastatin calcium and Telmisartan was found from the calibration curve.

Figure 17.HPTLC chromatogram of assay of tablet samples (400 ng/spot)

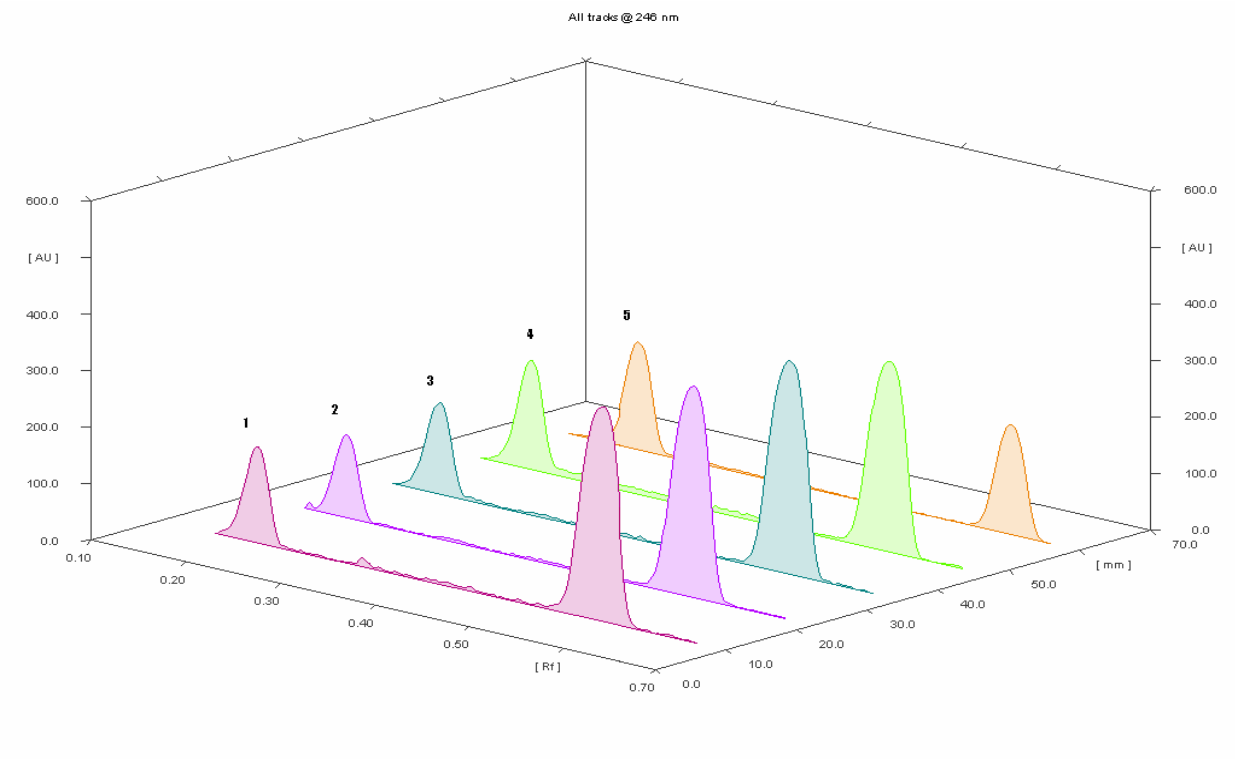


Figure 16.HPTLC chromatogram of assay of tablet samples (400 ng/spot)

Track 1: Telsartan™-ATR (Telmisartan-40mg, Atorvastatin calcium-10mg)

Track 2: Arbitel-AV (Telmisartan-40mg, Atorvastatin calcium-10mg)

Track 3: TELDAY AV (Telmisartan-40mg, Atorvastatin calcium-10mg)

Track 4: TELEACT ST 40 (Telmisartan-40mg, Atorvastatin calcium-10mg)

Track 5: TELEACT ST 20 (Telmisartan-20mg, Atorvastatin calcium-10mg)

5.3.5 Conclusion:

By the virtue of the developed method, it can be concluded that High performance thin layer chromatography method is a reliable technique for the analysis of commercial formulations of Atorvastatin calcium and Telmisartan, The developed method is simple sensitive and specific which renders it suitable for routine analysis of Atorvastatin calcium and Telmisartan from its combined dosage form. A good % recovery for both the drugs shows that the developed method is free of the interference of excipients used in the formulation.

5.4 DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF ATORVASTATIN CALCIUM AND TELMISARTAN IN THEIR COMBINED DOSAGE FORMS

5.4.1 Abstract

A simple, specific, accurate reversed phase liquid chromatographic method was developed for the determination of Atorvastatin calcium and Telmisartan in their pharmaceutical dosage forms. A phenomenex column (150 mm×4.6mm i.d., 5µm) was used, and U.V detection at 246 nm. The linearity for Atorvastatin calcium and Telmisartan was in the range of 0.5-16 µg/ml. Five dosage forms of Atorvastatin calcium and Telmisartan were successfully analyzed using the developed method. High percentage recovery shows that there is no interference of excipients in the dosage form. The developed method was found to be precise, selective and rapid for simultaneous estimation of Atorvastatin calcium and Telmisartan in their combined dosage forms.

5.4.2 Instrumentation

High performance liquid chromatography

- **Model**: JASCO 200 Series
- **Manufacturer**: JASCO, Inc. JAPAN
- **Pump**: JASCO PU-2080 plus
- **Mixer** : JASCO MX-2080-31
- **Injector**: Rheodyne model 7125 with 20 µl fixed loop
- **Detector**: JASCO-UV-2075 PLUS
- **Software**: Borwin software version 1.50 was used

P^H METER

- **Model**: 111E/101E
- **Manufacture**: Analabs scientific instruments ltd.
- **PH:** 0 to 14
- **Resolution**: ±0.01 ph
- **Accuracy**: ±0.01 P^H±0.01 P^H±14 digit

Analytical Balance

- **Model:** KEROY®
- **Manufacturer**: Keroy (Balance) Pvt. Ltd.

- **Capacity:** 0.0001 to 100 g

Sonicator

- **Model:** Trans-O-Sonic, D- compact
- **Capacity**: 2 L

5.4.3 Materials and methods

5.4.3.1 Chemicals and Materials

- Methanol (HPLC Grade, S.D. Fine chemicals Ltd., Mumbai, India)
- Acetronitrile (HPLC Grade, S.D. Fine chemicals Ltd., Mumbai, India)
- Triple distilled water
- Tablets containing Atorvastatin calcium (10 mg) and Telmisartan (40 mg).
    1. (Brand name: **Telsartan™-ATR;** Name of manufacturer: **DR.REDDY'S)**
    2. (Brand name: **Arbitel-AV;** Name of manufacturer: **MICRO LABS LIMITED)**
    3. (Brand name: **TELDAY AV;** Name of manufacturer: **TORRENT PHARMACEUTICALS LTD.)**
    4. (Brand name: **TELEACT ST 40;** Name of manufacturer: **RANBAXY LAB. LTD.)**
- Tablets containing Atorvastatin calcium (10 mg) and Telmisartan (20 mg).
    1. (Brand name: **TELEACT ST 20;** Name of manufacturer: **RANBAXY LAB. LTD.)**
- API Atorvastatin calcium (gift sample from the Alembic Pharmaceutical Ltd.)
- API Telmisartan (gift sample from the Torrent Pharmaceutical Ltd.)

5.4.3.2 Chromatographic Conditions

- **Stationary phase:** Phenomenex column (150 mm × 4.6mm., i.d., 5µm)

Optimization of the chromatographic condition was studied by checking the effect of chromatographic variables such as temperature, back pressure, flow rate and solvent ratio. The resulting chromatograms were recorded and the chromatographic parameters which give best peak resolution were selected for further study.

5.4.3.4 Preparation of Standard Stock Solution of Atorvastatin calcium and Telmisartan

100 mg Atorvastatin calcium and Telmisartan was weighed accurately and dissolved each standard drug in separately in 100 ml methanol in different volumetric flasks. 1 ml aliquots of the above solutions were diluted to 10 ml with methanol in different volumetric flasks to produce 100 µg/ml of Atorvastatin calcium and 100 µg/ml of Telmisartan.

5.4.3.5 Preparation of Standard Solution of Atorvastatin calcium and Telmisartan

Aliquots of 0.5, 1, 2, 4, 8, 10, 12, 14, 16 µg/ml were prepared by using this stock solution of Atorvastatin calcium and aliquots of 2, 4, 6, 8, 10, 12, 16 µg/ml were prepared by using this stock solution of Telmisartan, for preparation of calibration curve.

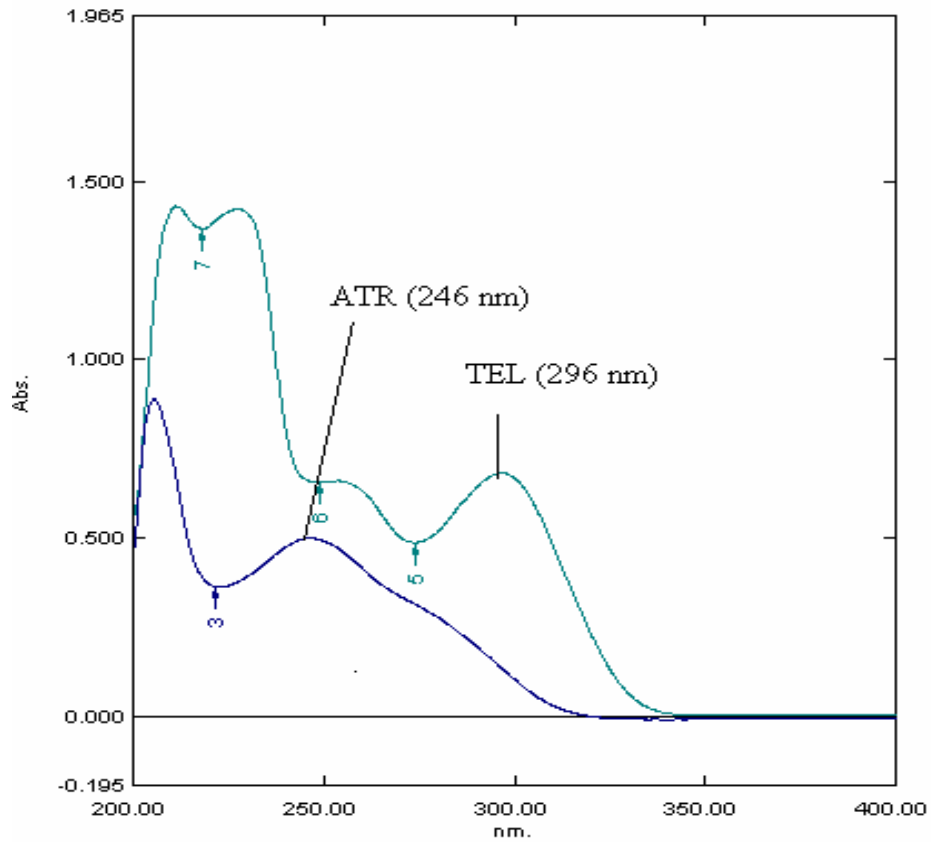
5.4.3.5 Preparation of binary mixtures of Atorvastatin calcium and Telmisartan

Suitable aliquots of standard stock solution of Atorvastatin calcium and Telmisartan are mixed and diluted to volume with methanol to obtain different binary mixture solutions containing Atorvastatin calcium and Telmisartan in 1:1 ratio. Concentration of solution in the range 0.5 -16 µg/ml was prepared for the calibration curve of both drugs.

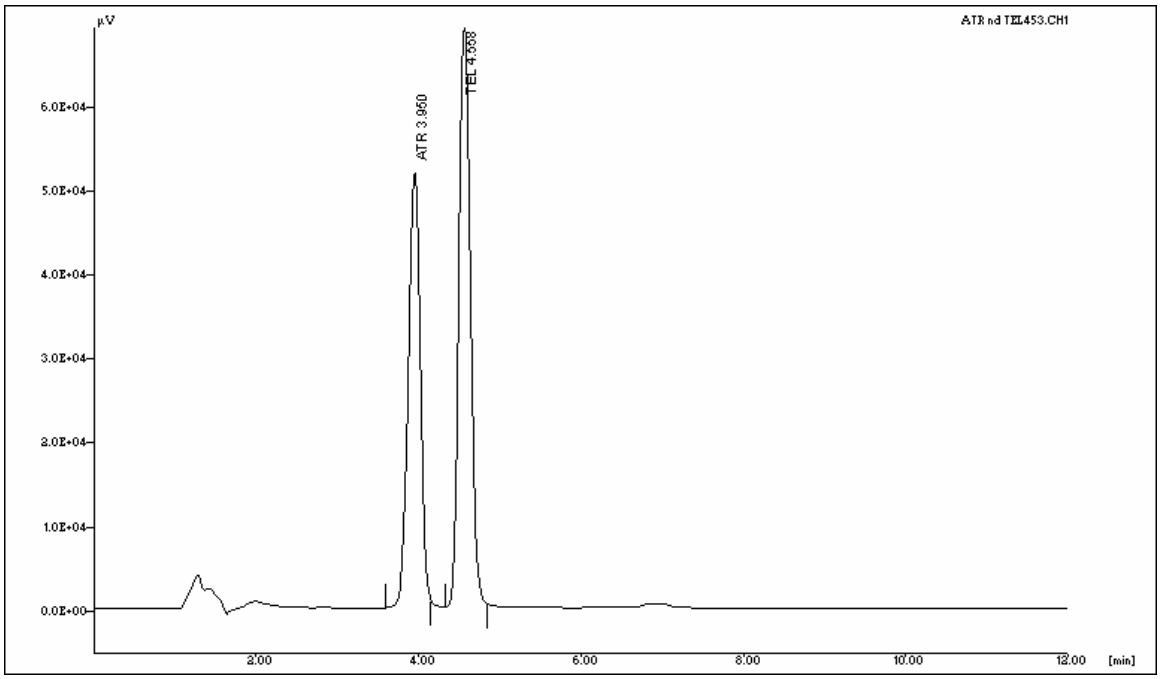
5.4.3.6 Selection of wavelength for detection

The standard solution 10 µg/ml of Atorvastatin calcium and Telmisartan was prepared in methanol. The drug solution is scanned in the UV region of 200-400 nm using SHIMADZU 2450 double beam spectrophotometer, version 2.21 and the spectra were recorded. Here we selected 246 nm Wavelength for detection. At this wavelength both drugs show considerable absorbance.

Figure 18.Overlain spectra of Atorvastatin calcium and Telmisartan



5.4.3.7 Optimization of mobile phase



5.4.3.8 Method validation

1) Preparation of Linearity Curve

For estimation of Atorvastatin calcium, calibration curve (n=6) was plotted in the range of 0.5-16 µg/ml. For estimation of Telmisartan, calibration curve (n=6) was plotted in the range of 0.5-16 µg/ml.

2) Precision

For Atorvastatin calcium, intraday precision was carried out by taking three different concentrations (4, 8, 10 µg/ml) repeated for 3 times in a day and interday precision was carried out by taking three different concentrations (4, 8, 10 µg/ml) repeated on three different days. For Telmisartan, intraday precision was carried out by taking three different concentrations (4, 8, 10 µg/ml) repeated for 3 times in a day and interday precision was carried out by taking three different concentrations (4, 8, 10 µg/ml) repeated on three different days.

3) LOD and LOQ

For this determination Calibration curve for both the drugs was repeated six times. The LOD & LOQ were measured by using mathematical equations given below.

LOD = 3.3 x σ/S

LOQ = 10 x σ/S

Where,

σ = Standard deviation of the Intercept

S = Slope of calibration curve

4) Accuracy

4 µg/ml standard solution for Atorvastatin calcium and 4 µg/ml standard solution for Telmisartan. After that accuracy of the method was determined by standard addition method at three different levels (80%, 100% and 120%).

5.4.3.9 Analysis of Tablet Samples.

Total 20 tablets were weighed accurately and powdered. An amount equivalent to one tablet (containing 10 mg of Atorvastatin calcium and 40 mg of Telmisartan) was taken and dissolved in 100 ml methanol in 100 ml volumetric flask. The solution was sonicated for

15 minutes and then diluted with methanol to the mark. The solution was filtered by using Whatmann filter No.41. From this solution, 0.32 ml of sample solution was taken and diluted with 10 ml methanol to get the final solution containing 3.2 µg/ml concentration of Atorvastatin calcium and 12.8 µg/ml concentration of Telmisartan .

5.4.4 Result and discussion

5.4.4.1. Validation Parameters

1) Linearity

Linearity range of Atorvastatin calcium and Telmisartan were found to be 0.5 - 16 µg/ml with correlation co-efficient 0.9990 and 0.9990 respectively.

Table 47.Calibration data of Atorvastatin calcium by RP-HPLC with UV detection

Sr. No.	Concentration (µg/ml)	Peak Area	
		Mean ± SD*	%RSD
1	0.5	19478 ±41.01	0.21
2	1	50991 ±622.254	1.20
3	2	96283 ±1109.45	1.14
4	4	199832 ±269.40	0.13
5	8	396629 ±1060.66	0.26
6	10	499580 ±707.10	0.14
7	12	627852 ±7071.068	1.11
8	16	839688 ±7141.78	0.84

*indicates (n=3)

Figure 24.Linearity curve for Atorvastatin calcium

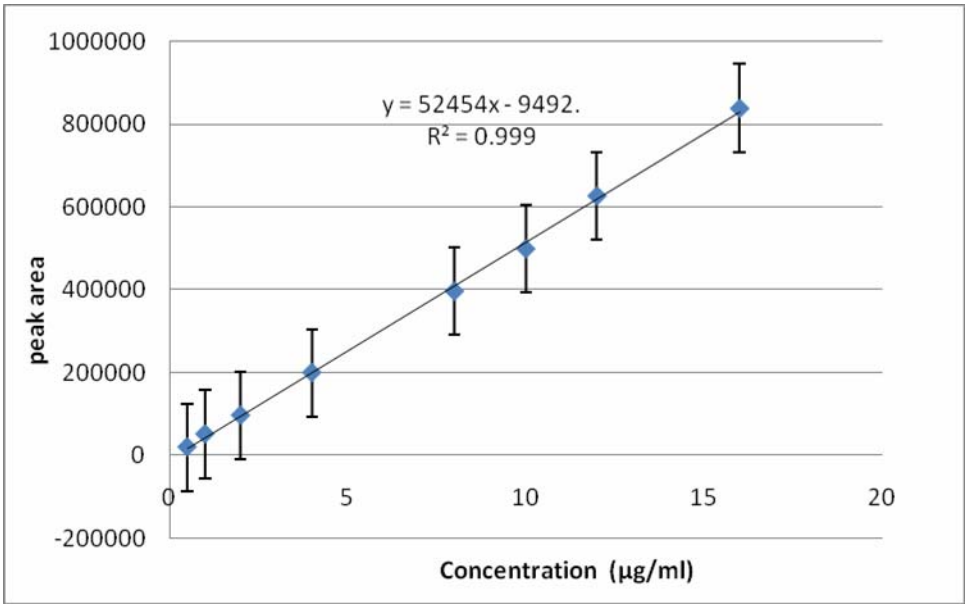


Table 48.Calibration data of Telmisartan by RP-HPLC with UV detection

Sr. No.	Concentration (µg/ml)	Peak Area	
		Mean ± SD*	%RSD
1	0.5	19846±74.95	0.37
2	1	64297±707.10	1.09
3	2	121191±2126.27	1.73
4	4	242382±2758.42	1.12
5	8	489398±1376.73	0.28
6	10	606809±7071.06	1.15
7	12	764454±3181.98	0.41
8	16	1012124±5656.85	0.55

*indicates (n=3)

Figure 25.Linearity curve for Telmisartan

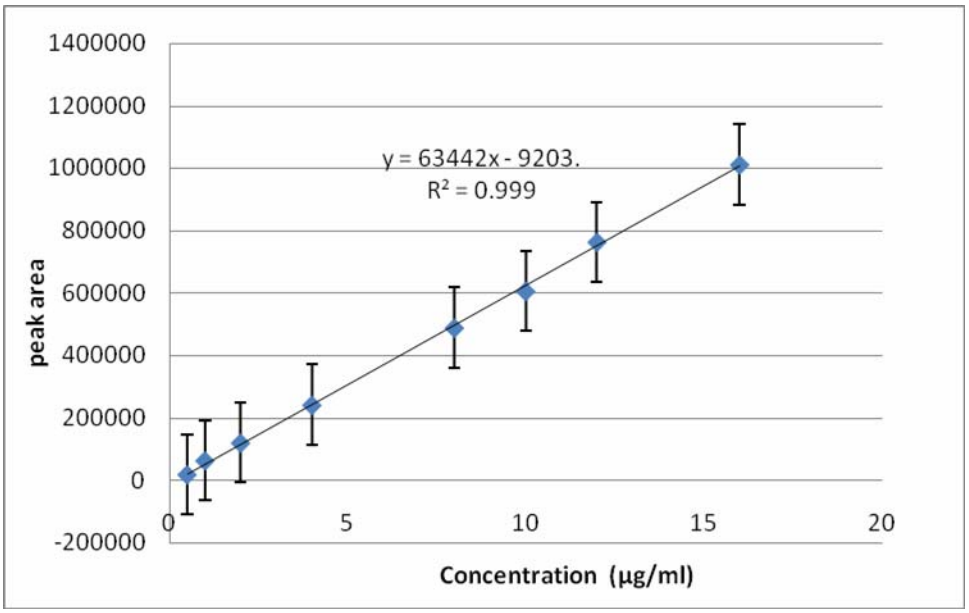
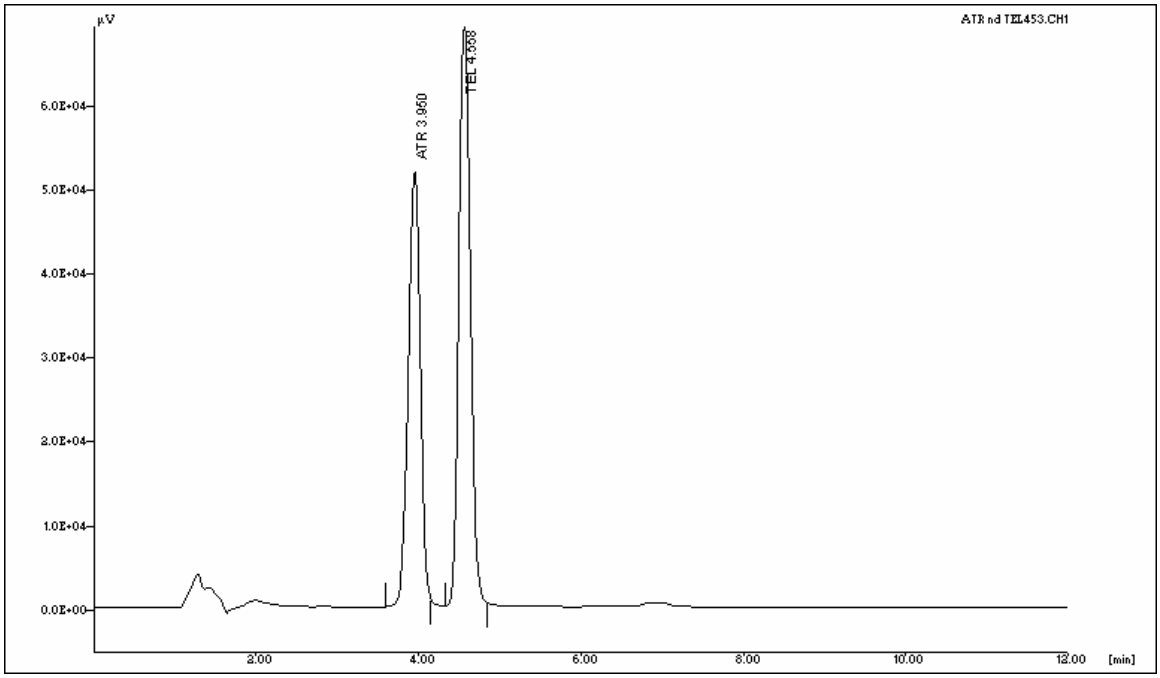


Figure 20.Rp-HPLC chromatogram of Atorvastatin calcium (R_T = 4.0) and Telmisartan (R_T = 4.6) standard mixture.



2) Repeatability

Table 49.Repeatability data of Atorvastatin calcium

Conc.	Peak area							Mean± S.D	%RSD
	1	2	3	4	5	6	7		
4	199832	199956	199854	198545	199485	198741	198474	197484 ± 659.89	0.33
8	393890	395074	395388	395023	395348	395074	403443	396177.1±3242.9	0.81
10	499580	499885	499854	499874	498741	499851	498854	499519.9±505.57	0.10

Table 50.Repeatability data of Telmisartan

Conc.	Peak area							Mean± S.D	%RSD
	1	2	3	4	5	6	7		
4	242382	245898	246895	248785	248978	245632	248421	246713±1488.64	0.603
8	489398	487898	485698	485687	487887	485696	489563	487403.9±1627.9	0.334
10	606809	606845	606320	608945	609845	606785	606531	607440±1472.84	0.242

3) Intraday and Interday Precision:

Intraday precision for both the drugs was done by analyzing three different concentrations (ng/ml) within linearity range, three times in a day (3*3 determinations).

Interday precision for both the drugs was done by analyzing three different concentrations (ng/ml) within linearity range, on three consecutive days.

Table 51.Intraday precision data of Atorvastatin calcium

Sr. No.	Concentration (µg/ml)	Peak Area	
		Mean ± SD*	%RSD
1	4	199125.5±670.62	0.33
2	8	396263.8±567.07	0.14
3	10	498908.3±856.37	0.17

*indicates (n=3)

Table 52.Interday precision data of Atorvastatin calcium

Sr. No.	Concentration (µg/ml)	Peak Area	
		Mean ± SD*	%RSD
1	4	199394.3±1418.14	0.71
2	8	393790.8±4804.9	1.22
3	10	496639.5±4674.4	0.94

*indicates (n=3)

Table 53.Intraday precision data of Telmisartan

Sr. No.	Concentration (µg/ml)	Peak Area	
		Mean ± SD*	%RSD
1	4	246076.5±2718.14	1.10
2	8	395392.3±3787.92	0.95
3	10	497946.5±1386.36	0.27

*indicates (n=3)

Table 54.Interday precision data of Telmisartan

Sr.	Concentration	Peak Area

No.	(µg/ml)	Mean ± SD*	%RSD
1	4	245480.5±3071.83	1.25
2	8	393645±4604.86	1.16
3	10	496282.5±4395.79	0.88

*indicates (n=3)

3) Accuracy

Accuracy of the measurement of Atorvastatin calcium and telmisartan was determined by standard addition method. Standard addition was done at three levels, 80%, 100% and 120% of a concentration in the linearity range.

Table 55.Accuracy data of Atorvastatin calcium by RP-HPLC with UV detection

Initial conc. (ng/spot) (A)	Std. Added (%) (ng/spot) (B)	Conc. After spiking (A + B)	accuracy	
			Conc. Recovered Mean±S.D.*	%Recovery Mean±S.D*
4	80%	7.2	7.18±0.020	99.90±0.212
4	100%	8	8.07±0.193	100.95±2.42
4	120%	8.8	8.78±0.035	99.80±0.39

*indicates (n=3)

Table 56.Accuracy data of Telmisartan by RP-HPLC with UV detection

Initial conc. (ng/spot) (A)	Std. Added (%) (ng/spot) (B)	Conc. After spiking (A + B)	accuracy	
			Conc. Recovered Mean±S.D.*	%Recovery Mean±S.D*
4	80%	7.2	7.19±0.04	99.40±0.65
4	100%	8	8.05±0.193	100.38±1.00
4	120%	8.8	8.80±0.035	99.91±0.42

*indicates (n=3)

Table 57.System suitability parameter

Sr. No	Parameters	Atorvastatin calcium	Telmisartan
1	Retention time	3.94±0.067 min	4.55±0.020min
2	Tailing factor	0.91	1.12
3	Capacity factor	2.11 min	2.59 min
4	Resolution factor	2.58	–
5	Theoretical plates	4310	6200

Table 58.Summary of Validation parameters

Sr. No	Parameters	Atorvastatin calcium	Telmisartan
1	Linearity range (µg/ml)	0.5 - 16	0.5 - 16
2	Regression equation	y = 52454x-9492.7	y = 63442x-9203.2
3	Correlation coefficient (r^2)	0.9990	0.9990
4	Precision Intraday % RSD (n = 3) Interday % RSD (n = 3) Repeatability of measurements % RSD	 0.14% - 0.33% 0.71% - 1.22% 0.10% - 0.81%	 0.27% - 1.10% 0.88% - 1.25% 0.94% - 1.65%
5	Limit of detection(µg/ml)	0.0089	0.0064
6	Limit of quantification (µg/ml)	0.027	0.019
7	% Recovery	99.80±0.39% to 100.95±2.42	99.40±0.65% to 100.38±1.00

5.4.4.2 Analysis of Tablets

The developed method was used to estimate Atorvastatin calcium and Telmisartan in the tablet dosage form. Five different brands of tablet formulations were procured from the market for analysis by the proposed method. The percentage of Atorvastatin calcium and Telmisartan was found from the calibration curve.

5.4.5 Conclusion:

By the virtue of the developed method, it can be concluded that reverse phase high performance liquid chromatography method is a reliable technique for the analysis of commercial formulations of Atorvastatin calcium and Telmisartan, The developed method is simple sensitive and specific which renders it suitable for routine analysis of Atorvastatin calcium and Telmisartan from its combined dosage form. A good % recovery for both the drugs shows that the developed method is free of the interference of excipients used in the formulation.

5.5 COMPARISON OF U.V, HPTLC AND RP-HPLC METHOD

5.5.1 Comparison of Developed Chromatographic Methods

Comparison of developed Chromatographic methods (Spectrophotometric, HPTLC and RP-HPLC) was performed by applying Student-ANOVA-test (single factor)

Table 60.Comparison of three methods

Brand name	Drugs	% Assay results		
		U.V	HPTLC	RP-HPLC
Telsartan-ATR	Atorvastatin calcium	98.45	98.32	98.25
		98.33	98.23	98.66
		98.56	98.19	98.62
	Telmisartan	98.65	98.12	98.32
		98.7	98.59	99.36
		98.60	98.56	98.62
Arbitel-AV	Atorvastatin calcium	98.56	98.41	98.26
		98.43	98.16	98.63
		98.52	98.47	98.54
	Telmisartan	99.01	99.45	99.65
		98.6	99.43	99.55
		99.52	99.32	99.25
TELDAY AV	Atorvastatin calcium	99.01	98.56	98.69
		98.36	98.33	98.33
		98.54	98.24	98.35
	Telmisartan	99.01	99.45	99.65
		98.6	99.43	99.55
		99.52	99.32	99.25
TELEACT ST 40	Atorvastatin calcium	98.06	98.14	98.35
		98	98.03	98.63
		98.51	98.52	98.24
	Telmisartan	98.98	98.87	98.65
		99.5	99.45	99.38
		99.18	99.51	99.25
TELEACT ST 20	Atorvastatin calcium	98.9	98.5	98.65
		98.6	98.4	99.06
		98.23	98.69	98.45
	Telmisartan	98.64	98.52	98.24
		98.65	99.5	99.79
		99.08	99.06	99.01

Table 61.ANOVA TEST

Brand name	Drugs	ANOVA:SINGLE FACTOR						
		Source of variation	SS	df	MS	F	P-VALUE	F crit
Telsartan-ATR	Atorvastatin calcium	Between Groups	0.11335	2	0.056678	2.472613	0.16473	5.1432
		Within groups	0.13753	6	0.022922			
		Total	0.25088	8				
	Telmisartan	Between Groups	0.18286	2	0.091433	0.765631	0.50565	5.1432
		Within groups	0.71653	6	0.119422			
		Total	0.8994	8				
Arbitel-AV	Atorvastatin calcium	Between Groups	0.04215	2	0.021078	0.920427	0.44808	5.1432
		Within groups	0.1374	6	0.0229			
		Total	0.17955	8				
	Telmisartan	Between Groups	0.32775	2	0.163878	1.886061	0.23146	5.1432
		Within groups	0.52133	6	0.086889			
		Total	0.84908	8				
TELDAY AV	Atorvastatin calcium	Between Groups	0.1064	2	0.0532	0.882743	0.46126	5.1432
		Within groups	0.3616	6	0.060267			
		Total	0.468	8				

	Telmisartan	Between Groups	0.13548	2	0.163878	1.886061	0.23146	5.1432
		Within groups	0.040933	6	0.006822			
		Total	0.176422	8				
TELEACT ST 40	Atorvastatin calcium	Between Groups	0.079756	2	0.039878	0.649358	0.55553	5.1432
		Within groups	0.368467	6	0.061411			
		Total	0.448222	8				
	Telmisartan	Between Groups	0.052867	2	0.026433	0.229611	0.80151	5.1432
		Within groups	0.690733	6	0.115122			
		Total	0.7436	8				
TELEACT ST 20	Atorvastatin calcium	Between Groups	0.058822	2	0.029411	0.381907	0.69803	5.1432
		Within groups	0.462067	6	0.077011			
		Total	0.520889	8				
	Telmisartan	Between Groups	0.106067	2	0.053033	0.175866	0.84290	5.1432
		Within groups	1.809333	6	0.301556			
		Total	1.9154	8				

5.5.2 Conclusion

ANOVA was performed by using Microsoft excel and graph pad instat, Version 3.05, 32 bit. The Atorvastatin calcium and Telmisartan in combination tablets are not reported by any methods or not in any official standard book. So it was compared results of both drugs for all three developed methods by ANOVA. Results are show that the F_{cal} value is lower than F_{tab} value. Hence, it was concluded that the methods do not differ significantly and can be successfully applied for the analysis of Atorvastatin calcium and Telmisartan in pharmaceutical dosage forms.

CHAPTER 6
SUMMARY

Spectrophotometric, High Performance Thin Layer Chromatography (HPTLC) and RP-HPLC methods were developed for estimation of Atorvastatin calcium and Telmisartan in pharmaceutical dosage forms. The methods were validated statistically and by recovery study.

- For estimation of Atorvastatin calcium and Telmisartan by simultaneous equation. The method was found most sensitive as it obeys Beer's law. Molecular weight of Atorvastatin calcium and Telmisartan is 1155.36 and 514.63 respectively. The lowest quantifiable amount of Atorvastatin calcium and telmisartan was found to be 0.80 µg/ml and 0.29 µg/ml respectively.
- A HPTLC method was developed for estimation of Atorvastatin calcium and Telmisartan. The method was found most sensitive as it obeys Beer's for both Atorvastatin calcium and Telmisartan.
- For estimation of Atorvastatin calcium and Telmisartan by RP-HPLC, A phenomenex column (150 mm×4.6mm i.d., 5µm) was used, and U.V detection at 246 nm. The method was found most sensitive as it obeys Beer's for both Atorvastatin calcium and Telmisartan. The lowest quantifiable amount of Atorvastatin calcium and Telmisartan was found to be 0.027 µg/ml and 0.019 µg/ml respectively.

- High % recoveries 98-100 % for both the methods show that the methods are free from the interference of excipients used in the formulation.
- The methods were compared statistically by ANOVA test. The results show that there is no significant statistical difference between the results obtained by above mentioned methods. In the cases, F_{stat} is less than $F_{critical.}$

CHAPTER 7

REFERENCES

1. Maton A, Jean H, Charles W, McLaughlin, Susan J; Maryanna Quon Warner, David LaHart, Jill D. Wright (1993);" Human Biology and Health"; Englewood Cliffs, New Jersey, USA: Prentice Hall. Section 1: 220-229.
2. Carretero OA, Oparil S; "Essential hypertension. Part I: definition and etiology"; (2009): 329–35.
3. Oparil S, Zaman MA, Calhoun DA; "Pathogenesis of hypertension". Ann. Intern. Med; N (2003): 761–76.
4. Hall, John E, Guyton, Arthur C; "Textbook of medical physiology"; St. Louis, Mo: Elsevier Saunders: 228.
5. http://emedicine.medscape.com
6. Pierdomenico SD, Di Nicola M, Esposito AL; "Prognostic Value of Different Indices of Blood Pressure Variability in Hypertensive Patients"; American J Hyper: 842–7.
7. Guyton & Hall; "Textbook of Medical Physiology"; 7th edition, (2005), Elsevier-Saunder publication: 220.
8. Chobanian AV, Bakris GL, Black HR; "Seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure"; (2003): 1206–52.
9. http://www.hypertensionlibrary.com/is-low-intensity-exercise-a-superior-blood-pressure-reducer-than-high-intensity-exercise.
10. Klaus D, Bohm M, Halle M; "Restriction of salt intake in the whole population promises great long-term benefits"; (2009):134.
11. W. L. T. Addison; "The Use of Sodium Chloride, Potassium Chloride, Sodium Bromide, and Potassium Bromide in Cases of Arterial Hypertension which are Amenable to Potassium Chloride" ; Can Med Assoc J; (1928): 281–285.
12. Benson, Herbert, M.D; "The Relaxation Response"; New York: Benson, Herbert, M.D; "The Relaxation Response"; New York: (2000): 369-376
13. "CG34 Hypertension - quick reference guide"; National Institute for Health and Clinical Excellence. (2006): 280-285.

14. Kragten JA; Dunselman PHJM; "Nifedipine gastrointestinal therapeutic system (GITS) in the treatment of coronary heart disease and hypertension"; Expert Rev Cardiovascular; (2007): 643-653.

15. Piller LB, Davis BR, Cutler JA; "Validation of Heart Failure Events in the Antihypertensive and Lipid Lowering Treatment to Prevent Heart Attack Trial (ALLHAT) Participants Assigned to Doxazosin and Chlorthalidone". Current Control Trials Cardiovascular; (2002): 10.

16. Mayor S;"NICE removes beta blockers as first line treatment for hypertension"; (2006). 8-10.

17. George L. Bakris; "Presentation on Direct Renin Inhibitors as Antihypertensive Drugs"; 7th edition, Elsevier-Saunder publication: (2010): 225.

18. Maton A., Roshan L., Jean Hopkins, Charles William McLaughlin, Susan Johnson, Maryanna Quon Warner, David LaHart, Jill D. Wright; Human Biology and Health. Englewood Cliffs, New Jersey, USA; (1993): 231-238.

19. Fabricant CG, Fabricant J; "Atherosclerosis induced by infection with Marek's disease herpesvirus in chickens". Am Heart J; (1999): 465–468.

20. Hsu HY, Nicholson AC, Pomerantz KB, Kaner RJ, Hajjar DP (1995). "Altered cholesterol trafficking in herpesvirus-infected arterial cells"; J Biol Chem: 19630–7.

21. Glagov S, Weisenberg E, Zarins CK, Stankunavicius R, Kolettis GJ (1987). "Compensatory enlargement of human atherosclerotic coronary arteries". N. Engl. J. Med. 316 (22): 1371–5.

22. Nissen; Paul M. Ridker, M.D., M.P.H., Nader Rifai "Effect of Very High-Intensity Statin Therapy on Regression of Coronary Atherosclerosis–The ASTEROID Trial"; Volume 344; (2001):1959-1965

23. Blankenhorn DH, Hodis HN; "Atherosclerosis--reversal with therapy"; Western J med (1993): 172–9.

24. Mitchell, Richard Sheppard, Kumar Vinay, Abbas, Abul K.; Fausto, Nelson (2007). Robbins Basic Pathology: With student consult Online Access. Philadelphia: Saunders. 8th edition: 345.

25. H.P.Rang; M.M.Dale; "Pharmacology"; published by Elsevier, fifth edition, 2003: 306-313.

26. http://chemicalland21.com/lifescience/phar/atorvastatin%20calcium.htm

27. http://en.wikipedia.org/wiki/file:atorvastatin

28. http://en.wikipedia.org/wiki/file:telmisartan

29. K. Florey; "Analytical profiles of drug substances"; 1st edition; Vol.13: 1-25.

30. Sweetman S. C. Eds; "Martindale: The complete drug reference";35th Edition; Published by Pharmaceutical press, (2006): 1266.

31. http://www.medicinenet.com/telmisartan/article.htm.

32. http://www.pdrhealth.com/drug_info/rxdrugprofiles/drugs/mic1592.html.

33. Nickenig G, Baumer AT, Temur, Y; "Statin-sensitive dysregulated AT_1 receptor function and density in hypercholesterolemic men"; (1999): 2131–2134.

34. Wassmann S, Laufs U, Baumer AT, "Inhibition of geranylgeranylation reduces angiotensin II–mediated free radical production in vascular smooth muscle cells: involvement of angiotensin AT_1 receptor expression and Rac1 GTPase. Mol Pharmacol. (2001); 59: 646–654.

35. Dickstein K, Kjekshus J, "OPTIMAAL Steering Committee of the OPTIMAAL Study Group. Effects of losartan and captopril on mortality and morbidity in high-risk patients after acute myocardial infarction: the OPTIMAAL randomised trial: Optimal Trial in Myocardial Infarction with Angiotensin II Antagonist Losartan"; (2002): 752–760.

36. DeFronzo RA, Ferrannini E. Insulin resistance: a multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. Diabetes Care. (1991): 173–194.

37. Nickenig G, Sachinidis A, Michaelsen F, et al. Upregulation of vascular angiotensin II receptor gene expression by low-density lipoprotein in vascular smooth muscle cells. Circulation. (1997): 473–478.

38. Lusis AJ; "Atherosclerosis"; Nature. (2000): 233–241.

39. Zimmet P, Alberti KG, Shaw J. Global and societal implications of the diabetes epidemic. Nature. (2001): 782–787.

40. Pitt B, Poole-Wilson PA, Segal R, et al. Effect of losartan compared with captopril on mortality in patients with symptomatic heart failure: randomised trial: the Losartan Heart Failure Survival Study ELITE II. Lancet. (2000): 1582–1587.

41. Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). Lancet. (1994): 1383–1389.

42. Mishra P; Gupta A; Shah K; "Stability indicating RP-HPLC estimation of atorvastatin calcium and amlodipine besylate in pharmaceutical formulations"; Indian J Pharm sci; **(**2008), Volume-70, Issue - 6: 754-760.

43. Shah D. A, Bhatt K. K., Mehta R. S., Shankar M. B., Baldania S. L., "RP-HPLC Method for the Determination of Atorvastatin calcium and Nicotinic acid in Combined Tablet Dosage Form"; Indian J Pharm sci; (2007), vol. 69: 700-703.

44. N. Jain R., Raghuwanshi, and Deeti Jain; "Development and Validation of RP-HPLC Method for Simultaneous Estimation of Atorvastatin Calcium and Fenofibrate in Tablet Dosage Forms"; Indian J Pharm sci; (2008): 263–265.

45. Ertürk S, Sevinç Aktaş E., Ersoy L., Ficicioglu S; "An HPLC method for the determination of Atorvastatin and its impurities in bulk drug and tablets"; J Pharm Biomed Anal; (2003): 1017-23.

46. G. F. Patel; "RP-HPLC Estimation of Aspirin and Atorvastatin Calcium in Combined Dosage Forms"; J Pharm Res; (2009); Vol 2, No 8 .

47. Samy E**.,** Kashyap K. Bhatt; **"**Application of UV-Spectrophotometry and RP-HPLC for Simultaneous Determination of Atorvastatin Calcium and Ezetimibe in Pharmaceutical Dosage Form"; Eurasian J Anal Chem; Volume 1, Number 1; (2006).

48. Dr. Rafiq Zakaria Campus, Rauza Bagh Aurangabad; "Stability-indicating high performance liquid chromatographic determination of Atorvastatin calcium in pharmaceutical dosage form"; African J Pharm and Pharmacol; (2008) Vol. 2(10): 204-210.

49. Hiral J. Panchal, Bhanubhai N. Suhagia, Natvarlal J. Patel; "Simultaneous HPTLC analysis of atorvastatin calcium, ramipril, and aspirin in a capsule dosage form"; J Chromatogr; (2009), Volume 22: 265-271.

50. Yadav Savita S., Mhaske Deepali V., kakad A. B., Patil B. D., Kadam S. S., Dhaneshwar S. R., "A simple and sensitive HPTLC method for the determination of content uniformity of Atorvastatin calcium tablets"; Indian J Pharm sci, (2005) vol. 67: 182-186.

51. Chaudhari B. G., Patel N. M., Shah P. B., Modi K. P., "Development and validation of a HPTLC method for the simultaneous estimation of Atorvastatin calcium and ezetimibe"; Indian J Pharm sci; (2006), vol. 68: 793-796.

52. P. B. Deshpande, G. Shridharan, Libi Anandi, D. Jadhav, M. C. Damle, S. V. Gandhi; "Validated Method Development for Estimation of Atorvastatin Calcium and Fenofibrate in Fixed Dose Combination by HPTLC"; kongposh Publications Pvt. Ltd; 2008: 151

53. Naresh V. Nakarani, Kashyap K. Bhatt, Rutvad. Patel, and Hemaxi S. Bhatt; "Estimation of Atorvastatin Calcium and Fenofibrate in Tablets by Derivative Spectrophotometry and Liquid Chromatography"; J AOAC Inter; Vol. 90, NO. 3, (2007).

54. "Indian Pharmacopoeia"; Indian Pharmacopoeia Commission; central Indian pharmacopoeia laboratory, Govt. of India, Ministry of Health & Family Welfare, volume-2:130-132.

55. MS Palled, M Chatter, PMN Rajesh, AR Bhat, "Difference spectrophotometric determination of telmisartan in tablet dosage form"; Indian J Pharm sci ; (2006): 68-5

56. Zonghui Qin, Weifen Niu, Rong Tan, "spectrophotometric determination of telmisartan with congo red"; J Anal chem.; May (2009): 64-5.

57. Alaa EG., Samy E., Ahmed M; "spectrophotometric method for estimation of telmisartan in tablets"; Inter Res J Pure Appl Chem; (2008); vol.25(3).

58. Lories I. Bebawy, Samah S. Abbas, Laila A. Fattah; "simultaneous determination of telmisartan and hydrochlorthiazide in plasma and dosage form"; II Farmaco (2005), vol-60 (10), 859-867.

59. VP Kurade, MG Pai, R Gude; "RP-HPLC determination of telmisartan in tablet dosage form"; Indian J Pharm Sci; (2005):67.

60. SB Wankhede, MR Tajne, KR Gupta, SG Wadodka; "RP-HPLC method for determination of telmisartan and hydrochlorthizide in tablet dosage form"; Indian J Pharm sci; (2000): 69-72.

61. Shah SA., Rathod IS., Shishoo CJ., Savale SS., Satia MC., Bhat KM., "RP-HPLC method for determination of telmisartan and ramipril in tablet dosage form"; Indian J Pharm sci (2009): 71-72.

62. Pengfei Li, Yingwu Wang, Yan Wang, Yunbiao Tang; "LC-MS method for the simultaneous quantitation of telmisartan and hydrochlorthizide in human plasma"; J Pharm Biomed Anal; (2004): 1225-1229.

63. N. Torrealday, L. González, R. M. Alonso, R. M. Jiménez, E. Ortiz Lastra, "The quantitation of the angiotensin II receptor antagonist telmisartan in urine by HPLC-fluorimetric method"; J Pharm Biomed Anal; (2003): 847-857.

64. Kiran R. Patil, Vipul P. Rane, Jaiprakash N. Sangshetti, Devanand B. Shinde; "A stability indicating LC method for simultaneous determination of telmisartan and ramipril in dosage form"; J Anal chem; April (2009): 67-68.

65. NJ Shah, BN Suhagia, RR Shah, PB Shah, "Development and validation of HPLTC method for simultaneous estimation of telmisartan and hydrochlorthizide in tablet dosage form" J chromatogr; (2008), 67 (7), 575-582.

66. J. mendham; RC Denney; "VOGEL'S Text book of quantitative chemical analysis"; published by Pearson education; 6th edition; 679-680.

67. Zlatkis A, Kaiser E. R; "HPTLC-High Performance Thin-Layer Chromatography"; Elsevier, Amsterdam, Netherlands; (1977): 12.

68. Burger K; "Instrumental Thin-Layer Chromatography/Planar Chromatography"; Brighton (1989): 33–44.

69. Kaiser R.E; "Instrumental Thin-Layer Chromatography/Planar Chromatography, Proceedings of the International Symposium"; (1989): 251–262.

70. Janchen D., Issaq J. H., "Liq. Chromatography"; (1988): 1941–1965.

71. Janchen D; "Handbook of Thin Layer Chromatography"; (1996): 144.

72. Wall P.E; "Thin layer Chromatography- A Modern practical approach"; (2005); 1: 126-175.

73. Frank Settle, Phyllis Brown, Kathryn; "High Performance Liquid Chromatography"; Prentice Hall, (1997): 147-164.

74. http://www.chemicool.com/definition/high_performance_liquid_chromatography_hplc.html.

75. Skoog, Holler, Nieman; "Principles of Instrumental Analysis"; 5th Edition. Saunders College Publishing; (1998): 673-697, 725-766.

76. http://ull.chemistry.uakron.edu/analytical/Chromatography.

77. Hadjicostas E, wenclawiak, B.W. koch., hadjicostas, E., Springer-Verlag,; "validation of analytical method" in Quality assurance in analytical chemistry"; (2004): 201-219.

78. International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use, Validation of analytical procedures: definitions and terminology, Geneva (1996).

79. International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use, Validation of analytical procedures: Methodology, adopted in (1996), Gene.

80. International Conference on Harmonization (ICH Q2A), Guideline on validation of analytical procedure: Definition and terminology; availability. Federal register 60: 11260-11262.

81. Vessman J; "Validation of analytical methods from the perspective of an analytical chemist in the pharmaceutical industry"; J. Pharm & Biomed Analysis, (1996); 14: 867–869.

82. Maffat A.C., Lin., Clerk's isolation and Identification of Drug's analytical techniques II; (1998): 230.