"Development and Validation of Two Analytical Methods for Simultaneous Estimation of Ramipril and Hydrochlorothiazide"

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in

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BY

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GUIDE

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APRIL 2010

DECLARATION

I declare that the thesis entitled "DEVELOPMENT AND VALIDATION OF TWO ANALYTICAL METHODS FOR SIMULTANEOUS ESTIMATION OF RAMIPRIL AND HYDROCHLOROTHIAZIDE" 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.

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CERTIFICATE

This is to certify that Mr. ANKIT R. MODH has prepared his thesis entitled "DEVELOPMENT AND VALIDATION OF TWO ANALYTICAL METHODS FOR SIMULTANEOUS ESTIMATION OF RAMIPRIL AND HYDROCHLOROTHIAZIDE", 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.

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Dedicated

to

My Parents

&

Friends

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Date: 20/04/2004 ***Ankit R. Modh***

CONTENTS

1.Introduction

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. It is common. About 90-95% of hypertension is essential hypertension.[2][3] Secondary hypertension indicates that the high blood pressure is a result of 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.[4] 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. 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

The variation in pressure in the left ventricle and the aorta over two cardiac cycles ("heart beats"), showing the definitions of systolic and diastolic pressure.

A recent classification recommends blood pressure criteria for defining normal blood pressure, prehypertension, hypertension (stages I and II), and isolated systolic hypertension, which is a common occurrence among the elderly. These readings are based on the average of seated blood pressure readings that were properly measured during 2 or more office visits. In individuals older than 50 years, hypertension is considered to be present when a person's blood pressure is consistently at least 140 mmHg systolic or 90 mmHg diastolic. Patients with blood pressures over 130/80 mmHg along with Type 1 or Type 2 diabetes, or kidney disease require further treatment.[5]Resistant hypertension is defined as the failure to reduce blood pressure to the appropriate level after taking a three-drug regimen (include thiazide diuretic).

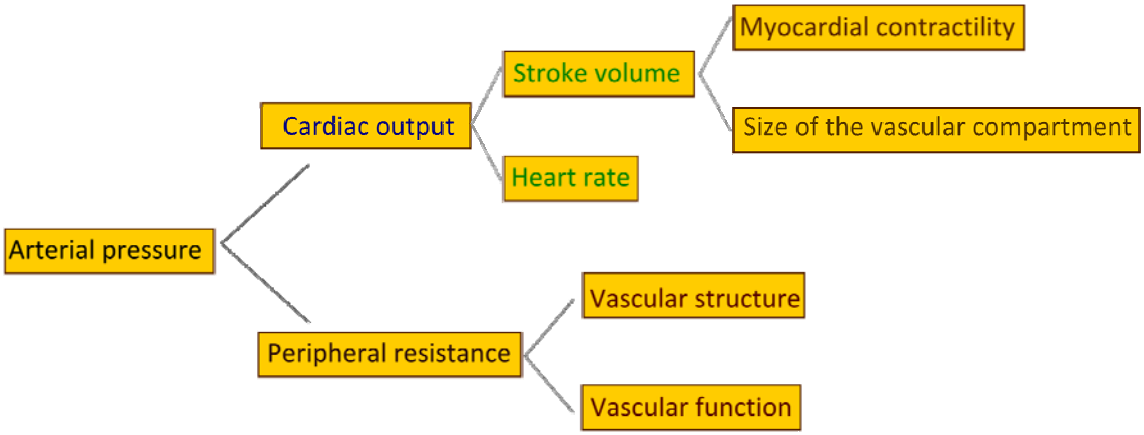
Excessive elevation in blood pressure during exercise is called exercise hypertension.[7] The upper normal systolic values during exercise reach levels between 200 and 230 mm Hg. Exercise hypertension may be regarded as a precursor to established hypertension at rest.[8]

Signs and symptoms

Mild to moderate essential hypertension is usually asymptomatic.[9][10] Accelerated hypertension is associated with headache, somnolence, confusion, visual disturbances, and nausea and vomiting (hypertensive encephalopathy). Retinas are affected with narrowing of arterial diameter to less than 50% of venous diameter, copper or silver wire appearance, exudates, hemorrhages, or papilledema.[11] Some signs and symptoms are especially important in infants and neonates such as failure to thrive, seizure, irritability or lethargy, and respiratory distress.[12] While in children hypertension may cause headache, fatigue, blurred vision, epistaxis, and bell palsy.[12]

Pathophysiology

Figure 1: factors affecting arterial pressure



Most of the mechanisms associated with secondary hypertension are generally fully understood. However, those associated with essential (primary) hypertension are far less understood. What is known is that cardiac output is raised early in the disease course, with total peripheral resistance (TPR) normal; over time cardiac output drops to normal levels but TPR is increased. Three theories have been proposed to explain this:

- Inability of the kidneys to excrete sodium, resulting in natriuretic factors such as Atrial Natriuretic Factor being secreted to promote salt excretion with the side effect of raising total peripheral resistance.
- An overactive Renin-angiotensin system leads to vasoconstriction and retention of sodium and water. The increase in blood volume leads to hypertension.[13][14]
- An overactive sympathetic nervous system, leading to increased stress responses.[15]

It is also known that hypertension is highly heritable and polygenic (caused by more than one gene) and a few candidate genes have been postulated in the etiology of this condition. [16]

Medication Treatment

There are many classes of medications for treating hypertension, together called antihypertensives, which — by varying means — act by lowering blood pressure. Evidence suggests that reduction of the blood pressure by 5–6 mmHg can decrease the risk of stroke by 40%, of coronary heart disease by 15–20%, and reduces the likelihood of dementia, heart failure, and mortality from vascular disease.

The aim of treatment should be blood pressure control to <140/90 mmHg for most patients, and lower in certain contexts such as diabetes or kidney disease (some medical professionals recommend keeping levels below 120/80 mmHg).[17] Each added drug may reduce the systolic blood pressure by 5–10 mmHg, so often multiple drugs are often necessary to achieve blood pressure control.

Commonly used drugs include the typical groups of:[4]

- **ACE inhibitors** such as captopril, enalapril, fosinopril, lisinopril, 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, amlodipine, diltiazem, verapamil
- **Diuretics:** eg, bendroflumethiazide, chlorthalidone, hydrochlorothiazide (also called HCTZ).

Other additionally used groups include:

- Additional diuretics such a furosemide or 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.
- **Beta blockers** such as atenolol, labetalol, metoprolol, propranolol.[18]
- **Direct renin inhibitors** such as aliskiren.

Finally several agents may be given simultaneously:

- Combination products (which usually contain HCTZ and one other drug). The advantage of fixed combinations resides in the fact that they increase compliance with treatment by reducing the number of pills taken by the patients. A fixed combination of the ACE inhibitor perindopril and the calcium channel blocker amlodipine, recently been proved to be very effective even in patients with additional impaired glucose tolerance and in patients with the metabolic syndrome.

Drug profile of Ramipril[19,20,21]

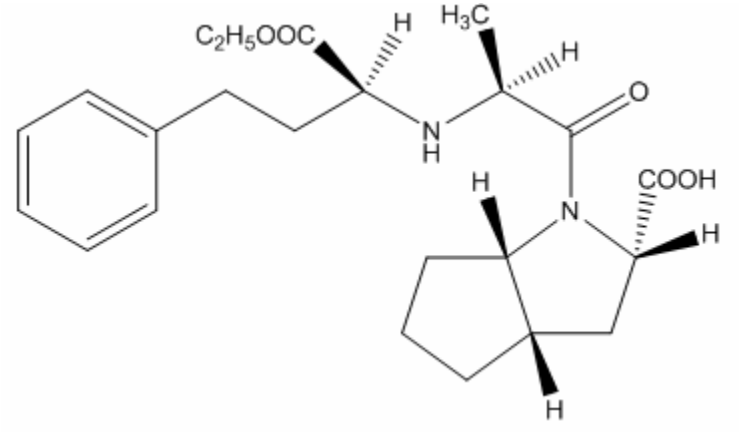
Category: it is a angiotensin converting enzyme inhibitor

Chemical name: it is chemically (2S,3aS,6aS)1-[(S)N[(S)1-Carboxy3phenylpropyl] alanyl] octahydrocyclopenta[b]pyrrole2carboxylic acid, 1ethyl ester.

Empirical formula: $C_{23}H_{32}N_2O_5$

Molecular weight: 416.51

Structure:[22]



Physicochemical properties:

Appearance: white, crystalline substance soluble in polar organic solvents and buffered aqueous solutions

Solubility: soluble in dilute ammonia,NaOH, water; freely soluble in methanol, ethanol and acetone.

Melting point: 105-112 ° C

Reported UV wavelength:

In methanol: 210 nm

In water: 210 nm

Clinical pharmacology:

Mechanism of Action:

ACE is important because it is an enzyme responsible for producing the chemical, angiotensin II. Angiotensin II causes muscles in most arteries, including the arteries of the heart, to contract, thereby narrowing the arteries and elevating blood pressure. ACE inhibitors such as ramipril lower blood pressure by reducing the production of angiotensin II, thereby relaxing arterial muscle and enlarging arteries. When the blood pressure is lower, the heart - including the failing heart - does not have to work as hard to pump blood. The arteries supplying the heart with blood also enlarge during treatment with ACE inhibitors. This increases the flow of blood and oxygen to the heart, further improving the ability of the heart to pump blood.

Indications:[23]

Indications for its use include:

- Hypertension;
- Congestive heart failure
- Following myocardial infarction in patients with clinical evidence of heart failure;
- Susceptible patients over 55 years: prevention of myocardial infarction, stroke, cardiovascular death or need of revascularization procedures.

Diabetic nephropathy with microalbuminuria

Pharmacokinetics:[24,25]

Absorption

Extent of absorption in GI tract is at least 50% to 60%. T_{max} is 1 h (parent compound) or 2 to 4 h (metabolite, ramiprilat). Bioavailability is 28% (ramipril) or 44% (ramiprilat).

Distribution

Protein binding is about 73% (parent) or about 56% (metabolite). Plasma concentrations of ramiprilat decline in a triphasic manner: initial rapid decline (representing distribution into peripheral compartment), apparent elimination phase, and terminal elimination phase.

Metabolism

In liver to active metabolite ramiprilat, which had 6 times the ACE inhibitory activity.

Elimination

Eliminated in urine (60% of parent and metabolites) and feces (40%). Less than 2% of drug recovered in urine is unchanged. The $t_{½}$ is less than 50 h (ramiprilat).

Dosage and administration:

Heart Failure Post-Myocardial Infarction

Adults

PO 2.5 mg twice daily. Switch to 1.25mg twice daily if hypotension occurs. Titrate to target dose of 5mg twice daily.

Hypertension

Adults

PO Initial dose is 2.5 mg every day initially. Maintenance dose is 2.5 to 20mg/day as single dose or in 2 equally divided doses.

Patients with Renal Impairment

PO 1.25 mg every day in patients with Ccr below 40mL/min (serum creatinine higher than 2.5mg/dL; max, 5mg/day).

Side effects:[26]

- may cause swelling of the mouth, tongue, or throat
- low blood sugar in patients taking other medicine for diabetes which shows as sweating or shakiness
- Dry cough may develop, dizziness, and light-headedness due to low blood pressure. (It is recommended to start treatment with the lowest dose.)
- decreased sex drive
- tiredness and fatigue especially in the early stages
- mouth dryness in the early stages

Drug interactions:[26]

Table 1: Drugs interaction with ramipril

Drugs	**Interactions**
Amiloride	Increased risk of hyperkaliemia
Drospirenone	Increased risk of hyperkaliemia
Lithium	The ACE inhibitor increases serum levels of lithium
Potssium	Increased risk of hyperkaliemia
Spironolactone	Increased risk of hyperkaliemia
Tizanidine	Tizanidine increases the risk of hypotension with the ACE inhibitor
Triamterene	Increased risk of hyperkaliemia

Food interactions: Take without regard to meals. Avoid alcohol

Drug profile of Hydrochlorothiazide

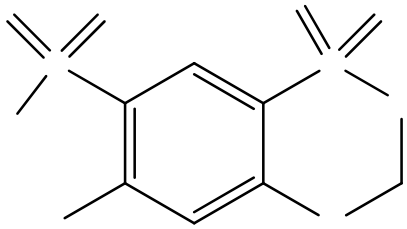
Category: it is a thiazide diuretic[27]

Chemical name: it is chemically 6-chloro-3,4-dihydro-2 H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide

Empirical formula- $C_7H_8ClN_3O_4S_2$

Molecular weight: 297.73

Structure:[28]



Physicochemical Properties:

Appearance: white or practically white, practically odourless, crystalline powder, slightly bitter in the taste

Solubility: soluble in water and methanol; freely soluble in sodium hydroxide solution, n-butylamine and dimethylformamide; and insoluble in ether, chloroform and in dilute mineral acids.[29]

Melting point: 273-275 °C[30]

Density: 1.68±0.01 gm/cm ^{3}

Dissociation constant: pka 7.0, 9.2

Partition coefficient: log P(octanol/water)=-1

Reported UV wavelength:

In methanol: 226, 271 nm

In water: 226, 270, 315 nm

In 0.01 N NaOH: 272, 323 nm

Clinical pharmacology:

Mechanism of Action:

As a diuretic, hydrochlorothiazide inhibits active chloride reabsorption at the early distal tubule via the Na-Cl cotransporter, resulting in an increase in the excretion of sodium, chloride, and water. Thiazides like hydrochlorothiazide also inhibit sodium ion transport across the renal tubular epithelium through binding to the thiazide sensitive sodium-chloride transporter. This results in an increase in potassium excretion via the sodium-potassium exchange mechanism. The antihypertensive mechanism of hydrochlorothiazide is less well understood although it may be mediated through its action on carbonic anhydrases in the smooth muscle or through its action on the large-conductance calcium-activated potassium (KCa) channel, also found in the smooth muscle.[31]

Indications:

HCTZ is often used in the treatment of hypertension, congestive heart failure, symptomatic edema and the prevention of kidney stones. It is effective for nephrogenic diabetes insipidus and is also sometimes used for hypercalciuria, Dent's disease and Meniere's disease.

Hypokalemia, an occasional side effect, can be usually prevented by potassium supplements or by combining hydrochlorothiazide with a potassium-sparing diuretic.

Thiazides are also used in the treatment of osteoporosis. Thiazides decrease mineral bone loss by promoting calcium retention in the kidney, and by directly stimulating osteoblast differentiation and bone mineral formation.

Pharmacokinetics:

Absorption

Bioavailability is 65% to 75%, C_{max} is 70 to 490 ng/mL (dose dependent), and T_{max} is 1 to 5 h. Food reduces the bioavailability 10% and the C_{max} 20%; increases the T_{max} from 1.6 to 2.9 h. Plasma concentrations are linearly related to administration doses.

Distribution

Protein binding is 40% to 68% and crosses the placenta, but not the blood brain barrier. It is also excreted in breast milk.

Metabolism

Hydrochlorothiazide is not metabolized.

Elimination

Hydrochlorothiazide is eliminated primarily by renal pathways (as unchanged by the kidneys; 55% to 77% or the administration dose appear in urine with more than 95% of the absorbed dose excreted in urine unchanged). Plasma $t_{½}$ is 5.6 to 14.8 h.

Dosage and administration:

Edema

Adults

PO 25 to 100 mg/day. Rarely patients may require 200 mg/day.

Hypertension

Adults

PO 25 to 50 mg/day as single dose or 2 divided doses.

Children (2 to 12 yr of age)

PO 37.5 to 100 mg/day in 2 doses.

Infants (6 mo to 2 yr of age)

PO 12.5 to 37.5 mg/day in 2 doses.

Infants (younger than 6 mo)

PO Up to 3.3 mg/kg/day in 2 doses.

Side effects:[32]

- Hypokalemia
- Hypomagnesemia
- Hyperuricemia and gout
- High blood sugar
- Hyperlipidemia
- Hypercalcemia
- Headache
- Nausea/vomiting
- Photosensitivity
- Weight Gain

Drug interactions:[33,34]

Table 2: Table 1: Drugs interaction with hydrochlorothiazide

Drug	Interaction
Amantadine	The diuretic increases the adverse effect of amantadine
Deslanoside	Possible electrolyte variations and arrhythmias
Diazoxide	Significant hyperglycemic effect
Digitoxin	Possible electrolyte variations and arrhythmias
Digoxin	Possible electrolyte variations and arrhythmias
Dofetilide	Increased risk of cardiotoxicity and arrhythmias
Lithium	The thiazide diuretic increases serum levels of lithium

Food interactions:

- Avoid alcohol.
- Avoid excess salt/sodium unless otherwise instructed by your physician.
- Avoid natural licorice.

- Do not take calcium, aluminum, magnesium or Iron supplements within 2 hours of taking this medication.
- Increase potassium intake; add a banana or orange juice; unless instructed otherwise.
- Take with food.

Rationale for combination[35]

ACE inhibitors prevent the severe heart failure by preventing the conversion of angiotensin I to angiotensin II, thereby counteracting salt and water retention, peripheral arterial and venous vasoconstriction and activation of the sympathetic nervous system. They also prevent the undesirable activation of the renin-angiotensin system caused by diuretic therapy. The major benefit of ACE inhibitor therapy is a reduction in afterload as well as preload and modest increase in the plasma potassium concentration. Therefore treating the heart failure with a combination of a potassium losing diuretic like thiazide diuretics(e.g. Hydrochlorthiazide) and an ACE inhibitors(e.g. Ramipril) has potential advantages.

2. Literature Review

Table 3: Reported Methods for Determination of Ramipril

SR. NO	TITLE	MATRIX	DESCRIPTION	REF NO.
1	**Estimation of ramipril**	Capsules and tablets	**Method:** HPLC **Column:** stainless steel column 12.5 cm *4.6 cm packed with octadecyldilyl bonded to porous silica(5 µm) **Mobile Phase:** acetonitrile:1.4 % w/v of sodium perchlorate+0.58% w/v of orthophosphoric acid **pH:** 2.1 **Flow rate:** 1 ml/min **UV detection:** 210 nm	36
2	**Estimation of ramipril**	Dosage forms	**Method:** Potentiometric Titration 0.3 gm in 25 ml methanol and 25 ml water. Titrate with 0.1 M NaOH. **Factor:** 1 ml of 0.1 nm NaOH equivalent to 41.65 mg of ramipril	37
3	**Estimation of ramipril**	Dosage forms	**Method:** HPLC **Column:** 4 mm * 25 cm contains 3 µm packing L1 **Mobile Phase:** Sodium perchlorate:acetonitrile(variable proportion) **Flow rate:** 1 ml/ min **UV detection:** 210 nm	38
4	**RP-HPLC estimation of ramipril and telmisartan**	Tablets	**Method:**RP-HPLC **Column:** Genesis C18 column (250 mm × 4.6 mm i.d., 5 µm) **Mobile phase:** 0.01 M potassium dihydrogen phosphate buffer (adjusted to	39

			pH 3.4 using orthophosphoric acid): methanol:acetonitrile (15:15:70 v/v/v) **Flow rate:** 1 ml/min **UV detection:** Wavelength amplitude: 210 nm LOD:- ramipril-0.5 µg/ml HCTZ-1.5 µg/ml	
5	**RP-LC for separation of antihypertensive formulations(enalapril maleate + amlodipine besilate and ramipril + hydrochlorothiazide**	Dosage forms	**Method:** RP-HPLC **Column-** C18 **Mobile Phase:** methanol-water (60:40 v/v) **UV detection:-** ➢ 215 nm for enalapril maleate + amlodipine besilate ➢ 220 nm for ramipril+ hydrochlorothiazide	40
6	**Simultaneous estimation of ramipril and metoprolol tartrate**	Dosage Forms	**Method:** UV **1. Simultaneous Equation Method** Ramipril: 205 nm Metoprolol: 222.5 nm **2. Area under curve method** Ramipril: 200 to 210 nm Metoprolol: 217.5 to 227.5 nm **3. Graphical absorbance ratio** at isoabsorptive point for the drugs(216.5 nm) and abs. maximum of metoprolol (222.5 nm) **4. Derivative Spectrophotometric method**	41
7	**Simultaneous Estimation of Atorvastatin Calcium, Ramipril and Aspirin**	Capsule	**Method:** RP-LC **Column:** Phenomenex Luna C_{18} (250 mm, 4.6 mm i.d., 5 µm) **Mobile phase:** 0.1%, orthophosphoric acid	42

			buffer:acetonitrile:methanol (45:50:5 *v*/*v*/*v*) pH: 3.3 **Flow rate:** 1 ml/min **UV detection:** Amplitude wavelength: 210 nm **Retention times:** Atenolol Cal.-12.19 min Ramipril: 2.35 min Aspirin: 3.95 min	
8	**Simultaneous Estimation of Atorvastatin Calcium and Ramipril**	Capsule	**Method:** UV Spectrophotometry(First order derivative) **First order amplitude wavelength:** Atorvastatin Calcium: 294 nm Ramipril: 229 nm	43
9	**Simultaneous Estimation of Amlodipine and Ramipril**	Tablet	**Method:** UV Spectrophotometry (simultaneous equation method) **Amplitude Wavelength:** Ramipril: 210 nm Amlodipine: 238 nm **Beer's law limit:** 15- 35 µg/ml for ramipril 5-25 µg/ml for amlodipine	44
10	**Stability-Indicating LC Method for the Simultaneous Determination of Telmisartan and Ramipril**	Dosage forms	**Method:** HPLC **Column:** ACE 5 C_{18}, 25-cm analytical column **Moblie Phase:** Buffer(0.1 M sodium perchlorate monohydrate in double distilled water pH adjusted 3.0 with trifluoroacetic acid)–acetonitrile (55:45 *v/v*) **Flow Rate:** 1.5 ml/min **Detection:** At 215 nm with PDA detector	45

11	**Kinetic spectrophotometric determination of ramipril**	Pharmaceutical dosage forms	**Method:** Spectrofluorometric Method based upon reaction of drug with 1-chloro-2,4-dinitrobenzene (CDNB) in dimethylsulfoxide (DMSO). The reaction is followed spectrophotometrically by measuring the rate of change of the absorbance at 420 nm.	46
12	**HPLC and Chemometrically-Assisted Spectrophotometric Estimation of antihypertensive agents(felodipine+ ramipril)**	Binary mixtures	**1. Method:** HPLC **Stationary phase:** Hypersil BDS C_{18} 3 µm (150× 4.6) **Mobile phase:** 0.015 M 1-heptanesulfonic acid sodium salt-methanol-acetonitrile (35:40:25, v/v/v) **pH:** 2.5 **UV detection:** Amplitude wavelength: 210 nm **2. Chemometrically methods:** derivative ratio and partial least square method	47
13	**A stability-indicating LC method for the simultaneous determination of ramipril and hydrochlorothiazide**	Dosage forms	**Method:** HPLC **Column:** supelcosil™ LC-8 column (5 µm), 15 cm×4.6 mm i.d **Mobile phase:** Acetonitrile: sodium perchlorate solution (0.1 M) adjusted to pH 2.5±0.2 with phosphoric acid (46:54 v/v) **Internal standard:** clobazam **UV detection:** Amplitude wavelength: 210 nm	48

Table 4: Reported Methods for Determination of Hydrochlorothiazide

SR. NO	TITLE	MATRIX	DESCRIPTION	REF NO.
1	**Estimation of Hydrochlorothiazide**	Tablets	20 mg powder+100 ml 0.1 M NaOH. Take 0.5 ml and dilute to 100 ml with water and measure absorbance at 273 nm. Calculate the content of HCTZ taking 520 as sp.abs.at 273 nm.	49
2	**Estimation of Hydrochlorothiazide**	Dosage forms	**Method:** Potentiometric titration 0.120 gm in 50 ml DMSO. Titrate with 0.1 M tatrabutylammonium hydroxide in 2- propranolol Factor: 1 ml 0.1 M tetrabutylammonium hydroxide in 2- propranolol equivalent 14.88 mg of HCTZ	50
3	**Estimation of Hydrochlorothiazide**	Dosage forms	**Method:** HPLC **Column:**4.6mm * 25 cm contains 5 µm packing L1 **Mobile Phase:** Sodium perchlorate:acetonitrile(1:9) **pH:** 3 **Flow rate:** 1 ml/ min **UV detection:** 270nm	51
4	**A stability-indicating LC method for the simultaneous determination of ramipril and hydrochlorothiazide**	Dosage forms	**Method:** HPLC **Column:** supelcosil™ LC-8 column (5 µm), 15 cm×4.6 mm i.d **Mobile phase:** Acetonitrile: sodium perchlorate solution (0.1 M) adjusted to pH 2.5±0.2 with phosphoric acid (46:54 v/v) **Internal standard:** clobazam **UV detection:** Amplitude wavelength: 210 nm	48
5	**simultaneous**	Dosage forms	**Method:** RP-HPLC	52

	estimation of nebivolol hydrochloride and hydrochlorthiazide		**Column:** C18 Novapak column (150 x 3.9 nm; 4 μm) **Mobile Phase:** Acetonitrile: potassium dihydrogen phosphate buffer (50:50 v/v) **pH:** 3.2 **Internal standard**: Diazepam **Detection:**UV Detection (282 nm) **Retention time:** Nebivolol hcl: 6.66 mins HCTZ: 3.57 mins	
6	**Validated HPTLC method for simultaneous determination of quinapril hydrochloride and hydrochlorothiazide**	Tablets	**Method:** HPTLC **Stationary Phase:** silica gel 60 F_{254} **Solvent system:** ethyl acetate: acetone: acetic acid (6.5: 3: 0.5 v/v/v) **solvent:** methanol **detection:**UV detection at 208 nm **LOD:** HCTZ: 143.82 ng/spot Quinapril hcl: 123.02 ng/spot **Rf:** HCTZ: 0.76 Quinapril: 0.51	53
7	**HPTLC method for the simultaneous estimation of valsartan and hydrochlorothiazide**	Tablets	**Method:** HPTLC **Stationary Phase:** Precoated silica gel 60 F_{254} **Mobile phase:** chloroform: methanol: toluene: glacial acetic acid (6:2:1:0.1 v/v/v/v) **detection:** UV detection at 260 nm **LOD:** HCTZ: 30 ng/spot Valsartan: 100 ng/spot	54

8	**Ion pair-HPLC method for the simultaneous estimation of quinapril and hydrochlorothiazide**	Tablet	**Method**: HPLC **Mobile Phase:** 0.1% v/v triethylamine (pH 3.5), containing 1 mM of hexane sulphonic acid: acetonitrile (30:70% v/v) **Detection:** PDA Detection (220 nm)	55
9	**Simultaneous estimation of bisoprolol fumarate and hydrochlorothiazide RP-HPLC**	Tablets	**Method:** RP-HPLC **Mobile phase:** Water:acetonitrile:tetrahydrofuran (80:20:5 v/v/v) **Column:** lichrospher 100 C-18, 5 µm column 20 cm × 4.6 mm **Flow rate:** 1 ml/min **Detection:** UV Detection (225 nm) **LOD:** 3.5 µg/ml for bisoprolol fumarate 0.4 µg/ml for HCTZ	56
10	**Simultaneous spectrophotometric estimation of hydrochlorothiazide and bisoprolol fumarate**	Pharmaceutical dosage Forms	**Method:** UV spectrophotometry 1. **Simultaneous Equation Method** Amplitude wavelengths: 223 nm and 271.6 nm 2. **ratio spectra derivative method** 202.6 nm for bisoprolol fumarate difference at 212.6 nm and 230 nm for HCTZ	57

11	**RP-HPLC method for simultaneous estimation of telmisartan and hydrochlorothiazide**	Tablets	**Method:** RP-HPLC **Column:** ODS Hypersil C18 (25 cm×4.6 mm I.D) **Mobile phase:** acetonitrile:0.05 M KH_2PO_4 pH 3.0 (60:40) **Flow rate:** 1 ml/min **UV detection:** 218 nm **Rt:** Telmisartan: 5.19 min HCTZ: 2.97 min	58
12	**Simultaneous Spectrophotometric Determination of Losartan Potassium and Hydrochlorothiazide**	Tablets	**Method:** UV spectrophotometry 1. **Simultaneous equation method** at 206.6 nm and 270.6 nm 2. **Dual wavelength method** 206.6 and 261.4 for losartan and 270.6 for hydrochlorothiazide	59
13	**Back-propagation neural network Model for simultaneous spectrophotometric estimation of losartan potassium and hydrochlorothiazide**	Tablets	The development of multivariate calibration model with back-propagation neural network using calibration sets constructed from the spectral data of pure components is proposed for the simultaneous estimation of active components, losartan potassium and hydrochlorothiazide in tablet dosage.	60
14	**Simultaneous estimation of penbutolol sulfate and hydrochlorothiazide by RP-HPLC**	Tablets	**Method:** RP-HPLC **Column:** Chromegabond M C 18 column **Mobile Phase:** 50 mM Na2HPO4: acetonitrile (60:40 v/v) adjusted to pH 3.5 with orthophosphoric acid **Flow rate:** 1 ml/min **UV detection:** at 220 nm **Internal standard:** propranolol HCl	61

15	**Development and validation of a HPTLC method for the simultaneous estimation of telmisartan and hydrochlorothiazide**	Tablets	**Method:** HPTLC **Stationary phase:** precoated silica gel $60F_{254}$ **Mobile Phase:** chloroform: methanol: toluene (2:5:5 v/v/v) **UV detection:** at 272 nm **LOD:** Telmisartan: 75 ng/spot HCTZ: 55 ng/spot	62
16	**Development and validation of a simultaneous HPTLC method for the estimation of olmesartan medoxomil and hydrochlorothiazide**	Tablets	**Method:** HPTLC **Stationary phase:** precoated silica gel $60F_{254}$ **Mobile Phase:** acetonitrile:chloroform:glacial acetic acid (7:2:0.5, v/v/v) **UV detection:** at 254 nm **LOD:** Olmesartan medoxomil: 170 ng/spot HCTZ: 30 ng/spot	63
17	**Development and validation of a HPTLC method for the simultaneous estimation of irbesartan and hydrochlorothiazide**	Tablets	**Method:** HPTLC **Stationary phase:** precoated silica gel $60F_{254}$ **Mobile Phase:** acetonitrile: chloroform: glacial acetic acid (7:3:0.1 v/v/v) **UV detection:** at 260 nm **LOD:** irbesartan: 30 ng/spot HCTZ: 25 ng/spot	64
18	**Simultancous estimation of captopril and hydrochlorothiazide in two component tablets by ultra violet absorption spectrophotometry.**	Tablets	**Method:** UV spectrophotoetry **Amplitude Wavelength:** Captopril: 238 and 260 nm HCTZ: 322 nm	65

19	**Simultaneous estimation of losartan potassium and hydrochlorthiazide**	Dosage forms	**Method:** UV spectrophotoetry (Simultaneous Equation Method) **Amplitude Wavelength:** Losartan: 236 nm HCTZ: 270 nm **Beer's law limit:** Losartan: 2-20 µg/ml HCTZ: 1-50 µg/ml	66
20	**Derivative and Q-analysis spectrophotometric methods for estimation of hydrochlorothiazide and olmesartan medoxomil**	Tablets	**Method:** UV spectrophotoetry 1. Q- analysis Method (Abs. Ratio method) **Wavelength amplitude:** Olmesartan medoxomil: 264 nm (isobestic point) HCTZ: 271 nm 2. derivative spectrophotometric method	67
21	**Simultaneous determination of valsartan and hydrochlorothiazide in tablets by RP-HPLC**	Tablets	**Method:** RP-HPLC **Column:** C 18 column(200 × 4.6 mm i.d) **Mobile Phase:** methanol:acetonitrile:water: isopropylalcohol (22:18:68:2; adjusted to pH 8.0 using triethylamine; v/v) **Flow rate:** 1 ml/min **UV detection:** at 270 nm **Rt:** Valsartan: 3.42 min HCTZ: 8.43 min	68
22	**New spectrophotometric method for simultaneous determination of metoprolol tartarate and hydrochlorthiazide**	Tablets	**Method:** UV spectrophotometry **(Dual wavelength method)** 257..8 and 282.9 for metoprolol tartarate and 315 nm for hydrochlorothiazide	69

23	**Ratio Spectra Derivative and Zero-Crossing Difference Spectrophotometric Determination of Olmesartan Medoxomil and Hydrochlorothiazide**	Pharmaceutical dosage form	**Method:** UV spectrophotometry **1. First order derivtive Wavelength amplitude:** Olmesartan Medoxomil:231 nm HCTZ: 271 nm **2. zero-crossing difference spectrophotometric method Wavelength amplitude:** 257.8 nm and 240.2 nm **Beer's law limit:** Olmesartan Medoxomil: 08–24 µg/mL HCTZ: 05–15 µg/mL	70

3. Aim of Present Work

Ramipril – an angiotensin coverting enzyme inhibitor – prevents the severe heart failure by preventing the conversion of angiotensin I to angiotensin II, thereby counteracting salt and water retention, peripheral arterial and venous vasoconstriction and activation of the sympathetic nervous system. It also prevents the undesirable activation of the rennin – angiotensin system caused by diuretic therapy. The major benefit of ACE inhibitor therapy is a reduction in afterload as well as preload and modest increase in the plasma potassium concentration. Therefore treating the heart failure with a combination of a potassium losing diuretic like thiazide diuretics(e.g. Hydrochlorothiazide) and an ACE inhibitors(e.g. Ramipril) has potential advantages.

The literature review revealed that chromatographic, spectroscopic, titrametric etc. methods are reported for the determination of Ramipril and Hydrochlorothiazide in their combined dosage forms.

The reported chromatographic methods for estimation of Ramipril and Hydrochlorothiazide are time consuming and costly. So, it was thought to develop a rapid and cost-effective UV and HPTLC methods for estimation of Ramipril and Hydrochlorothiazide in pharmaceutical dosage forms.

The prime objective of work was to develop and validate simple, rapid and cost efficient UV and HPTLC methods for estimation of Ramipril and Hydrochlorothiazide in pharmaceutical dosage forms.

4. Introduction to method development

Drugs have become an essential part of life. Everyone has been taking various drugs since its birth. The quality of these drugs is an essential feature as it directly affects the life of the consumer and the quality of any drug products can be judged by analyzing it only.

Quantitative analysis of the drug is an important tool to assure its quality. Quality control is an integral part of all modern industrial process and pharmaceutical industry with no exception. For assuring the quality of the drug products there is a crucial need of specific analytical methods and because drugs have direct impact on human lives so lots of care should be taken during selection of the method.

The aim of the analytical studies is to obtain quantitative and qualitative information about the compounds of interest (analyte) in a sample. Pharmaceutical formulations are formulated with more than one drug, typically referred to as combination products. These are intended to meet desired patient need by combining their therapeutic effects of two or more drugs in one product. These combination products can present challenges to the analytical chemists responsible for the development and validation of the analytical method for their analysis.

Testing a pharmaceutical product involves chemical, instrumental and sometimes microbiological analysis. Simultaneous estimation of drugs in combination can be carried out by using spectrophotometric and spectrofluorimetric methods and some chromatographic techniques like HPLC, HPTLC, SFC, LC/MS etc.

Planner chromatography is a multistage distribution process. It is a form of liquid chromatography in which the stationary phase is supported on a planer surface rather than a column. High performance thin layer chromatography has developed to the extent that separation and quantitation can provide results that are comparable with other analytical methods such as HPLC. HPTLC is a modern separation technique which is accepted

world wide as an extremely flexible, reliable, and cost efficient method. Compared to techniques like HPLC it has features like flexibility, parallel analysis of many samples, simplified sample preparation because of single used of stationary phase and possibility of multiple evaluation of the plate with different parameters because all fractions of the sample are stored on the plates. HPTLC technique is most suited technique for content uniformity test and impurity profiling of the drugs as per compendial specification.

4.1.1 INTRODUCTION TO HPTLC METHOD

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.[71]

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.[72-74] 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.[75] 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.[76]

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

Table 5: 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 nutes

4.1.2 Steps involved in HPTLC [76]

- 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 6: 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.

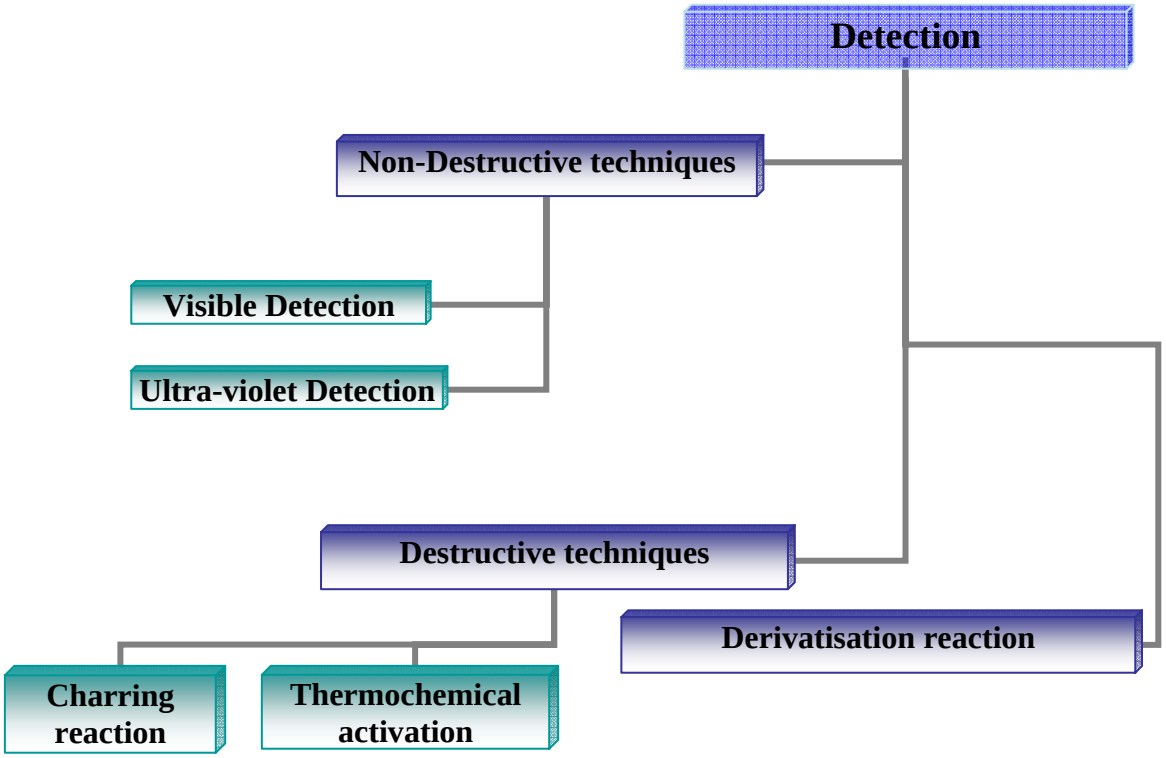
The types of HPTLC chambers are

- Nu-chamber
- Ns-chamber
- Twin-through chamber

- Su-chamber
- Ss- chamber
- Horizontal chamber
- Automatic development chamber (ADC)
- Vario chambers

Detection

Figure 2: Techniques of 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, 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.

4.2 VALIDATION OF HPTLC 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

4.2.1 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

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

Where,

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

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

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

4.2.2 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

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

4.2.3 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.

$$\mathbf{y = mx + c}$$

where, y = measured signal
x = concentration of sample
c = intercept
m = slope of line

4.2.4 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]

4.2.5 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.

4.2.6 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

4.2.7 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.[82] 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 7: 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:

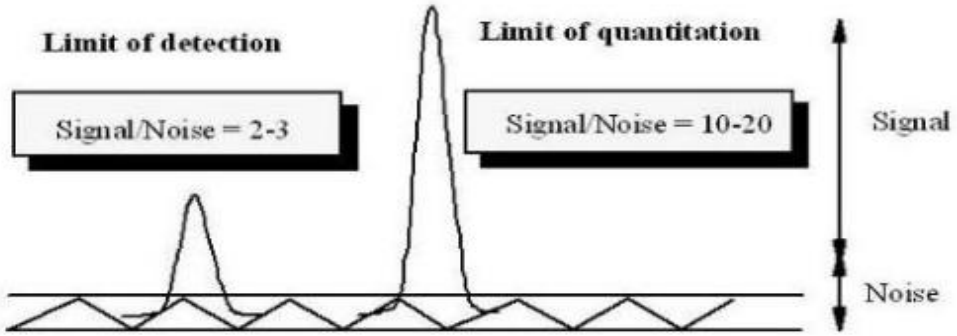
1. **Based on the standard deviation of the blank**

   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.

Figure 3: Signal/Noise for LOD and LOQ



4.2.8 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]

4.2.9 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.

4.3 INTRODUCTION TO UV METHOD FOR ANALYSIS

Ultraviolet-visible spectroscopy 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

At present, dual-wavelength spectrophotometry is probably the least well known of the techniques of absorption spectrophotometry. However, recent improvements in application techniques have shown that for various organic and inorganic materials dual-wavelength spectrophotometry can provide higher sensitivity and selectivity than conventional spectrophotometry.

A dual wavelength spectrophotometer produces a relatively small, high power, high duty cycle light spot from a single relatively low power multi-chromatic light source. A Xenon arc lamp light source is focused by an ellipsoidal mirror onto a rotating partially reflective optical chopper. The chopper comprises a wheel having mirrored segments alternately separated by transparent segments. Light reflected by the mirrored segments passes through a first monochromator which produces a first monochromatic light beam. Light transmitted through the transparent segments passes through a second monochromator and emerges as a second monochromatic light beam having a wavelength different from the wavelength of said first monochromatic light beam. The first and second monochromatic light beams are recombined into a single dual wavelength light beam that is reflected through a sample to be analyzed. Reflective front surfaces are employed throughout the system in order to minimize power loss.

4.4 INTRODUCTION TO 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.

4.4.1 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.

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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".[83-85]

4.4.3 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. [86]

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.

$$y = mx + c$$

4.4.4 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. [86]

4.4.5 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.[86]

Selectivity is ability of analytical method to differentiate various substances in the sample.[86] 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.

4.4.6 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.[86] It is expressed in terms of concentration units. Limit of Detection values are always specific for a particular set of experimental conditions.

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.[86]

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APPROACH	LIMIT OF DETECTION	LIMIT OF QUANTIFICATION
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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

5. Experimental Work

5.1 IDENTIFICATION OF DRUG

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 9: comparison of melting points

Drugs	Standard M.P.	Obtained M.P.
Ramipril	109 ° C	111 – 114 ° C
Hydrochlorothiazide	274 ° C	270 – 273 ° C

5.1.2 Determination of UV spectra

UV-spectrum of Ramipril (10 µg/ml) and Hydrochlorothiazide (10 µg/ml) in distilled water was taken.

Figure 4: UV spectrum of Ramipril (10 µg/ml) in distilled water

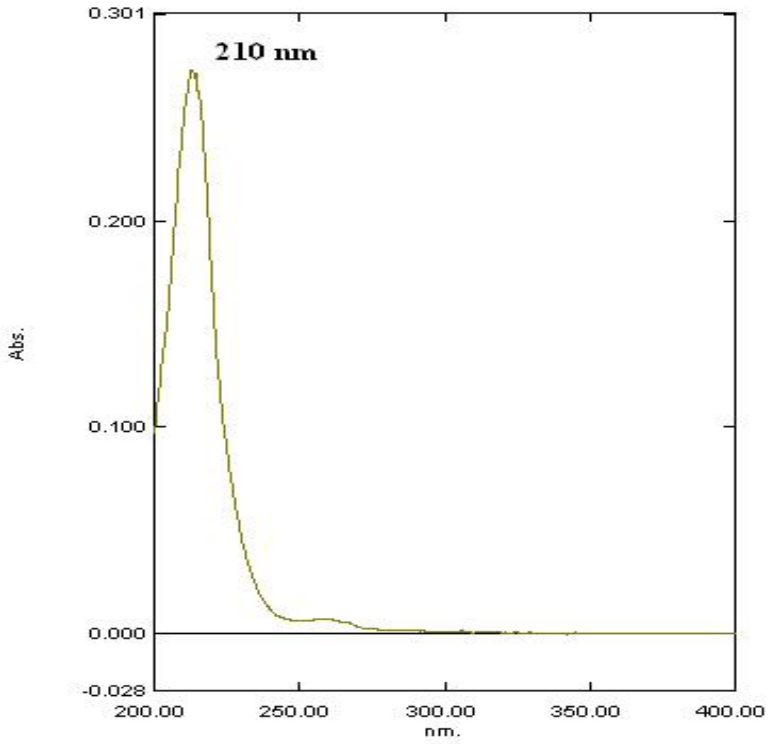


Figure 5: UV spectrum of Hydrochlorothiazide (10 µg/ml) in distilled water

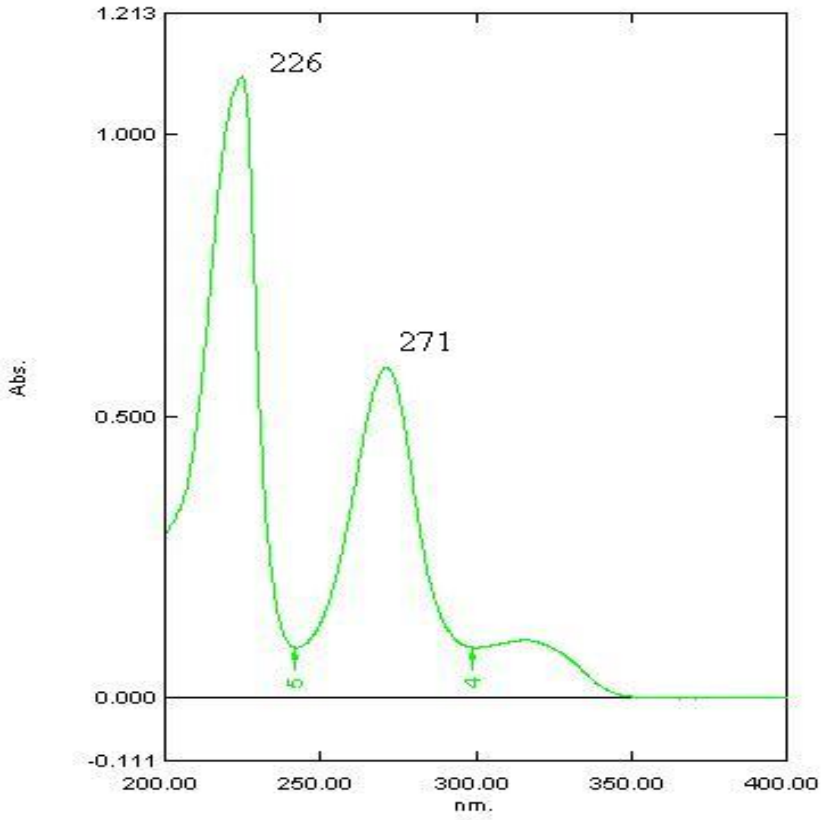


Table 10: Wavelength Maxima for Ramipril and Hydrochlorothiazide

DRUG NAME	REPORTED PEAK (NM)	PEAK OBTAINED (NM)
Ramipril	210	210
Hydrochlorothiazide	226,271	226,271

5.1.3 Determination of Raman Spectra

Figure 6: Raman Spectra of Ramipril

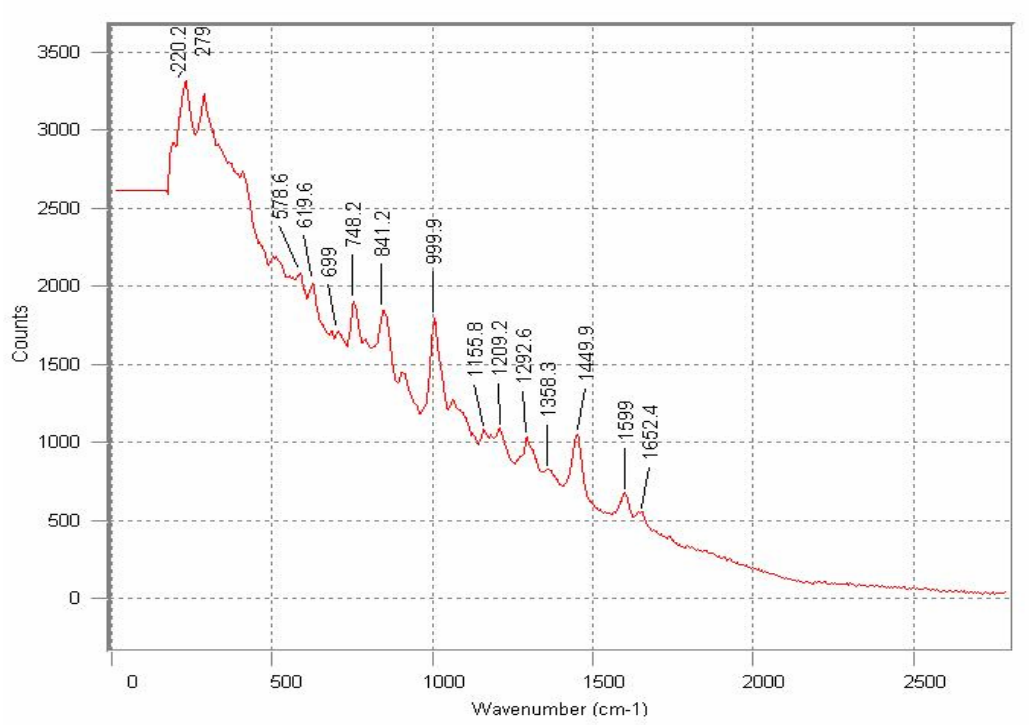


Table 11: Specification of Raman spectrum of Ramipril

Specification of Raman Peak for Ramipril	**Recorded Wave Number(cm-1)**
Mono substituted benzene	619.6
Symmetric C-N-C stretch	841.2
Mono substituted benzene	999.9
CH_2 in phase twist	1292.6
CH_3,CH_2 deformation	1449.9
Benzene derivatives	1599
Symmetric C=O stretch	1652.4

Figure 7: Raman Spectra of Hydrocholrothiazide

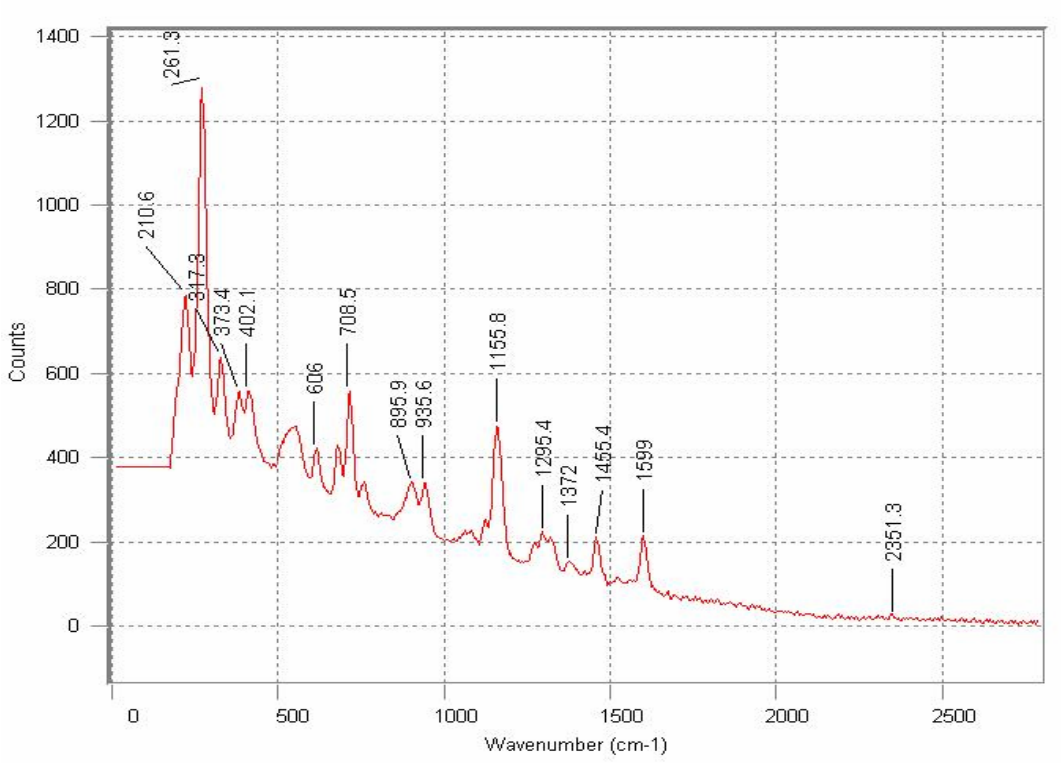


Table 12: Specification of Raman spectrum of Hydrochlorothiazide

Specification of Raman Peak for Hydrochlorothiazide	**Recorded Wave Number(cm-1)**
CS stretch	708.5
Symmetric C-N-C stretch	895.9
CC stretch	1155.8
CH_3,CH_2 deformation	1455.4
NH_2 scissors	1599

5.2 HPTLC METHOD DEVELOPMENT FOR SIMULTANEOUS ESTIMATION OF RAMIPRIL AND HYDROCHLOROTHIAZIDE IN COMBINED DOSAGE FORM

5.2.1 EXPERIMENTAL WORK:

5.2.1.1 Apparatus and Instruments:

- Camag – 100 µl Applicator syringe (Hamilton, Bonaduz, Schweiz)
- Camag Applicator-Linomat V
- Camag – Twin trough chamber (20 × 20) with stainless steel Lid
- Camag TLC scanner3
- UV cabinet with dual wavelength UV lamp (254 nm and 366 nm)
- Balance Model: KEROY®, Keroy (Balance) Pvt. Ltd.
- Ultra Sonicator, Trans-o-sonic, India
- Amber coloured volumetric flask - 100ml, 50ml, 10ml.

5.2.1.2 Reagents and Materials:

- Ramipril standard
- Hydrochlorothiazide standard
- Methanol, AR grade, s.d. fine chemicals, Mumbai
- Chloroform, AR grade, s.d. fine chemicals, Mumbai
- Ethyl acetate, AR grade, s.d. fine chemicals, Mumbai
- Glacial acetic acid (GAA), AR grade, s.d. fine chemicals, Mumbai
- Double distilled water
- Tablets containing Ramipril and Hydrochlorothiazide.

5.2.2 Preparation of stock solution of Ramipril:

Standard Ramipril was accurately weighed and transferred to 100 ml volumetric flask. It was dissolved properly and diluted up to mark with Methanol to obtain final concentration. This solution was used to prepare standard mixture.

5.2.3 Preparation of stock solution of Hydrochlorothiazide:

Standard Hydrochlorothiazide was accurately weighed and transferred to 100 ml volumetric flask. It was dissolved properly and diluted up to mark with Methanol to obtain final concentration. This solution was used to prepare standard mixture.

5.2.4 Preparation of working standard mixture:

From 1 mg/ml of both solution, 1 ml was taken from both solution, mixed and diluted up to 10 ml with methanol to obtain final concentration of both drugs. This solution was used as working standard mixture.

5.2.5 Pre-treatment of pre-coated plates:

TLC plate was placed in twin trough glass chamber containing methanol as mobile phase. Methanol was allowed to run up to upper edge of plate (ascending method). Plate was removed and allowed to dry in oven at 60^0C for 20 min. For the actual experiment the plate was allowed to come to room temperature and used immediately.

5.2.6 Calibration curve for standard Ramipril and Hydrochlorothiazide:

From the working standard mixture(100 µg/ml), aliquots were spotted on the TLC plate under nitrogen stream using Linomat V to obtain final concentration range.

Standard	Application volume (µl)	Conc. Per spot (ng)
Standard 1	5	500
Standard 2	7	700
Standard 3	9	900
Standard 4	11	1100
Standard 5	13	1300
Standard 6	15	1500
Standard 7	17	1700
Standard 8	19	1900

5.2.7 Analysis of prepared standards:

The plates were developed in Twin trough developing chamber (20 × 20 cm) with stainless steel Lid, previously saturated with the mobile phase for 15 min. The plates were removed from the chamber after development and were dried in Hot air oven at 60^0C for 15 mins.

The plates were scanned and quantified at 210 nm in absorbance mode with camag TLC scanner 3. The calibration curve was constructed by plotting peak area vs. concentration (ng/spot) corresponding to each spot and regression equation was calculated.

5.2.8 Quantification of Ramipril and Hydrochlorothiazide in Tablet:

5.2.8.1 Preparation of test stock solution:

To determine the content of Ramipril and Hydrochlorothiazide in tablet, the contents of 20 tablets were weighed and their mean weight determined and finely powdered. An equivalent weight of the tablet content was transferred into a 100 ml volumetric flask containing 100 ml Methanol, sonicated for 15 mins and supernatant was filtered through whatman filter paper.

5.2.8.2 Analysis of prepared samples:

12 µl from these solutions were spotted on the TLC plate under nitrogen stream using Linomat V. The plate was dried in air, and then the plate was developed in Twin trough developing chamber, previously saturated with the mobile phase for 15 min. The plates were removed from the chamber after development and were dried.

The plates were scanned and quantified at 210 nm in absorbance mode with camag TLC scanner 3.The concentration of sample solution was found from calibration curve of Ramipril and Hydrochlorothiazide respectively. Recovery studies were performed by standard addition method.

5. 3 Result and Discussion:

5.3.1 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 Rf values, practical problems such as diffusion of spots, tailing, proper peak shape after scanning.
- The solvent methanol was used initially for preparation of solution of both drugs which gave good solubility for both drugs and also give good resolution so it was selected as a solvent for preparation of levodopa, carbidopa solutions for the experimental work.

5.3.2 Validation Parameters

5.3.2.1. Linearity

Linearity range of Ramipril and Hydrochlorothiazide were found to be 500 – 1900 ng/spot with correlation co-efficient 0.9923 and 0.9963 respectively.

Table 14: Calibration data of Ramipril by HPTLC with UV detection

Sr. No.	Concentration (µg/spot)	Peak Area	
		Mean ± SD	%RSD
1	500	3040.87 ± 5.38	0.18
2	700	3864.57 ± 9.77	0.25
3	900	4540.23 ± 10.79	0.24
4	1100	5322.6 ± 8.25	0.16
5	1300	6049.17 ± 11.69	0.19
6	1500	6535.2 ± 12.14	0.19
7	1700	7056.77 ± 11.46	0.16
8	1900	7622.47 ± 11.33	0.15

Figure 8: Linearity curve for Ramipril

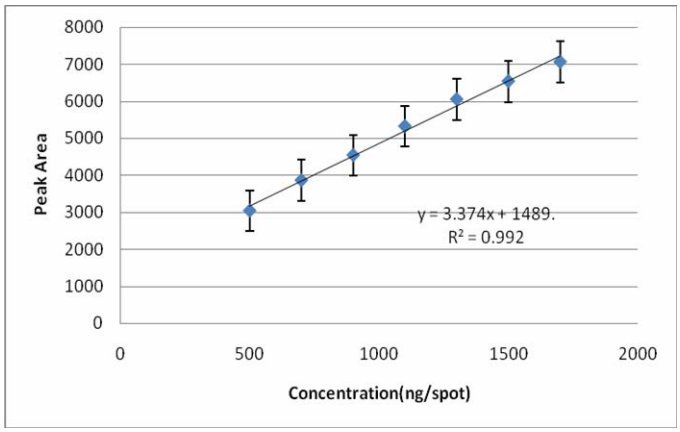


Figure 9. Linearity curve for Ramipril from winCATS software

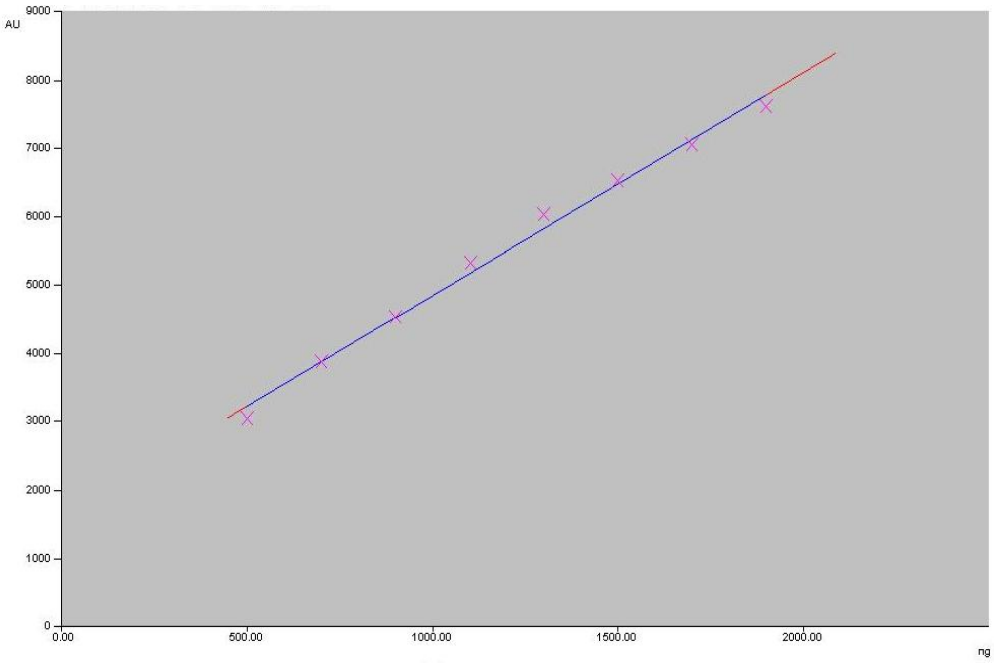


Table 15: Calibration data of Hydrochlorothiazide by HPTLC

Sr. No.	Concentration (µg/spot)	Peak Area	
		Mean ± SD	%RSD
1	500	10869.63 ± 15.7	0.14
2	700	12204.33 ± 48.80	0.40
3	900	14262.43 ± 16.80	0.12
4	1100	15872.43 ± 53.60	0.34
5	1300	17187.43 ± 17.32	0.10
6	1500	18245.4 ± 31.58	0.17
7	1700	19630.83 ± 33.37	0.17
8	1900	20785.2 ± 27.07	0.13

Figure 10: Linearity curve for Hydrochlorothiazide

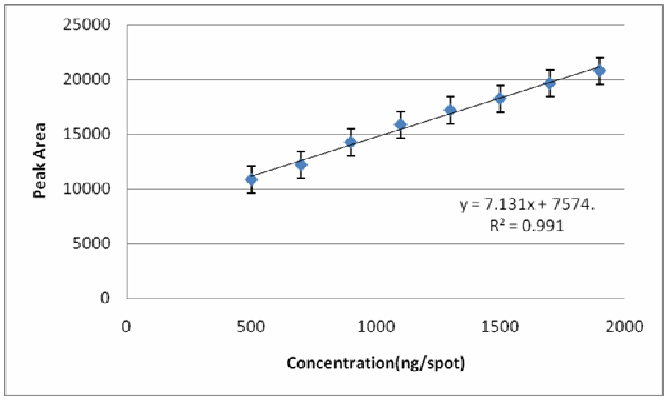


Figure 11: Linearity curve for Hydrochlorothiazide from winCATS software

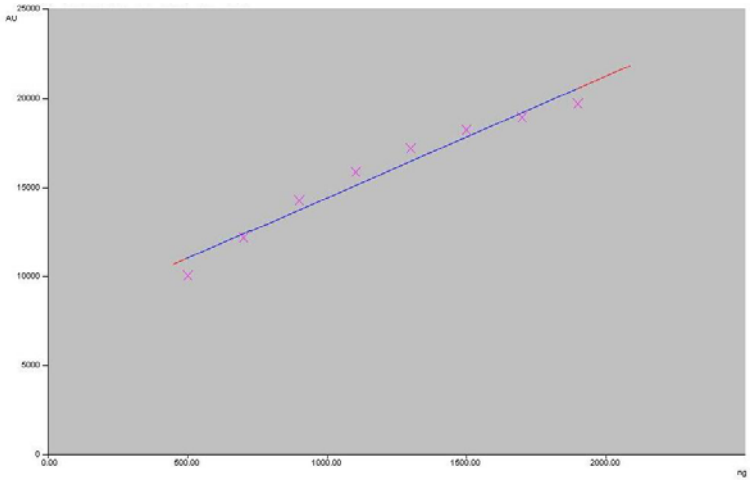


Figure 12: HPTLC chromatogram of Ramipril (1000 ng/spot; R_f = 0.28) in methanol

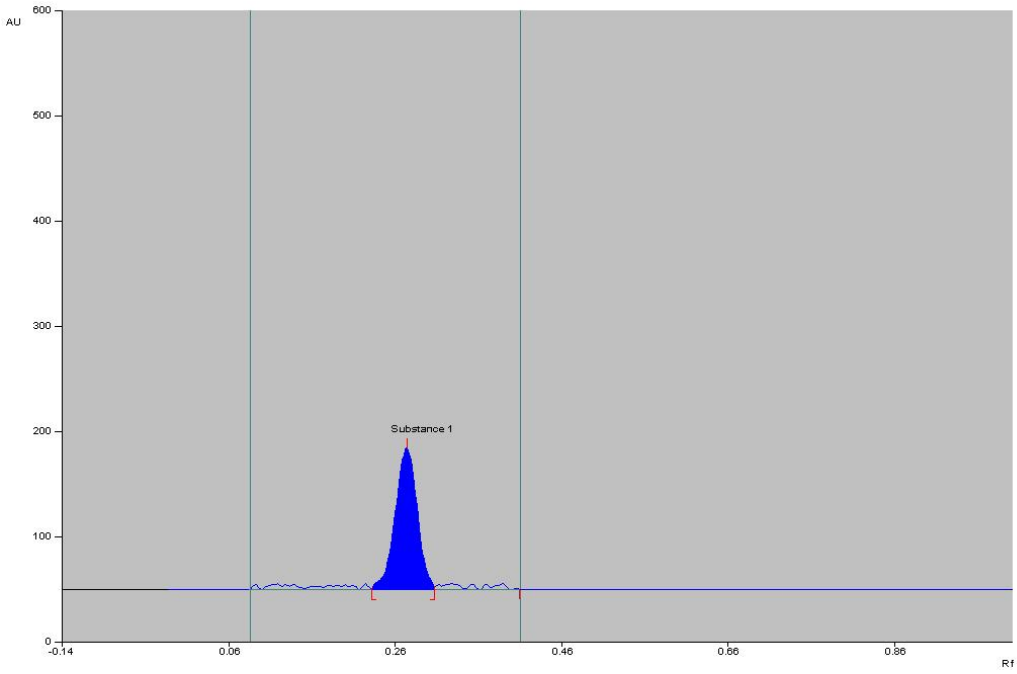


Figure 13: HPTLC chromatogram of Hydrochlorothizide (1000 ng/spot; R_f = 0.49) in methanol

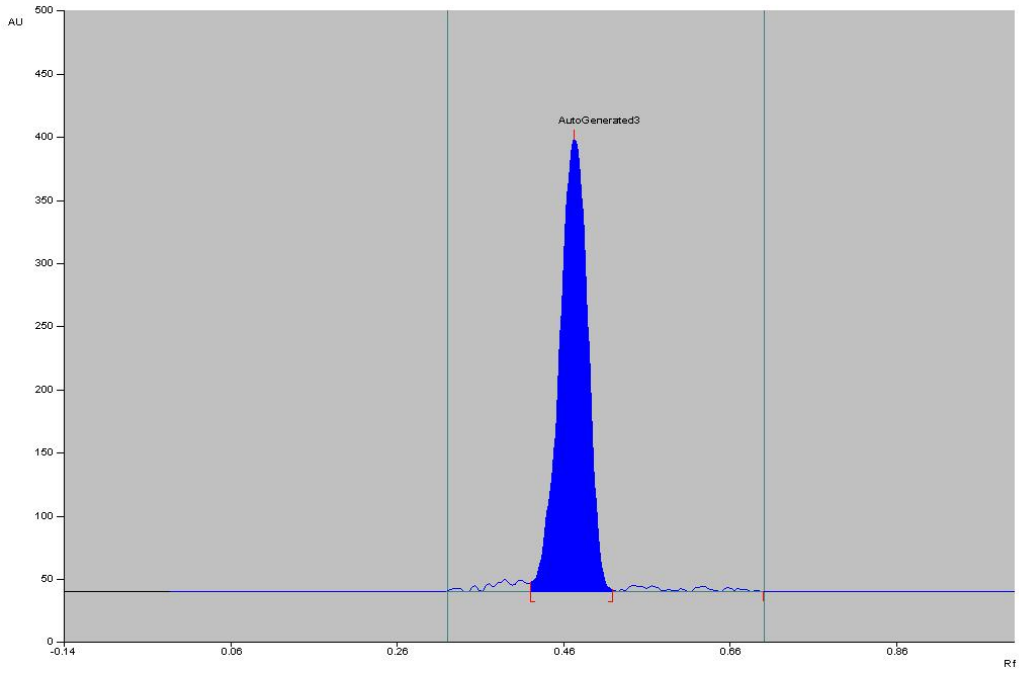


Figure 14: HPTLC chromatogram of standard mixture of Ramipril(1000 ng/spot; R_f=0.28) and Hydrochlorothiazide(1000 ng/spot; R_f=0.49)

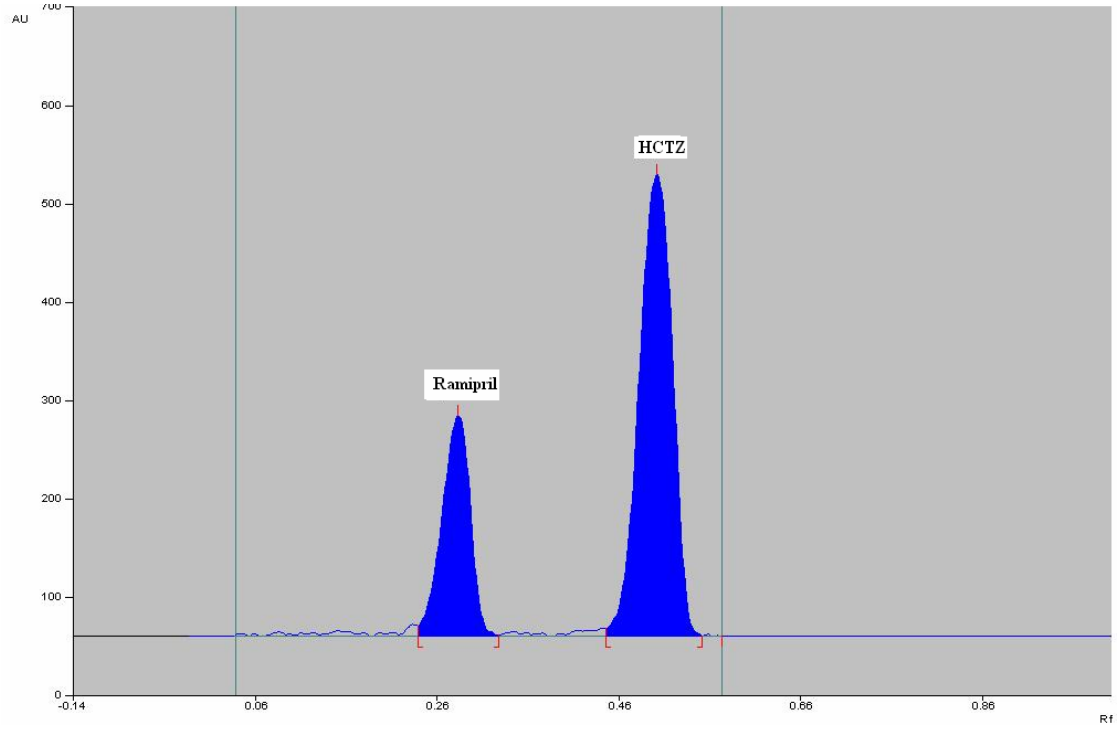
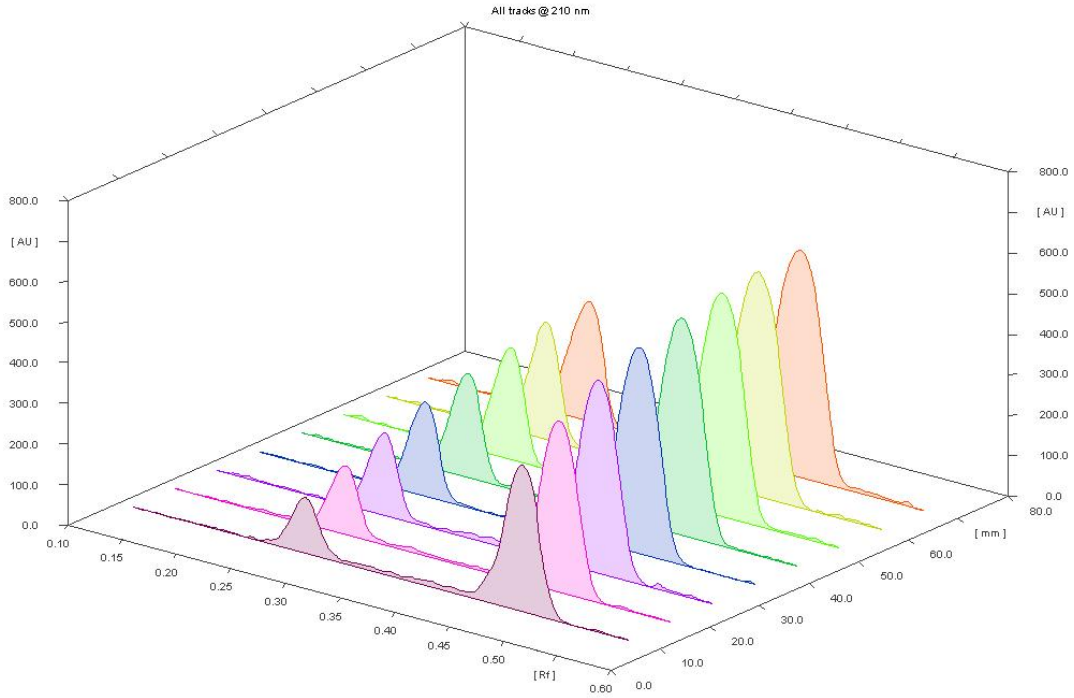


Figure 15: HPTLC chromatogram (3D view) for linearity of Ramipril and Hydrochlorothiazide



5.3.2.2 Precision

5.3.2.2.1. Repeatability

Table 16: Repeatability data of Ramipril (900 ng/spot) by HPTLC with UV detection

Time	Peak Area
1st	4530
2nd	4539.2
3rd	4551.5
4th	4515.6
5th	4561.9
Mean	4539.64
S.D.	18.08
%RSD	0.40

Table 17: Repeatability data of Hydrochlorothiazide(900 ng/spot) by HPTLC with UV detection

Time	Peak Area
1st	14262.3
2nd	14279.3
3rd	14245.7
4th	14298.7
5th	14229.8
Mean	14263.16
S.D.	27.12
%RSD	0.190

5.3.2.2.2 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 18: Intraday precision data of Ramipril by HPTLC with UV detection

Sr. No.	Concentration (ng/spot)	Peak Area	
		Mean ± SD	%RSD
1	900	4542.33 ± 14.89	0.33
2	1100	5334.83 ± 20.29	0.38
3	1300	6056.87 ± 28.73	0.47

Table 19: Interday precision data of Ramipril by HPTLC with UV detection

Sr. No.	Concentration (ng/spot)	Peak Area	
		Mean ± SD	%RSD
1	900	4544.47 ± 22.97	0.50
2	1100	5339.13 ± 16.13	0.30
3	1300	6059.70 ± 15.9	0.26

Table 20: Intraday precision data of Hydrochlorothiazide by HPTLC with UV detection

Sr. No.	Concentration (ng/spot)	Peak Area	
		Mean ± SD	%RSD
1	900	14267 ± 27.67	0.19
2	1100	15860.03 ± 58.22	0.37
3	1300	17179.67 ± 30.03	0.17

Table 21. Interday precision data of Hydrochlorothiazide by HPTLC with UV detection

Sr. No.	Concentration (ng/spot)	Peak Area	
		Mean ± SD	%RSD
1	900	14280.53 ± 21.38	0.15
2	1100	15754.4 ± 132.56	0.84
3	1300	17173.63 ± 16.01	0.09

5.3.2.3 Accuracy

Accuracy of the measurement of Ramipril and Hydrochlorothiazide 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 22: Accuracy data of Ramipril by HPTLC with UV detection

Initial conc. (ng/spot) (A)	Quantity of std. Added (ng/spot)(B)	Total Amount (A + B)	Accuracy	
			Peak area Mean ± S.D.	%Recovery Mean ± S.D
500	400	900	4543.4 ± 16.71	99.12 ± 1.21
500	500	1000	4942.35 ± 76.67	99.78 ± 3.25
500	600	1100	5376.83 ± 22.67	98.45± 4.54

Table 23: Accuracy data of Hydrochlorothiazide by HPTLC with UV detection

Initial conc. (ng/spot)(A)	Quantity of std. Added (µg/spot)(B)	Total Amount (A + B)	Accuracy	
			Peak area Mean ± S.D.	%Recovery Mean ± S.D
500	400	900	14288.75 ± 9.95	99.89 ± 1.54
500	500	1000	14986.35 ± 54.56	100.9 ± 2.54
500	600	1100	15773.47 ± 111.51	98.56 ± 3.65

5.3.2.4 Limit of detection

Limit of detection for Ramipril and Hydrochlorothiazide was found as per the procedure given in section 7.4

The minimum detectable concentration of Ramipril was found to be **4.24 ng/spot**

The minimum detectable concentration of Hydrochlorothizide was found to be **8.99 ng/spot**

5.3.2.5 Limit of quantification

Limit of quantification for Ramipril and Hydrochlorothiazide was found as per the procedure given in section 7.5

The lowest quantifiable concentration of Ramipril was found to be **12.85 ng/spot**

The lowest quantifiable concentration of Hydrochlorothizide was found to be **27.23 ng/spot**

5.3.2.6 Specificity(Peak Purity)

Figure 16: Peak purity spectra of Ramipril and Hydrochlorothiazide

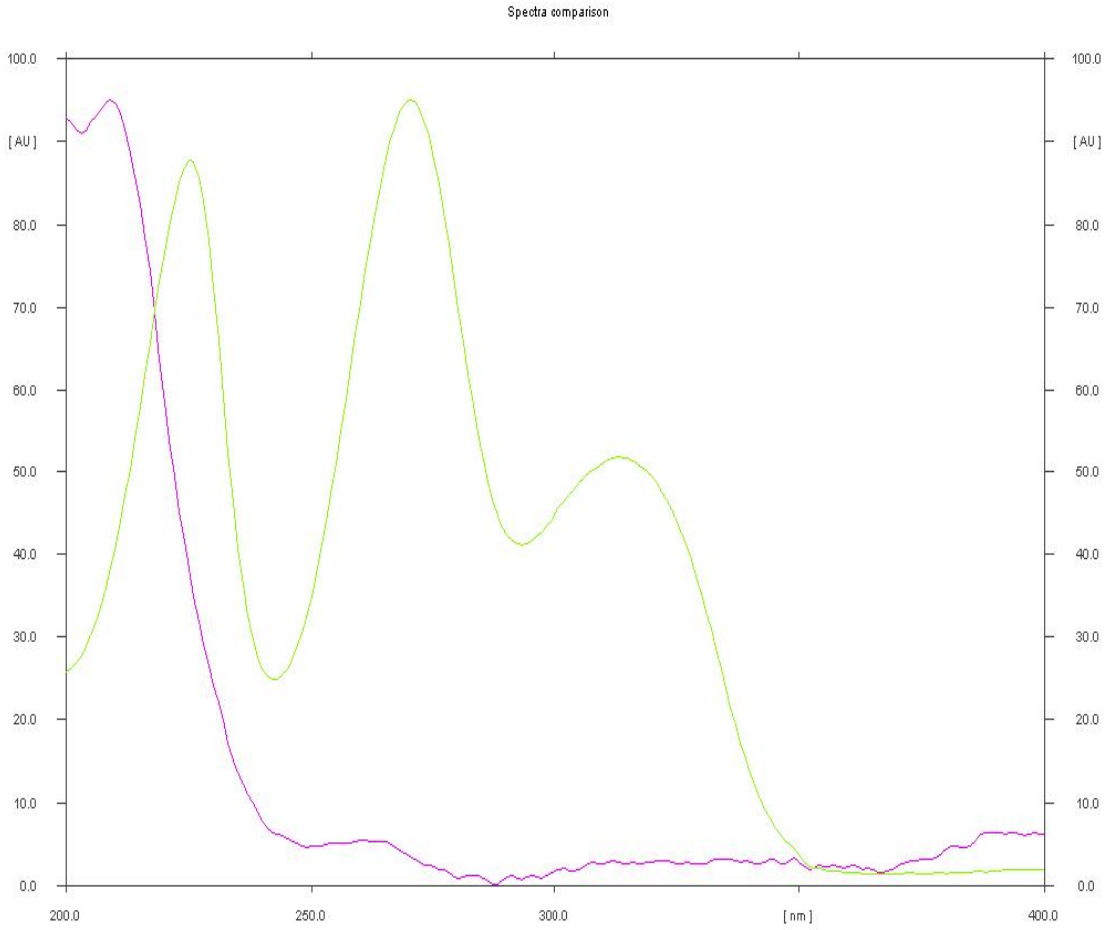


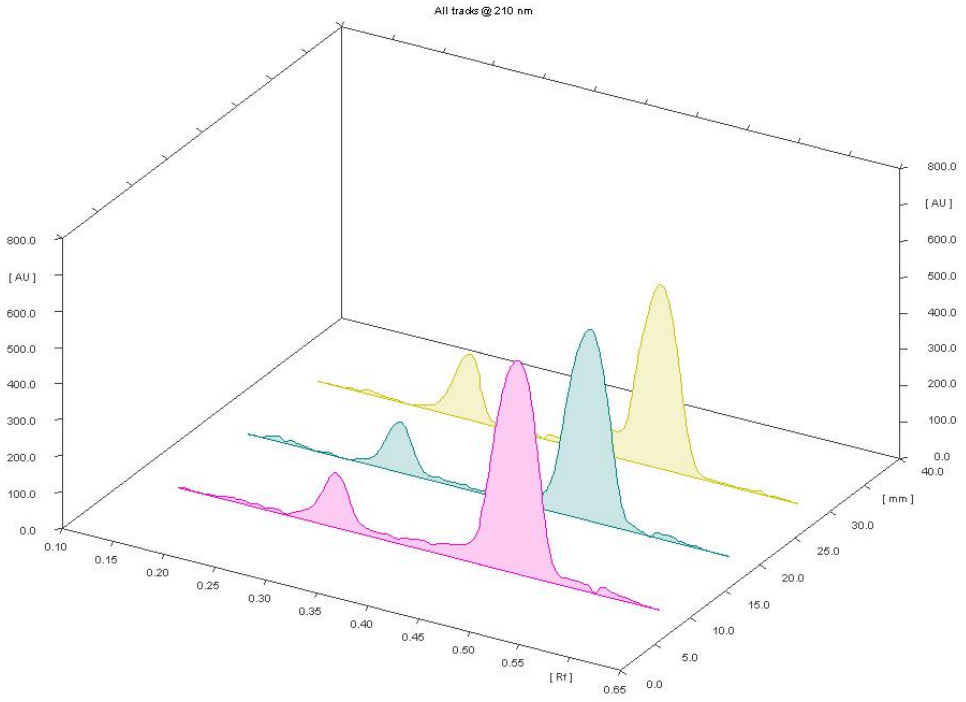
Table 24: Specificity data of Ramipril and Hydrochlorothiazide

Drugs	Correlation Coefficient	Purity
Ramipril	0.999915	Pass
Hydrochlorothiazide	0.999585	Pass

5.3.4 Estimation of Ramipril and Hydrochlorothiazide in marketed Tablet:

The developed method was used to estimate Ramipril and Hydrochlorothiazide in the tablet dosage form. Three different brands of tablet formulations were procured from the market for analysis by the proposed method. The percentage of Ramipril and Hydrochlorothiazide was found from the calibration curve of the standard drug respectively.

Figure 17. HPTLC chromatogram of assay of tablet samples 600ng/spot for Ramipril and 1500ng/spot for HCTZ



Track 1 : Ramihart H5

Track 2: Ramistar H5

Track 3: Ramipres H5

5.4 UV METHOD DEVELOPMENT FOR SIMULTANEOUS ESTIMATION OF RAMIPRIL AND HYDROCHLOROTHIAZIDE IN COMBINED DOSAGE FORM

5.4.1 Abstract

A UV absorption spetrophotometric method was developed for the simultaneous estimation of Ramipril and Hydrochlorothiazide in their combined dosage forms. The developed method was found to be precise, selective and rapid for simultaneous estimation of Ramipril and Hydrochlorothiazide in their combined dosage forms.

5.4.2 Materials and Methods

5.4.2.1 Reagents and chemicals:

- Distilled Water
- Tablets containing Ramipril (5 mg) and Hydrochlorothiazide (12.5 mg).
- (Brand name: **Ramipres H5;** Name of manufacturer: **Cipla Ltd**
  Ramistar H5 ; Name of manufacturer: **Lupin Ltd.**.
  Ramihart H5; Name of manufacturer: **Mankind Pharma Ltd**
  Race H2.5; Name of manufacturer: **Alkem Lab. Ltd.)**
- Ramipril API
- Hydrochlorothiazide API

5.4.2.2Instrumentation

(1) UV-Visible Double-Beam spectrophotometer:

Matched quartz cell (1cm)

Model: UV-2450

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: 100gm.

(3) Sonicator:

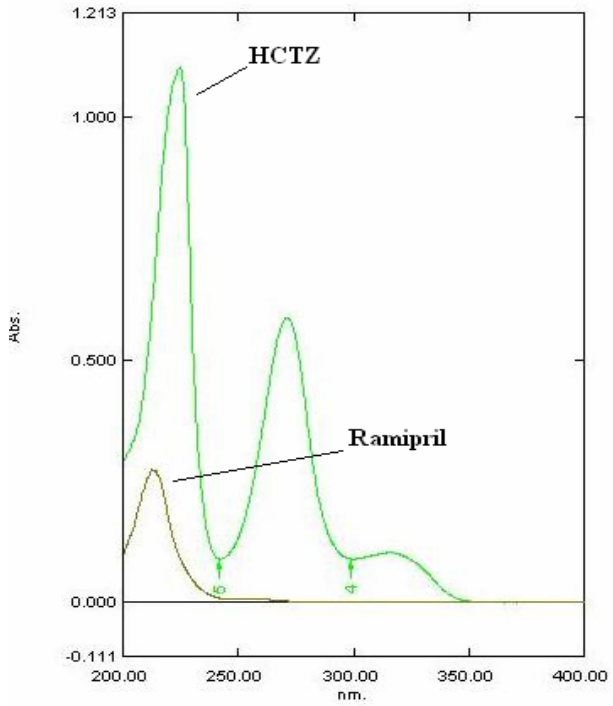
Model: TRANS-O-SONIC; D-compect.

Capacity: 2 Lit.

5.4.2.3 Preparation of Standard Stock Solution of Ramipril and Hydrochlorothiazide

UV analysis was done by using the standard stock solution of 1000 µg/ml of Ramipril and Hydrochlorothiazide by dissolving 100 mg of each standard drug separately in 100 ml distilled water in different volumetric flask.

Figure 18: Overlain spectra of Hydrochlorothiazide (10 ppm) and Ramipril (10 ppm)



Wavelength selection

From the overlay spectra, two wavelengths were selected for estimation of Ramipril (212.20 nm and 271 nm) where Hydrochlorothiazide shows similar absorbance or zero difference absorbance and the estimation of Hydrochlorothiazide on one wavelength (271 nm) was performed where Ramipril has no significant absorbance, so Hydrochlorothiazide can be estimated as single component.

5.4.2.4 Method validation

1) Preparation of Linearity Curve

For estimation of Ramipril, calibration curve (n=3) was plotted in the range of 2-12 µg/ml between absorbance difference at 212.20 nm and 271 nm. For estimation of Hydrochlorothiazide, calibration curve (n=3) was plotted in the range of 2-12 µg/ml at 271 nm. Both curve shows linearity in the range of 2-12 µg/ml for Ramipril and Hydrochlorothiazide.

Figure 19: Linearity curve of mixture of Ramipril and Hydrochlorothiazide (2 – 12 µg/ml)

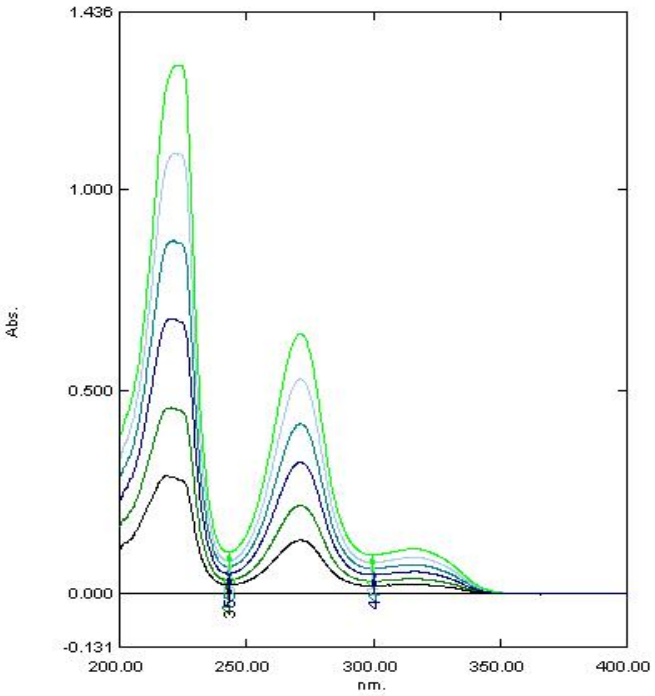


Figure 20: Linear Curve for Ramipril

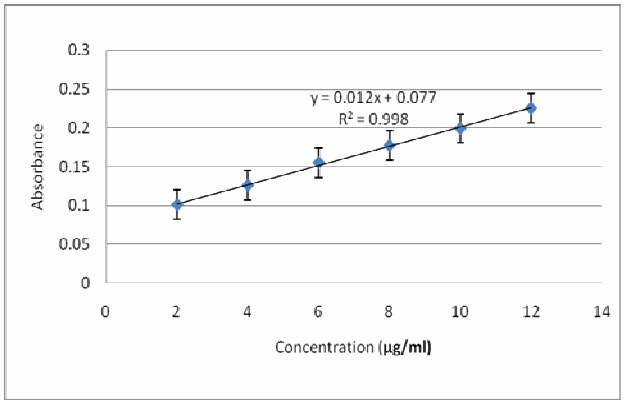
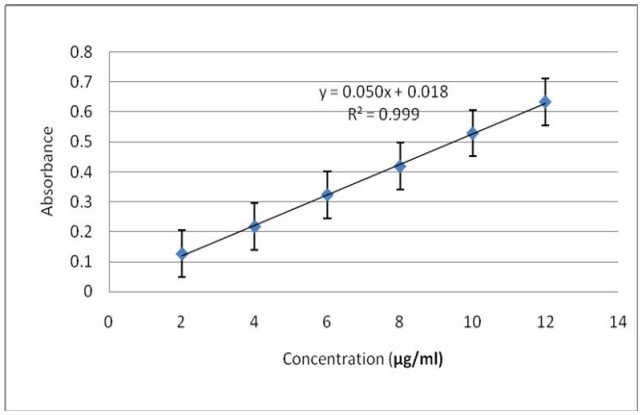


Figure 21: Linear Curve for Hydrochlorothiazide



2) Precision

For Ramipril, intraday precision was carried out by taking three different concentrations (4, 6, 8 µg/ml) repeated for 3 times in a day and interday precision was carried out by taking three different concentrations (4, 6, 8 µg/ml) repeated on three different days. For Hydrochlorothiazide, intraday precision was carried out by taking three different concentrations (4, 6, 8 µg/ml) repeated for 3 times in a day and interday precision was carried out by taking three different concentrations (4, 6, 8 µg/ml) repeated on three different days.

Table 30: Intraday precision for Ramipril

Conc. of Ramipril (µg/ml)	Amplitude at (212.20 - 271)	Mean ± S.D.	%RSD
4	0.125	0.124 ± 0.001155	0.93
	0.123		
	0.125		
6	0.156	0.156 ± 0.000577	0.37
	0.155		
	0.156		
8	0.179	0.179 ± 0.001528	0.85
	0.181		
	0.178		

Table 31: Intraday precision for Hydrochlorothiazide

Conc. of Hydrochlorothiazide(µg/ml)	Amplitude at 271 nm	Mean ± S.D.	%RSD
4	0.218	0.218 ± 0.000577	0.264
	0.217		
	0.218		
6	0.326	0.325 ± 0.001155	0.355
	0.324		
	0.324		
8	0.419	0.419 ± 0.000577	0.138
	0.418		
	0.419		

Table 32: Interday precision for Ramipril

Conc. of Ramipril(µg/ml)	Exp. Day	Amplitude at (212.20 - 271)	Mean ± S.D.	%RSD
4	1	0.124	0.124 ± 0.001155	0.93
	2	0.124		
	3	0.126		

6	1	0.157	0.156 ± 0.001155	0.74
	2	0.155		
	3	0.155		
8	1	0.178	0.177 ± 0.001732	0.98
	2	0.178		
	3	0.175		

Table 33: Interday precision for Hydrochlorothiazide

Conc. of HCTZ(µg/ml)	Exp. Day	Amplitude at 271 nm	Mean ± S.D.	%RSD
4	1	0.218	0.216 ± 0.002	0.93
	2	0.214		
	3	0.216		
6	1	0.330	0.329 ± 0.002309	0.7
	2	0.326		
	3	0.330		
8	1	0.419	0.418 ± 0.001155	0.28
	2	0.419		
	3	0.417		

Table 34: Repeatability for Ramipril

Conc (µg/ml)	Abs. of Ramipril at (212.20 – 271) nm					Avg.	±S.D
	1	2	3	4	5		
4	0.125	0.124	0.124	0.125	0.126	0.1248	0.000837
6	0.155	0.156	0.156	0.153	0.156	0.1552	0.001304
8	0.177	0.177	0.177	0.179	0.178	0.1776	0.000894

Table 35: Repeatability for Hydrochlorothiazide

Conc (µg/ml)	Abs. of HCTZ at 271 nm					Avg.	±S.D
	1	2	3	4	5		
4	0.215	0.214	0.215	0.218	0.217	0.2158	0.001643
6	0.331	0.331	0.330	0.332	0.332	0.3312	0.000837
8	0.419	0.415	0.418	0.419	0.418	0.4178	0.001643

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.

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

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

Where,

σ = Standard deviation of the intercept

S = Slope of calibration curve

Table 36: LOD and LOQ for Ramipril and Hydrochlorothiazide

Parameters	Ramipril	Hydrochlorothiazide
LOD	0.16 µg/ml	0.038 µg/ml
LOQ	0.48 µg/ml	0.12 µg/ml

4) Accuracy

Sample concentration was taken 4 µg/ml for Ramipril and Hydrochlorothiazide. After that accuracy of the method was determined by standard addition method at three different levels (80%, 100% and 120%).

Table 37: Accuracy observed for Ramipril

Sample Conc.(µg/ml)	Std. Addition(%)	Conc. after Spiking(µg/ml)	Conc.Recovered (µg/ml)	% Recovery±S.D
4	80%	7.2	7.16	99.38±0.16
	100%	8	7.96	
	120%	8.8	8.73	
4	80%	7.2	7.13	98.84±0.16
	100%	8	7.9	
	120%	8.8	8.69	
4	80%	7.2	7.15	98.52±1.09
	100%	8	7.92	
	120%	8.8	8.56	

Table 38: Accuracy observed for Hydrochlorothiazide

Sample Conc.(µg/ml)	Std. Addition(%)	Conc. after Spiking(µg/ml)	Conc.Recovered (µg/ml)	% Recovery±S.D
4	80%	7.2	7.17	99.77±0.3
	100%	8	7.97	
	120%	8.8	8.81	
4	80%	7.2	7.17	99.22±0.31
	100%	8	7.92	
	120%	8.8	8.72	
4	80%	7.2	7.13	98.97±0.43
	100%	8	7.95	
	120%	8.8	8.67	

5.4.2.8 Analysis of Tablet Samples.

Total 20 tablets were weighed accurately and powdered. An amount equivalent to one tablet (containing 5 mg of Ramipril and 12.5 mg of Hydrochlorothiazide) was taken and dissolved in 15 ml distilled water in 100 ml volumetric flask. The solution was sonicated for 15 minutes and then diluted with distilled to the mark. The solution was filtered by using Whatmann filter No.41. From this solution, 0.75 ml of sample solution was taken and diluted with 10 ml distilled water to get the final solution containing 3.75 µg/ml concentration of Ramipril and 9.375 µg/ml concentration of Hydrochlorothiazide.

5.5 Comparison of Both Methods by Student T-Test

Both the methods were found to be accurate, precise and selective for the simultaneous estimation of Ramipril and Hydrochlorothiazide in their combined dosage forms. After the application of both methods for simultaneous estimation, they were compared by the two paired sample t-test. By the help of t-test, we can say that both methods would differ significantly or not with the help of null hypothesis. The results of the t-test for both the drugs were summarized in table 41 and 42.

Table 42: Student T-test for Ramipril

T-TEST: PAIRED TWO SAMPLE FOR MEANS			
Brand Name	**Parameter**	**Ramipril**	
		UV	**HPTLC**
Ramipres H5	Mean	99.61667	99.77
	Variance	0.618033	0.0853
	Observations	3	3
	Pearson Correlation	0.494546	
	Df	2	
	T Stat (t_{cal})	-0.38485	
	P (T≤t) one-tail	0.368711	
	t Critical one tail	2.919987	
	P (T≤t) two-tail	0.737421	
	t Critical two tail (t_{crit})	4.302656	
Ramistar H5	Mean	99.86	100.2067
	Variance	0.7519	0.096133
	Observations	3	3
	Pearson Correlation	-0.99608	
	Df	2	
	T Stat (t_{cal})	-0.51046	
	P (T≤t) one-tail	0.330245	
	t Critical one tail	2.919987	
	P (T≤t) two-tail	0.660489	
	t Critical two tail (t_{crit})	4.302656	
Ramihart H5	Mean	100.1567	100.35
	Variance	0.825433	0.5449
	Observations	3	3
	Pearson Correlation	-0.5259	
	Df	2	
	T Stat (t_{cal})	-0.23242	
	P (T≤t) one-tail	0.418913	
	t Critical one tail	2.919987	
	P (T≤t) two-tail	0.837827	
	t Critical two tail (t_{crit})	4.302656	
Race H2.5	Mean	100.41	100.5267
	Variance	0.7651	0.343633
	Observations	3	3
	Pearson Correlation	0.046514	
	Df	2	
	T Stat (t_{cal})	-0.19617	
	P (T≤t) one-tail	0.4313	
	t Critical one tail	2.919987	
	P (T≤t) two-tail	0.862599	
	t Critical two tail (t_{crit})	4.302656	

Table 43: Student T-test for Hydrochlorothiazide

T-TEST: PAIRED TWO SAMPLE FOR MEANS			
Brand Name	**Parameter**	**Hydrochlorothiazide**	
		UV	**HPTLC**
Ramipres H5	Mean	100.2333	99.71333
	Variance	0.790833	0.519433
	Observations	3	3
	Pearson Correlation	-0.98048	
	Df	2	
	T Stat (t_{cal})	0.562138	
	P (T≤t) one-tail	0.31531	
	t Critical one tail	2.919987	
	P (T≤t) two-tail	0.63062	
	t Critical two tail (t_{crit})	4.302656	
Ramistar H5	Mean	99.98667	99.91667
	Variance	0.052233	0.837433
	Observations	3	3
	Pearson Correlation	0.560295	
	Df	2	
	T Stat (t_{cal})	0.149775	
	P (T≤t) one-tail	0.447341	
	t Critical one tail	2.919987	
	P (T≤t) two-tail	0.894682	
	t Critical two tail (t_{crit})	4.302656	
Ramihart H5	Mean	100.2133	99.64
	Variance	0.165433	0.1083
	Observations	3	3
	Pearson Correlation	-0.2839	
	Df	2	
	T Stat (t_{cal})	1.679188	
	P (T≤t) one-tail	0.117562	
	t Critical one tail	2.919987	
	P (T≤t) two-tail	0.235125	
	t Critical two tail (t_{crit})	4.302656	
Race H2.5	Mean	100.5067	99.34
	Variance	1.456933	1.3588
	Observations	3	3
	Pearson Correlation	-0.55906	
	Df	2	
	T Stat (t_{cal})	0.964557	
	P (T≤t) one-tail	0.218268	
	t Critical one tail	2.919987	
	P (T≤t) two-tail	0.436535	
	t Critical two tail (t_{crit})	4.302656	

5.6 Conclusion

By the virtue of the developed method, it can be concluded that High performance thin layer chromatography and UV methods are reliable techniques for the analysis of commercial formulations of Ramipril and Hydrochlorothiazide. The developed methods are simple, sensitive and specific which render they suitable for routine analysis of Ramipril and Hydrochlorothiazide from its combined dosage form. A good % recovery for both the drugs shows that the developed methods are free of the interference of excipients used in the formulation. The results of validation parameters are satisfactory level indicates the accuracy of proposed methods for estimation of Ramipril and Hydrochlorothiazide. After the performing of T-test for both the method, it was observed that T stated value < T critical value. Hence, it was concluded that both the methods do not differ significantly and can be successfully applied for the analysis of Ramipril and Hydrochlorothiazide in their pharmaceutical dosage forms.

6. Summary

- The HPTLC and UV methods were developed for the simultaneous determination of Ramipril and Hydrochlorothiazide in their combine dosage form. The developed methods were validated in terms of linearity, precision, accuracy, repeatability, limit of detection and limit of quantitation.

In HPTLC method:

- Saturation time was kept 15 minutes with run length of 80 mm.
- The drugs showed linearity with correlation coefficient of 0.9923 for Ramipril and 0.9916 for Hydrochlorothiazide. The method was found accurate, precise, specific, selective and repeatable. The minimum detectable concentration of Ramipril and Hydrochlorothiazide was found to be 4.24 ng/spot and 8.99 ng/spot respectively. The lowest quantifiable concentration of Ramipril and Hydrochlorothiazide was found to be 12.85 ng/spot and 27.23 ng/spot respectively.

In UV method:

- The method was found to be linear within range for both Ramipril and Hydrochlorothiazide. The method was found accurate, precise, specific, selective and repeatable. The minimum detectable concentration of Ramipril and Hydrochlorothiazide was found to be 0.16 µg/ml and 0.038 µg/ml respectively. The lowest quantifiable concentration of Ramipril and Hydrochlorothiazide was found to be 0.48 µg/ml and 0.12 µg/ml respectively.

- The developed methods were than employed for the assay of tablets containing Ramipril and Hydrochlorothiazide, of four different brands.

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