Development and Validation of Spectrophotometric and Stability indicating RP-HPLC assay methods for simultaneous estimation of Lercanidipine hydrochloride and Atenolol in pharmaceutical dosage form

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*I declare that the thesis entitled "**Development and Validation of Spectrophotometric and Stability indicating RP-HPLC assay methods for simultaneous estimation of Lercanidipine hydrochloride and Atenolol in pharmaceutical dosage form**" has been prepared by me under the guidance of Dr. Priti J. Mehta, Head & Associate Professor, Department of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University and Mr. Santajirao B. Patil, Team leader, Analytical development department (Formulation and development), Alembic Research Centre. No part of this thesis has formed the basis for award of any degree of fellowship previously.*

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INTRODUCTION

Analytical chemistry is the science of obtaining, processing and communicating information about the composition and structure of matter. In other words, it is the art and science of determining what matter is and how much of it exists. At present, modern analytical chemistry is on its new stage of change. Due to the demands resulted from the development of life sciences, environment science new materials as well as the introduction of biology, information science and computer technology, analytical chemistry has entered into new phase. The system of analytical studies has turned from simple system to complex ones.

1.1 INTRODUCTION TO HYPERTENSION AND ANGINA PECTORIS

1.1.1 HYPERTENSION[1,2,3]

Hypertension is a clinical condition in which the arterial blood pressure in rest exceeds constantly 140/90 mm Hg (as defined by the World Health Organization). The top number is your systolic pressure, the pressure created when your heart beats. It is considered high if it is consistently over 140. The bottom number is your considered high if it is consistently over 90. Hypertension is a risk factor for stroke, myocardial infarction (heart attack), and serious renal damage.

Table 1.1: Classification of Blood Pressure[4]

Optimal	< 120	< 80
Normal	< 130	< 85
High-Normal	130-139	85-89
Grade 1 Hypertension (mild)	140-159	90-99
Subgroup: Borderline	140-159	90-94
Grade 2 Hypertension (moderate)	160-179	100-109
Grade 3 Hypertension (severe)	≥ 180	≥ 110
Isolated Systolic Hypertension	≥ 140	< 90
Subgroup: Borderline	140-149	< 90

ADAPTED FROM:
2003 World Health Organization (WHO)/International Society of Hypertension (ISH) Statement on Management of Hypertension. *J Hypertens*. 2003,21:1983-92.

Chalmers et al. *J Hypertens*. 1999, 17:151-85.

SYMPTOMS OF HYPERTENSION: Chest pain, severe headache, confusion, ear noise or buzzing, irregular heartbeat, nosebleed, tiredness, vision changes.

CAUSES OF HYPERTENSION: Smoking, Obesity or being overweight, Diabetes, Sedentary lifestyle, Lack of physical activity, High levels of salt intake (sodium sensitivity), Insufficient calcium, potassium, and magnesium consumption, Vitamin D deficiency, High levels of alcohol consumption, Stress, Aging, Medicines such as birth control pills, Genetics and a family history of hypertension, Chronic kidney disease, Adrenal and thyroid problems or tumors.

TREATMENT OF HYPERTENSION

- Alpha blockers
- Angiotensin-converting enzyme (ACE) inhibitors
- Angiotensin receptor blockers (ARBs)
- Beta-blockers
- Calcium channel blockers
- Central alpha agonists
- Diuretics
- Renin inhibitors
- including aliskiren (Tekturna)
- Vasodilators

1.1.2 ANGINA PECTORIS [1,2,3]

Angina pectoris is the medical term for chest pain or discomfort due to coronary heart disease. Angina is a symptom of a condition called myocardial ischemia. It occurs when the heart muscle (myocardium) doesn't get as much blood (hence as much oxygen) as it needs. This usually happens because one or more of the heart's arteries (coronary blood vessels that supply blood to the heart muscle) is narrowed or blocked. Insufficient blood supply is called ischemia.

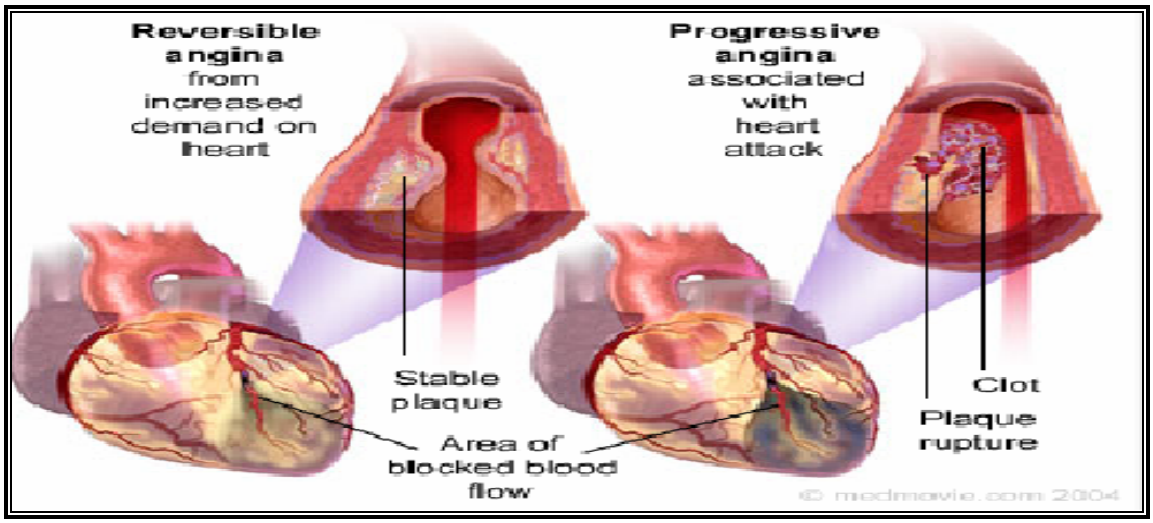


FIG 1.1: ANGINA PECTORIS

STABLE ANGINA:

People with stable angina (or chronic stable angina) have episodes of chest discomfort that are usually predictable. They occur on exertion or under mental or emotional stress.

UNSTABLE ANGINA:

In people with unstable angina, the chest pain is unexpected and usually occurs while at rest. The discomfort may be more severe and prolonged than typical angina or be the first time a person has angina. The most common cause is reduced blood flow to the heart muscle because the coronary arteries are narrowed by fatty buildups (atherosclerosis). An artery may be abnormally constricted or partially blocked by a blood clot. Inflammation, infection and secondary causes also can lead to unstable angina.

VARIANT ANGINA PECTORIS (PRINZMETAL'S ANGINA):

Variant angina pectoris is also called Prinzmetal's angina. Variant angina is due to transient coronary artery spasm. It usually occurs spontaneously, and unlike typical angina, it nearly always occurs when a person is at rest. It doesn't follow physical exertion or emotional stress, either. Attacks can be very painful and usually occur

between midnight and 8 a.m. About two-thirds of people with it have severe coronary atherosclerosis in at least one major vessel. The spasm usually occurs very close to the blockage.

SYMPTOMS OF ANGINA PECTORIS: Anxiety, Chronic chest pain, Increased or irregular heart rate, Paleness and cold sweat, Feeling of doom.

TREATMENT OF ANGINA PECTORIS:

- Nitrates
- Beta-blocking Drugs
- Calcium-channel Blocking Drugs

1.2 INTRODUCTION TO DRUG PROFILE

LERCANIDIPINE HYDROCHLORIDE [5-14]

- **Official status**: Not official in any pharmacopoeia.
- **Description**: Lercanidipine hcl is a calcium channel blocker of the dihydropyridine class.
- **Category**: Second generation dihydropyridine Ca^{++} channel blocker
- **Chemical name:** 1,4-Dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5 pyridinedicarboxylic acid 2-[(3,3-diphenylpropyl)methylamino]-1,1 dimethylethyl methyl ester hydrochloride
- **CAS Registry number:** 132866-11-6
- **Molecular weight**: 648.19 g/mol
- **Molecular formula**: $C_{36}H_{41}N_3O_6.HCl$
- **Structural formula:**

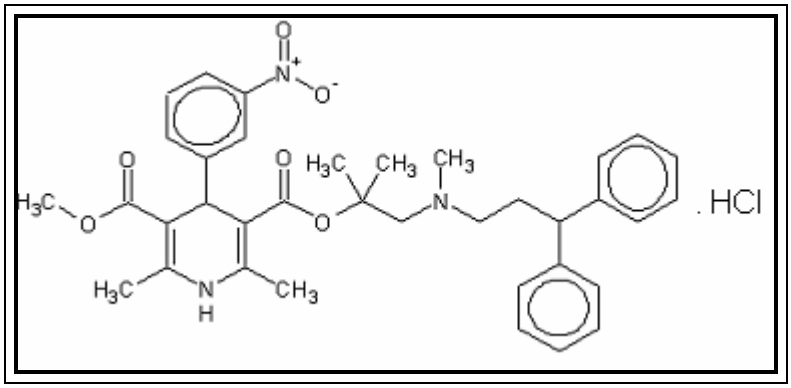


Figure 1.2: Structure of Lercanidipine hydrochloride

- **Physical and chemical Properties**
  - **Appearance:** Pale-Yellow Powder
- **Solubility**
  - Water : Practically insoluble
  - Chloroform, Methanol, Ethanol and Acetonitrile : Soluble
- **Log P/Hydrophobicity:** 6.42
- **Melting point:** 119°-123°C
- **BCS Class:** Class-II (High Permeability and Low Solubility)

- **Ionisation Constant**
  - pKa: 6.83 at 37^{o} C
- **Pharmacological Profile:**
  - **Therapeutic Category:** Antihypertensive and Anti-anginal agent (In Unstable or Prinzmetal's variant angina)
  - **Mode of Action:**

    By deforming the L-type of Ca^{+2}channel (inhibiting ion-control gating mechanism), Lercanidipine selectively inhibits the transmembrane influx of calcium into cardiac and vascular smooth muscle, with a greater effect on vascular smooth muscle than on cardiac smooth muscle. The decrease in intracellular calcium inhibits the contractile processes of the myocardial smooth muscle cells, causing dilation of the coronary and systemic arteries, increased oxygen delivery to the myocardial tissue, decreased total peripheral resistance, decreased systemic blood pressure, and decreased afterload.
- **Pharmacokinetic Profile**[9,10]
  - **Absorption**: Lercanidipine is completely absorbed after oral administration. The absolute bioavailability of lercanidipine is about 10%, because of high first pass metabolism.
  - **Distribution:** Distribution of lercanidipine from plasma to tissues and organs is rapid and extensive. Serum protein binding exceeds 98%.
  - **Biotransformation:** As for other dihydropyridine derivatives, lercanidipine is extensively metabolised by CYP3A4. It is predominantly converted to inactive metabolites; no parent drug is found in the urine or faeces. About 50% of the dose is excreted in the urine.
  - **Elimination:** The mean terminal elimination half-life of S- and R-lercanidipine enantiomers is 5.8 ± 2.5 and 7.7 ± 3.8 hours, respectively.
- **Indication:**
  - For the treatment of Hypertension and management of angina pectoris and Reynaud's syndrome.
- **Dosage and Dosage forms:**

- Adult: 10-20 mg daily as single dose orally.
- In hepatic and renal dysfunction: 20 mg with caution.

❖ **Formulation**[13,14]

- **ZANIDIP** (Lercanidipine tablets) developed by **Recordati Pharmaceuticals Ltd.** are available in 10 mg or 20 mg strengths.

- **LERCADIP** (lercanidipine film coated tablet) developed by **Incepta pharmaceutical Ltd.** are available in 10 mg strength.

ATENOLOL[15-21]

- **Official status**: Drug is official in USP, IP, BP and EP.
- **Description**: Atenolol is a drug belonging to the group of beta blockers, a class of drugs used primarily in various cardiovascular diseases.
- **Category**: Selective B_1 antagonist
- **Chemical name:** (RS)-4-(2-hydroxy-3-isopropylaminopropoxy)phenylacetamide
- **CAS Registry number:** 29122-68-7
- **Molecular weight**: 266.336 g/mol
- **Molecular formula**: $C_{14}H_{22}N_2O_3$
- **Structural formula:**

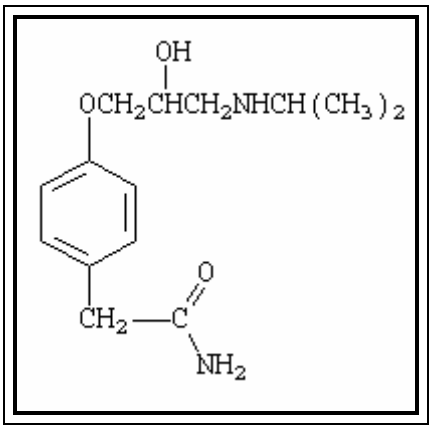


Figure 1.3: Structure of Atenolol[22]

- **Physical and chemical Properties**
  - **Appearance:** White powder, odorless and slightly bitter in taste
- **Solubility**
  - Water, 96% ethanol, Isoprpanol : Sparingly soluble
  - Methanol : Freely soluble
  - Acetic acid and Dimethylsulfoxide : Soluble
  - Chloroform, ethyl acetate : Practically insoluble
- **Log P/Hydrophobicity (octanol-water):** 0.23
- **Melting point:** 152°C – 156.5°C
- **BCS Class:** Class-III (High Solubility and Low Permeability).

- **Ionisation Constant(pKa):** 9.6
- **Dipole moment**: 5.71 ± 0.20 D (in propionic acid solution, 20 °C)
- **Optical rotation**: Atenolol molecule contains an asymmetric carbon atom but the commercial product is recemic mixture
- **Pharmacological Profile:**
  - **Therapeutic Category:** Antihypertensive and Anti-anginal agent
  - **Mode of Action:** Atenolol is $beta_1$-selective (cardioselective) beta-adrenergic receptor blocking agent without membrane stabilizing or intrinsic sympathomimetic (partial agonist) activities. This preferential effect is not absolute, however, and at higher doses, Atenolol inhibits $beta_2$-adrenoreceptors, chiefly located in the bronchial and vascular musculature.
- **Pharmacokinetic Profile**[20]
  - **Absorption**: Absorption of an oral dose is rapid and consistent but incomplete. Approximately 50% of an oral dose is absorbed from the gastrointestinal tract, the remainder being excreted unchanged in the feces.
  - **Bioavailability:** 45-55 %
  - **Plasma Half life:** 6 to 8 hours
  - **Plasma protein binding:** 6%-16%
  - **Distribution:** Atenolol is a hydrophilic drug. The concentration found in brain tissue is approximately 15% of the plasma concentration only. t_{cmax}= 2 to 4 hours after oral dosing (time elapsed before maximal concentration in the blood plasma is reached). The drug crosses the placenta barrier freely. In the milk of breastfeeding mothers, approximately 3 times the plasma concentrations are measured.
  - **Biotransformation:** By Hepatic metabolism. (<10%)
  - **Elimination:** The mean elimination halflife is 6 hours. However, the action of the usual oral dose of 25 to 100 mg lasts over a period of 24 hours. Atenolol is almost exclusively eliminated renally and is well removable by dialysis. A compromised liver function does not lead to higher peak-activity and/or a longer halflife with possible accumulation.

- **Dosage and Dosage forms:**
  - **For hypertention[19]**
    - Adult: 25-100mg daily as single dose orally.
    - Renal impairment: creatinine clearance, 15-35 ml/min 50mg daily by mouth or 10 mg once every 2 days IV; <15ml/min 25mg daily by mouth or 10 mg once every 4 days IV.
  - **For cardiac arrhythmias[20]**
    - Adult: 2.5mg IV at the rate of 1mg/ml may repeat over 5min if needed alternatively 150µg/kg to be infused over 20 minutes may repeat 12hrs if needed.
    - Renal impairment: creatinine clearance, 15-35 ml/min 50mg daily by mouth or 10 mg once every 2 days IV; <15ml/min 25mg daily by mouth or 10 mg once every 4 days IV.

- **Formulation[20]:**
  - **TENORMIN®** (Atenolol tablet) developed by **AstraZeneca Pharmaceuticals** is available as 25, 50 and 100 mg tablets for oral administration.

1.3 RATIONAL OF DRUG COMBINATION[21]

Lercanidipine hydrochloride, second generation dihydropyridine calcium channel blocker, and Atenolol, a beta blocker are established antihypertensive agents. A fixed dose tablet formulation of the Lercanidipine hydrochloride and Atenolol is mainly used combination for the treatment of special hypertensive patients not responding to monothrapy.

Special hypertensive patients includes

- Elderly hypertension
- Hypertensive patient with COPD and asthma
- Unstable angina
- Heart failure or Enlarged heart

1.4 INTRODUCTION TO STABILITY INDICATING ASSAY METHODS (SIAMs)

1.4.1 Stability Indicating Assay Methods (SIAMs)

The stability-indicating assay is a method that is employed for the analysis of stability samples in pharmaceutical industry. With the advent of International Conference on Harmonization (ICH) guidelines, the requirement of establishment of explicitly require conduct of forced decomposition studies under a variety of conditions, like pH, light, oxidation, dry heat, etc. and separation of drug from degradation products. The method is expected to allow analysis of individual degradation products.

Stability-indicating methods according to United States-Food and Drug Administration (US-FDA) stability guideline of 1987 were defined as the 'Quantitative analytical methods that are based on the characteristic structural, chemical or biological properties of each active ingredient of a drug product and that will distinguish each active ingredient from its degradation products so that the active ingredient content can be accurately measured.' This definition in the draft guideline of 1998 reads as: 'Validated quantitative analytical methods that can detect the changes with time in the chemical, physical, or microbiological properties of the drug substance and drug product, and that are specific so that the contents of active ingredient, degradation products, and other components of interest can be accurately measured without interference.[22]

Types of stability indicating assay method (SIAM) [23]

a) Specific Stability Indicating Assay Method

It can be defined as 'A method that is able to measure unequivocally the drug(s) in the presence of all degradation products, in the presence of excipients and additives, expected to be present in the formulation.'

b) Selective Stability Indicating Assay Method

Whereas it can be defined as 'A method that is able to measure unequivocally the drug(s) and all degradation products in the presence of excipients and additives, expected to be present in the formulation'.

1.4.2 REGULATORY REQUIREMENTS[24]

From a regulatory perspective, forced degradation studies provide data to support the following:

- Identification of possible degradants.
- Degradation pathways and intrinsic stability of the drug molecule.
- Validation of stability indicating analytical procedures.

Issues addressed in regulatory guidances include

- Forced degradation studies are typically carried out using one batch of material.
- Forced degradation conditions are more severe than accelerated stability testing such as 50 °C; ≥75% relative humidity; in excess of ICH light conditions; high and low pH, oxidation, etc.
- Photo stability should be an integral part of forced degradation study design[32].
- Degradation products that do not form in accelerated or long term stability may not have to be isolated or have their structure determined.
- Mass balance should be considered.

Issues not specifically addressed in regulatory guidance:

- Exact experimental conditions for forced degradation studies (temperatures, duration, extent of degradation, etc.) are not specified.
- Experimental design is left to the applicant's discretion.

There are guidances available from the FDA as well as from private industry on regulatory requirements for IND and NDA filings.[25]

SUMMARY OF REQUIREMENTS AT THE IND STAGE

The reporting of forced degradation study conditions or results is not required in Phase 1 or 2 INDs. However, preliminary studies are encouraged to facilitate the development of stability indicating methodology. It is recommended that forced degradation testing be conducted as early in the development of API and DP as possible. Studies can be conducted on the API and developmental formulations to examine for degradation by thermolysis, hydrolysis, oxidation, and photolysis to evaluate the potential chemical

behavior of the active. A draft guidance document suggests that results of one-time forced degradation studies should be included in Phase 3 INDs.[26]

SUMMARY OF REQUIREMENTS FOR MARKETING APPLICATION

Completed studies of the degradation of the API and DP are required at the NDA stage, including isolation and/or characterization of significant degradation products and a full written account of the degradation studies performed.[27]

Requirements at the time of registration include[28]:

- Forced degradation products should be accurately characterized and the reaction kinetics established.
- Structural elucidation of degradation products should be attempted, even if not successful, should be referenced in the NDA.
- Mass balance should be determined or at least attempted.
- Main band peak purity should be confirmed.
- Any degradants present in ICH stability samples which are greater than the identification threshold should be isolated and identified.

Information from these studies should provide for filing are:

- Degradation pathways of the API alone and in DP.
- Discussion of any possible polymorphic or enantiomeric substances.
- Differentiation between drug related degradation and excipient interferences

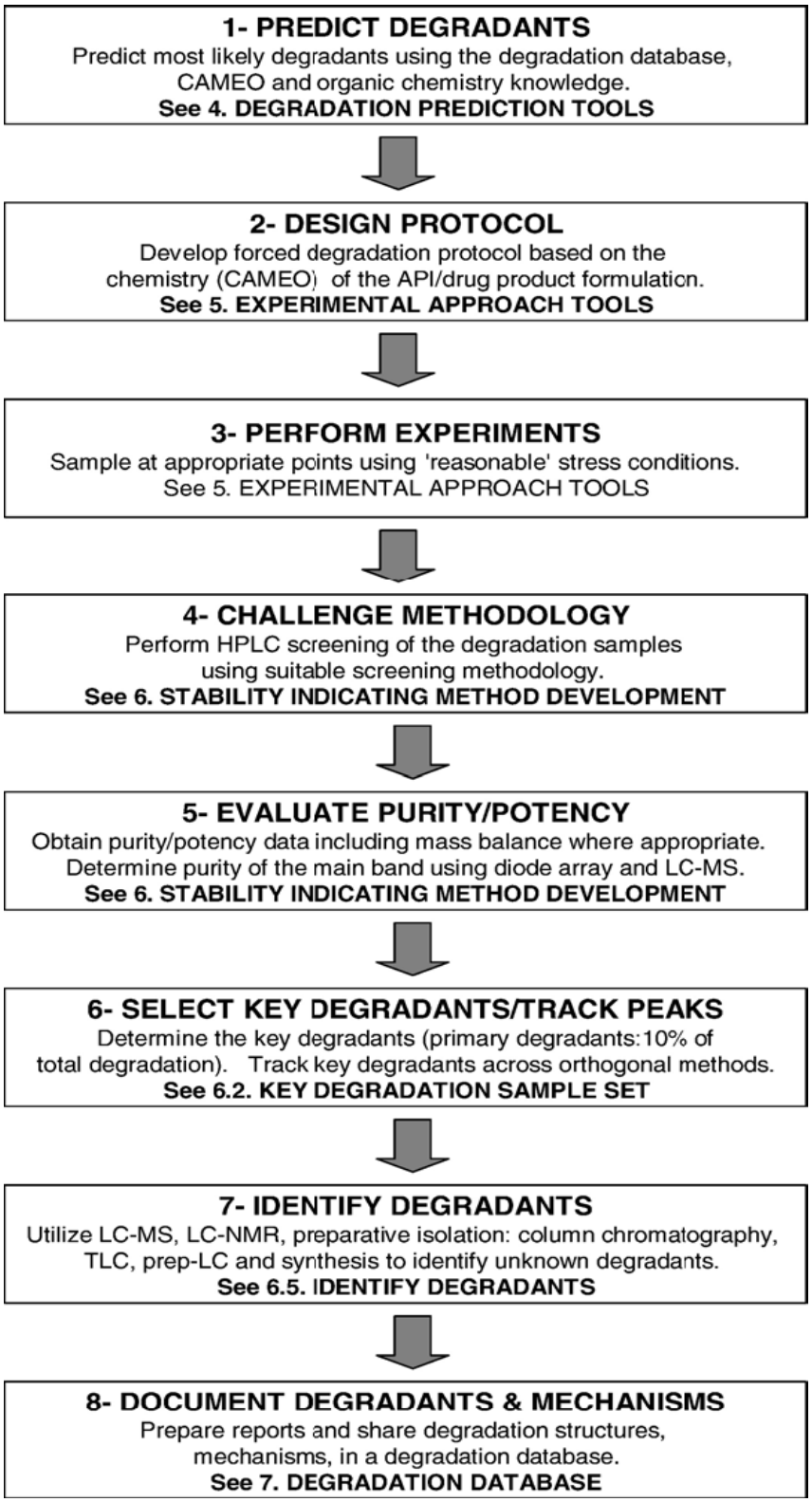


Fig 1.4: Forced degradation process flow map—prediction to documentation in a structure searchable global degradation database.[25]

1.4.3 OVERVIEW OF STRESS TESTING GUIDANCE DOCUMENTS:

ICH Q1A (R2) Stability Testing of New Drug Substances and Products[28]

Drug substance. Stress testing a drug substance can help identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stability of the molecule and validate the stability-indicating power of the analytical procedures used. The nature of the stress testing will depend on the individual drug substance and the type of drug product. Stress testing is likely to be carried out on a single batch of the drug substance. It should include the effect of temperatures (in 10 ^{0}C increments [*e.g.,* 50 ^{0}C, 60 ^{0}C] above that for accelerated testing), humidity (*e.g.,* 75% RH or greater) where appropriate, oxidation, and photolysis on the drug substance. Testing should evaluate the susceptibility of the drug substance to hydrolysis across a wide range of pH values when in solution or suspension. Photostability testing should be an integral part of stress testing. Examining degradation products under stress conditions is useful for establishing degradation pathways and developing and validating suitable analytical procedures. It may not be necessary, however, to examine for specific degradation products if previous studies have demonstrated that these products are not formed under accelerated or long-term storage conditions.

Drug product: The design of formal stability studies for a drug product should be based on the behavior and properties of the drug substance, the results from stability studies on the drug substance, and the experience gained from clinical formulation studies. The likely changes to storage conditions and the rationale for the selection of attributes to be tested in the formal stability studies should be stated. Photostability testing should be conducted on at least one primary batch of the drug product if appropriate. Standard conditions for photostability testing are described in ICH Q1B.

Any evaluation should take into account not only the assay but also the degradation products and other appropriate attributes. Where appropriate, attention should be paid to reviewing the adequacy of the mass balance and stability and degradation performance.

ICH Q1B: Stability Testing: Photostability Testing of New Drug Substances and Products[29]

Drug substance: Photostability testing should consist of two parts: forced-degradation testing and confirmatory testing. The purpose of forced-degradation testing is to evaluate the overall photosensitivity of the material for method-development purposes and/or degradation pathway elucidation. This testing may involve the drug substance alone and/or the substance in simple solutions and suspensions to validate the analytical procedures. In these studies, the samples should be in chemically inert and transparent containers. For forced-degradation studies, various exposure conditions may be used, depending on the photosensitivity of the drug substance and the intensity of the light sources. For development and validation purposes, it is appropriate to limit exposure and end the studies if extensive decomposition occurs. For photostable materials, studies may be terminated after an appropriate exposure level has been used. The design of these experiments is left to the applicant's discretion although the exposure levels used should be justified. Under forcing conditions, decomposition products may be observed that are unlikely to be formed under the conditions used for confirmatory studies. This information may be useful in developing and validating suitable analytical methods. If in practice it has been demonstrated that they are not formed in the confirmatory studies, these degradation products need not be further examined.

Light Source used for Photostability testing:

- ***Option 1***

  Any light source that is designed to produce an output similar to the D65/ID65 emission standard such as an artificial daylight fluorescent lamp combining visible and ultraviolet (UV) outputs, xenon, or metal halide lamp. D65 is the internationally recognized standard for outdoor daylight as defined in ISO 10977 (1993). ID65 is the equivalent indoor indirect daylight standard. For a light source emitting significant radiation below 320 nm, an appropriate filter(s) may be fitted to eliminate such radiation.

➢ ***Option 2***

For option 2 the same sample should be exposed to both the cool white fluorescent and near ultraviolet lamp.

- A cool white fluorescent lamp designed to produce an output similar to that specified in ISO 10977(1993) ; and
- A near UV fluorescent lamp having a spectral distribution from 320 nm to 400 nm with a maximum energy emission between 350 nm and 370 nm; a significant proportion of UV should be in both bands of 320 to 360 nm and 360 to 400 nm.

For confirmatory studies, samples should be exposed to light providing an overall illumination of **not less than 1.2 million lux hours** and an integrated near ultraviolet energy of **not less than 200 watt hours/square meter** to allow direct comparisons to be made between the drug substance and drug product, if no any degradation seen than expose the drug substance and drug product to light providing an overall illumination of **not less than 6 million lux hours,** and than also no any degradation seen than declare the drug substance and drug product to be photostable.

Requirements for relevant stress conditions:

- Should lead to the degradation of the main compound, but not more than 5–15%.
- Should lead to a good predictability of degradation pathways (*i.e.,* a low probability of "drastic "or "false "degradation).
- Should be conducted for no longer than three months.

Table 1.2: General protocol for stress degradation studies (API/DP) [24]

	Drug Substance		Drug Product	
Condition	Solid	Solution/ Suspension	Solid (Tablets, Capsules, Blends)	Solution (IV, Oral Suspension)
Acid/base		✓		X
Oxidative	X	✓	✓	✓
Photostability	✓	X	✓	✓
Thermal	✓		✓	✓
Thermal/humidity	✓		✓	

*✓ = recommended; X = optional, suggested for some compounds.

1.4.4 DEVELOPMENT OF VALIDATED STABILITY INDICATING ASSAY METHODS (SIAMs)

A stability-indicating method is defined as an analytical method that accurately quantitates the active ingredients without interference from degradation products, process impurities, excipients, or other potential impurities **or**

A method that accurately quantitates significant degradants may also be considered stability-indicating.

A proactive approach to developing a stability indicating HPLC method should involve forced degradation at the early stages of development with the key degradation samples used in the method development process. Forced degradation should be the first step in method development. If forced degradation studies are performed early, method development and identification of primary degradation products and unknown impurities can be run in parallel.

Step I: Critical study of the drug structure to assess the likely decomposition route(s)

This should be the first element whenever one takes up the project on establishment of a SIAM. Much information can simply be gained from the structure, by study of the functional groups and other key components. There are defined functional group categories, like amides, esters, lactams, lactones, etc. that undergo hydrolysis, others like thiols, thioethers, etc. undergo oxidation, and compounds like olefins, aryl halo derivatives, aryl acetic acids, and those with aromatic nitro groups, N-oxides undergo photo decomposition.[30]

Step II: Collection of information on physicochemical parameters

Before method development is taken up, it is generally important to know various physicochemical parameters like pKa, log P, solubility, absorptivity and wavelength maximum of the drug in question. The knowledge of pKa is important as most of the pH-related changes in retention occur at pH of the buffer to be used in the mobile phase. The knowledge of log P for the drug and the identified degradation products provides good insight into the separation behavior likely to be obtained on a particular stationary phase.

Step III: Stress (forced decomposition) studies[31]

The next step in the development of SIAM is the conduct of forced decomposition studies to generate degradation products of the drug. The ICH guideline Q1A suggests the following conditions to be employed: (i) 10 ^{0}C increments above the accelerated temperatures (e.g. 50 ^{0}C, 60 ^{0}C etc.), (ii) humidity where appropriate (e.g. 75 % or greater), (iii) hydrolysis across a wide range of pH values, (iv) oxidation and (v) photolysis. However, the guideline provides no details on how hydrolytic, photolytic and oxidative studies have to be actually performed. A comprehensive document providing guidance on the practical conduct and issues related to stress testing under variety of ICH prescribed conditions has been published lately. The decision trees have been developed to help in the selection of the right type of stress condition in a minimum number of attempts. Dependent upon the results, decision is taken on whether to increase the strength of the reaction conditions.

Step IV: Preliminary separation studies on stressed samples[32]

The stress samples so obtained are subjected to preliminary analysis to study the number and types of degradation products formed under various conditions. For doing so, the simplest way is to start with a reversed-phase octadecyl column, preferably a new or the one in a healthy condition. Well-separated and good quality peaks at the outset provide better confidence because of the unknown nature of products formed during stressing. It should be preferred to use water-methanol or water-acetonitrile as the mobile phase in an initial stage.

Step V: Final method development and optimization[33]

To separate close or co-eluting peaks, the method is optimized, by changing the mobile phase ratio, pH, gradient, flow rate, temperature, solvent type, and the column and its type.

Step VI: Identification and characterization of degradation products and preparation of standards

Before moving to the validation of a SIAM, it necessary to identify the drug degradation products and arrange for their standards. These are required to establish

specificity/selectivity of the method. The work on this aspect can even be initiated once an idea on the nature and number degradation products formed under different degradation conditions is obtained from preliminary separation studies. Peak purity of the active substance is checked (by photo-diode array detector) to verify that the method is selective, and a single component peak is quantified.

Step VI: Validation of Stability Indicating Assay Methods [34, 35]

Validation of analytical methods, in general, has been extensively covered in the ICH guidelines Q2A and Q2B, in the FDA guidance and by USP. There are several other reports in literature, which have reviewed the concept, either in general or specifically the validation of spectroscopic, non-chromatographic and chromatographic methods.

Overall, there are two stages in the validation of a Stability Indicating Assay Method. First stage is early in the development cycle when drug substance is subjected to forced decomposition studies and the SIAM is established based on the knowledge of drug degradation behavior. The main focus of validation at this stage is on establishment of specificity/selectivity, followed by other parameters like accuracy, precision, linearity, range, robustness, etc. The limits of detection and quantitation are also determined finds application in the analysis; of stability samples of bulk drug for determination of its retest or expiry period. In the second stage, when the SIAM so developed is extended to formulations or other matrices, the emphasis gets limited to just prove the pertinence of the established validation parameters in the presence of excipients or other formulation constituents.

1.5 METHOD DEVELOPMENT IN HPLC

DEVELOPING THE SEPARATION

Selecting an HPLC Method and Initial Conditions[36]

The first consideration when developing an HPLC method is to determine the solubility of the sample components. Knowing the nature of analyte will allow the most appropriate mode of HPLC to be selected. For the selection of a suitable chromatography method for organic compounds first Reversed-phase should be tried, if not successful, normal-phase should be taken into consideration.

Given figure, there is a temptation to quickly take an appropriate column, prepare a sample and suitable mobile phase, and run it on the HPLC system. This may work with some trial and error, but the key to efficient method development is planning. Figure 1.5 summarizes appropriate separation modes and mobile phases to consider for method development.

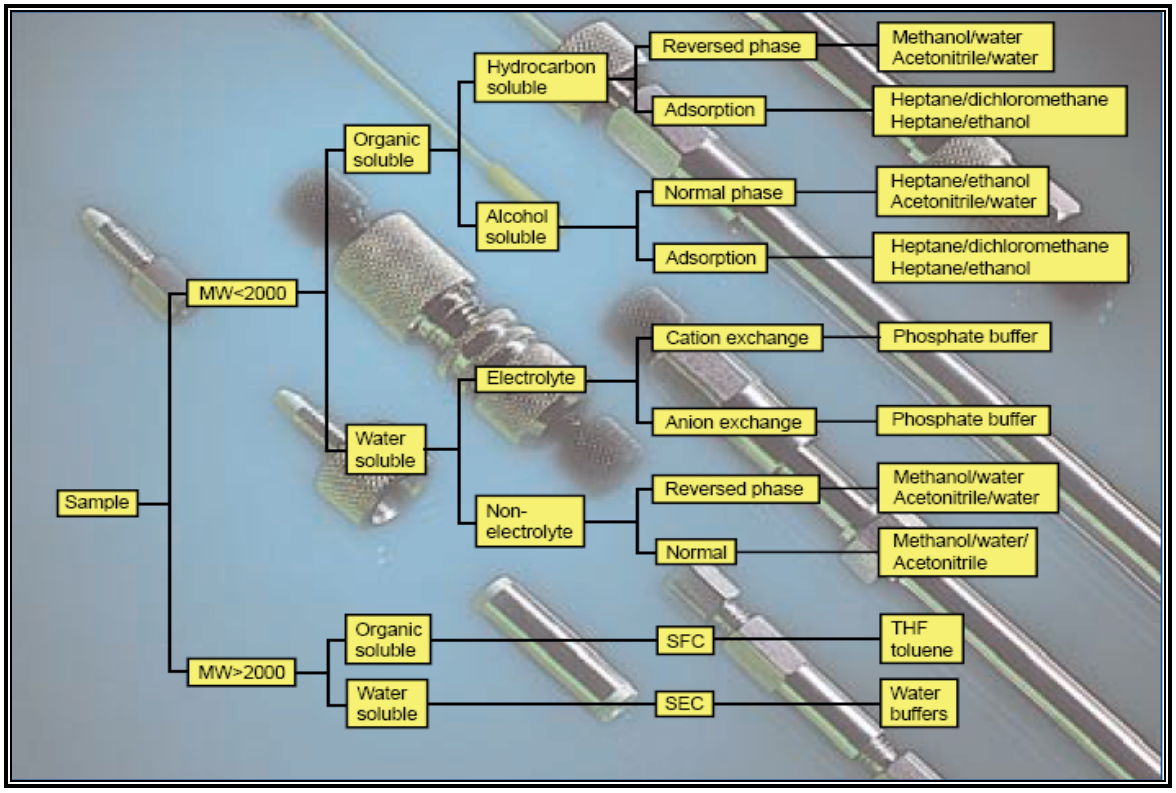
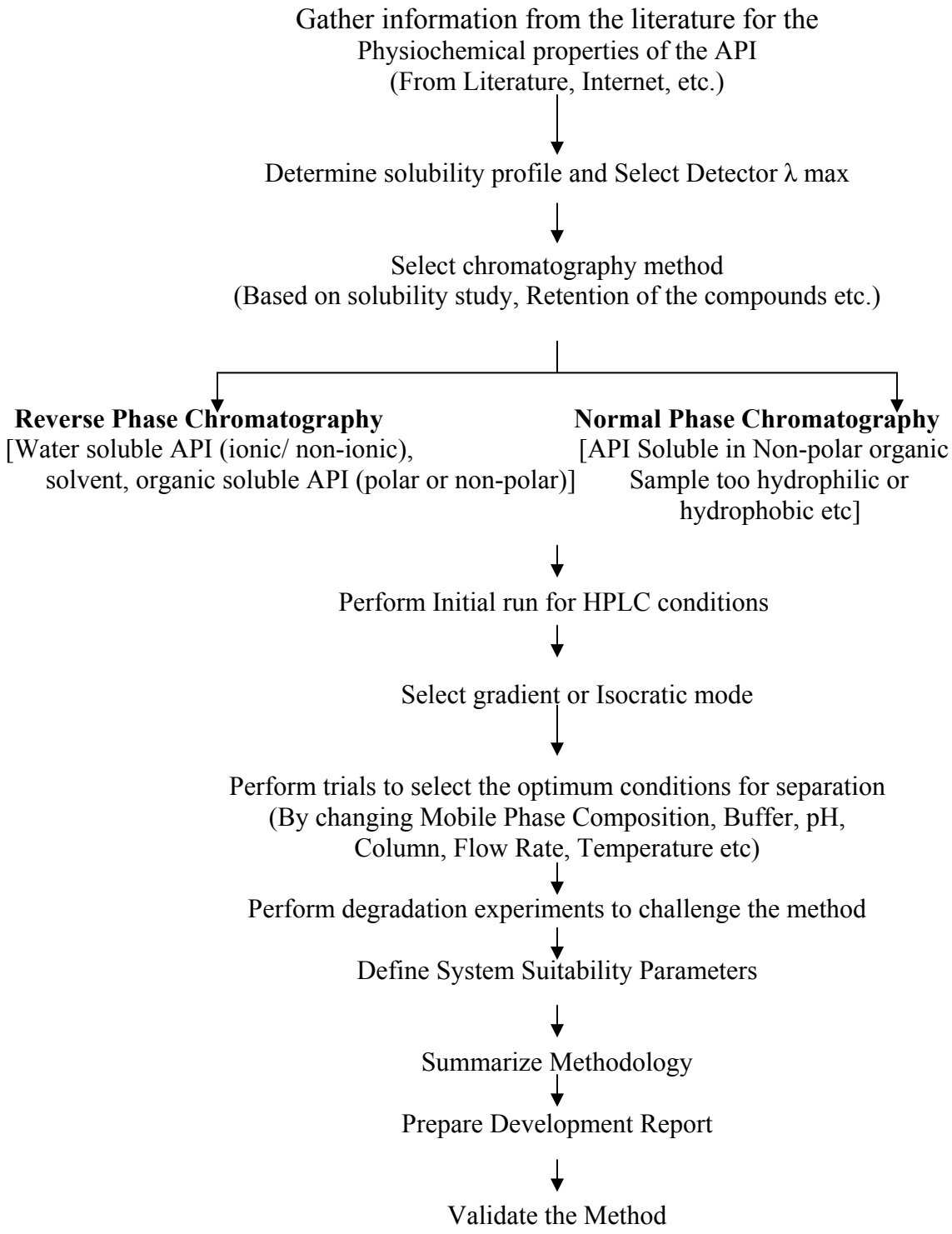


Figure 1.5: Phase Selection Process[36]

GENERAL CONSIDERATION FOR HPLC METHOD DEVELOPMENT

Flow Chart 1: Method Development Flow Chart



Getting Started on Method Development

"Best column, best mobile phase, best detection wavelength, efforts in separation can make a world of difference while developing HPLC method for routine analysis. Determining the ideal combination of these factors assures faster delivery of desired results – a validated method of separation." [37]

1.5.1 Selection of best mobile phase[36]:

Most separations can be achieved by the choosing the optimum mobile phase compositions of the aqueous and organic portions. Most widely used solvent for the reverse phase chromatography are Methanol and Acetonitrile. Tetrahydrofuran is also used, but to lesser extent. Mobile phase with Tetrahydrofuran are known to be susceptible to oxidation. Experiments should be conducted with the mobile phase having buffers of different pH and different organic phases to check the best possible separation between the impurities can be used for checking the separation with different mobile phase ratios. Alternatively the stressed solution of the drug product can also be used. Mobile phase composition selected should be able to separate all the possible impurities from API and should be rugged for variation of both the aqueous and organic phase by atleast 5%.

Experiments should be conducted with the mobile phase having buffers of different pH and different organic phases to check the best possible separation between the impurities. Drug solutions having different possible impurities can be used for checking the separation with different mobile phase ratios. Alternatively the stressed solution of the drug product can also be used.

If the sample is eluted with the mobile phase of 100% organic content, and there is no separation, the solvent strength should be decreased to get the retention. Generally the increase in organic content will shorten the run time but leads to increased band overlap. When the separations are complex, i.e. when many components are to be separated, and when solvent strength is decreased and still there is no resolution between two closely eluting peaks, another organic solvent of the different polarity or even a mixture of the two organic solvents may need to be tried to effect the separation.

Since the mobile phase governs solute-stationary phase interaction, its choice is critical.

- Practical considerations dictate that it should not degrade the equipment or the column packing. For this reason, strong acids, bases and halide solutions should be avoided.
- Chemical purity of solvents is an important factor. Since large volumes of solvent are pumped through the column, trace impurities can easily concentrate in column and eventually be detrimental to the results. Spectro or HPLC grade solvents are recommended.
- Volatility should be considered if sample recovery is required.
- Viscosity should be less than 0.5 centipoises, otherwise higher pump pressures are required and mass transfer between solvent and stationary phase will be reduced.

Table 1.3: Lower wavelength limit of UV transparency for the most typical solvents used in HPLC[38]

SOLVENT	UV CUT OFF	Viscosity	Miscibility in water
Water	190	1.00	-
Methanol	205	0.55	Y
THF	212	0.55	Y
Propanol	210	2.3	Y
Acetonitrile	190	0.38	Y
Hexane	195	0.31	N
Ethyl acetate	256	0.45	N
Chloroform	245	0.57	N

- Usually determined as the wavelength at which the absorbance of the neat solvent in a 1-cm cell is equal to 1 AU (Absorbance Unit) with water used as a reference.

Table 1.4: Some useful solvents for selection of mobile phase with order of increasing polarity[36]

Solvents	Polarity Index
n-heptane	0.0
n-hexane	0.0
Pentane	0.0
Cyclohexane	0.0
Isooctane	0.4
Trichloroethylene	1.0
Isopropylether	2.2
Toluene	2.3
Xylene	2.5
Benzene	2.7
Diethylether	2.8
Dichloromethane	3.4
Dichloroethane	3.7
1-Butanol	3.9
Butyl acetate	4.0
n-Propanol	4.0
Ethyl acetate	4.3
Chloroform	4.4
Methyl ethyl ketone	4.7
1,4-dioxane	4.8
Acetone	5.4
Methanol	5.1
ethanol	5.2
Acetonitirile	6.2
DimethylSulphoxide (DMSO)	7.2
Water	9.0

1.5.2 Selection of buffer[36]:

In reverse phase HPLC, the retention of analytes is related to their hydrophobicity. The more hydrophobic the analyte, the longer it is retained. When an analyte is ionized, it becomes less hydrophobic and, therefore, it retention decreases. When separating mixtures containing acid and/or bases by reversed phase HPLC, it is necessary to control the pH of mobile phase using appropriate buffer in order to achieve reproducible results.

Efficient separation of the acidic or basic components is achievable by controlling the pH of the mobile phase. The buffer should be UV transparent to below or at the wavelength of the organic solvent. Other properties such as solubility and stability of the buffer and its reactivity to analyte and hardware components of the chromatographic systems should be taken into consideration.

The buffer capacity is determined by pH, composition of the buffer and buffer concentration. Optimum buffering capacity occurs at a pH equal to the pKa of the buffer. In general, most buffers provide the adequate buffering capacity for controlling mobile phase pH only within ±1 unit of their pKa. .For RPC separation a buffer concentration of 10 to 50mM is usually adequate. This assumes that the volume of injection is small and the sample is not too heavily buffered at a pH quite different from that of the mobile phase. Higher buffer concentration (> 50mM) provide increased buffer capacity but may not be soluble in the mobile phase for high % of the strong solvent in the mobile phase. Higher buffer concentration also may adversely affect the operation of HPLC system. Reverse phase chromatography generally are carried out with C_8 or C_{18} bonded-phase silica based columns that are less stable outside the pH range 2 to 8.Therefore, the buffer should be able to control pH between 2.0 to 8.0.

Experiments should be conducted using the different buffers with the different strengths to obtain the required separation. Buffer that gives the separation of all the individual impurities form the API should be selected and effect of variation of buffer concentration should be studied. The selected buffer should be rugged for small changes in the strength say at least 2.0 %.

Buffers play an additional role in the reproducibility of a separation. The buffer salts reduce peak tailing for basic compounds by effectively masking silanols. They also reduce potential ion-exchange interactions with unprotonated silanols (Figure 1.6). To be

most effective, a buffer concentration range of 10 - 50 mM is recommended for most basic compounds.

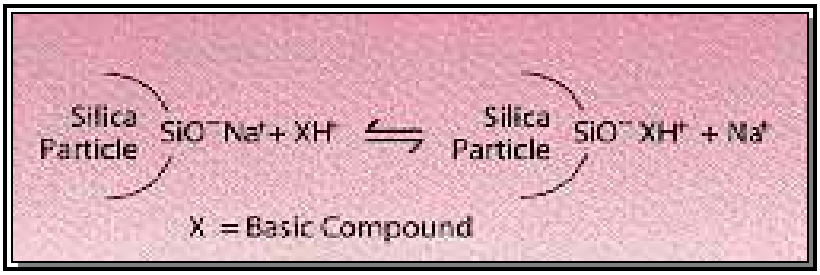


Figure 1.6: Peak Tailing Interaction[37]

1.5.3 Selection of pH of mobile phase[36]**:**

Depending on the pKa value of the drug molecules pH of the mobile phase can change retentions e.g. acids shows an increase in the retention as the pH is reduced while base shows a decrease. Mostly, all of the pH caused changes in the retention occur within $\pm$1.5 pH units of the pKa value, it is best to adjust the mobile phase to pH values atleast $\pm$1.5 pH units above or below the pKa to ensure practically 100% unionization for retention purposes. The pH range most often used for reversed-phase HPLC is 1 - 8 and can be divided into low pH (1 - 4) and intermediate pH (4 - 8) ranges. At the low pH (1-4) peak tailing is minimizes and method ruggedness is maximizes. For this reason, operating at low pH is recommended.

At a mobile phase pH greater than 7, dissolution of silica can severely shorten the lifetime of columns packed with silica-based stationary phases. The pKa value (acid dissociation [ionization] constant) for a compound is the pH at which equal concentrations of the acidic and basic forms of the molecule are present in aqueous solutions. Analytes may sometimes appear as broad or tailing peaks when the mobile phase pH is at, or near, their pKa values. A more rugged mobile phase pH will be nearby analyte pKa. This shifts the equilibrium so that 99% of the sample will be in one form. The result is consistent chromatography.

Dramatic changes in the retention and selectivity (peak spacing) of basic and acidic compounds can occur when the pH of the mobile phase is changed. This is often a result of different interactions between the column and the analytes when the ionization of these

compounds changes. It is important to evaluate these changes when a method is developed in order to select the mobile phase pH that provides the most reproducible results.

Table 1.5: Commonly used Buffers for reversed phase HPLC[36]

Buffer	P^{Ka} (25°C)	Maximum Buffer Range	UV Cutoff (nm)
TFA	0.3	-	210
Phosphate,p^{K}_1 H_2PO_4	2.1	1.1-3.1	< 200
Phosphate,p^{K}_2 HPO_4^{2-}	7.2	6.2-8.2	< 200
Phosphate,p^{K}_3 PO_4^{3-}	12.3	11.3-13.3	< 200
Citrate, p^{K}_1 $C_3H_5O(COOH)_2(COO)^{1-}$	3.1	2.1-4.1	230
Citrate, p^{K}_2 $C_3H_5O(COOH)_1(COO)^{2-}$	4.7	3.7-5.7	230
Citrate, p^{K}_3 $C_3H_5O(COO)^{3-}$	6.4	4.4-6.4	230
Carbonate, p^{K}_1 HCO_3^{1-}	6.1	5.1-7.1	< 200
Carbonate, p^{K}_2 CO_3^{2-}	10.3	9.3-11.3	> 200
Formate	3.8	2.8-4.8	210
Acetate	4.8	3.8-5.8	210
Tris	8.3	7.3-9.3	N/A
Ammonia	9.3	8.3-10.3	200
Borate	9.2	8.2-10.2	N/A
TEA	10.8	9.8-11.8	< 200

1.5.4 Selection of Column[37]:

HPLC column is the heart of the method and critical in performing the separation. The following parameters of the columns should be taken into consideration while choosing the column for the HPLC method:

- Column Packing Material
- Size and Shape of the particle
- Column length and diameter
- % carbon load
- Pore Volume and
- End capping etc.

For Reverse phase chromatography, a wide variety of the columns are available like C_8, C_{18} , Cyano group –CN and amino group like $–NH_2$ etc. It has to be remembered that no two column are same, they vary from manufacturer to manufacture with respect to above-mentioned parameters. The surface area of the bonded phase support is a major factor, as larger the surface area greater will be the retention. Sample retention normally increases for the bonded phases of greater length, $C_{18}>C_8>C_3>C_1$.As column length changes the column efficiency changes in direct proportion to the ratio of the column length. To select the type of column in the method, conduct the experiments using different columns with different mobile phases to get best possible separation. Based on the experimental data, select the column which gives separation of all the possible impurities and principal peak and which is rugged for the variation in mobile phase.

Table 1.6: Desired particle characteristics for HPLC analytical columns

Feature	Utility
5-µm totally porous particles	Most separation
3-µm porous particle	Fast separation
1.5-µm pellicular particles	Very fast separation
7 to 12 nm pores	Small molecule separation
15 to 100 nm pores	Macromolecular separation

1.5.5 Selection of the Column Temperature[36]:

Generally it is preferable to optimize the chromatographic conditions with the column temperature ambient. However if the peak symmetry is not achieved with any combination of the column and mobile phase at the ambient condition, then the column temperature above ambient can be adopted. If the column temperature is increased, it generally results in the reduction in the peak symmetry and peak retention time. Snyder reported that an increase in 1^{o} C would decrease retention by 1 to 2 %**.** When necessary, column temperature can be increased from 30^{o}C to 80^{o}C. Change in temperature may be more effective tool for the separations in the ionization of the sample and this changes pH and pKa values. If the column temperature is necessary for the separation, then the packing material selected should be able to withstand such temperatures and robustness within $\pm 5^{o}$C should be established.

1.5.6 The Best Column Length[38]:

Method development can be streamlined by starting with shorter columns; 150, 100 or even 50mm long. This is simply because they have proportionally shorter run times as illustrated in Figure

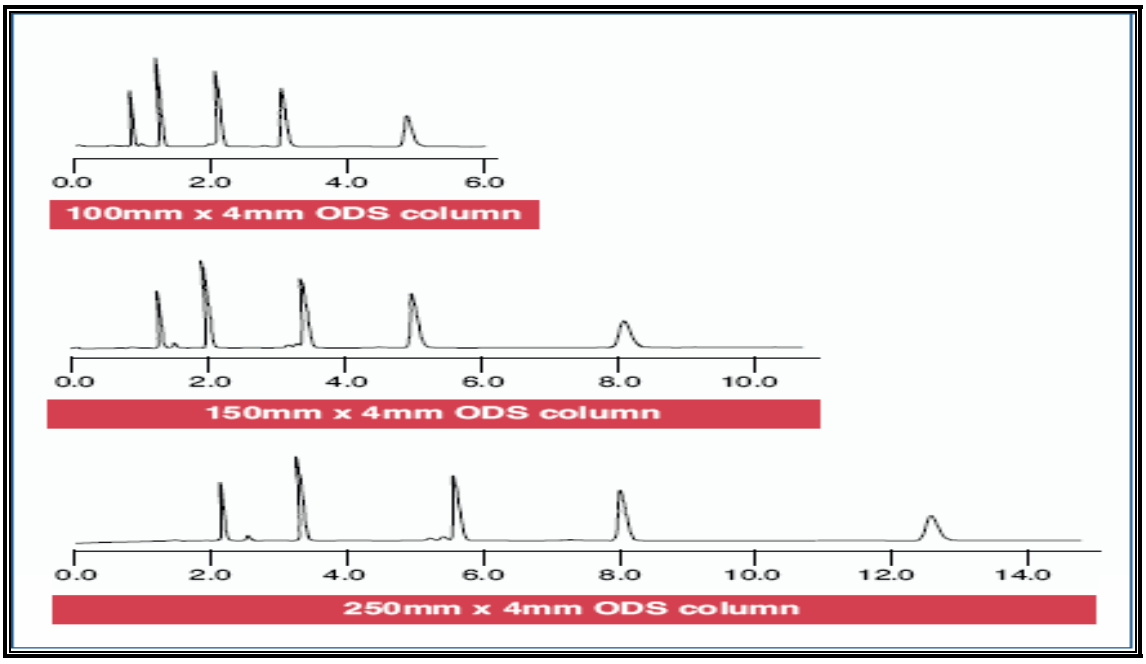


Figure 1.7: Effect of Column length

1.5.7 Selection of detector[37]:

UV-visible detectors are the most popular as they can detect a broad range of compounds and have a fair degree of selectivity for some analytes. Table summarizes some of the available options.

Table 1.7: Detector options

Detector	Analytes	Solvent Requirements	Comments
UV-visible	Any with chromophores	UV-grade non-UV absorbing solvents	Has a degree of selectivity and is useful for many RRLC applications
Fluorescence	Fluorescent compounds	UV-grade non-UV absorbing solvents	Highly selective and sensitive. Often used to analyze derivatized compounds
Refractive Index (RI)	Compounds with a different RI to the mobile phase	Cannot run mobile phase gradients	Virtually a universal detector but has limited sensitivity
Electrochemical	Readily oxidized or reduced compounds, especially biological samples	Mobile phase must be conducting	Very selective and sensitive
Evaporative Light Scattering (ELSD)	Virtually all compounds	Must use volatile solvents and volatile buffers	A universal detector which is highly sensitive. Not selective

1.5.8 Filter compatibility:

If the filter paper is used before injecting the sample, it should be checked for the adsorption of the drug(s). For this prepare the standard solutions and the sample solutions and filter them using 2-3 different types of the filter paper. Compare the filtered standard solution results to the unfiltered standard solution and filtered sample solutions to the centrifuged/unfiltered sample solutions. For the filters to be acceptable for the use, the results of the filtered portions should be within ±2.0% of the unfiltered standard solution and the centrifuged/unfiltered sample solution.

1.5.9 Method of extraction:

Generally used methods of extraction are sonication, rotary shaking or both. In some cases heating can be adapted if the substance is stable and should not precipitate on cooling to the room temperature.

Conduct experiments to optimize the extraction of API in presence of excipients at the different test concentration. Prepare the test solution in the diluent chosen based on solubility and carry out the extraction at the different time intervals of sonication or rotatory shaking or both and select the time of sonication and test concentration at which the maximum extraction takes place.

1.5.10 Selection of test concentration and injection volume:

The selected test concentration depends upon the response of the API at the selected wavelength. The test concentration should be finalized only after it is proved that the API is completely extractable at the selected test concentration.

Generally, the injection volume of the 10 µl to 20 µl is recommended but injection volume can be increased up to 50 µl-100 µl. Before selecting high injection volume, ensure that with the selected higher injection volume, column is not overloaded, resolution and peak symmetry are not compromised.

After the finalization of test concentration and the diluent, prepare the standard solution at the selected concentration and in the selected diluent and check it for the turbidly / precipitation for at least 24 hours, wherever applicable.

1.6 ANALYTICAL MEHOD VALIDATION [30, 31, 34, 35, 39]

1.6.1 TERMINOLOGY

"Doing thorough method validation can be tedious, but the consequences of not doing it right are wasted time, money, and resources."

Validation is a process of establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics.

Method validation is the process of demonstrating that analytical procedures are suitable for their intended use and that they support the identity, quality, purity, and potency of the drug substances and drug products. Simply, method validation is the process of proving that an analytical method is acceptable for its intended purpose. A successful Validation guarantees that both the technical and regulatory objectives of the analytical methods have been fulfilled.

1.6.2 Type of analytical procedures to be validated

Validation of analytical procedures is directed to the four most common types of analytical procedures.

- Identification test.
- Quantitative test for impurities content.
- Limit test for the control of impurities.
- Quantitative test of the active moiety in samples of drug substance on drug product on other selected components in the drug product.

1.6.3 Objective of validation

The objective of validation of analytical procedure is to demonstrate that it is suitable for its intended purpose. Validation is documented evidence, which provide a high degree of assurance for specific method. Any developed method may be influenced by variables like different elapsed assay times, different days, reagents lots, instruments, equipments, environmental conditions like temperature, etc so it is expected that after the

method has been developed and before it is communicated or transferred from one lab to the other, it is properly validated and the result of validity tests reported.

For analytical method validation of pharmaceuticals, guidelines from the International Conference on Harmonization (ICH), United States Food and Drug Administration (US FDA), American Association of Official Analytical Chemists (AOAC)United States Pharmacopoeia (USP), and International Union of Pure and Applied Chemists (IUPAC) provide a framework for performing such validations in a more efficient and productive manner.

1.6.4 Method validation is required when:

1. A new method is been developed
2. Revision established method
3. When established methods are used in different laboratories and different analysts etc.
4. Comparison of methods
5. When quality control indicates method changes.

The different parameters which are to be considered in analytical method validation of an as per USP **(1225)**, CDER and ICH guidelines can be summarized as follows.

Table 1.8: Method Validation Parameters

USP (1225)	ICH	CDER
Accuracy	Accuracy	Accuracy
Precision	Precision	Precision
Repeatability	Repeatability	Repeatability
----------	Intermediate Precision	Intermediate Precision
Reproducibility	Reproducibility	Reproducibility
Specificity	Specificity	Specificity
Limit Of Detection	Limit Of Detection	Limit Of Detection
Limit Of Quantitation	Limit Of Quantitation	Limit Of Quantitation
Linearity	Linearity	Linearity
Range	Range	Range
Ruggedness	----------	----------
Robustness	Robustness	Robustness
----------	----------	Stability Of Solution

Data Elements Required for Assay Validation

Both the USP and ICH recognize that is it not always necessary to evaluate every analytical performance parameter. The type of method and its intended use dictates which parameters needed to be investigated, as illustrated in Table 2.3

Table 1.9: ICH Validation Guideline[39]

Type of analytical procedure Characteristics	Identification	TESTING FOR IMPURITIES Quantitative	Limit	ASSAY -dissolution (measurement only) -content/potency
Accuracy	-	+	-	+
Precision				
Repeatability	-	+	-	+
Interm.Precision	-	+(1)	-	+(1)
Reproducibility	-	- (2)	-	- (2)
Specificity (3)	+	+	+	+(4)
Detection Limit	-	-	+	-
Quantitation Limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

- Signifies that this characteristic is not normally evaluated.

+ Signifies that this characteristic is normally evaluated.

(1) Intermediate precision is not needed in some case, when reproducibility is checked.

(2) May be needed in some cases.

(3) Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s).

(4) May not be needed in some cases.

VALIDATION PARAMETERS

The different parameters of analytical method development are discussed below as per ICH guideline:-

1) SPECIFICITY

Definition

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

Method

- When the impurities are available: Spiking of pure substance (drug substance or drug product) with appropriate levels of impurities/excipients and demonstrate the result is unaffected.
- When the impurities are not available: Comparing the test results of sample containing impurities or degradation product to second well-characterized procedure. These comparisons should include sample under relevant stress condition.
- In chromatographic method: Peak purity test to be done by diode array and mass spectrometry.

Expression/calculation

- Proof of discrimination of analyte in the presence of impurities. E.g. for chromatography chromatogram should be submitted.
- Peak purity test helps in demonstrating that the peak is not attributable to more than one component.
- For assay two results should be compared and for impurity tests two profiles should be compared.

Acceptance criteria: No interference peaks from blank or placebo at the R.T. of the drug and peak purity should pass (purity angle should be less than the purity threshold).

It includes followings,

Identification test:

Suitable identification tests should be able to discriminate between compounds of closely related structures which are likely to be present. The discrimination of a procedure may be confirmed by obtaining positive results (perhaps by comparison with a known reference material) from samples containing the analyte, coupled with negative results from samples which do not contain the analyte. In addition, the identification test may be applied to materials structurally similar to or closely related to the analyte to confirm that a positive response is not obtained.

Assay: Demonstrate that the results are unaffected by spiked impurities or excipients.

Impurities:

For the assay, this should involve demonstration of the discrimination of the analyte in the presence of impurities and/or excipients; practically, this can be done by spiking pure substances (drug substance or drug product) with appropriate levels of impurities and/or excipients and demonstrating that the assay result is unaffected by the presence of these materials. If impurity or degradation product standards are unavailable, specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to a second well-characterized procedure e.g.: pharmacopoeial method or other validated analytical procedure.

Placebo interference:

The placebo consists of all the excipients, coatings and capsule shell without the active ingredient. Placebo interference may be determined by weighing the sample of placebo blend, equal to or greater than the highest and lowest strength and preparing the solution and analyzing as per the selected test method. There should not be any interference from the placebo solution at the retention time of the principal peak and the impurities.

Forced degradation or stress study:

Forced degradation should be performed to demonstrate discriminating ability of the analytical method for the stability indicating method. The degradation study is performed in different condition on API/drug products and placebo to optimize the condition for the degradation of the drug. To select the optimum degradation conditions, trial and error

experiments should be performed on the API, Drug products and Placebo to find the proper combinations of the stress agent concentration and time to effect degradation, preferably in the range of 10-30% (Refer Table).

Achieving 100% degradation will be too strenuous and could cause secondary degradation, giving degradation products of the degradation products, which are not possible to be formed during the normal storage conditions.

Evaluation of the degradation mixture is generally performed by using the PDA (photodiode array) detector or LCMS. The peak purity of the major peak must be assessed to rule out any degradation peak under or unresolved from the major peak of interest. All the degradation products peaks' should have baseline resolution i.e. the minimum resolution of 1.5 should be achieved or as required for a particular product.

Table 1.10: Suggested outline for performing Forced Degradation Studies

Decide / Select matrix for degradation							
		Degradation Conditions					
Product/ Matrix	**Degradation**	**Acid**	**Base**	**Peroxide**	**Bisulfite**	**Photo stability**	**Temperature**
Drug Product	Yes	✓	✓	✓	✓	✓	✓
Placebo/Vehicle	Yes	✓	✓	✓	✓	✓	✓
API/ Raw material	Yes	✓	✓	✓	✓	✓	✓
Decide / Select degradation conditions/ agents							
Medium		**Conditions (Strive for 10 – 30% degradation)**					
1 N HCl, 10 ml		Reflux 30 Minutes, Neutralize with base					
0.1 N Sodium Hydroxide, 10 ml		Reflux 30 Minutes, Neutralize with acid					
3 % Hydrogen Peroxide, 10 ml		Reflux 30 Minutes					
10% Sodium Bisulfite, 10 ml		Reflux 30 Minutes					
Light		Light Chamber, 1 lumens (92.9 lux = 1000 ft-candles)/ (1 million lux.hours), 7 days					
Temperature		80°C, 7 Days					

2) LINEARITY

Definition

The linearity of an analytical procedure is its ability (within given range) to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample.

Method

Drug (different dilution) and/or separately weighed synthetic mixture. Measurement of response and plot response vs. concentration of analyte and demonstration of linearity by

- Visual inspection of plot
- Appropriate statistical methods

Recommendation

- Minimum of 5 concentrations are recommended

Expression/calculation

- Correlation coefficient, y-intercept, slope of regression line, residual sum of squares.

Acceptance criteria: Correlation coefficient should not be less than 0.999.

3) RANGE

Definition

The range of analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

Method

Drug (different dilution) and/or separately weighed synthetic mixture. Measurement of response and plot response vs. concentration of analyte and demonstration of linearity by

- Visual inspection of plot
- Appropriate statistical methods

Recommendation

- Assay of drug/finished product: 80 – 120% of test concentration.
- For content uniformity: 70 – 130% of test concentration.
- For dissolution testing: ± 20% over specified range.
- For impurity: from reporting level to 120% of specification.

Expression/calculation

- Correlation coefficient, y-intercept, slope of regression line, residual sum of squares.

4) ACCURACY

Definition

The accuracy of analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness.

Method

- Application of procedure to analyze synthetic mixture of known purity.
- Comparison of result with already established procedure.
- Accuracy may be inferred once precision, linearity and specificity have been established.

Recommendation

Minimum of nine determinations

- Low concentration of range × 3 replicates
- Medium concentration of range × 3 replicates
- High concentration of range × 3 replicates

Expression/calculation

- Percent recovery by the assay of known added amount of analyte
- Mean – Accepted true value with confidence interval

Acceptance criteria: % Recovery should be in the range of 98-102%.

5) PRECISION

Definition

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between the series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

Method

- Determination of % relative standard deviation (RSD) of response of multiple aliquots

Recommendation:

a) Repeatability (Same operating condition over short interval of time):

Minimum of nine determinations

- Low concentration of range × 3 replicates
- Medium concentration of range × 3 replicates
- High concentration of range × 3 replicates

  Or

  At target concentration × 6 determinations

b) Intermediate precision (within laboratory variation):

- Different Days
- Different Analysts
- Different Equipment etc.

Expression/calculation

- Standard deviation % RSD and confidence interval

Acceptance criteria: % Relative standard deviation should be less than 2.

6) DETECTION LIMIT

Definition

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantitated under stated experimental conditions.

Method

1. By visual evaluation
2. Based on S/N ratio
   - Applicable to procedure, which exhibit baseline noise.
   - Actual lowest concentration of analyte detected in compared with blank response
3. Based on S.D. of response and slope

$$\mathbf{LOD = 3.3\ \sigma/s}$$

s = Slope of calibration curve

σ = S.D. of response; can be obtained by

- Standard deviation of blank response
- Residual standard deviation of the regression line
- Standard deviation of the y-intercept of the regression line
- $S_{y/x}$ i.e. standard error of estimate

Expression/calculation

- If based on visual examination or S/N ratio – relevant chromatogram is to be presented.
- If by calculation/extrapolation – estimate is validated by analysis of suitable no. of samples known to be near or prepared at detection limit.

Acceptance criteria

- S/N ratio > 3 or 2:1; not specified in other cases

7) QUANTITATION LIMIT

Definition

The quantitation limit of an individual analytical procedure is defined as the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy.

Method

1. By visual evaluation
2. Based on S/N ratio

- Applicable to procedure, which exhibit baseline noise.
- Actual lowest concentration of analyte detected in compared with blank response

3. Based on S.D. of response and slope

$$LOQ = 10\ \sigma/s$$

s = Slope of calibration curve

σ = S.D. of response; can be obtained by

- Standard deviation of blank response
- Residual standard deviation of the regression line
- Standard deviation of the y-intercept of the regression line
- $S_{y/x}$ i.e. standard error of estimate

Recommendation

- Limit should be validated by analysis of suitable no. of samples known to be near or prepared at quantitation limit.

Expression/calculation

- Limits of quantitation and method used for determining should be presented.
- Expresses as analyte concentration.

Acceptance criteria

- S/N ratio > 10:1; not specified in other cases

8) ROBUSTNESS

Definition

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Method

It should show the reliability of an analysis with respect to deliberate variations in method parameters.

In case of liquid chromatography, examples of typical variations are

- Influence of variations of pH in a mobile phase,
- Influence of variations in mobile phase composition,

- Different columns (different lots and/or suppliers),
- Temperature,
- Flow rate.

Recommendation

- Robustness should be considered early in the development of a method.
- If the results of a method or other measurements are susceptible to variations in method parameters, these parameters should be adequately controlled and a precautionary statement included in the method documentation.

Expression/calculation

- Effect of these changed parameters on system suitability parameters.

Acceptance criteria

- The method must be robust enough to withstand slight changes and allow routine analysis of sample.

10) SOLUTION STABILITY:

During the earlier validation studies, the method developer gained some information on the stability of reagents, mobile phases, standards, and sample solutions. For routine testing in which many samples are prepared and analyzed each day, it is often essential that solutions are stable enough to allow for delays such as instrument breakdowns or overnight analyses using auto-samplers.

Stability has not been given due importance in ICH guidelines but the USFDA has discussed stability parameters for bio samples. It is important to determine the stability of an analyte in a particular matrix by comparison with freshly prepared standards.

Samples and standards should be tested over at least a 48 h period, and the quantitation of components should be determined. If the solutions are not stable over 48 h, storage conditions or additives should be identified that can improve stability.

Acceptance criteria: The difference in the peak area should not exceed than 2%.

Table 1.11: System Suitability Parameters and their recommended limits[34]

PARAMETER	RECOMMENDATION
Capacity factor (k')	The peak should be well-resolved from other peaks and the void volume, generally K' > 2
Repeatability	RSD ≤ 2% (N ≥ 5 is desirable)
Relative retention	Not essential as the resolution is stated.
Resolution(r_s)	R_s of > 2 between the peak of interest and the closest eluting potential interferent (impurity, excipients, degradation product, internal standard, etc.)
Tailing factor(t)	T ≤ 2
Theoretical plates(n)	In general should be > 2500

Table 1.12: Characteristics to be validated in HPLC[39]

CHARACTERISTICS	ACCEPTANCE CRITERIA
Accuracy/trueness	Recovery 98-102% (individual)
Precision	RSD < 2%
Repeatability	RSD < 2%
Intermediate precision	RSD < 2%
Specificity/ selectivity	No interference
Detection limit	S/N > 2 or 3
Quantitation limit	S/N > 10
Linearity	Correlation coefficient r^2 > 0.999
Range	80 –120 %
Stability	> 24 h or >12 h

1.7 HPLC - HIGH PERFORMANCE LIQUID CHROMATOGRAPHY[40]

HPLC (High Performance Liquid Chromatography): The acronym *HPLC*, coined by the late Prof. Csaba Horvath for his 1970 Pittcon paper, originally indicated the fact that high pressure was used to generate the flow required for liquid chromatography in packed columns. In the beginning, pumps only had a pressure capability of 500 psi [35 bars]. This was called *high pressure* liquid chromatography, or HPLC. The early 1970s saw a tremendous leap in technology. These new HPLC instruments could develop up to 6,000 psi [400 bar] of pressure, and incorporated improved injectors, detectors, and columns. HPLC really began to take hold in the mid-to late-1970s. With continued advances in performance during this time [smaller particles, even higher pressure], the acronym HPLC remained the same, but the name was changed to *high performance* liquid chromatography.

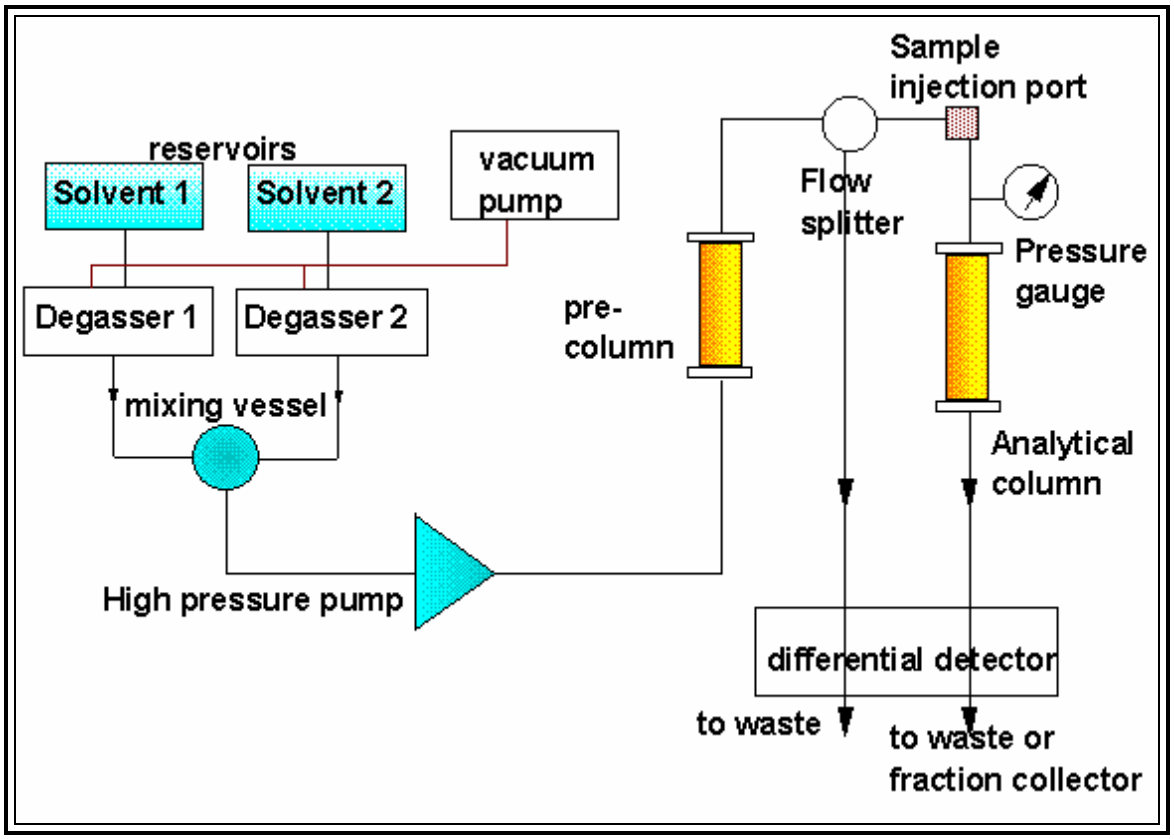


Figure 1.8: High Performance Liquid Chromatography[41]

1.7.1 TERMS ASSOCIATED WITH HPLC:

Ion-pairing agents[42]: Ion-pairing agents are ionic compounds that contain a hydrocarbon chain that imparts certain hydrophobicity so that the ion pair can be retained on a reversed-phase column. Ion Pairing agents are added at concentrations of 0.05 to 0.2 %. All ion-pairing agents are potentially capable of ion pairing with the positively charged basic residues of peptides or proteins, thus reducing hydrophilicity and increasing their retention time.

The use of ion pair reagent is suggested only when the adequate separation could not be obtained with the reverse phase chromatography. The reverse phase HPLC and reverse phase HPLC with ion pairing are similar except that the latter contains an ion pair reagent in the mobile phase to improve the selectivity of the ionic samples. However, reverse phase HPLC should be utilized first before for the ion-pair reagent unless any reference is available in the literature.

The solubility of the ion-pair reagent may be affected depending upon the organic solvent used in the mobile phase. Methanol is generally preferred over THF and Acetonitrile. In reverse phase HPLC with ion-pair, a suitable buffer is chosen at a concentration of about 25mM, the pH and ion-pair concentration are varied to provide the optimum selectivity to the separation. The pH of the mobile phase is closely associated with the ion pairing, whether the ion-pair is positively charged (Tetrabutylammonium, TBA+) or negatively charged (C5- or C6- sulfonate) and dependent on whether the analyte is an acid or base. For the cationic samples (protonated base) or bases, use the pentane hexane or higher hydrocarbon sulfonate ion-pair reagent and for the anionic samples (ionized acids) or acids, tetraethyl-ammonium hydroxide can be used as ion-pair reagent. Their optimization is the pH dependent.

Mechanism:

They form an “ion pair” with the charged molecule in solution that becomes one long nonpolar pseudomolecule with a masked charge couple in the center. The pseudomolecule then partitions with the bonded phase as if the charges did not exist. Instead of eluting at the void volume like the ionized species, the pseudomolecule is retained longer even than the free amine ionized species.

An alternate theory of ion pair action says that the ion pair reagent first interacts with the bonded phase, forming a nonbonded ion-exchange column. This modified bonded phase column then interacts with the compounds in solution through a mixed partition/ion-exchange mode. The longer the nonpolar chain of the ion pairing reagent, the longer this retention peak takes to come off. This allows us to position the retention time of an ionized species in a separation by controlling chain length of the ion pair.

Table 1.13: Commonly used Ion pairing agents

Class	Ion Pair Reagents	Used For Compound Class	pH Of Mobile Phase	Concentration Of Ion-Pair Reagent
Alkyl Sulfonate (Sulfonic Acid Alkyl Salts)	Pentane Sulfonate Hexane Sulfonate Heptane Sulfonate Octane Sulfonate	Cationic Samples (Protonated Bases) Basic Compounds	3.5	0.005 M
Alkyl Ammonium Salts	Tetrabutylammonium Hydrogen Sulphate Tetrabutylammonium Phosphate	Anionic Samples (Ionized acids) Acidic Compounds	7.5	0.005 M

The advantages of ion pair chromatography over ion exchange are[43]:

- Simple preparation of buffers
- Wide choice of carbon chain lengths for improved retention and separation
- Significantly reduced separation time
- Simultaneous separation of both ionized and nonionized solutes
- Highly reproducible results
- Improved peak shape

Organic modifiers[42]: A water miscible organic solvent which is added to an aqueous mobile phase to effect separations in reversed-phase HPLC.

In RP chromatography, water is the weak solvent, and acetonitrile, the strong solvent is added gradually to generate a gradient elution. Acetonitrile is the reverse phase solvent of choice because the UV cut off for acetonitrile is 190 nm, allowing detection at lower wavelengths. It is less viscous than methanol, thus causing less fluctuation in pressure.

Less bubble formation occurs when it is mixed with water. Isopropanol is used either alone or in combination with acetonitrile to elute large or hydrophobic proteins.

PEAK PURITY DETERMINATION WITH A DIODE ARRAY DETECTOR[44]:

Peak purity analysis is also a useful addition to routine quality control procedures, especially in the analysis of pharmaceuticals and food products, for which contamination and quality of results are critical.

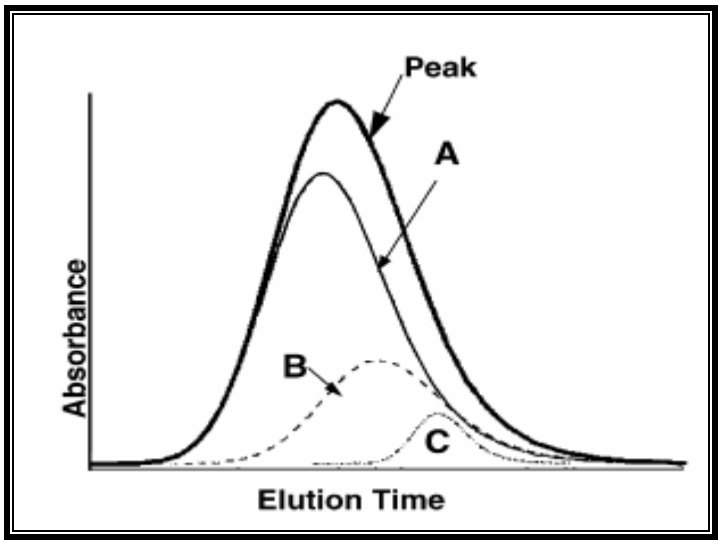


Figure 1.9: Coelution of three compounds A, B, and C in the chromatographic peak. No shoulders, valleys, or excessive tailing are seen.[44]

Peak purity analysis is designed to detect the presence of an impurity that is coeluting with the analyte peak. For impurity detection with a single-wavelength UV/Vis detector, one must see a shoulder, valley, or excessive tailing to suspect the presence of an impurity. The absence of these features on the chromatographic peak is not a foolproof assurance of peak purity. The impurity may not be seen simply because the chromatographic resolution is low (Fig. 1.15). A photodiode array detector can provide additional information by using the acquisition of UV/Vis spectra to determine peak purity. A proper peak purity determination requires access to a significant portion of the eluting compound's spectrum without interrupting the separation. For this reason, peak purity analysis is performed using a multisignal UV/Vis diode array spectrophotometer as the HPLC detector.

Principle of PDA:

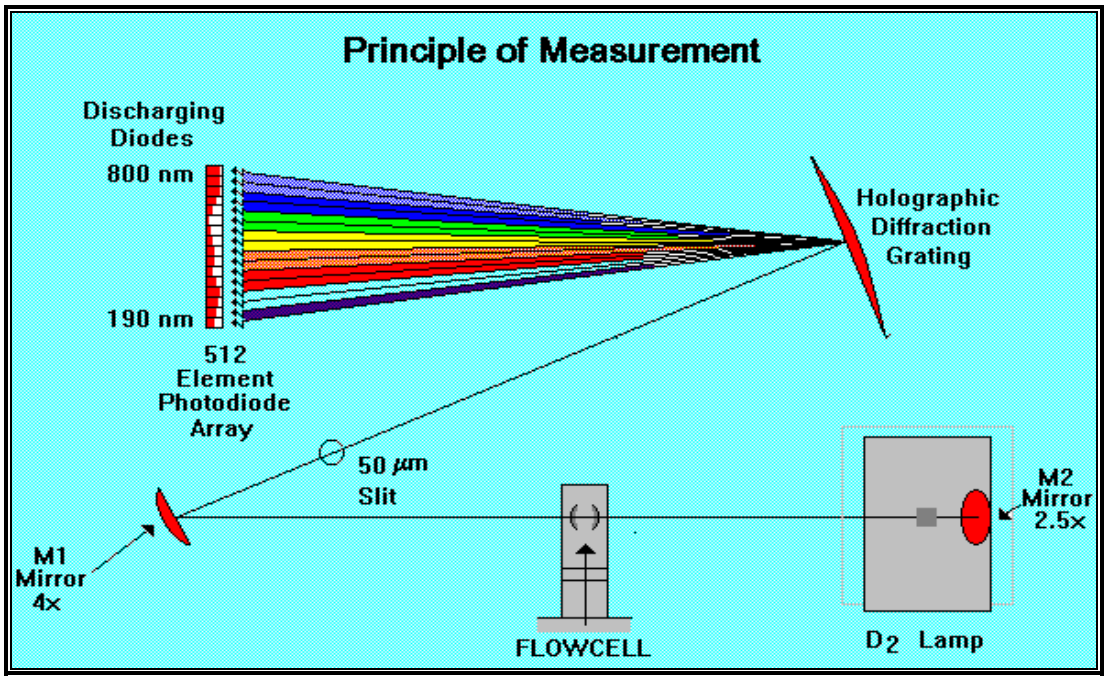


Figure 1.10: Optics unit components of PDA

The diode array spectrometer illuminates the sample with the entire spectrum of wavelengths emitted by the light source. Light transmitted by the sample is then broken into its component wavelengths by a diffraction grating and directed to a bank of photodiodes, each of which is dedicated to measure a narrow band of the spectrum. As no mechanical scanning is required, spectral acquisition can be accomplished in as little as 12 msec, well within the precision limits for HPLC peak elution. The rapid spectral acquisition makes it possible to perform peak purity determinations using selected multiple spectra as inputs. Therefore, the absence of any mechanical action in the acquisition of spectra enhances the reproducibility and the accuracy of the peak purity analysis. For coeluting peaks, a DAD makes it possible to differentiate both compounds, even when their spectral absorption overlaps the entire range of captured wavelength data. [80, 81]

DIFFICULTIES OF PEAK PURITY CONFIRMATION

Before the quantitative information contained in a chromatographic peak can be used, the purity of the peak should be confirmed. Only after we are sure that no coeluting impurity

was present, which could have contributed to the peak response, can we convert the peak's area or height into quantitative information based on the equivalent response of a pure standard. This peak purity analysis can be based on the comparison of the various spectra recorded during the elution of the peak. If the peak is pure, then, apart from concentration differences, the spectra taken at several points during a peak's elution should all be identical and the match scores obtained should be very close to the perfect match scores. If significant deviations are encountered, this can be seen as an indication of impurity.

Unfortunately, the inverse is not necessarily true. If the spectra are identical, based on the algorithm used for comparison, the peak can still be impure for one or several of three possible reasons:

1) The impurity is present at a much lower concentration than the main compound and is not detectable.

2) The impurity has the same or a very similar spectrum, compared to the main compound.

3) The impurity exhibits the same peak profile as does the main compound; that is, it completely coelutes with the main peak, across the entire peak.

One of the most important aspects of peak purity analysis that is often overlooked is the fact that any peak purity algorithm can only confirm the presence of impurities, but can never unambiguously prove that a peak is pure.

REVIEW OF LITERATURE

Lercanidipine hydrochloride is not official in any Pharmacopoeias.

2.1 REPORTED METHODS FOR DETERMINATION OF LERCANIDIPINE HCL

Table 2.1: Reported methods for determination Lercanidipine hydrochloride in formulation and in biological fluids

Sr. No.	Description and Matrix	Method	Chromatographic Details	References
1	New spectrophotometric method for estimation of Lercanidipine hydrochloride in bulk and formulation	U.V Spectrophoto-meteric (Colorimetric)	**Method A:** Colour formation by species condensation reaction with Vanillin (λ_{max}= 600 nm) **Method B:** Colour formation by reaction with citric acid /acetic anhydride(λ_{max}= 555 nm) **Method C:** Colour formation by reaction with Chloranil-acetaldehyde (λ_{max}= 585 nm)	**45**
2	Development and Validation of RP-HPLC Method for Estimation of Lercanidipine Hydrochloride in Bulk and Formulations	HPLC with ultraviolet detection.	**Column:** *C18* (125 mm* 4.6 mm, 5 µm) **Mobile Phase:** Acetonitrile:10 mM potassium dihydrogen phosphate buffer (60:40 v/v), pH 4. **Flow:** 1mL/min **Detection By UV at 240 nm.**	**46**

3	LC Determination of Lercanidipine and Its Impurities Using Drylab Software and Experimental Design Procedures	RP-HPLC	**Column:** C18 (3.5 µm, 20×4.6 mm I.D.) **Mobile Phase:** Acetonitrile: 1.5% aque. TEA (35:65), pH 3 by adding H3PO4 **Flow-rate:**1.0 mL/min **Detection:** 240 nm.	**47**
4	Determination of Lercanidipine Hydrochloride and Its Impurities in Tablets	RP-HPLC	**Column:** C18 Zorbax SB (5 µm, 250×4.6 mm I.D.) **Mobile Phase:** Acetonitrile:Water: TEA (55:44.8:0.2 v/v/v) **Flow-rate:**1.0 mL/min **Detection:** 240 nm.	**48**
5	A selective HPLC method for determination of Lercanidipine in tablets	RP-HPLC	**Column:** C18 Zorbax SB (5 µm, 250×4.6 mm I.D.) **Mobile Phase:** Acetonitrile:Water: TEA (55:44.8:0.2 v/v/v) **Flow-rate:**1.0 mL/min **Detection:** 356 nm.	**49**

6	Investigation on the photochemical stability of Lercanidipine and its determination in tablets by HPLC–UV and LC–ESI–MS/MS	HPLC–UV and LC–ESI–MS/MS	**Light source**: UV-A radiations (Xe-arc lamp) **Column:** Phenomenex LunaC18 (5 µm, 2.0×150mm) **Mobile phase**: methanol/TEA buffer (0.01 M) 60:40 (v/v), adjusted to pH 4 with acetic acid **flow rate:** 1 ml/min **UV detection**: 265nm **Detection**: ESI/MS/MS	**50**
7	Selective and rapid liquid chromatography-mass spectrometry method for the determination of Lercanidipine in human plasma	LC–MS/MS	**Column:** C8 **Mobile Phase:** Aque. Acetic acid and TEA in methanol **Source:** An ion trap mass spectrometer equipped with electrospray ionization (ESI) source	**51**
8	Enantioselective determination of Lercanidipine in human plasma for pharmacokinetic studies by normal-phase liquid chromatography–tandem mass spectrometry	LC–MS/MS	**Column:** Chiralpak® AD (250 ×4.6 mm,10µm) **Mobile Phase:** Hexane–Ethanol–DEA (95: 5: 0.1 v/v/v) **Flow-rate:**1.3 ml/min **Detection:** ESI/MS/MS	**52**

9	Differential Pulse Voltammetric Assay of Lercanidipine in Tablets	Differential Pulse Polarography	**Reagent:** Ethanol: 0.04M Britton Robinson buffer (20: 80) **Electrode:** Glassy carbon electrode **pH:** 4	**53**

Atenolol is official in all Pharmacopoeias like I.P, B.P, U.S.P, and E.P,

2.2 REPORTED OFFICIAL METHOD FOR DETERMINATION OF ATENOLOL

Table 2.2: Reported pharmacopoeial methods for determination of Atenolol in formulation

Sr. No.	Description and Matrix	Method	Chromatographic Details	References
1	Assay of Atenolol	Potentio-meteric	**Titrant:** 0.1 M perchloric acid **Titrate:** 0.2 g in 80 ml anhy. Acetic acid R **Each ml of 0.1 M $HClO_4$ ~ 26.63 mg of Atenolol**	**54, 55**
2	Assay of Atenolol	Rp-HPLC	**Column:** C18 (5 µm, 300×3.9 mm I.D.) **Mobile Phase:** Acetonitrile:Water: TEA (55:44.8:0.2 v/v/v) **Flow-rate:**0.6 ml/min **Detection:** 226 nm.	**56,57**

2.3 REPORTED METHOD FOR DETERMINATION OF ATENOLOL

Table 2.3: Reported methods for determination of Atenolol in formulation and in biological fluids

Sr. No.	Description and Matrix	Method	Chromatographic Details	Ref.
1	New spectrophotometric (Colorimetric) analysis of Atenolol in pure and pharmaceutical formulations	U.V Spectrophotometer	**Bulk drug:** Oxidation of the drug with excess quantities of oxidants, N-bromosuccinimide(NBS) and excess of NBS is determined using a dye celestine CB blue **μ = 540 nm** **Formulation:** Oxidation of the drug with excess quantities of oxidants, nitrous acid and excess of NBS is determined using a dye cresyl fast violet acetate CFVA **μ = 555 nm**	**58**
2	Spectrofluorimetric method for the determination of Atenolol in tablet dosage forms	Spectro-fluorimetric	**Solvent system:** Reaction of Atenolol with 0.1 N sodium hydroxide solutions in boiling water bath, which shows strong fluorescence **μ_{Ex} = 278 nm, μ_{Em} = 302nm**	**59**

3	Development of a HPLC method for the simultaneous determination of Losartan potassium and Atenolol	RP-HPLC	**Column:** Supelcosil C18 (5 µm, 250×4.6 mm I.D.) **Mobile Phase:** acetonitrile - 25 mM potassium dihydrogen phosphate (45:55 v/v, pH 3.00±0.05) **Flow-rate:**1.2 mL/min **Detection:** 227 nm. **Retention time:** 2.72 min	**60**
4	Enantioselective analysis of Atenolol in biologic fluids: comparison of liquid–liquid and solid-phase extraction methods	RP-HPLC	**Column:** Chiralcel OD-H **Mobile Phase:** hexane–ethanol (85:15, v/v) plus 0.1% diethylamine **Flow-rate:**1.0 ml/min **Detection:** 227 nm.	**61**
5	Reversed-phase high-performance liquid chromatographic analysis of Atenolol enantiomers in rat hepatic microsome after chiral derivatization with 2,3,4,6-tetra-O-acetyl-β--glycopyranosyl isothiocyanate	RP-HPLC	**Column:** Shimadzu CLC-C18 (100×4.6 mm,10 µm) derivatized with 2,3,4,6-tetra-*O*-acetyl-β-D -glycopyranosyl isothiocyanate at 35°C for 30 min **Mobile Phase:** phosphate buffer(pH 4.6)–methanol–acetonitrile (50:20:30, v/v) **Flow-rate:** 0.5 ml/min **Detection:** 254 nm.	**62**

6	Rapid high-performance liquid chromatographic method for the measurement of Atenolol in plasma using UV detection	HPLC–UV	**Column:** Cyano (6 µm) **Mobile Phase:** Acetonitrile: ammonium dihydrogen phosphate (4:96, v/v) containing triethylamine (0.25%) **Flow-rate:**1.0 ml/min **Detection:** 224 nm.	**63**
7	Cassette analysis of eight beta-blockers in bovine eye sclera, choroid–RPE, retina, and vitreous by liquid chromatography–tandem mass spectrometry	LC–MS/MS	**Column:** Hypersil-ODS C18 column (100 mm × 2.1 mm, 3.9 µm) **Mobile Phase:** **gradient mixture** of (A) 5 mM ammonium formate in water (pH 3.5 adjusted with formic acid) (B) acetonitrile: methanol (75:25, v/v) containing 0.02% TEA (pH 4.0; adjusted with formic acid) **Flow:** 0.4 ml/min	**64**
8	Simultaneous determination of Atenolol and Amlodipine in tablets by high-performance thin-layer chromatography mass spectrometry	HPTLC	**Stationary phase:** precoated silica gel 60F254 **Mobile phase**: Methylene chloride: methanol: ammonia solution (25% NH_3) (8.8:1.3:0.1; v/v/v) **Detection:** 230 nm **Retention factor:** 0.33	**65**

9	Determination of the β-blocker Atenolol in plasma by capillary zone electrophoresis	capillary zone electrophoresis	**Stationary phase:** Uncoated silica capillary (50 cm×75µm I.D.) **Mobile phase**: 50 m*M* H_3BO_3–50 m*M* $Na_2B_4O_7$ (50:50 v/v) **pH 9** **voltage :** +25 kV	**66**

2.4 REPORTED METHOD FOR DETERMINATION OF LERCANIDIPINE HCL AND ATENOLOL

Table 2.4: Reported method for simultaneous determination of Lercanidipine hydrochloride and Atenolol in tablet formulation

Sr. No.	Description and Matrix	Method	Chromatographic Details	Ref.
1	Simultaneous TLC-densitometric analysis of Atenolol and Lercanidipine hydrochloride in tablets	TLC	**Stationary phase:** Aluminum foil plates precoated with silica gel $60F_{254}$ **Mobile phase**: Toluene: methanol: TEA 3.5: 1.5: 0.1 (v/v/v) **Detection:** 275 nm **Retention factor:** 0.24 (Aten) 0.68 (Lerca)	**67**

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 has 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 multicomponents dosage forms becomes difficult due to cumbersome extraction or isolation procedures.

Stability testing of an active substance or finished product provide evidence on how the quality of a drug substance or drug product varies with time influenced by a variety of environmental factors such as pH, temperature, humidity and light. Knowledge from stability studies enables understanding the long-term effects of the environment on the drugs. Stability testing provides information about degradation mechanisms, potential degradation products, possible degradation pathways of drug as well as interaction between the drug and the excipients in drug product.[24, 68]

Although the concept of stress testing is not new to the pharmaceutical industry, the procedure was not clearly defined until the International Conference on Harmonization (ICH) provided a definition in its guidance on stability. The ICH guideline indicates that stress testing is designed to help "determine the intrinsic stability of the molecule by establishing degradation pathways in order to identify the likely degradation products and to validate the stability indicating power of the analytical procedures used."[28, 29]

Lotensyl-AT is the recently approved combination tablet of Lercanidipine hydrochloride and Atenolol, widely use in second-generation anti-hypertensive patients.

The literature survey reveals that a various spectrophotometric, chromatographic, fluorometric, electrophoresis, LC-MS etc. methods had been reported for individual drug, while TLC- densitometric analysis method had been reported for simultaneous estimation of Lercanidipine hydrochloride and Atenolol in pharmaceutical tablet dosage for. This all analytical methods are more complex and tedious.

No any stability indicating assay method by Rp-HPLC or Spectrophotomeric method had yet been reported in literature for simultaneous estimation of Lercanidipine hydrochloride and Atenolol combination.

SO, THE AIM OF PRESENT STUDY WAS

1) To develop and validate a simple, accurate, precise, specific, selective and rugged stability indicating RP-HPLC assay method for simultaneous estimation of Lercanidipine hydrochloride and Atenolol in presence of degradation products in stability samples as a part of in house research and development work in industry.
2) To develop and validate simple, rapid, accurate, precise and selective 1^{st} derivative spectrophotometric method for simultaneous estimation of Lercanidipine hydrochloride and Atenolol in pharmaceutical tablet dosage form.

4.1. UV SPECTRA OF LERCANIDIPINE HYDROCHLORIDE AND ATENOLOL

UV-spectrum of Lercanidipine hydrochloride (10 µg/ml) and Atenolol (50 µg/ml) in methanol was taken.

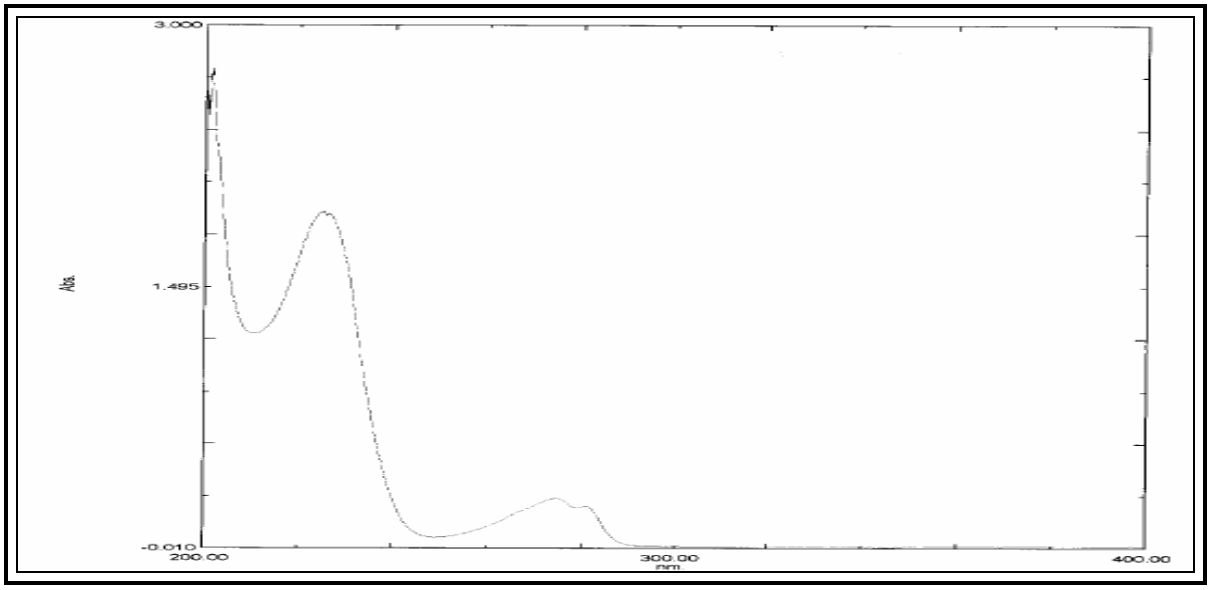


Figure 4.1: UV- spectra of Atenolol (50 µg/ml) in solvent mixture

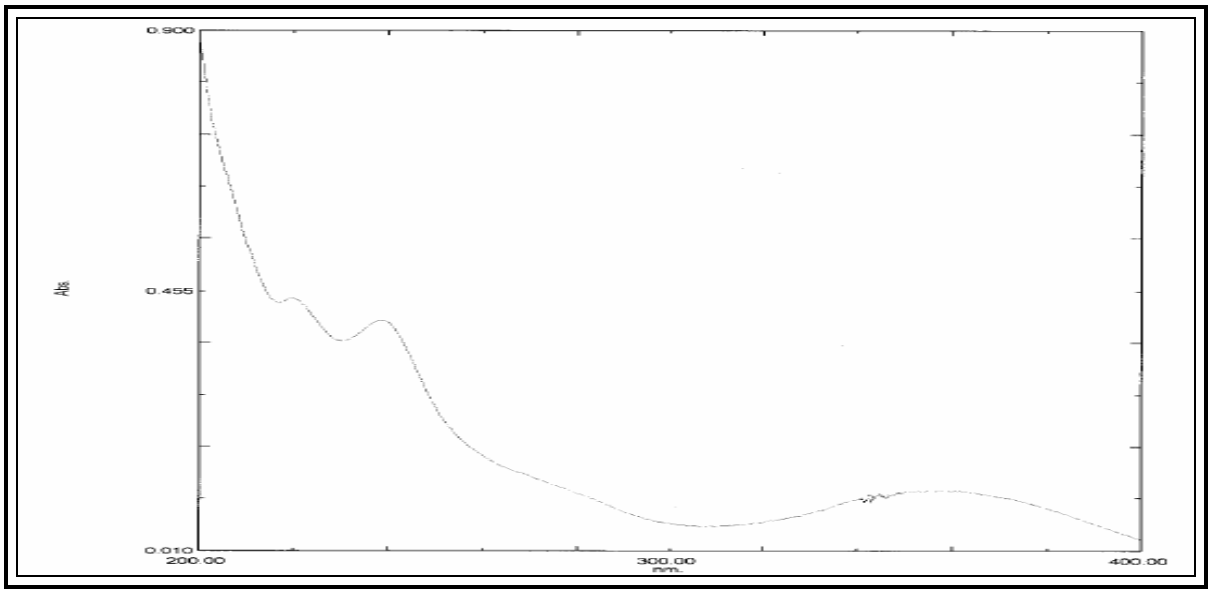


Figure 4.2: UV- spectra of Lercanidipine hydrochloride (10 µg/ml) in solvent mixture

Table 4.1: Comparison of Reported λ_{max} with Obtained λ_{max}

Drugs	Reported λ_{max}	Obtained λ_{max}
LER	238 nm, 354 nm[46]	238 nm, 354 nm
ATN	282 nm, 275 nm, 226 nm[54]	281.8 nm, 275 nm, 226 nm

4.2 MELTING POINT

Melting point of Lercanidipine hydrochloride and Atenolol had been determined by using melting point apparatus. The melting point of compounds was taken by open capillary method.

A small amount of drug sample was transferred in to capillary tube. Then capillary was placed in melting point test apparatus and noted down the temperature at which the drug started melting and was completely melted and corrected.

Table 4.2: Comparison of melting point of Lercanidipine hydrochloride and Atenolol with Reported melting point

Drug	Reported melting point	Observed melting point (°C)
LER	119°-123°C[7]	120°-122°C
ATN	152°-156.5°C[54]	151.9°-154.2°C

5.1 DEVELOPMENT AND VALIDATION OF METHOD FOR SIMULTANEOUS ESTIMATION OF LERCANIDIPINE HYDROCHLORIDE AND ATENOLOL IN PHARMACEUTICAL DOSAGE FORM BY FIRST ORDER DERIVATIVE SPECTROSCOPY.

5.1.1 EXPERIMENTAL:

5.1.1.1 Materials

1) **Lercanidipine hydrochloride and Atenolol (API)**: - Working standard grade APIs were supplied by Alembic Research Centre (Baroda) and its claimed purity was 99.00%.
2) **Lotensyl-AT Tablet** (label claim **10 mg Lercanidipine hydrochloride and 50 mg Atenolol**, manufactured by Sun pharmaceutical Ltd. (Baroda).

5.1.1.2 Reagents and Chemicals

1) Acetonitrile: -HPLC grade, Spectrochem pvt. Ltd., Mumbai, India.
2) Ortho-Phosphoric Acid (88%): - AR grade, Merck, India.
3) Milli-Q water: - It was purified by Millipore Corporation's system.

5.1.1.3 Instruments, Apparatus and equipment

1) A double beam UV-visible spectrophotometer having two matched cells with 1cm light path: - UV- 2450, Shimadzu, Japan.
2) Analytical Balance: - Mettler Toledo, Schwerzenland.
3) pH Meter: - Thermo electron corporation, Orion 420 A+.
4) Sonicator: - OSCAR Ultrasonics, India.

5.1.2 METHODS

5.1.2.1 Standard preparation:

Weigh and transfer about 10 mg of Lercanidipine HCl and 50 mg of Atenolol working standard to 100 ml amber colored volumetric flask. Add about 30 ml of diluent, sonicate to dissolve, make up the volume with the diluent. Further take 1 ml of this solution and make up the volume 10 ml with the diluent. (10 ppm Lercanidipine HCl and 50 ppm Atenolol)

5.1.2.2 Sample preparation:

Weigh accurately 20 tablets and crush the tablet transfer the powder equivalent to 10 mg of Lercanidipine HCl and 50 mg of Atenolol into 100 ml amber colored volumetric flask add about 30 ml of diluent, sonicate at 25°C for about 10 min with intermittent shaking, make up to volume with the diluent. Filter through 0.45µ PVDF filter, first discard about 5 ml of the filtrate further dilute 1ml of the solution to 10ml with solvent mixture. (10 ppm Lercanidipine HCL and 50 ppm Atenolol)

5.1.2.3 Blank preparation (diluent):

Add 1 ml of Ortho-Phosphoric Acid in to 1000 ml of Milli-Q water to obtain 0.1% OPA, mix it well. Mix 10% OPA and acetonitrile in the proportion of 55:45, sonicate it for proper mixing.

5.1.3 Method Validation:

5.1.3.1 Linearity and Range:

5.1.3.2 Accuracy (Recovery):

(Standard addition method)

Procedure

Accuracy was determined over the range 80% to 120% of the sample concentration. Samples were prepared in triplicate at each level and measure it at the sampling wavelength of LER and ATN.

Acceptance criteria: % Recovery should be in the range of 98-102%.

5.1.3.3 Precision:

a) Method Precision (Repeatability)

Procedure

Method precision should be established by analyzing six sample preparations under same conditions as per test procedure for assay using tablet sample. Individual assay value, mean assay value and % RSD shall be calculated for the results obtained and recorded.

b) Intraday precision:

Procedure

Intraday precision should be established by analyzing 3 different concentration of tablet sample prepared under same conditions as per test procedure for assay. Individual assay value, mean assay value and % RSD for tablet sample at three different times in a day shall be calculated for the results obtained and recorded.

c) Interday precision:

Procedure

Interday precision should be established by analyzing 3 different concentration of tablet sample prepared under same conditions as per test procedure for assay. Individual assay value, mean assay value and % RSD for tablet sample at three different days shall be calculated for the results obtained and recorded.

Acceptance criteria: % Relative standard deviation should be less than 2.

5.1.3.4 Solution Stability:

Procedure

Standard API and sample preparation was prepared as per test procedure and assay of standard and sample was determined as per method. Standard and sample solution was stored for 48 hours at room temperature. Assay of standard and sample solution after 48 hours was determined using freshly prepared standard. The assay obtained was compared with the initial assay value and recorded.

Acceptance criteria: The difference in the absorbance should not be exceed than 2% .

5.1.3.5 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 given in section.

5.1.3.6 Specificity:

(Interference from blank, placebo and impurities)

Procedure

- Blank solution was scanned.
- Placebo solution was scanned as per the method.

Acceptance criteria: The difference in the absorbance should not be more than 2%.

5.1.4 CALCULATION FOR ASSAY:

Procedure:

Prepare sample (tablet) solution as mentioned in sample preparation (As per section 5.2.2.2) and measure the concentration of both the drugs at sampling wavelength of Lercanidipine hydrochloride and Atenolol.

Acceptance criteria: % Assay should be in the range of 98-102%.

5.2 DEVELOPMENT AND VALIDATION OF STABILITY-INDICATING RP-HPLC ASSAY METHOD FOR ESTIMATION OF LERCANIDIPINE HYDROCHLORIDE AND ATENOLOL IN PHARMACEUTICAL DOSAGE FORM

5.2.1 EXPERIMENTAL:

5.2.1.1 Materials

1) **Lercanidipine hydrochloride and Atenolol (API)**: - Working standard grade APIs were supplied by Alembic Research Centre (Baroda) and its claimed purity was 99.00%.
2) **Lotensyl-AT Tablet** (label claim **10 mg Lercanidipine hydrochloride and 50 mg Atenolol**, manufactured by Sun pharmaceutical Ltd. (Baroda)
3) **Placebo** sample was supplied by Alembic Research Centre (Baroda)

5.2.1.2 Reagents and Chemicals

1) Ortho-Phosphoric Acid (88%): - AR grade, Merck, India.
2) Acetonitrile: -HPLC grade, Spectrochem pvt. Ltd., Mumbai, India.
3) Milli-Q water: - It was purified by Millipore Corporation's system.
4) Hydrochloric acid: - S.R. Enterprise, Baroda.
5) Sodium hydroxide: - S.R. Enterprise, Baroda.
6) Hydrogen Peroxide (30%):- S.R. Enterprise, Baroda.

5.2.1.3 Instruments, Apparatus and equipment

1) High performance Liquid chromatography system (HPLC): Waters 2695 (Separation module) with Waters 2696 PDA (Photodiode array detector).
2) Chromatographic software:– Empower-pro
3) A double beam UV-visible spectrophotometer having two matched cells with 1cm light path: - UV- 2450, Shimadzu, Japan
4) Analytical Balance: - Mettler Toledo, Schwerzenland
5) pH Meter: - Thermo electron corporation, Orion 420 A+
6) Sonicator: - OSCAR Ultrasonics, India

7) Hot air oven: - Cintex industrial corporation, dadar (W), Mumbai, India
8) Photo stability chamber: - QLPH-2014
9) Melting Point Apparatus:- MP-DS

5.2.2 METHODS

5.2.2.1 Standard preparation:

Weigh and transfer about 10 mg of Lercanidipine HCL and 50 mg of Atenolol working standard to 100 ml amber colored volumetric flask. Add about 30 ml of diluent, sonicate to dissolve, make up the volume with the diluent. Further take 1 ml of this solution and make up the volume 10 ml with the diluent. (10 ppm Lercanidipine HCL and 50 ppm Atenolol)

5.2.2.2 Sample preparation:

Weigh accurately 20 tablets and crush the tablet transfer the powder equivalent to 10 mg of Lercanidipine HCL and 50 mg of Atenolol into 100 ml amber colored volumetric flask add about 30 ml of diluent, sonicate at 25°C for about 10 min with intermittent shaking, make up to volume with the diluent. Filter through 0.45µ PVDF filter, first discard about 5 ml of the filtrate further dilute 1ml of the solution to 10ml with solvent mixture. (10 ppm Lercanidipine HCL and 50 ppm Atenolol)

5.2.2.3 Blank preparation (diluent):

Add 1 ml of Ortho-Phosphoric Acid in to 1000 ml of Milli-Q water to obtain 0.1% OPA, mix it well. Mix 10% OPA and acetonitrile in the proportion of 55:45, sonicate it for proper mixing.

5.2.2.5 Placebo Preparation:

Weighed accurately about 140 mg tablet placebo powder (As Average weight of tablet is 200 mg) and transferred to 100 ml amber colored volumetric flask. Add about 30 ml of diluent, sonicate to dissolve, make up the volume with diluent. Further take 1 ml of this solution and make up the volume 10 ml with diluent and record the chromatogram.

5.2.3 RP-HPLC Method development and optimization:

The standard solution of Lercanidipine hydrochloride and Atenolol was used for method development trials to optimize the assay method for determination of Lercanidipine hydrochloride and Atenolol.

5.2.3.1 Selection of detection wavelength:

The standard solution of Lercanidipine hydrochloride (10 µg/ml) and Atenolol (50 µg/ml) were scanned over the range of 200 nm to 400 nm wavelengths. It showed Iso-absorptive point at 239 nm. So the wavelength for the determination of Lercanidipine hydrochloride and Atenolol was selected as 239 nm.

5.2.3.2 Selection of Column:

From different trials, Inertsil ODS 3V column was selected. After the optimization of mobile phase different size of columns were tried. At last Inertsil ODS 3V (250*4.6 mm, 5µ) column was selected for assay method.

5.2.3.6 Selection of oven temperature:

Column temperature 25°C was optimized, which minimized day-to-day variation of retention time due to fluctuations in the ambient temperature; along with it imparted sharp symmetrical peak.

5.2.3.7 System suitability:

Inject Blank preparation in single injection, standard preparation in five replicate, record the chromatogram and calculate the system suitability parameters as given below:

- Theoretical plate for peak in five replicate standard injections : NLT 2500
- % RSD of peak area for five replicate standard injections : NMT 2.0
- Tailling Factor of peak in five replicate standard injections : NMT 2.0
- % RSD of retention time of peak in five replicate standard injections : NMT 2.0

If system suitability passes then inject sample preparation in duplicate.

5.2.4 Forced degradation study:

In order to establish whether the analytical method for the assay was stability-indicating, pure active pharmaceutical ingredient (API), tablet placebo and tablet of Lercanidipine hydrochloride and Atenolol were subjected to various stress conditions to conduct forced degradation studies. Stress studies were carried out under the conditions of acid/base hydrolysis, oxidation, Thermal as mentioned in ICH Q1A (R2). UV light degradation of drug substances and drug product was performed in the solid state as well as liquid state as mentioned in ICH Q1B. Several trials with different severity of each stressed condition were conducted, so that upto 5- 30% degradation is achieved.

5.2.4.1 *Acidic degradation:*

Procedure:

For API:

- Weigh and transfer accurately about 10 mg Lercanidipine hydrochloride and 50 mg Atenolol in to 100 ml amber colored volumetric flask.
- Add about 5 ml of 1 N HCL in it.
- Heat it at 80 °C for 5 hours, protecting it from the light as to prevent degradation by light.
- Cool the solution to room temperature and neutralize it by adding 5 ml of 1 N NaOH.
- Add 30 ml of diluent to it, and sonicate the solution for about 10 mins with occasional shaking.
- Make the volume up to mark with diluent and mix.
- Filter the solution with 0.45 µ PVDF filter discarding the first 5 ml of solution.
- From above solution dilute 1 ml to 10 ml with the diluent.
- Record the chromatogram.

For Placebo:

- Weigh and transfer accurately about 140 mg of placebo in to 100 ml amber colored volumetric flask. (As the average weight of the tablet is 200 mg and it contains 60 mg of API)

- Follow same procedure as followed for API.

For Tablet sample:

- Weigh and transfer accurately about 200 mg of tablet powder in to 100 ml amber colored volumetric flask. (As the average weight of the tablet is 200 mg.)
- Follow same procedure as followed for API.

5.2.4.2 *Alkali degradation:*

Procedure:

For API:

- Weigh and transfer accurately about 10 mg Lercanidipine hydrochloride and 50 mg Atenolol in to 100 ml amber colored volumetric flask.
- Add about 5 ml of 1 N NaOH in it.
- Store the solution for 6 hours at room temperature, protecting it from the light as to prevent degradation by light.
- Neutralize the solution by adding 5 ml of 1 N HCL.
- Add 30 ml of diluent to it, and sonicate the solution for about 10 mins with occasional shaking.
- Make the volume up to mark with diluent and mix.
- Filter the solution with 0.45 µ PVDF filter discarding the first 5 ml of solution.
- From above solution dilute 1 ml to 10 ml with the diluent.
- Record the chromatogram.

For Placebo:

- Weigh and transfer accurately about 140 mg of placebo in to 100 ml amber colored volumetric flask. (As the average weight of the tablet is 200 mg and it contains 60 mg of API)
- Follow same procedure as followed for API.

For Tablet sample:

- Weigh and transfer accurately about 200 mg of tablet powder in to 100 ml amber colored volumetric flask. (As the average weight of the tablet is 200 mg.)
- Follow same procedure as followed for API.

5.2.4.3 ***Neutral degradation:***

Procedure:

For API:

- Weigh and transfer accurately about 10 mg Lercanidipine hydrochloride and 50 mg Atenolol in to 100 ml amber colored volumetric flask.
- Add about 5 ml of Milli-Q water in it.
- Heat it at 80 °C for 8 hours, protecting it from the light as to prevent degradation by light.
- Cool the solution to room temperature.
- Add 30 ml of diluent to it, and sonicate the solution for about 10 mins with occasional shaking.
- Make the volume up to mark with diluent and mix.
- Filter the solution with 0.45 µ PVDF filter discarding the first 5 ml of solution.
- From above solution dilute 1 ml to 10 ml with the diluent.
- Record the chromatogram.

For Placebo:

- Weigh and transfer accurately about 140 mg of placebo in to 100 ml amber colored volumetric flask. (As the average weight of the tablet is 200 mg and it contains 60 mg of API)
- Follow same procedure as followed for API.

For Tablet sample:

- Weigh and transfer accurately about 200 mg of tablet powder in to 100 ml amber colored volumetric flask. (As the average weight of the tablet is 200 mg.)
- Follow same procedure as followed for API.

5.2.4.4 ***Peroxide degradation:***

Procedure:

For API:

- Weigh and transfer accurately about 10 mg Lercanidipine hydrochloride and 50 mg Atenolol in 100 ml amber colored volumetric flask.
- Add about 5 ml of 3% H_2O_2 in it.
- Store the solution for 2 hours at room temperature, protecting it from the light as to prevent degradation by light.
- Add 30 ml of diluent to it, and sonicate the solution for about 10 mins with occasional shaking.
- Make the volume up to mark with diluent and mix.
- Filter the solution with 0.45 µ PVDF filter discarding the first 5 ml of solution.
- From above solution dilute 1 ml to 10 ml with the diluent.
- Record the chromatogram.

For Placebo:

- Weigh and transfer accurately about 140 mg of placebo in 100 ml amber colored volumetric flask. (As the average weight of the tablet is 200 mg and it contains 60 mg of API)
- Follow same procedure as followed for API.

For Tablet sample:

- Weigh and transfer accurately about 200 mg of tablet powder in 100 ml amber colored volumetric flask. (As the average weight of the tablet is 200 mg.)
- Follow same procedure as followed for API.

5.2.4.5 ***Thermal degradation:***

Procedure:

For API:

- Weigh and transfer accurately about 10 mg Lercanidipine hydrochloride and 50 mg Atenolol in to 100 ml amber colored volumetric flask.
- Store it at 100°C for 24 hours in hot air oven, protecting it from the light as to prevent degradation by light.
- Cool it to room temperature.

- Add 30 ml of diluent to it, and sonicate the solution for about 10 mins with occasional shaking.
- Make the volume up to mark with diluent and mix.
- Filter the solution with 0.45 µ PVDF filter discarding the first 5 ml of solution.
- From above solution dilute 1 ml to 10 ml with the diluent.
- Record the chromatogram.

For Placebo:

- Weigh and transfer accurately about 140 mg of placebo in to 100 ml amber colored volumetric flask. (As the average weight of the tablet is 200 mg and it contains 60 mg of API)
- Follow same procedure as followed for API.

For Tablet sample:

- Weigh and transfer accurately about 200 mg of tablet powder in to 100 ml amber colored volumetric flask. (As the average weight of the tablet is 200 mg.)
- Follow same procedure as followed for API.

5.2.4.6 *Photolytic degradation:*

Procedure:

For API:

- Weigh and transfer accurately about 10 mg Lercanidipine hydrochloride and 50 mg Atenolol in 100 ml amber colored volumetric flask.
- Solid drugs (Mixture) were spread in 1 mm thickness uniform layer on a Petridish and exposed it with the light of energy 1.2 million lux/hours in photo stability chamber for 7 days.
- While liquid sample was exposed direct in sun light for 30 min.
- Add 30 ml of diluent to it, and sonicate the solution for about 10 mins with occasional shaking.
- Make the volume up to mark with diluent and mix.
- Filter the solution with 0.45 µ PVDF filter discarding the first 5 ml of solution.

- From above solution dilute 1 ml to 10 ml with the diluent.
- Record the chromatogram.

For Placebo:

- Weigh and transfer accurately about 140 mg of placebo in to 100 ml amber colored volumetric flask. (As the average weight of the tablet is 200 mg and it contains 60 mg of API)
- Follow same procedure as followed for API.

For Tablet sample:

- Weigh and transfer accurately about 200 mg of tablet powder in to 100 ml amber colored volumetric flask. (As the average weight of the tablet is 200 mg.)
- Follow same procedure as followed for API.

5.2.5 Method Validation:

5.2.5.1 Specificity:

(Interference from blank, placebo and impurities)

- Blank solution was injected. There should be no interference from the (if peak arising) blank at the R.T. of both LER and ATN and degradation products.
- Placebo solution was injected as per method.
- Standard working solution was injected as per the method.
- Sample (Tablet) solution was injected as per method.
- A sample was analyzed as per method.

Acceptance criteria: No interference peaks from blank or placebo at the RT of known Lercanidipine hydrochloride and Atenolol and peak purity should pass (Purity angle should be less than the Purity threshold).

Procedure

Prepare blank preparation, placebo preparation, standard preparation and sample preparation for Lotensyl-AT tablet as per method.

Placebo preparation: As per **5.3.2.5.**

5.2.5.2 Linearity and Range:

The linearity was determined at seven levels over the range of 50% to 150% of sample concentration. Each linearity level preparation was injected in triplicate.

Stoke solution preparation (100 µg/ml of LER and 500 µg/ml of ATN):

- Weigh and transfer accurately about 10 mg LER and 50 mg ATN in 100 ml amber colored volumetric flask.
- Add 30 ml of diluent in it, sonicate it for 10 min.
- Make the volume up to mark with diluent and mix well.

Dilution preparation:

- Appropriate volumes of aliquots from stoke solution of LER and ATN were transferred to 10 ml volumetric flask. Volumes were adjusted to mark with the diluent to obtain concentration of 5:25, 7:35, 9:45, 10:50, 11:55, 13:65 and 15:75 µg/ml of LER and ATN respectively in the mixtures.
- Inject dilution solutions as per test procedure at each level in triplicate.
- Plot a graph of mean area versus concentration in ppm. Calculate and record value of correlation co-efficient (r), y-intercept and slope of regression line.

Acceptance criteria: Correlation coefficient should not be less than 0.999.

5.2.5.3 Accuracy (Recovery):

(Placebo method)

Procedure

Accuracy was determined over the range 50% to 150% of the sample concentration. Samples were injected in triplicate at each level and record the chromatogram.

Acceptance criteria: % Recovery should be in the range of 98-102%.

5.2.5.4 Precision:

a) Method Precision (Repeatability)

Procedure

Method precision should be established by analyzing six sample preparations under same conditions as per test procedure for assay using tablet sample. Individual assay value, mean assay value and % RSD shall be calculated for the results obtained and recorded.

b) Intraday precision:

Procedure

Intraday precision should be established by analyzing 3 different concentration of tablet sample prepared under same conditions as per test procedure for assay. Individual assay value, mean assay value and % RSD for tablet sample at three different times in a day shall be calculated for the results obtained and recorded.

c) Interday precision:

Procedure

Interday precision should be established by analyzing 3 different concentration of tablet sample prepared under same conditions as per test procedure for assay. Individual assay value, mean assay value and % RSD for tablet sample at three different days shall be calculated for the results obtained and recorded.

Acceptance criteria: % Relative standard deviation should be less than 2.

5.2.5.5 Solution Stability:

Procedure

Standard API and sample preparation was prepared as per test procedure and assay of standard and sample was determined as per test method. Standard and sample solution was stored in dark for 48 hours at room temperature. Assay of standard and sample solution after 48 hours was determined using freshly prepared standard. The assay obtained was compared with the initial assay value and recorded.

Acceptance criteria: The difference in the peak area should not exceed than 2%.

5.2.5.6 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 given in section.

5.2.5.7 Robustness:

The following parameters were changed one by one and their effect was observed on system suitability.

1. Flow rate of mobile phase (± 10%) to 0.45 ml/min and 0.55 ml/min.
2. Column oven temperature (± 5°C absolute) to 20°C and 30°C.
3. pH (± 0.2 absolute) to 2.3 and 2.7.

5.2.6 CALCULATION FOR ASSAY:

Procedure:

Inject the sample (tablet) solution, prepared as per section 5.3.2.2 and record the chromatogram. Measure the concentration of both LER and ATN.

Acceptance criteria: % Assay should be in the range of 98-102%.

6.1 DEVELOPMENT AND VALIDATION OF METHOD FOR SIMULTANEOUS ESTIMATION OF LERCANIDIPINE HYDROCHLORIDE AND ATENOLOL IN PHARMACEUTICAL DOSAGE FORM BY FIRST ORDER DERIVATIVE SPECTROSCOPY.

METHOD DEVELOPMENT

Selection of wavelengths for simultaneous estimation of Lercanidipine hydrochloride and Atenolol from overlay 1st derivative spectra:

Spectra:

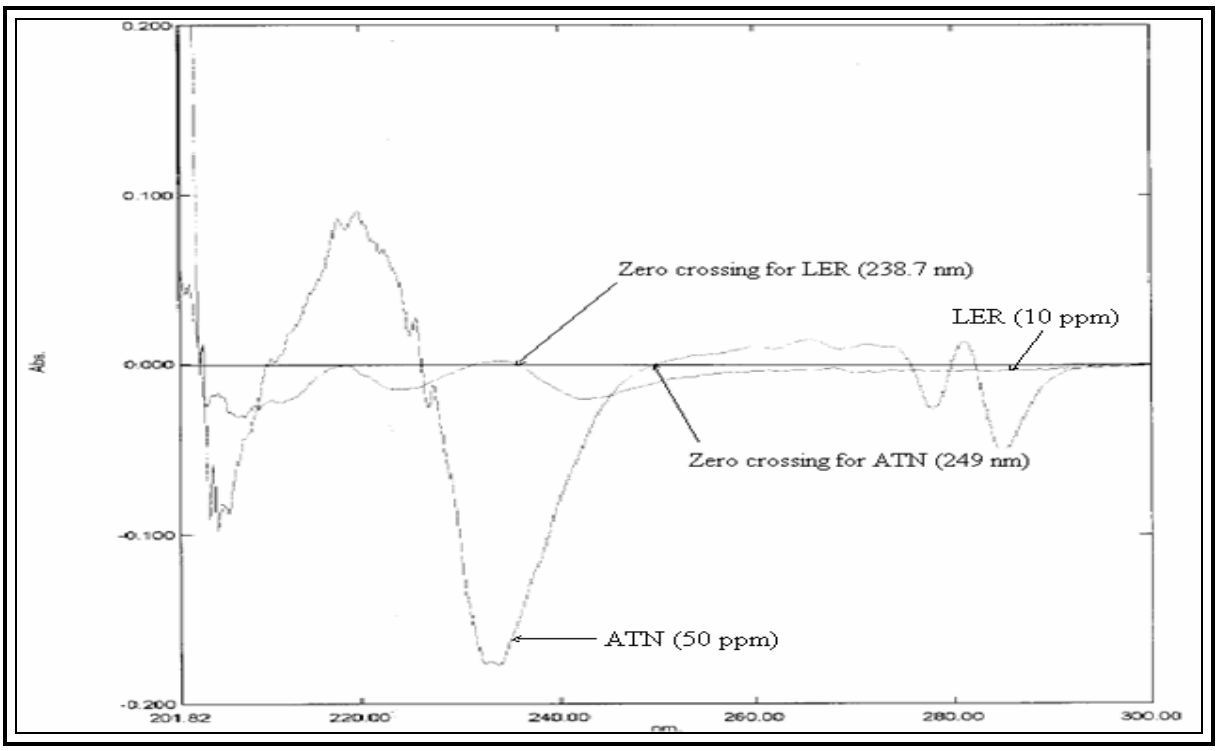


Figure 6.1: Overlay 1st derivative spectra of Lercanidipine hydrochloride and Atenolol

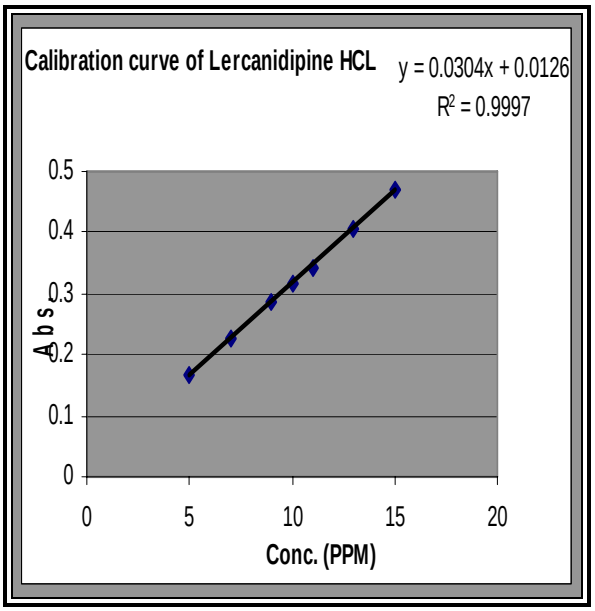
Discussion: For determination of sampling wavelength 10 µg/ml of LER and 50 µg/ml of ATN were scanned individually in 200-400 nm range and sampling wavelengths were optimized as 249 nm for LER estimation, where ATN showed zero crossing point and 238.7 nm for ATN estimation, where LER showed zero crossing point from overlapped first order derivative spectra of both LER and ATN.

6.1.1 Method Validation:

6.1.1.1 Linearity and Range: [N= 3]

LERCANIDIPINE HCL		
Conc.(PPM)	Absorbance ± SD	% RSD
5	0.167 ± 0.0019	1.13
7	0.225 ± 0.0028	1.24
9	0.285 ± 0.0016	0.56
10	0.316 ± 0.0022	0.69
11	0.344 ± 0.0031	0.90
13	0.408 ± 0.0027	0.66
15	0.471 ± 0.0017	0.36

ATENOLOL		
Conc.(PPM)	Absorbance ± SD	% RSD
25	0.397 ± 0.0025	0.62
35	0.542 ± 0.003	0.55
45	0.682 ± 0.0023	0.33
50	0.758 ± 0.0015	0.19
55	0.848 ± 0.0028	0.33
65	0.994 ± 0.0032	0.32
75	1.147 ± 0.0022	0.19



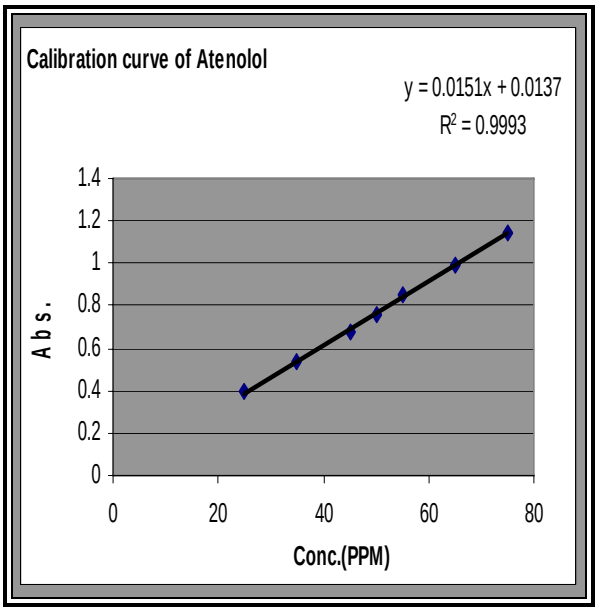


Fig 6.2: Calibration curve of Lercanidipine hydrochloride and Atenolol

Conclusion: Meets acceptance criteria.

6.1.1.2 Accuracy (Recovery):

Observation:

LERCANIDIPINE HCL (STANDARD ADDITION METHOD)					
Spiked level	**Absorbance**	**Abs * D.F(2)**	**Conc. (PPM)**	**% Recovery**	**Avg %Recovery**
80%	0.28	0.56	18.00	100.03	
80%	0.282	0.564	18.13	100.76	**100.04 ± 0.73**
80%	0.278	0.556	17.87	99.30	
100%	0.307	0.614	19.782	98.91	
100%	0.306	0.612	19.71	98.58	**99.02 ± 0.5**
100%	0.309	0.618	19.91	99.57	
120%	0.337	0.674	21.75	98.89	
120%	0.337	0.674	21.75	98.89	**99.29 ± 0.69**
120%	0.341	0.682	22.02	100.08	

ATENOLOL (STANDARD ADDITION METHOD)					
Spiked	**Absorbance**	**Abs * D.F(2)**	**Conc. (PPM)**	**% Recovery**	**Avg %Recovery**
80%	0.694	1.388	91.01	101.12	
80%	0.688	1.376	90.21	100.24	**100.97 ± 0.66**
80%	0.697	1.394	91.41	101.56	
100%	0.739	1.478	99.07	99.07	
100%	0.741	1.482	99.34	99.34	**98.99 ± 0.41**
100%	0.735	1.47	98.54	98.54	
120%	0.842	1.684	110.61	100.55	
120%	0.837	1.674	109.95	99.95	**100.07 ± 0.43**
120%	0.835	1.67	109.69	99.71	

Conclusion: Meets acceptance criteria.

6.1.1.3 Precision:

a) Repeatability:

REPEATABILITY (TABLET SAMPLE)				
N	**LER (10 ppm)**	**% Assay Of LER**	**ATN (50 ppm)**	**% Assay Of ATN**
1	0.312	101.07	0.75	98.43
2	0.312	101.07	0.749	98.42
3	0.31	100.43	0.769	100.94
4	0.314	101.72	0.751	98.66
5	0.314	101.71	0.77	101.2
6	0.31	100.43	0.767	100.68
Mean	**-**	**101.07**	**-**	**99.72**
Std. dev.	**-**	**0.574**	**-**	**1.34**
% RSD	**-**	**0.57**	**-**	**1.35**

Conclusion: Meets acceptance criteria.

b) Intraday precision:

INTRADAY PRECISION						
Drug	**ATN**			**LER**		
Conc.	**25 ppm**	**50 ppm**	**75 ppm**	**5 ppm**	**10 ppm**	**15 ppm**
10 a.m.	0.364	0.771	1.082	0.152	0.302	0.445
2 p.m.	0.361	0.769	1.081	0.15	0.302	0.444
6 p.m.	0.36	0.752	1.08	0.149	0.299	0.444
mean	0.361	0.764	1.081	0.150	0.301	0.445
S.D	0.0017	0.010	0.0008	0.0012	0.0014	0.0004
% RSD	0.46	1.36	0.07	0.82	0.46	0.10

Conclusion: Meets acceptance criteria.

c) Interday precision:

INTERDAY PRECISION						
Drug	**ATN**			**LER**		
Conc.	**25 ppm**	**50 ppm**	**75 ppm**	**5 ppm**	**10 ppm**	**15 ppm**
Day 1	0.376	0.771	1.081	0.161	0.314	0.455
Day 2	0.373	0.766	1.075	0.158	0.31	0.451
Day 3	0.367	0.752	1.069	0.155	0.305	0.448
mean	0.372	0.763	1.075	0.158	0.309	0.451
S.D	0.0037	0.009	0.0048	0.0024	0.0036	0.0028
% RSD	1.01	1.29	0.45	1.55	1.18	0.63

Conclusion: Meets acceptance criteria.

6.1.1.4 Solution Stability

SOLUTION STABILITY (API)				
Time (hrs)	**LER**		**ATN**	
	Absorbance	**% Difference**	**Absorbance**	**% Difference**
Initial	0.308	-	0.771	-
12 hrs	0.305	-0.003	0.766	-0.005
24 hrs	0.304	-0.004	0.759	-0.012
48 hrs	0.302	-0.006	0.748	-0.023
SOLUTION STABILITY (TABLET SAMPLE)				
Time (hrs)	**LER**		**ATN**	
	Absorbance	**% Difference**	**Absorbance**	**% Difference**
Initial	0.314	-	0.767	-
12 hrs	0.310	-0.004	0.762	-0.005
24 hrs	0.309	-0.005	0.754	-0.013
48 hrs	0.307	-0.007	0.747	-0.02

Conclusion: Both the drugs are practically stable at room temperature in mentioned diluents.

6.1.1.5 Limit of detection (LOD) and Limit of quantitation (LOQ):

DRUG	LOD (ppm)	LOQ (ppm)
LER	**0.09**	**0.27**
ATN	**0.28**	**0.84**

6.1.1.6 Specificity:

- The absorbance of placebo sample was found to be 0.007, which is less than 2% to the absorbance of standard LER and ATN solutions.

Conclusion: Meets acceptance criteria.

6.1.3 Conclusion

The proposed 1st derivative spectrophotometric method was accurate, precise, rapid and cost-efficient for determination of Lercanidipine hydrochloride and Atenolol in pharmaceutical dosage form. High %Recovery shows that the method is free from the interference from excipients used in the commercial pharmaceutical preparations. Hence, it can be successfully applied for routine estimation for Lercanidipine hydrochloride and Atenolol in quality control laboratories. The results of validation parameters are satisfactory level indicates the accuracy of proposed method for estimation of Lercanidipine hydrochloride and Atenolol in different dosage forms.

6.2 DEVELOPMENT AND VALIDATION OF STABILITY-INDICATING RP-HPLC ASSAY METHOD FOR ESTIMATION OF LERCANIDIPINE HYDROCHLORIDE AND ATENOLOL IN PHARMACEUTICAL DOSAGE FORM

6.2.1 METHOD DEVELOPMENT TRIALS:

1. Selection of detection wavelength:

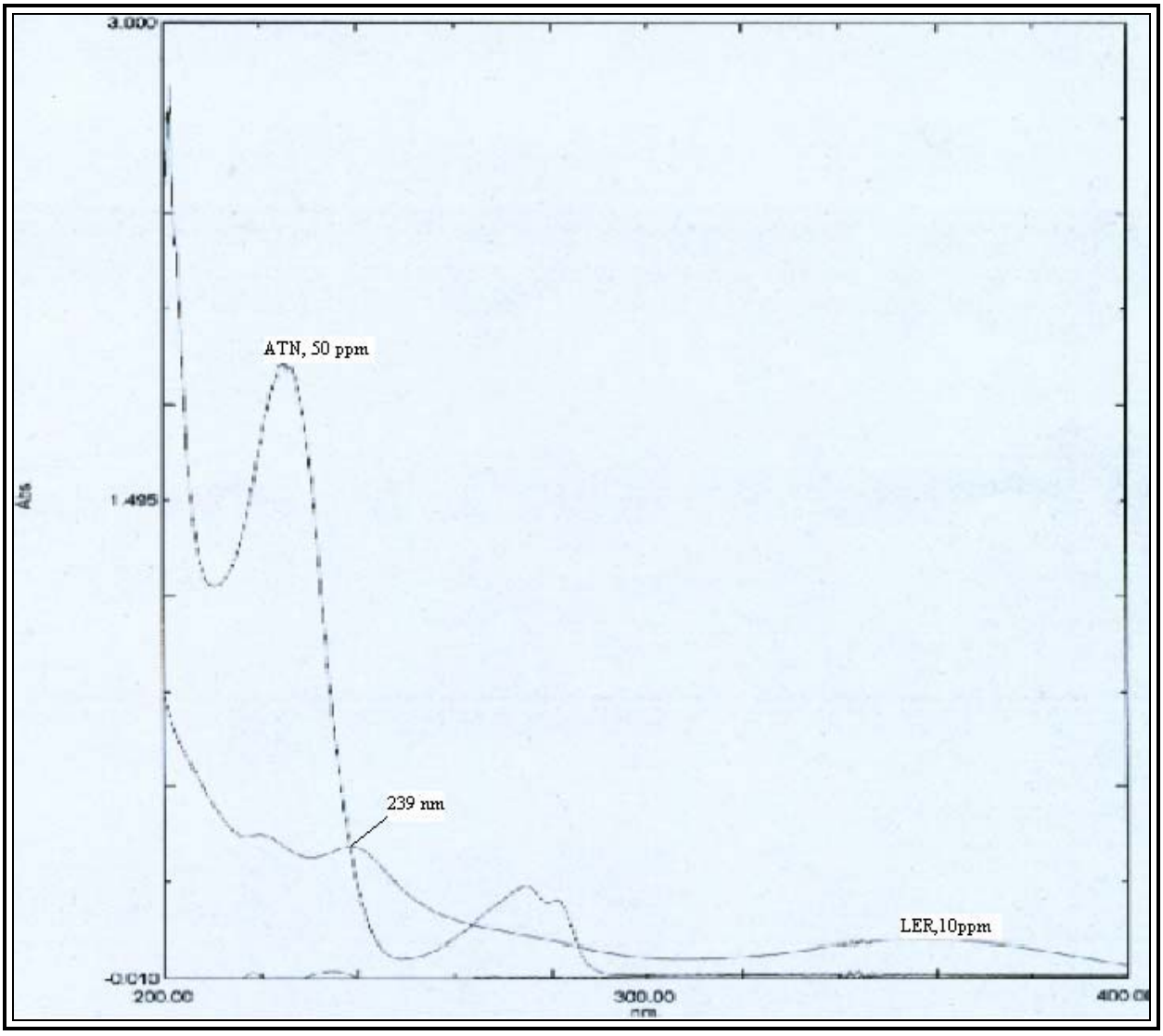


Figure 6.3: Overlay spectra of Lercanidipine hydrochloride (10ppm) and Atenolol (50ppm) in Diluent

1. Chromatographic conditions:

- Instrument : Waters 2695-Separation module
- Mobile Phase : 0.1% OPA: Acetonitrile (70: 30)
- Column : Inertsil ODS 3V (250*4.6 mm, 5 µ)
- Flow Rate : 1 ml/min
- Wavelength : 239 nm
- Column oven Temp : 25°C
- Injection Volume : 20µL
- Diluent : 0.1% OPA: Acetonitrile (70: 30)
- Run time: 15 min (Isocratic)

Chromatogram: -

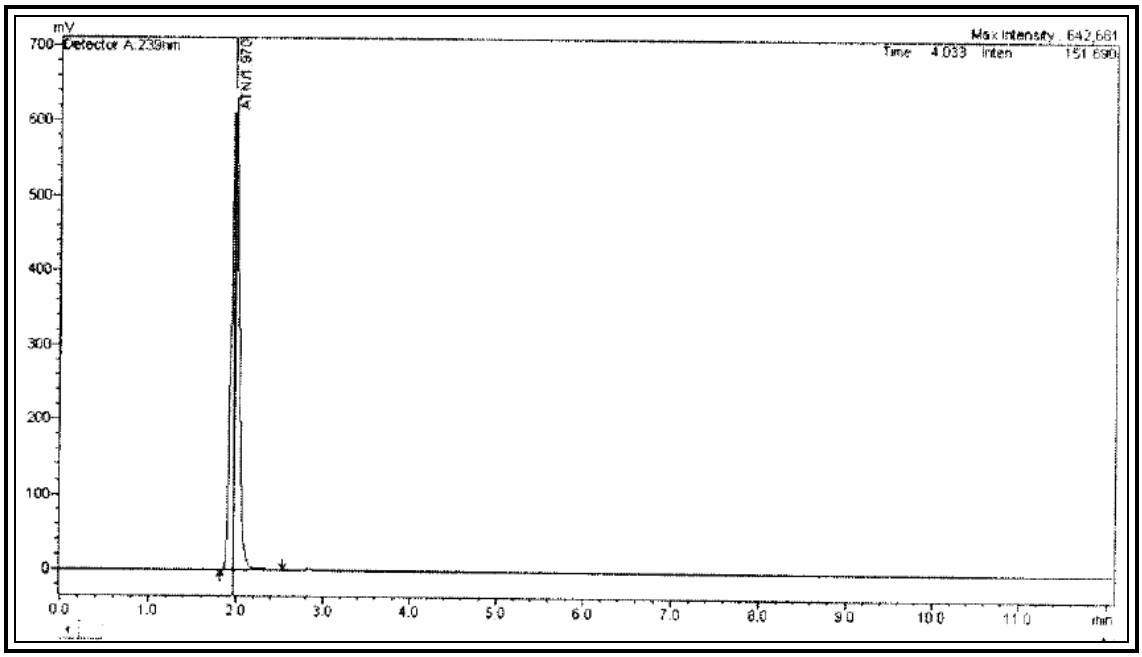


Figure-6.4: Chromatograms of standard preparation of LER and ATN (Concentration 10 ppm and 50 ppm respectively, Mobile Phase: Mixture of 0.1% OPA: acetonitrile (70: 30), Flow Rate: 1 ml/min, WL: 239 nm)

Observation: - Peak of Lercanidipine hydrochloride was not observed, hence the present method need to be optimized.

2. Chromatographic conditions:

- Instrument : Waters 2695-Separation module
- Mobile Phase : 0.1% OPA: Acetonitrile (50:50)
- Column : Inertsil ODS 3V (250*4.6 mm, 5 µ)
- Flow Rate : 1 ml/min
- Wavelength : 239 nm
- Column oven Temp : 25°C
- Injection Volume : 20µL
- Diluent : 0.1% OPA: Acetonitrile (50:50)
- Run time : 20 min (Isocratic)

Chromatogram: -

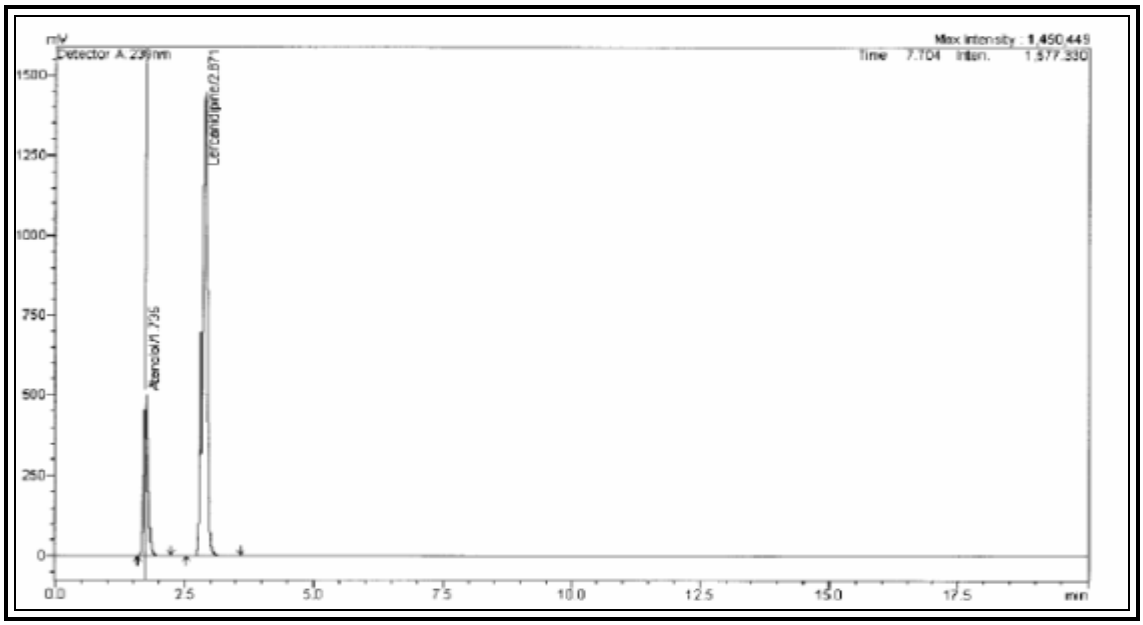


Figure 6.5: Chromatograms of standard preparation of LER and ATN (Concentration 10 ppm and 50 ppm respectively, Mobile Phase: Mixture of 0.1% OPA: Acetonitrile (50: 50), Flow Rate: 1 ml/min, WL: 239 nm)

Observation: - Peaks of both Lercanidipine hydrochloride and Atenolol were observed, but the resolution between 2 peaks was too low, so more separation between 2 peaks should be desired, hence the present method need to be optimized.

3. Chromatographic conditions:

- Instrument : Waters 2695-Separation module
- Column : Inertsil ODS 3V (250*4.6 mm, 5 µ)
- Flow Rate : 0.5 ml/min
- Injection volume : 20 µL
- Column temperature : 25°C
- Sample cooler Temperature : 20°C
- Detection : 239 nm
- Mobile Phase : 0.1% OPA: Acetonitrile (50: 50)
- Diluent : 0.1% OPA: Acetonitrile (50:50)
- Blank prep. : Diluent
- Run time : 20 minutes (Isocratic)

Chromatogram: -

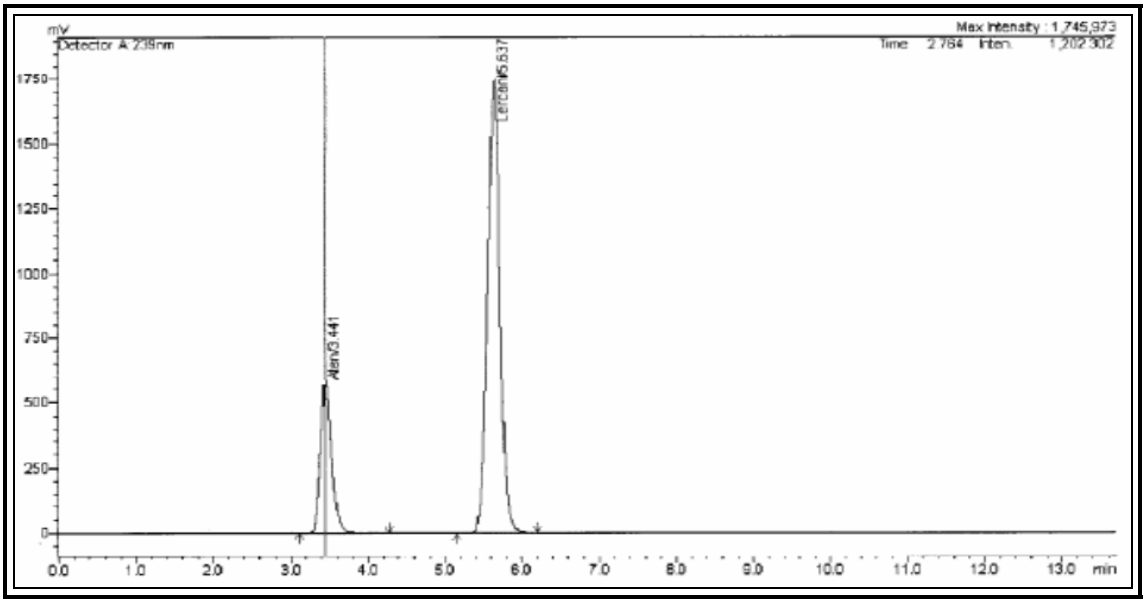


Figure 6.6: Chromatograms of standard preparation of LER and ATN (Concentration 10 ppm and 50 ppm respectively, Mobile Phase: Mixture of 0.1% OPA: Acetonitrile (50: 50), Flow Rate: 0.5 ml/min, WL: 239 nm)

Observation: - Peaks of both Lercanidipine hydrochloride and Atenolol were observed, the resolution between 2 peaks was also optimum, but as I have to develop stability indicating

method, it was thought that 2 peaks should be more separated, so that I can achieve better separation between generated degradation products, hence the present method need to be optimized.

4. Optimized Chromatographic conditions:

- Instrument : Waters 2695-Separation module
- Column : Inertsil ODS 3V (250*4.6 mm, 5 µ)
- Flow Rate : 0.5 ml/min
- Injection volume : 20 µL
- Column temperature : 25°C
- Sample cooler Temperature : 20°C
- Detection : 239 nm
- Mobile Phase : 0.1% OPA: Acetonitrile (55: 45)
- Diluent : 0.1% OPA: Acetonitrile (55: 45)
- Blank prep. : Diluent
- Run time : 20 minutes (Isocratic)

Chromatogram: -

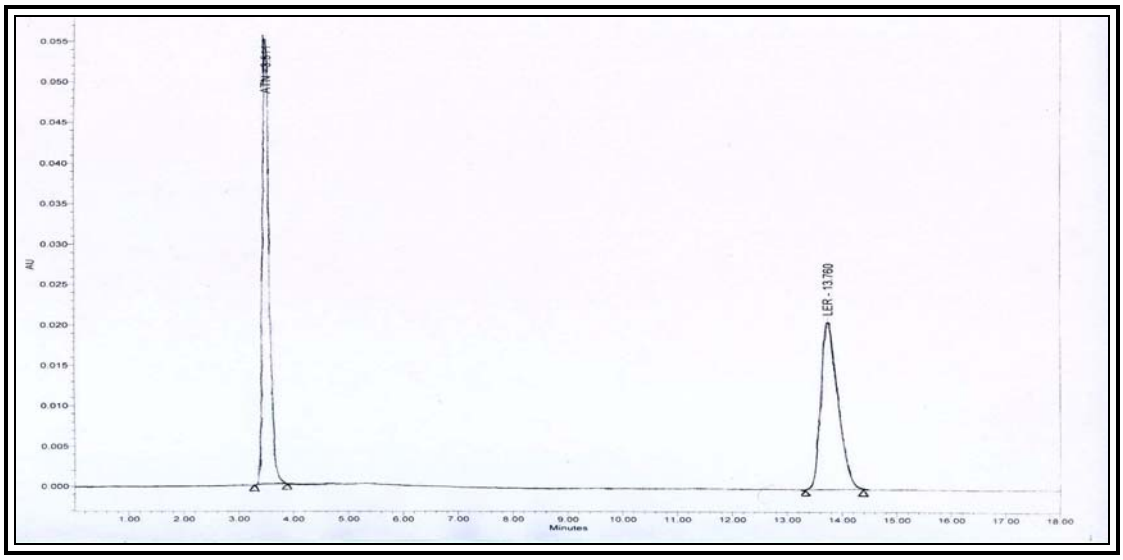


Figure 6.7: Chromatograms of standard preparation of LER and ATN (Concentration 10 ppm and 50 ppm respectively, Mobile Phase: Mixture of 0.1% OPA: Acetonitrile (55: 45), pH 2.5 adjusted with OPA, Flow Rate: 0.5 ml/min, WL: 239 nm)

Observation: - Separation between both the peaks of Lercanidipine hydrochloride and Atenolol was optimum; hence the present method was selected as optimized method for development of Stability indicating assay method. As per USP run time in stability indicating assay method should be 3 times to the last generated peak, so run time was optimized as 45 mins for present study.

6.2.2 System suitability:

Injection	Theoretical		Area		Tailing factor		Retention time	
Drug	**LER**	**ATN**	**LER**	**ATN**	**LER**	**ATN**	**LER**	**ATN**
1	8976.3	3828.3	451857	476467	1.288	1.28	13.907	3.6399
2	8887.6	3798.0	455661	479364	1.283	1.279	13.981	3.653
3	8905.9	3808.6	454474	478255	1.29	1.280	13.965	3.641
4	8878.7	3768.9	453867	477756	1.281	1.283	13.945	3.646
5	8826.1	3805.7	454796	480102	1.283	1.283	13.936	3.643
Mean	**8894.92**	**3801.9**	**3801.9**	**478388.8**	**1.285**	**1.281**	**13.96**	**3.4**
Std.dev.	**54.30**	**21.56**	**1426.67**	**1414.012**	**0.003808**	**0.0018**	**0.0283**	**0.0046**
% RSD	**0.61**	**0.56**	**0.31**	**0.29**	**0.29**	**0.14**	**0.202**	**0.11**

6.2.3 Specificity:

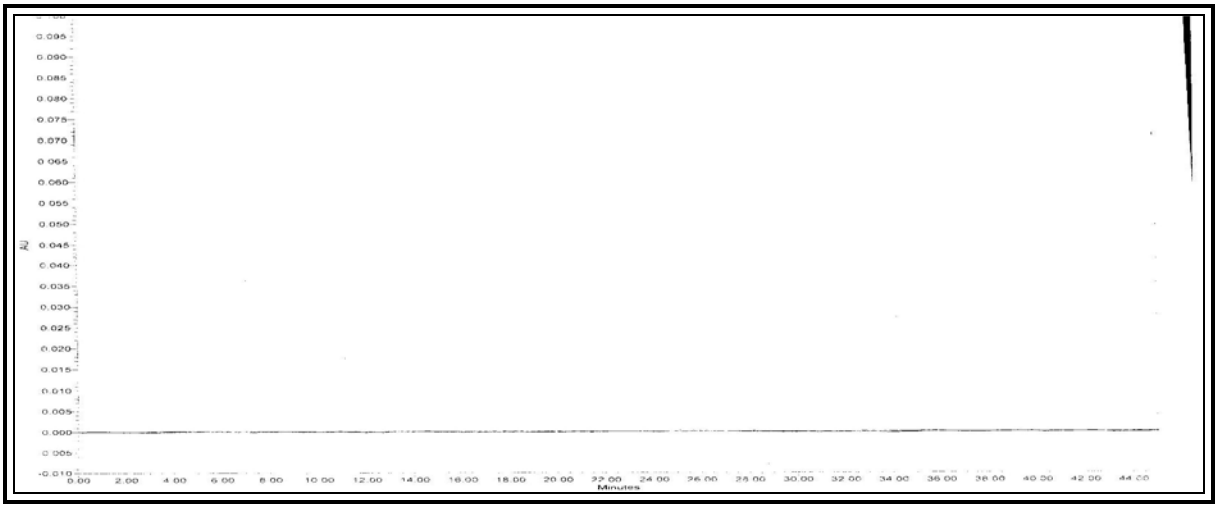


Figure 6.8: Chromatograms of blank preparation

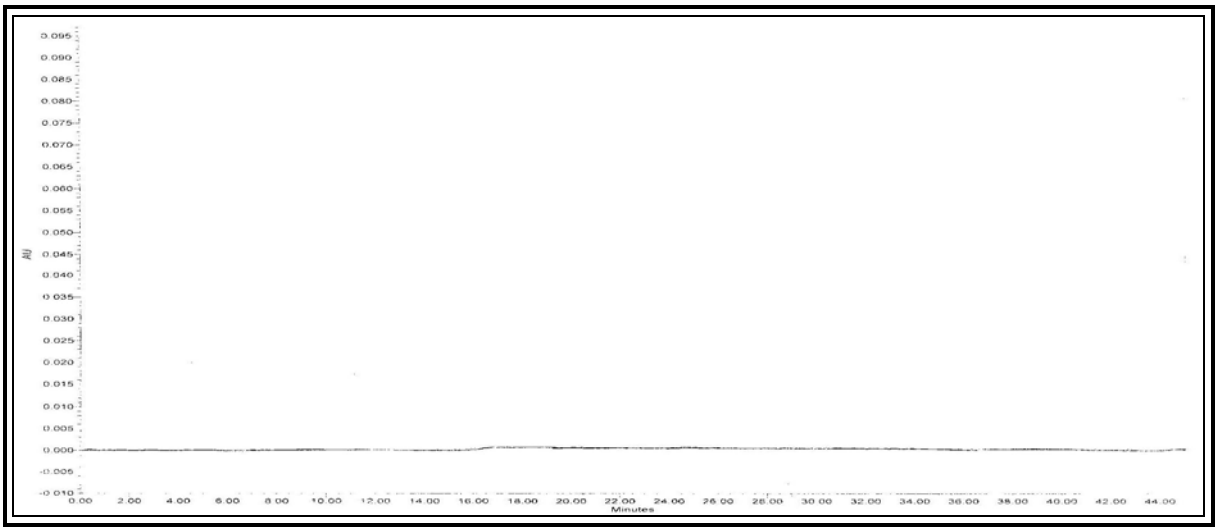


Figure 6.9: Chromatograms of placebo preparation

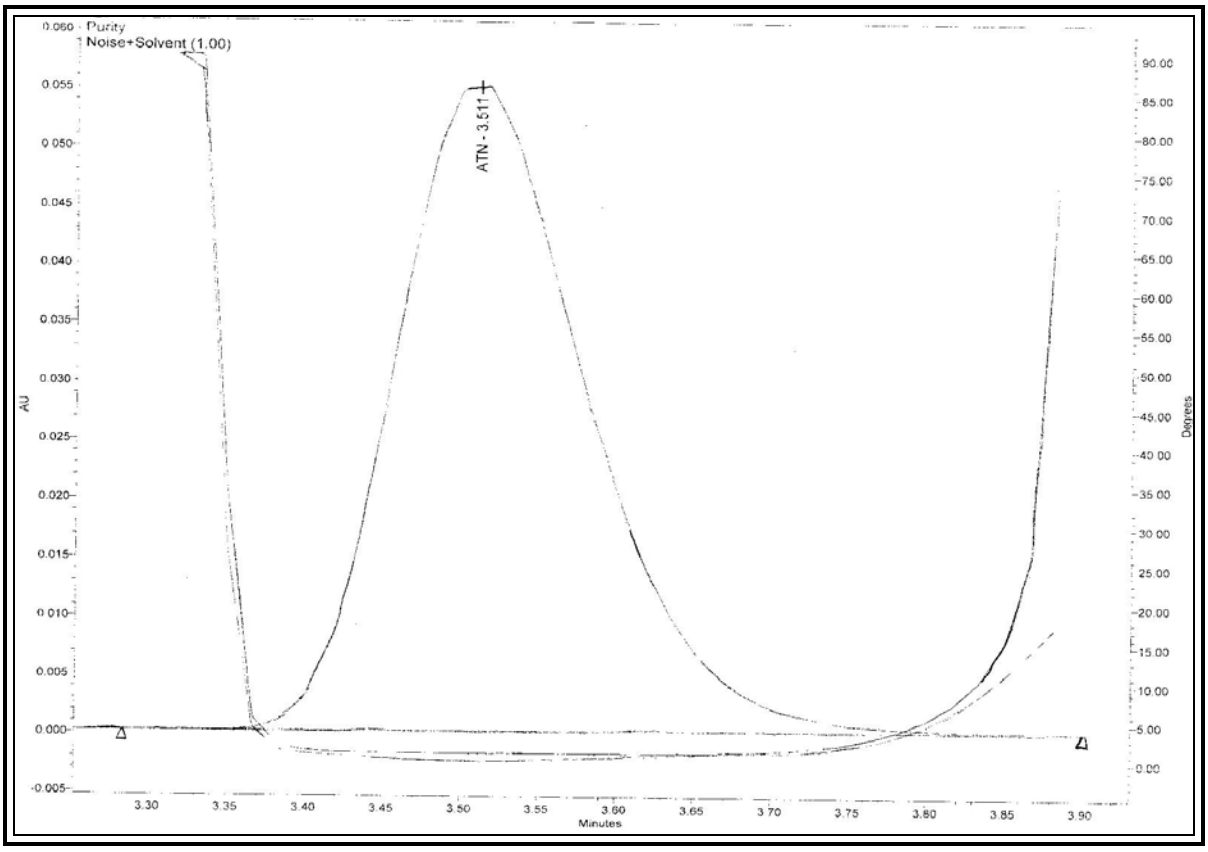


Figure 6.10: Peak purity plot of Atenolol

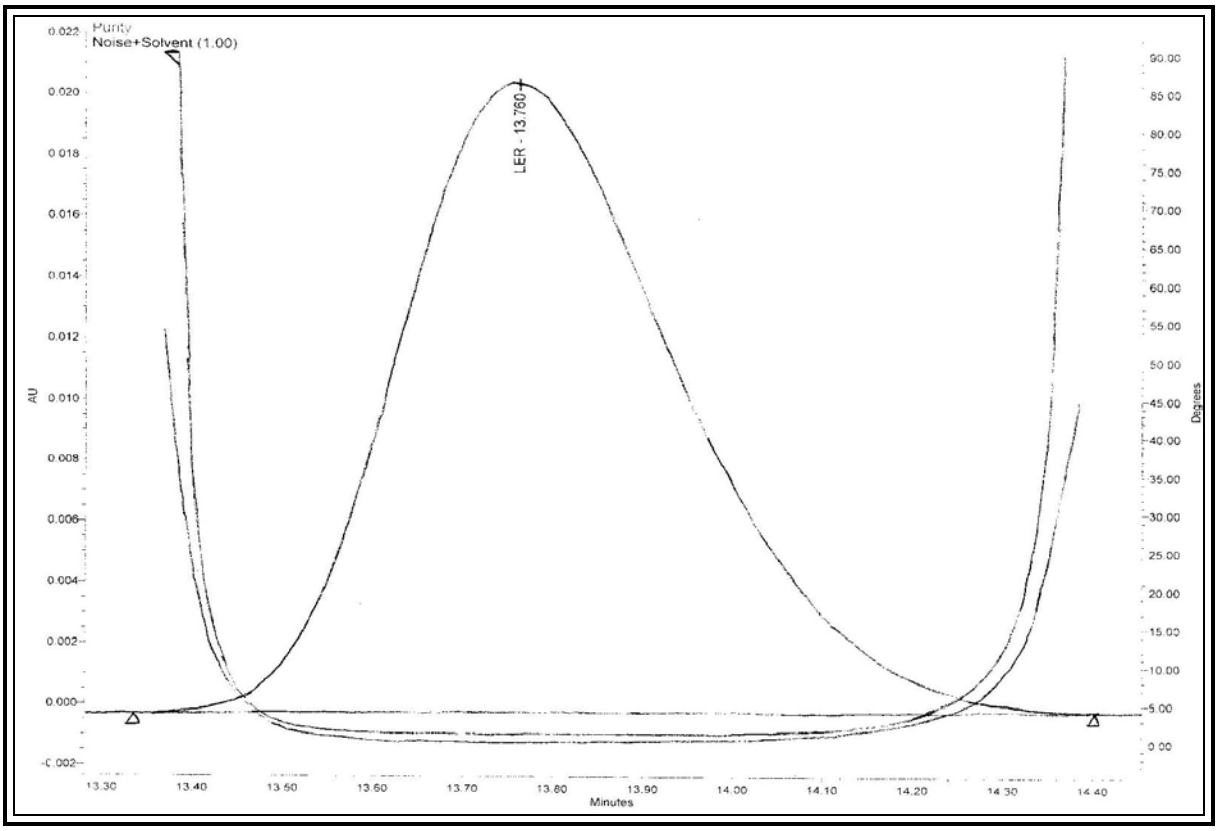


Figure 6.11: Peak purity plot of Lercanidipine hydrochloride

Observation:

N	DRUG	PURITY ANGEL	PURITY THRESHOLD	PURITY CRITERIA
1	**ATN**	0.294	1.025	Pass
2	**LER**	0.223	1.145	Pass

Conclusion: No any interfering peak from blank or placebo at the RT of Lercanidipine hydrochloride and Atenolol was observed and purity angle for both the peaks of Lercanidipine hydrochloride and Atenolol was less than purity threshold, hence both the peaks were concluded as pure. So the developed method is specific for estimation of both Lercanidipine hydrochloride and Atenolol.

6.2.4 Forced degradation study:

6.2.4.1 *Acidic degradation:*

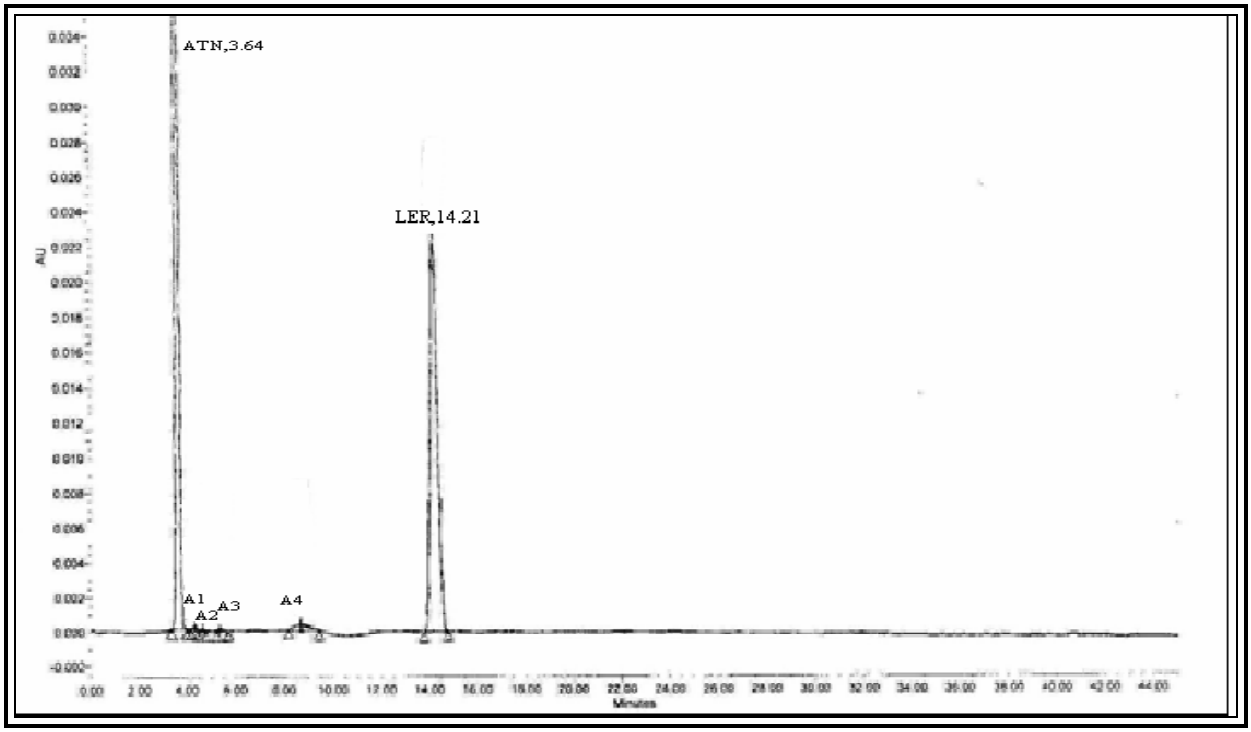


Figure 6.12: Chromatograms of acid degraded API sample of LER and ATN (Concentration 10 ppm and 50 ppm respectively, 1N HCL, 80°C for 5 hours)

	Name	R.T.	Purity Angel	Purity Threshold	Area (µV*sec)	Purity criteria	USP Resolution	USP Tailing	USP Plate-count
1	ATN	3.64	1.030	1.296	468330	Pass	-	1.1	3275
2	DEG_ATN_1	4.286	12.038	17.331	951	Pass	2.1	1.2	7683
3	DEG_ATN_2	4.678	-	-	300	-	1.8	1.2	14579
4	DEG_ATN_3	5.360	21.811	22.286	953	Pass	2.2	2.2	3915
5	DEG_ATN_4	8.690	7.299	7.834	12563	Pass	9.7	1.2	6541
6	LER	14.21	0.251	1.141	458109	Pass	17.3	1.2	10633

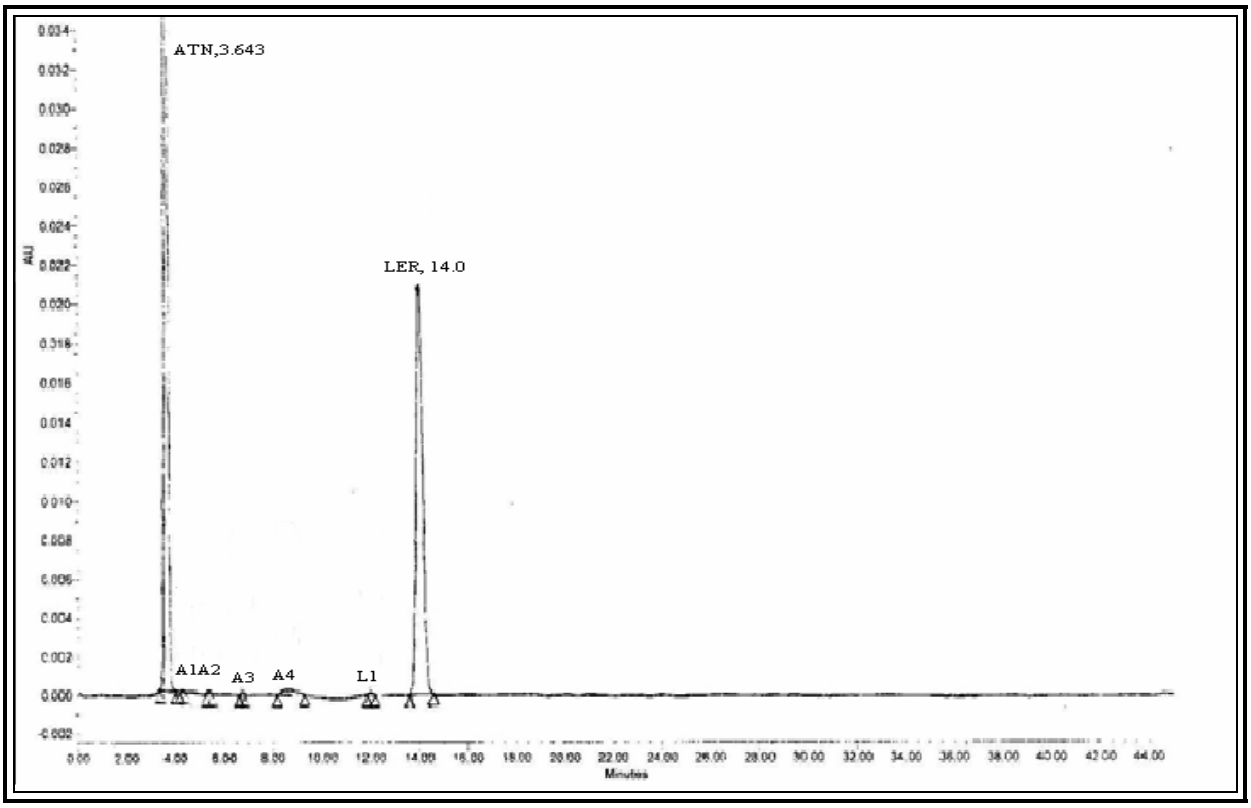


Figure 6.13: Chromatograms of acid degraded tablet sample of LER and ATN (Concentration 10 ppm and 50 ppm respectively, 1N HCL, 80°C for 5 hours)

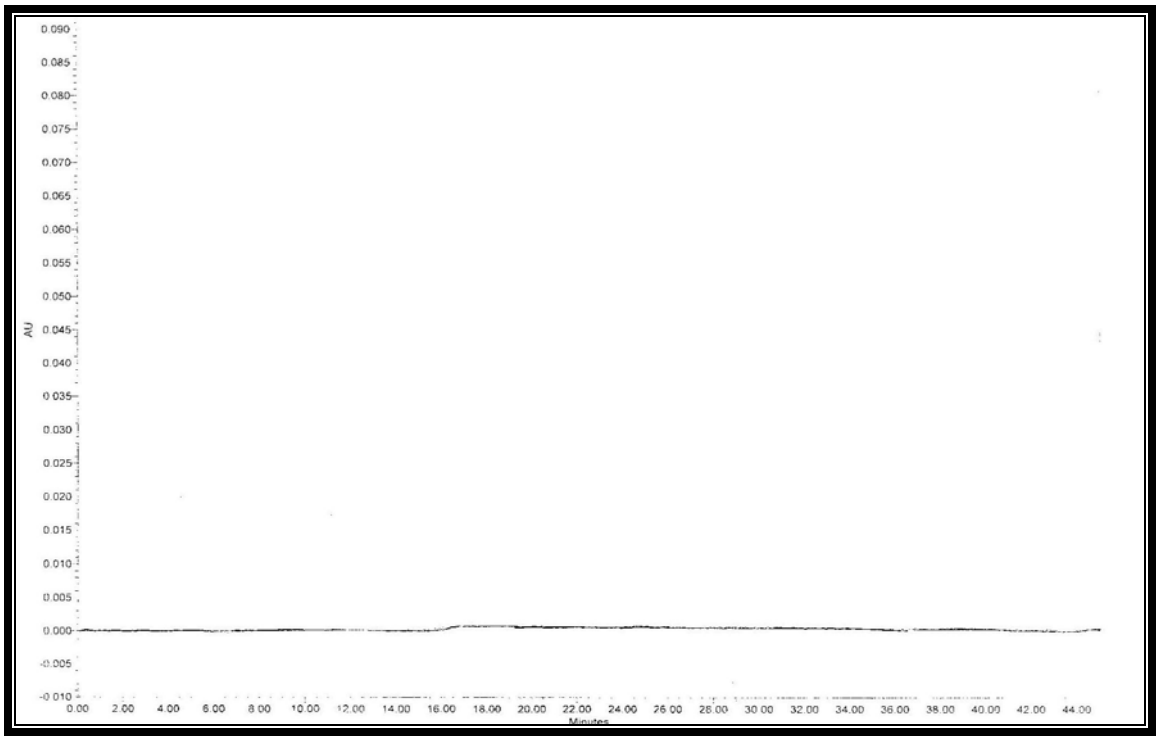


Figure 6.14: Chromatograms of acid degraded placebo sample of LER and ATN (Concentration 10 ppm and 50 ppm respectively, 1N HCL, 80°C for 5 hours)

Observation:

- ✓ Atenolol amongst both the drugs in API sample gets partially degraded (5% degradation of ATN) in strongly acidic conditions over a period of time, on heating at 80 °C in 1 M HCl (5 h), the height of the Atenolol peak decreased slightly, while no any significant change observed in peak height of Lercanidipine hydrochloride.
- ✓ In tablet sample 7% degradation of Atenolol, while 2% degradation of Lercanidipine hydrochloride was observed.
- ✓ In placebo sample, no any significant peak was observed.

Conclusion:

This study indicates that both the drugs are practically acid labile.

6.2.4.2 *Alkali degradation:*

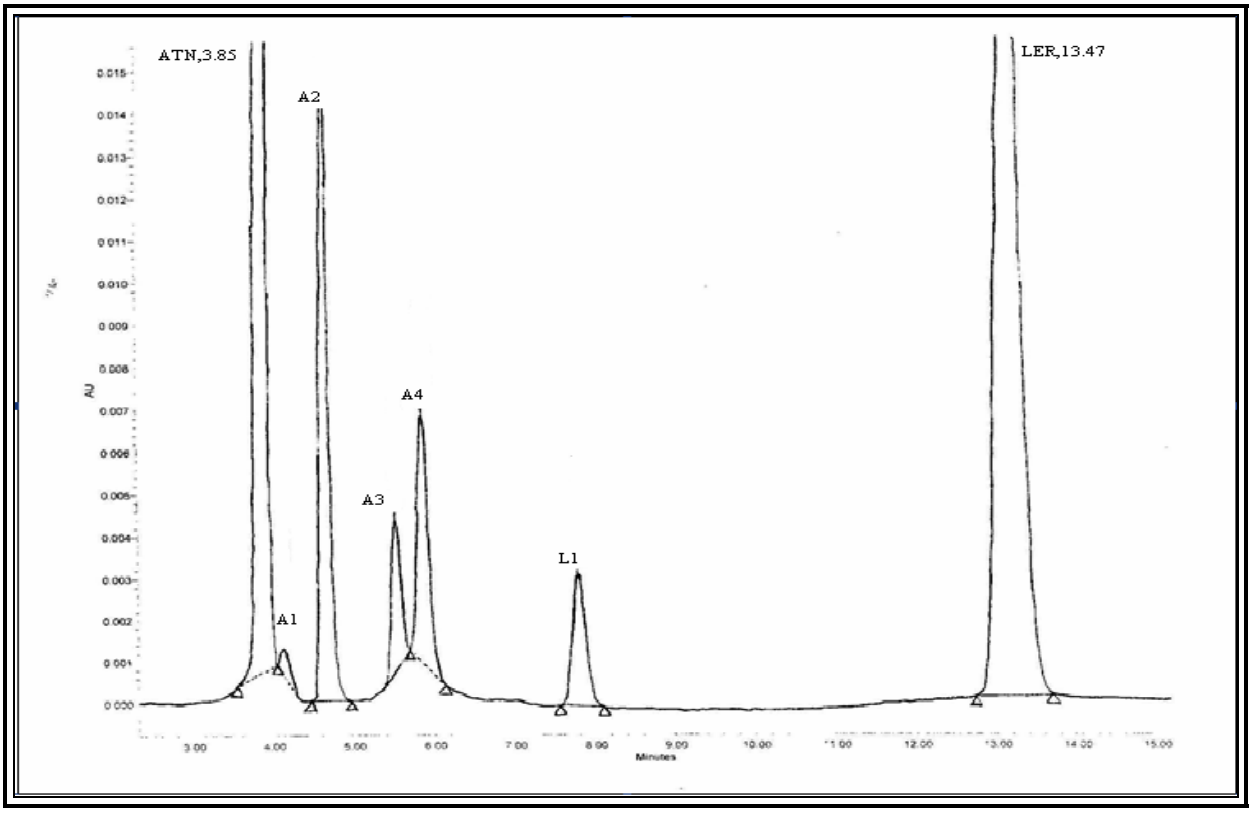


Figure 6.15:- Chromatograms of alkali degraded API sample of LER and ATN (Concentration 10 ppm and 50 ppm respectively, 1N HCL, R.T. for 6 hours)

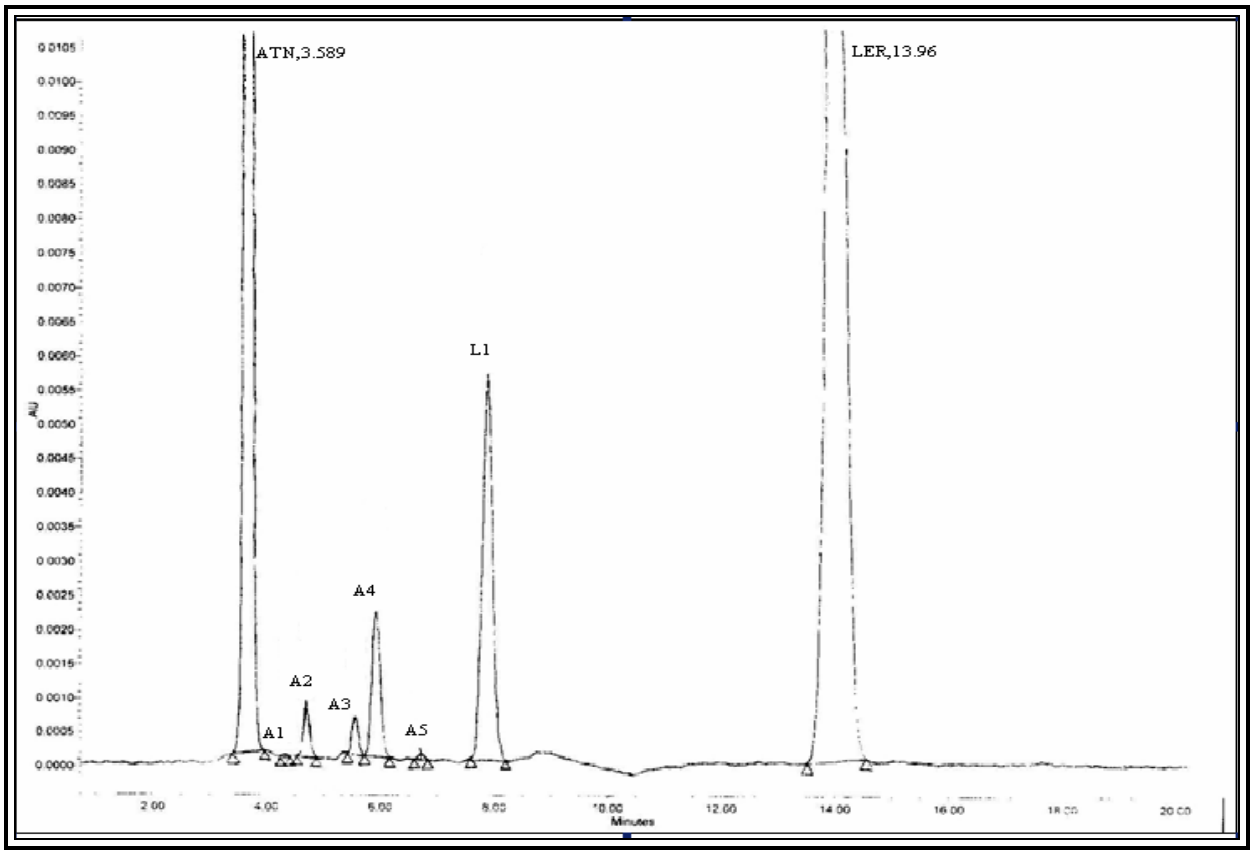


Figure 6.16:- Chromatograms of alkali degraded tablet sample of LER and ATN (Concentration 10 ppm and 50 ppm respectively, 1N NaOH, R.T. for 6 hours)

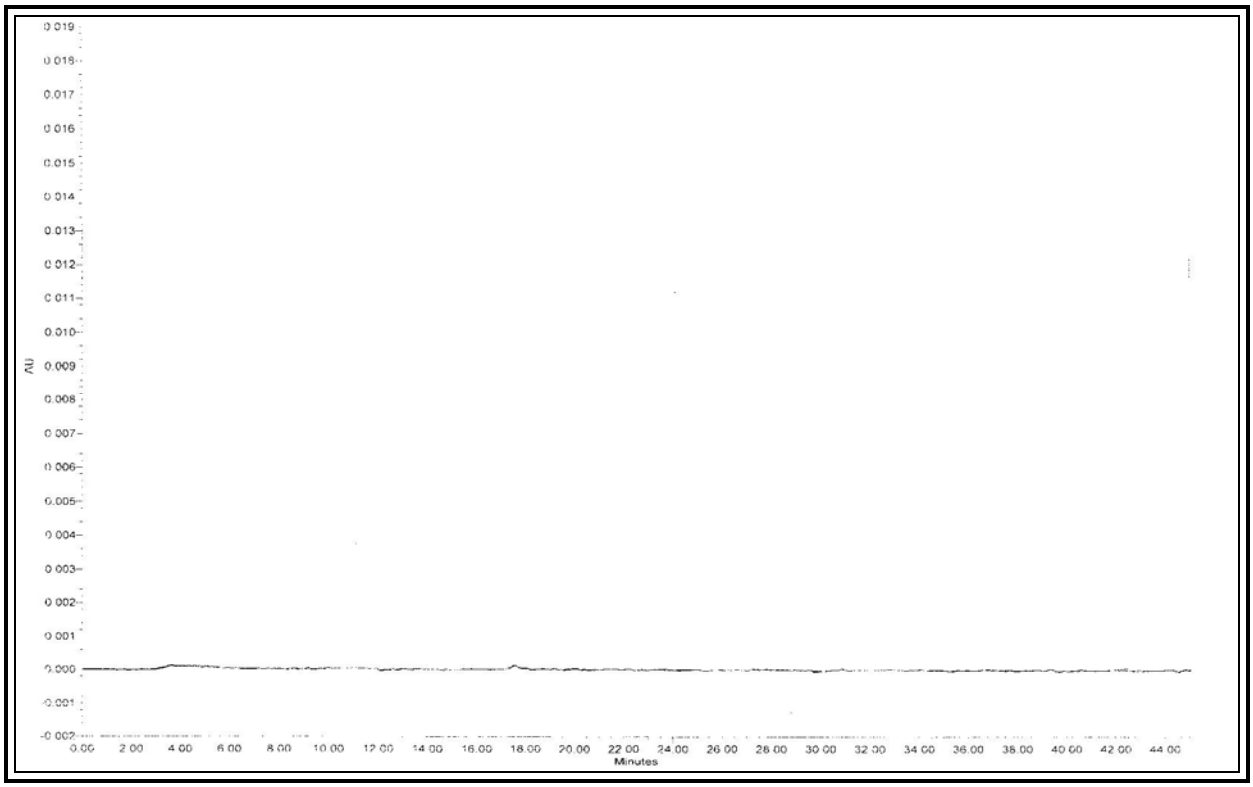


Figure 6.17: Chromatograms of alkali degraded placebo sample of LER and ATN (Concentration 10 ppm and 50 ppm respectively, 1N NaOH, R.T. for 6 hours)

Observation:

- ✓ In API sample, 25% degradation of Atenolol, while 5 % degradation of Lercanidipine hydrochloride was observed in 1 M NaOH (6 h) at room temperature.
- ✓ In tablet sample, 16% degradation of Atenolol, while 16 % degradation of Lercanidipine hydrochloride was observed.
- ✓ Degradation of Lercanidipine hydrochloride was increased while degradation of Atenolol was decreased in tablet sample as compared to API indicates drug excipients interaction.
- ✓ In placebo sample, no any significant peak was observed.

Conclusion:

This study indicates that both the drugs are practically alkali labile.

6.2.4.3 *Neutral degradation:*

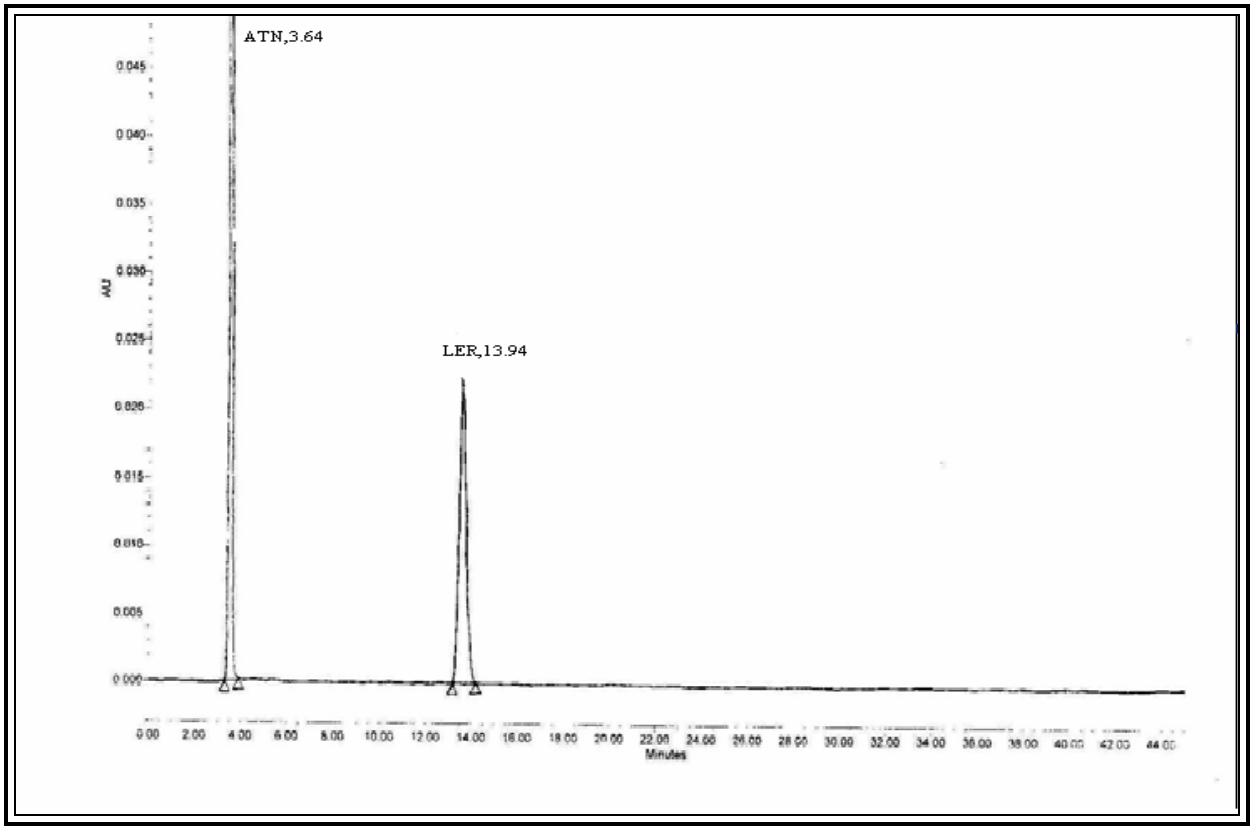


Figure 6.18:- Chromatograms of neutral degraded tablet sample of LER and ATN (Concentration 10 ppm and 50 ppm respectively, Milli-Q water, 80 °C *for 8 hours)*

	Name	R.T.	Purity Angel	Purity Threshold	Area (µV*sec)	Purity criteria	USP Resolution	USP Tailing	USP Plate-count
1	ATN	3.64	1.030	1.296	499876	Pass	-	1.1	3875
2	LER	13.94	0.251	1.141	458109	Pass	24.6	1.2	8868

Observation:

No any significant degradation occurred in both API or tablet sample in neutral condition over a period of time at 80 °C for 8 hours in Milli-Q water.

Conclusion:

This study indicates that both the drugs are practically stable at neutral condition.

6.2.4.4 *Peroxide degradation:*

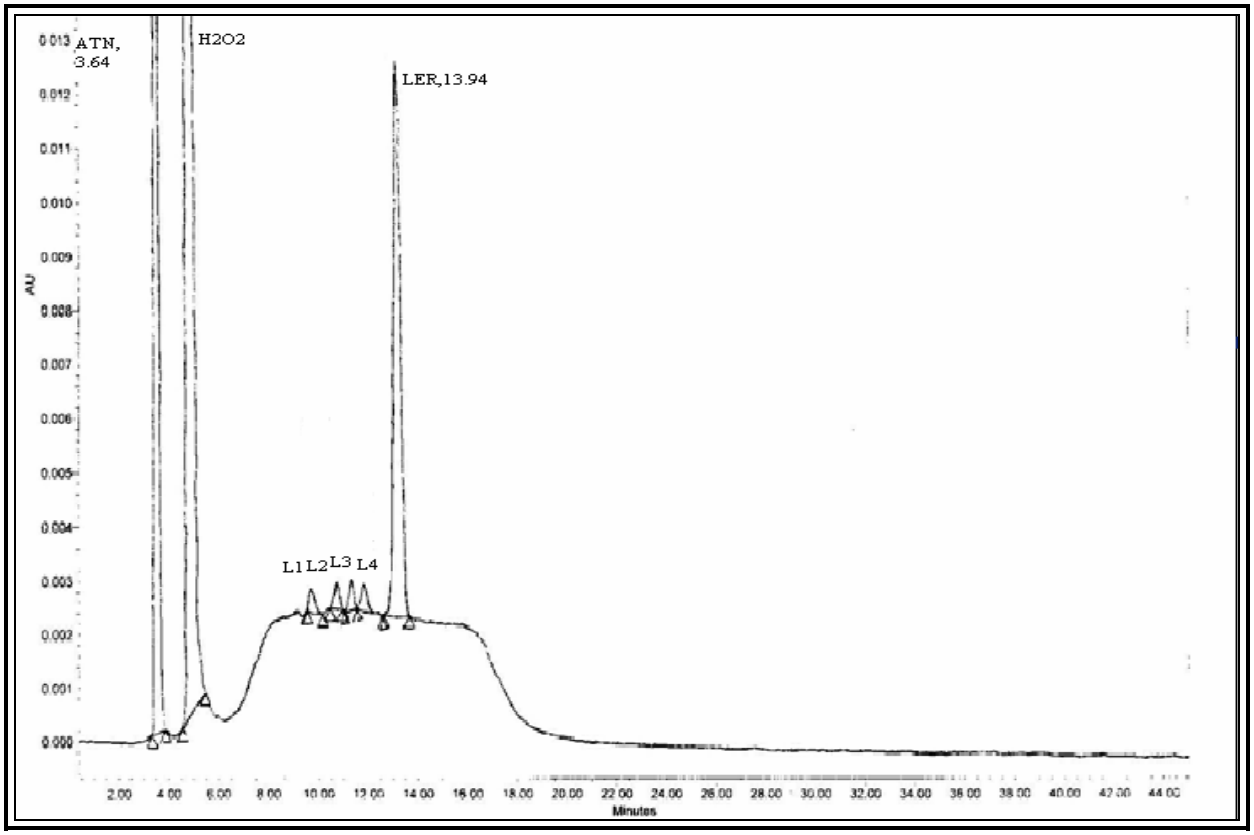


Figure- 6.19:- Chromatograms of peroxide degraded API sample of LER and ATN (Concentration 10 ppm and 50 ppm respectively, 3% H_2O_2*, R.T. for 2 hours)*

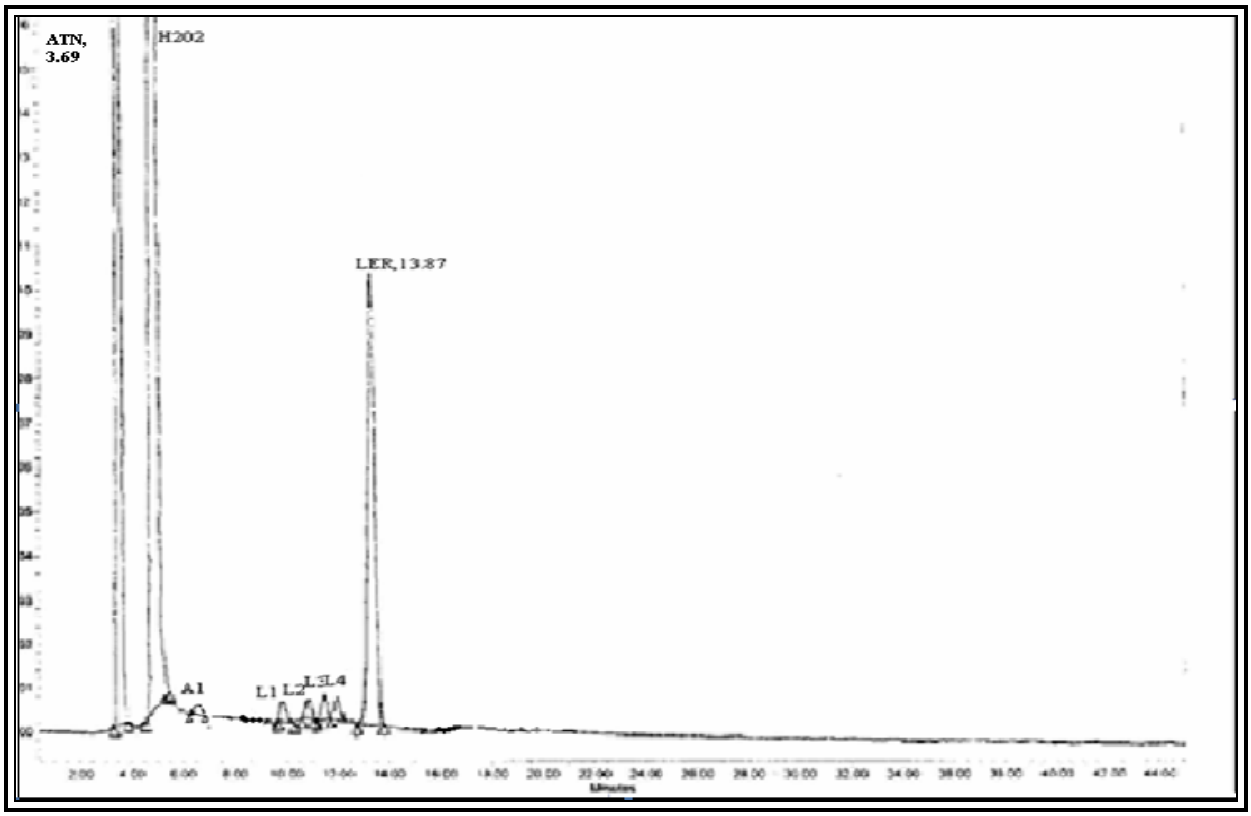


Figure- 6.20:- Chromatograms of peroxide degraded tablet sample of LER and ATN (Concentration 10 ppm and 50 ppm respectively, 3% H_2O_2*, R.T. for 2 hours)*

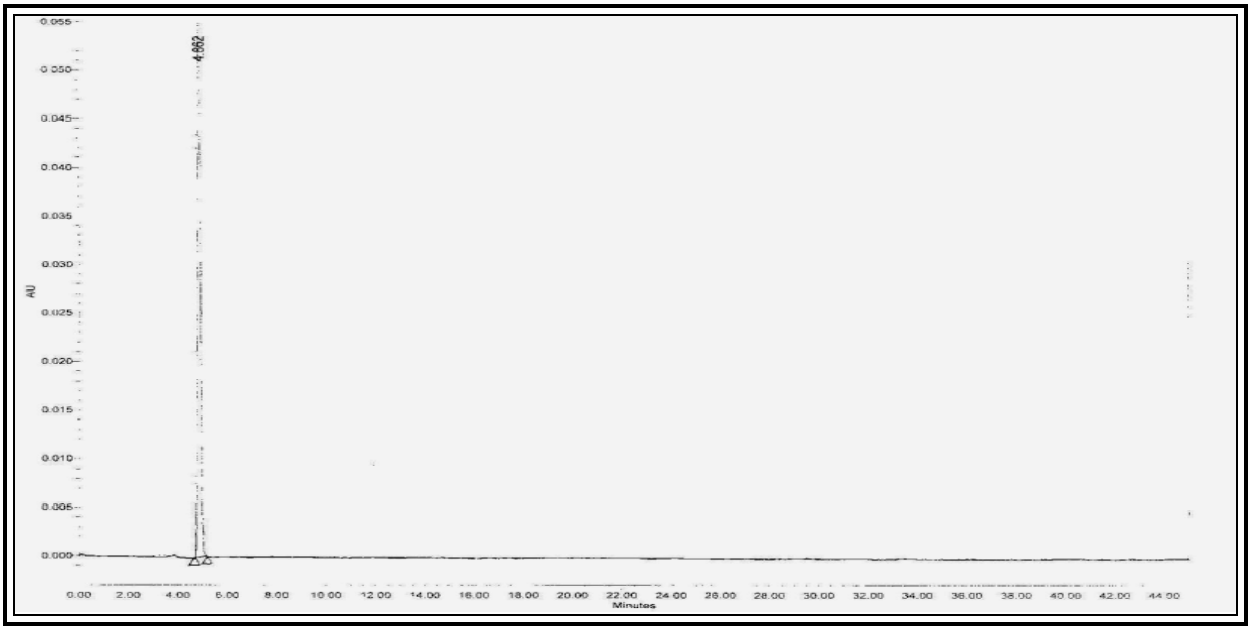


Figure- 6.21:- Chromatograms of peroxide degraded Placebo sample of LER and ATN (Concentration 10 ppm and 50 ppm respectively, 3% H_2O_2*, R.T. for 2 hours)*

Observation:

- ✓ In API sample, 2% degradation of Atenolol, while 13 % degradation of Lercanidipine hydrochloride was observed in 3% H_2O_2 (*2 h)* at room temperature.
- ✓ In tablet sample, 7% degradation of Atenolol, while 15 % degradation of Lercanidipine hydrochloride was observed in 3% H_2O_2 (*2 h)* at room temperature.
  (As time of exposure of 3% H_2O_2 was increasing, significant degradation of Atenolol was also observed, i.e. 53% degradation of Atenolol and 93% degradation of Lercanidipine hydrochloride was observed when exposed the tablet sample to 3% H_2O_2 for 22 hours at room temperature.)
- ✓ In placebo sample, no any significant peak was observed.

Conclusion:

This study indicates that both the drugs are practically labile to oxidative stress.

6.2.4.5 *Thermal degradation:*

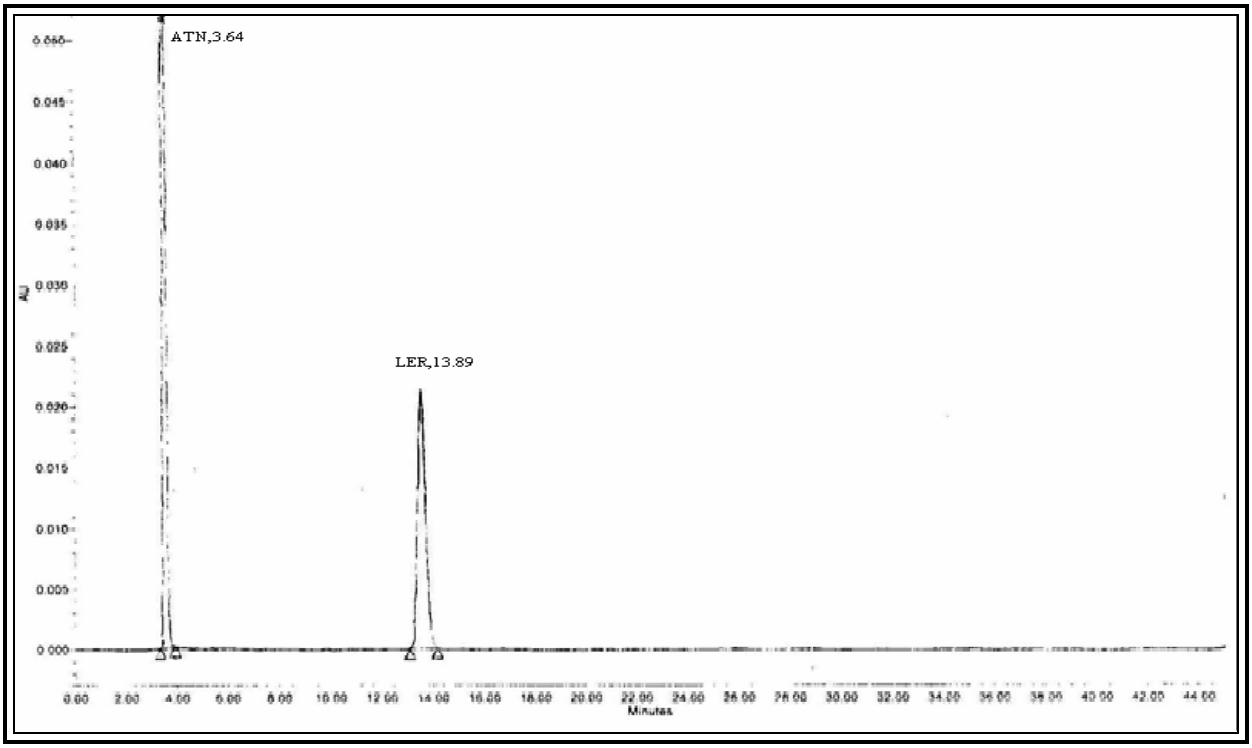


Figure 6.22:- Chromatograms of thermal degraded tablet sample of LER and ATN (Concentration 10 ppm and 50 ppm respectively, 100 °C *for 24 hours)*

	Name	R.T.	Purity Angel	Purity Threshold	Area (µV*sec)	Purity criteria	USP Resolution	USP Tailing	USP Plate-count
1	ATN	3.73	1.16	1.24	499989	Pass	-	1.3	3804
2	LER	13.89	0.235	1.85	460986	Pass	24.3	1.2	8889

Observation:

No any significant degradation occurred in both API as well as tablet sample in thermal condition over a period of time at 100°C for 24 hours in hot air oven.

Conclusion:

This study indicates that both the drugs are practically stable to thermal stress condition.

6.2.4.6 *Photolytic degradation:*

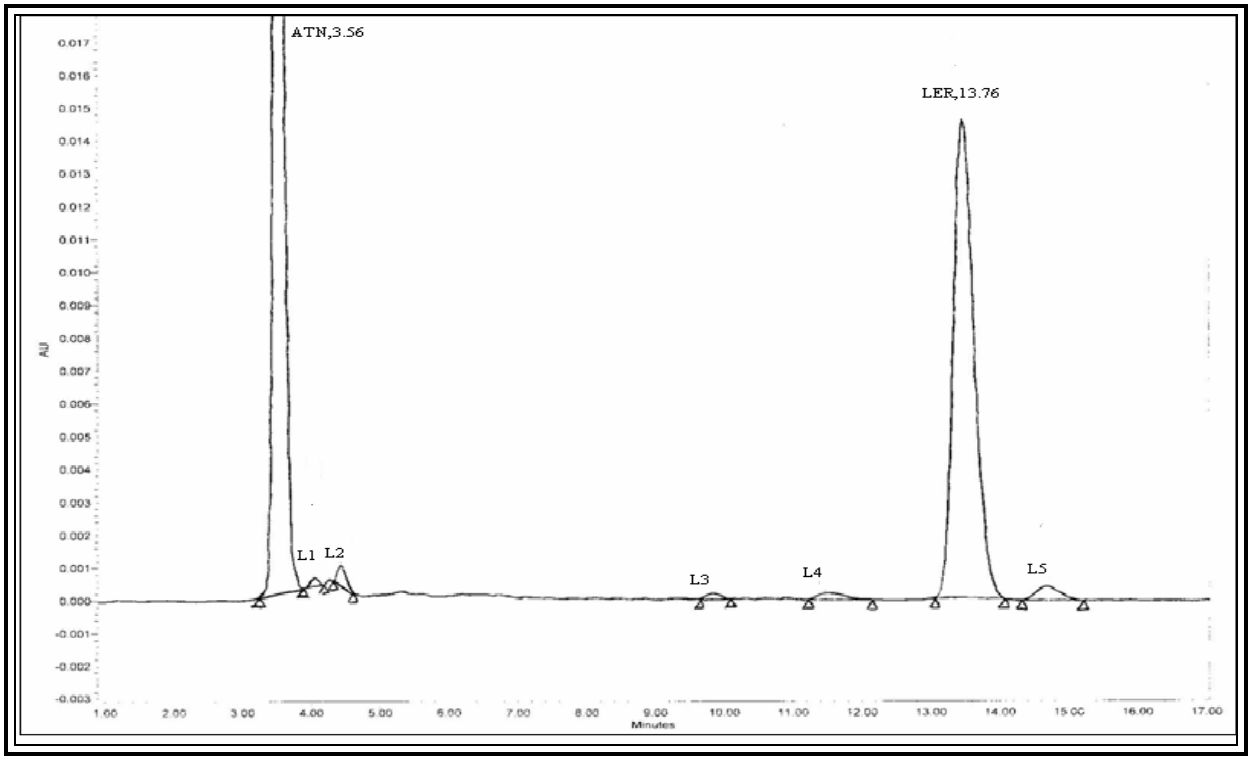


Figure 6.23:- Chromatograms of Photo-degraded tablet sample of LER and ATN (Solid state degradation, Concentration 10 mg and 50mg respectively, 1.2 million lux.hours*, R.T. for 7days)*

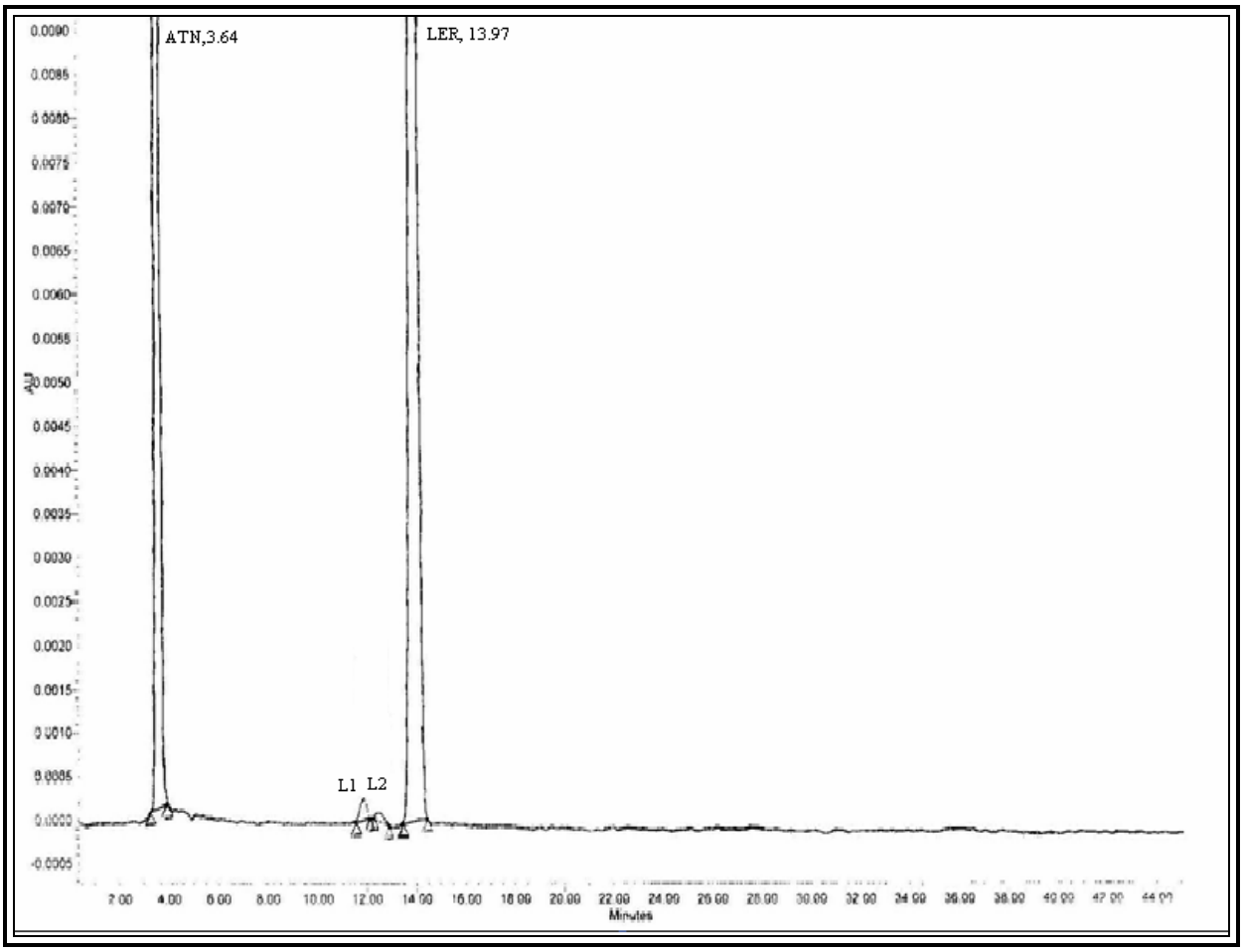


Figure 6.24:- Chromatograms of Photo-degraded API sample of LER and ATN (Liquid state degradation, Concentration 10 ppm and 50 ppm respectively, sun light, 30 min*)*

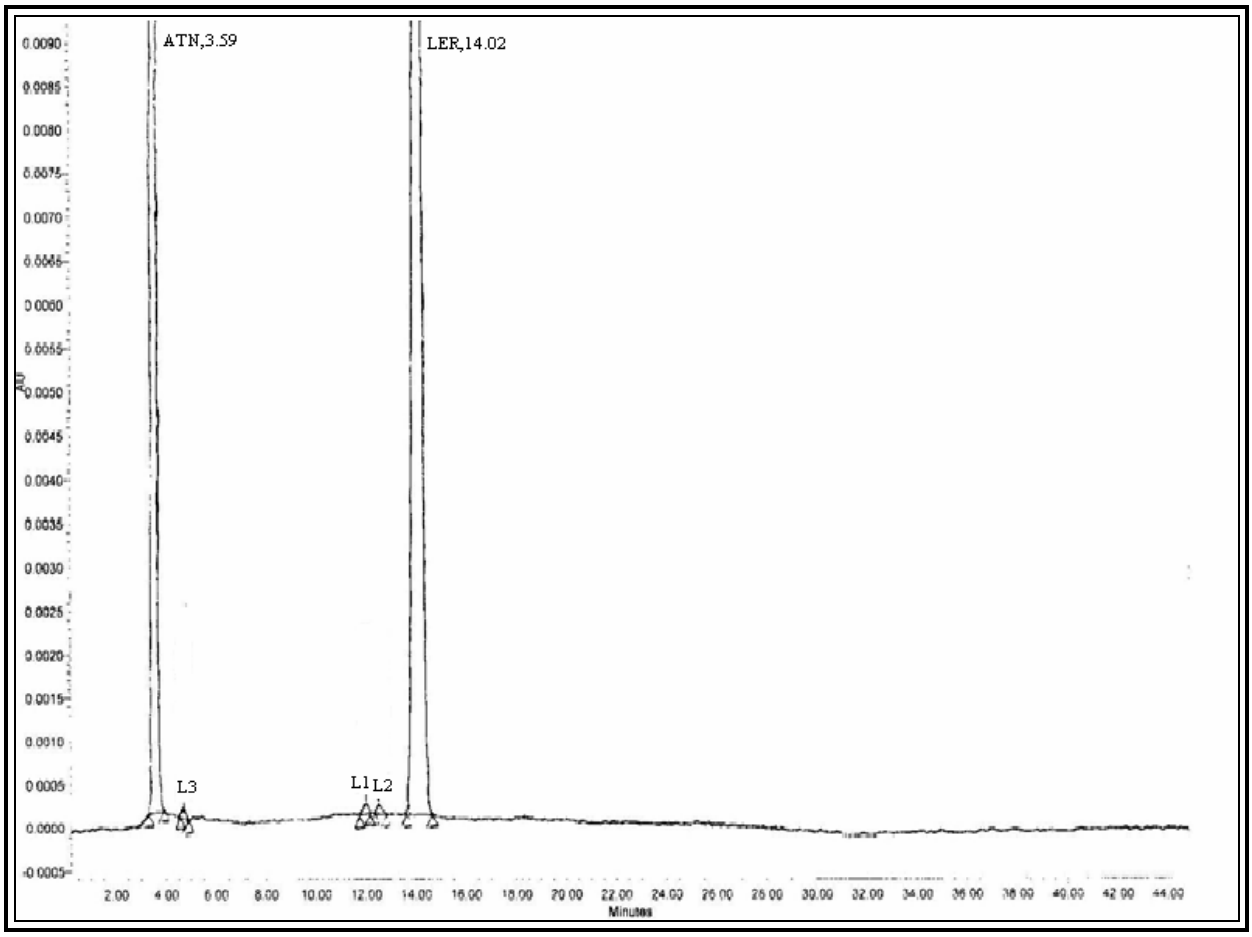


Figure 6.25:- Chromatograms of Photo-degraded tablet sample of LER and ATN

(Liquid state degradation, Concentration 10 ppm and 50 ppm respectively, sun light, 30 min*)*

Observation:

Lercanidipine hydrochloride amongst both the drugs gets degraded in photolytic conditions over a period of time. On exposing the sample in photo-stability chamber (Solid sample, 34% degradation of LER, 1.2 million lux.hours, 7 days) or direct in sun light (Liquid sample, 3% degradation in sunlight, 30 min), the height of the Lercanidipine hydrochloride peak decreased significantly, while no any significant change observed in peak height of Atenolol. It was also observed that at 11.5 R.T., Lercanidipine hydrochloride show significant degradation product and as the sample was exposed more in sun light, significant increase in the area of degradation product, while decrease in the area of Lercanidipine hydrochloride was observed.

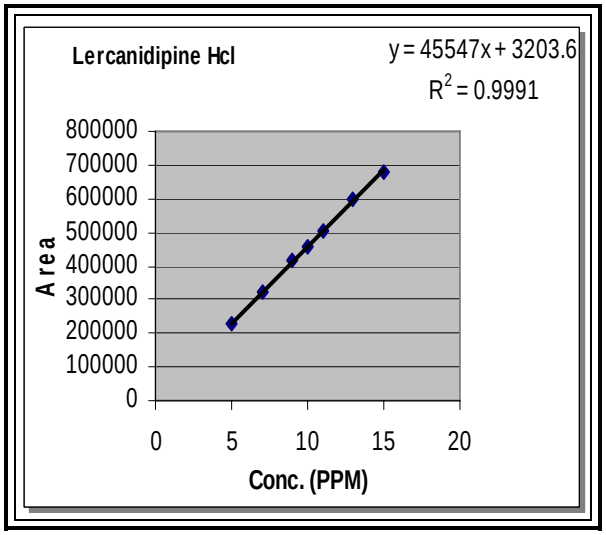
Conclusion:

This study indicates that Lercanidipine hydrochloride is photo labile, while Atenolol is photo stable.

6.2.5 Method Validation:

6.2.5.1 Linearity and Range: [N= 3]

LERCANIDIPINE HCL		
Conc.(PPM)	Area ± SD	% RSD
5	225870.3 ± 529.3	**0.23**
7	320546.7 ± 635.24	0.21
9	417734.7 ± 517.26	0.12
10	459693.7 ± 433.15	0.09
11	505759.7 ± 1412.41	0.27
13	605230.7 ± 1581.25	0.26
15	680736 ± 1338.61	0.19



ATENOLOL		
Conc.(PPM)	Area ± SD	%RSD
25	257104 ± 1170.5	0.45
35	357246 ± 1507.2	0.42
45	454831 ± 2087.19	0.45
50	497898 ± 3671.14	0.73
55	558390 ± 4989.32	0.89
65	660363 ± 1428.57	0.21
75	751752 ± 4804.66	0.63

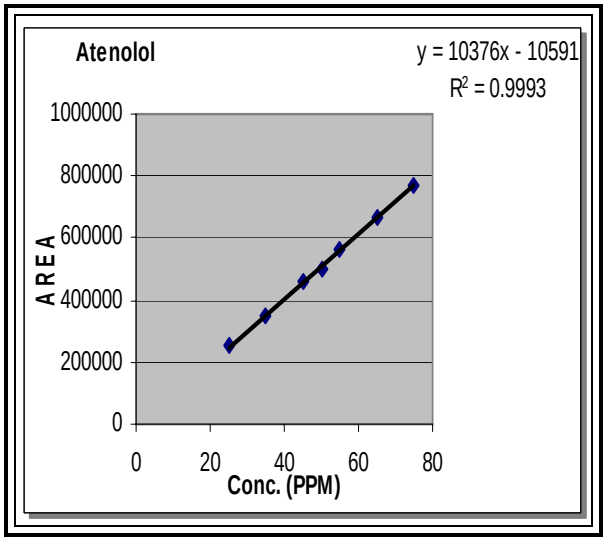


Fig 6.26: Calibration curve of Lercanidipine hydrochloride and Atenolol

Conclusion: Meets acceptance criteria.

6.2.5.2 Accuracy (Recovery):

Observation:

LERCANIDIPINE HCL (PLACEBO METHOD)				
Spiked level	**Area**	**Conc. (PPM)**	**% Recovery**	**Avg %Recovery**
50%	229855	4.976209	99.52418	
50%	234696	5.082495	101.6499	**99.82 ± 1.69**
50%	227075	4.915173	98.30347	
100%	453651	9.889727	98.89727	
100%	456421	9.950543	99.50543	**99.62 ± 0.8**
100%	460802	10.04673	100.4673	
150%	679082	14.83914	98.92761	
150%	680082	14.8611	99.07398	**99.16 ± 0.29**
150%	682926	14.92354	99.49026	

ATENOLOL (PLACEBO METHOD)				
Spiked level	**Area**	**Conc. (PPM)**	**% Recovery**	**Avg %Recovery**
50%	253690	25.47041	101.8816	
50%	253654	25.46694	101.8678	**101.8 ± 0.11**
50%	253173	25.42059	101.6823	
100%	499727	49.18254	98.36507	
100%	498965	49.1091	98.2182	**98.31 ± 0.08**
100%	499714	49.18128	98.36257	
150%	770208	75.25048	100.334	
150%	761415	74.40305	99.20406	**99.37 ± 0.89**
150%	756559	73.93504	98.58006	

Conclusion: Meets acceptance criteria.

6.2.5.3 Precision:

a) Repeatability:

REPEATABILITY (TABLET SAMPLE)				
N	**LER (10 ppm)**	**% Assay Of LER**	**ATN (50 ppm)**	**% Assay Of ATN**
1	459735	100.233	507246	99.81438
2	463792	101.1238	507112	99.78855
3	452790	98.70824	504765	99.33616
4	462352	100.8076	505037	99.38859
5	453595	98.88498	505717	99.51966
6	465054	101.4008	517456	101.7824
Mean	**-**	**100.19**	**-**	**99.93**
Std. dev.	**-**	**1.150**	**-**	**0.925**
% RSD	**-**	**1.14**	**-**	**0.9**

Conclusion: Meets acceptance criteria.

b) Intraday precision:

INTRADAY PRECISION						
Drug	**ATN**			**LER**		
Conc.	**25 ppm**	**50 ppm**	**75 ppm**	**5 ppm**	**10 ppm**	**15 ppm**
10 a.m.	261557	493250	749031	235988	458936	684811
2 p.m.	262246	496104	754282	234838	460471	689074
6 p.m.	260830	505621	749168	233009	459159	685433
Mean	**261544**	**498325**	**750827**	**234611**	**459522**	**686439**
S.D	**578.142**	**5288.98**	**2443.692**	**1226.65**	**677.19**	**1880.22**
% RSD	**0.229**	**1.069**	**0.323**	**0.52**	**0.14**	**0.27**

Conclusion: Meets acceptance criteria.

b) Interday precision:

INTERDAY PRECISION						
Drug	**ATN**			**LER**		
Conc.	**25 ppm**	**50 ppm**	**75 ppm**	**5 ppm**	**10 ppm**	**15 ppm**
Day 1	262555	504166	738405	235827	460953	685601
Day 2	262536	502248	737741	236953	461095	689510
Day 3	261928	499865	732882	237561	459994	685671
Mean	**262339**	**502093**	**736342**	**236780**	**460680**	**686927**
S.D	**291.19**	**1759.29**	**2462.02**	**718.35**	**488.99**	**1826.44**
% RSD	**0.116**	**0.355**	**0.335**	**0.30**	**0.10**	**0.26**

Conclusion: Meets acceptance criteria.

6.2.5.4. Solution Stability:

SOLUTION STABILITY (API)				
Time (hrs)	**LER**		**ATN**	
	Area	**% Difference**	**Area**	**% Difference**
Initial	456753	-	498785	-
12 hrs	455987	-766	498104	-681
24 hrs	452924	-3829	496721	-2064
48 hrs	449824	-6929	494629	-4156
SOLUTION STABILITY (TABLET SAMPLE)				
Time (hrs)	**LER**		**ATN**	
	Area	**% Difference**	**Area**	**% Difference**
Initial	459735	-	507246	-
12 hrs	458876	-859	507135	-111
24 hrs	457659	-2076	504561	-2685
48 hrs	456249	-3486	499873	-7373

Conclusion: Both the drugs are practically stable at room temperature in mentioned diluents.

6.2.5.5 Limit Of Detection and Limit Of Quantitation:

DRUG	LOD (ppm)	LOQ (ppm)
LER	0.007	0.021
ATN	0.015	0.045

6.2.5.6 Robustness:

- **Change the flow rate of Mobile Phase:**

Standard repetitions	0.45 ml/min		0.5 ml/min		0.55 ml/min	
	LER	**ATN**	**LER**	**ATN**	**LER**	**ATN**
Mean[N=5]	447592	490393	454131	498388.8	447666.4	491701
% Assay	99.51	99.33	100.97	100.95	99.53	99.6
% RSD	0.91	0.21	0.63	0.59	0.46	1.70
Th. Plates	8665.6	3798.1	8894.2	3801.9	8747.74	3750.3
R.T.	13.77 ± 0.3	3.57 ± 0.7	13.68 ± 0.6	3.48 ± 0.9	13.57 ± 1.2	3.39 ± 0.3
Tailing factor	1.39	1.40	1.28	1.28	1.34	1.32
Resolution	24.67		24.5		24.3	

- **Change the Column Oven Temperature:**

Standard repetitions	20°C		25°C		30°C	
	LER	**ATN**	**LER**	**ATN**	**LER**	**ATN**
Mean[N=5]	445054.6	491773.4	454131	498388.8	446648.4	490761.6
% Assay	98.96	99.61	100.97	100.95	99.30	99.41
% RSD	1.38	0.63	0.63	0.59	1.56	1.05
Th. Plates	8673.74	3775.3	8894.2	3801.9	8597.6	3763.1
R.T.	13.76 ± 0.9	3.55 ± 0.7	13.68 ± 0.6	3.48 ± 0.9	13.61 ± 1.2	3.41 ± 0.9
Tailing factor	1.38	1.35	1.28	1.28	1.42	1.48

Resolution	24.9	24.5	23.7

- **Change in pH of mobile phase:**

Standard repetitions	**pH: 2.3**		**pH: 2.5**		**pH: 2.7**	
	LER	**ATN**	**LER**	**ATN**	**LER**	**ATN**
Mean[N=5]	451455.6	470529	454131	498388.8	451271.6	491683.4
% Assay	100.37	99.36	100.97	100.95	100.33	99.59
% RSD	0.76	0.65	0.63	0.59	1.02	1.18
Th. Plates	8837.3	3805.5	8894.2	3801.9	8874.2	3793.9
R.T.	13.65 ± 1.3	3.44 ± 1.42	13.68 ± 0.6	3.48 ± 0.97	13.72 ± 0.89	3.53 ± 0.98
Tailing	1.76	1.63	1.28	1.28	1.56	1.59
Resolution	24.64		24.5		24.57	

Conclusion: From this study, it was concluded that the presented is robust with respect to change in pH of mobile phase, change in column temperature and change in flow rate of mobile phase.

6.2.7 CONCLUSION:

This study is a typical example of development of a stability-indicating assay, established following the recommendations of ICH guidelines. It is one of the rare studies where forced decomposition studies were done under all different suggested conditions and the products were resolved in a single isocratic run. In literature, most of the studies on development of stability-indicating assays involve either forced decomposition studies under only one or two conditions, [69-71] or separation of drug from major degradation products whose standards were available. [72-74]

The developed method is simple, accurate, precise, specific, selective and robust. It is proposed for analysis of both the drugs and degradation products in stability samples in industry. The method, however, is not suggested to establish mass balance between the extent of drug decomposed and formation of degradation products, as some of the products are shown to decompose further in a complex reaction scheme while others may indicated to be nonchromophoric or volatile at higher temperature.

A new finding of this study is that Significant degradation of both Lercanidipine hydrochloride and Atenolol was found to be occurred in alkaline and oxidative stress conditions, while Lercanidipine hydrochloride was found to be degraded in presence of light. Mild degradation of Atenolol was found to be occurred in acidic condition, while no degradation was found to be occurred in neutral condition. Lercanidipine hydrochloride show significant degradation product at R.T. of 11.5, and as the sample was exposed more in sun light, significant increase in the area of degradation product, while decrease in the area of Lercanidipine hydrochloride was observed. So need to isolate and identify this degradation product and analyze for its biological action.

6.3 COMPARISON OF SPECTROPHOTOMETRIC AND RP-HPLC METHODS FOR ESTIMATION OF LERCANIDIPINE HYDROCHLORIDE AND ATENOLOL IN PHARMACEUTICAL DOSAGE FORM

Comparison of developed Spectrophotometric and RP-HPLC methods was performed by applying Student-t-test (Paired Two Sample for means)

ASSAY RESULTS OF LOTENSYL-AT TABLETS

DRUG	%ASSAY RESULTS OF METHODS	
	SPECTROPHOTOMETRIC (U.V.) METHOD	RP-HPLC
LERCANIDIPINE HYDROCHLORIDE	101.07	100.233
	101.07	101.124
	100.43	98.7082
	101.72	100.808
	101.71	98.885
	100.43	101.401
ATENOLOL	98.43	99.8144
	98.42	99.7886
	100.94	99.3362
	98.66	99.3886
	101.2	99.5197
	100.68	101.782

RESULTS OF STUDENT T-TEST

T-TEST: PAIRED TWO SAMPLE FOR MEANS					
Brand Name	**Parameter**	**LERCANIDIPINE HYDROCHLORIDE**		**ATENOLOL**	
		U.V.	**HPLC**	**U.V.**	**HPLC**
LOTENSYL-AT	**Mean**	101.0717	100.1931	99.72167	99.93829
	Variance	0.330257	1.323986	1.815617	0.8557
	Observations	6	6	6	6
	Pearson Correlation	-0.07876		0.217156	
	Hypothesized Mean Difference	0		0	
	df	5		5	
	T Stat (t_{cal})	1.622955		-0.36357	
	P (T≤t) one-tail	0.082763		0.365521	
	t Critical one tail	2.015049		2.015049	
	P (T≤t) two-tail	0.165526		0.731042	
	t Critical two tail (t_{crit})	2.570578		2.570578	

RESULTS OF VALIDATION PARAMETERS

Parameters		Lercanidipine hydrochloride	Atenolol	Lercanidipine hydrochloride	Atenolol
		SPECTROPHOTOMETRIC METHOD		RP-HPLC METHOD	
Range (µg/ml)		5- 15	25- 75	5- 15	25- 75
Regression equation y=mx+c		Y= 0.0304X + 0.0126	Y= 0.0151X + 0.0137	Y= 45547X + 3203.6	Y= 10376X - 10591
Slope		0.0304	0.0151	45547	10376
Intercept		0.0126	0.0137	3203.6	10591
Correlation coefficient (r^2)		0.9997	0.9993	0.9991	0.9993
LOD (µg/ml)		0.09	0.28	0.007	0.015
LOQ(µg/ml)		027	0.84	0.021	0.045
%Recovery (accuracy)± SD, n=3 for each level	Level 1	100.04 ± 0.73	100.97 ± 0.66	99.82 ± 1.69	101.8 ± 0.11
	Level 2	99.02 ± 0.5	98.99 ± 0.41	99.62 ± 0.8	98.31 ± 0.08
	Level 3	99.29 ± 0.69	100.07 ± 0.43	99.16 ± 0.29	99.37 ± 0.89
Repeatability (%RSD) (n=6)		0.57	1.35	1.14	0.9
Interday (%RSD) (n = 3)		0.46	1.36	0.14	1.06
Intraday (%RSD) (n = 3)		1.18	1.29	0.10	0.36
% Assay ± SD (n= 3)		99.56 ± 0.98	100.89 ± 0.401	99.56 ± 0.57	99.28 ± 0.42

RESULTS OF DEGRADATION STUDY

Stress condition/ duration/ state	% Assay		% Assay (Tablet)		Peak Purity	
	LER	ATN	LER	ATN	LER	ATN
Acidic / 1N HCl/ 80°C/5hr/ solution	100	95	98	93	Pass	Pass
Alkaline/1N NaOH / RT / 6 hrs /solution	95	75	85	84	Pass	Pass
Neutral/ Milli-Q water/80°C/8 hr/ solution	100	100	100	100	Pass	Pass
Oxidative/ 3% H_2O_2 / RT/ 2 hr/solution	87	98	85	93	Pass	Pass
Light/ 1.2 million lux.hours/ 7 days/ solid	-	-	66	100	Pass	Pass
Sun light/ 30 min/ liquid	95	100	97	100	Pass	Pass
Thermal/100°C/24 hr/solid	100	100	100	100	Pass	Pass

7.1. 1ST DERIVATIVE SPECTROPHOTOMETRIC METHOD:

Lercanidipine hydrochloride and Atenolol are used in combination for treatment of hypertension. The present study deals with simple spectrophotometric method development for simultaneous estimation of Lercanidipine hydrochloride (LER) and Atenolol (ATN) from Tablet Dosage Form. The method employed first order derivative spectroscopy. For determination of sampling wavelength 10 µg/ml of Lercanidipine hydrochloride and 50 µg/ml of Atenolol were scanned in 200-400 nm range and sampling wavelengths were 249 nm for Lercanidipine hydrochloride were Atenolol showed zero crossing point and 238.7 nm for Atenolol were Lercanidipine hydrochloride showed zero crossing point in first order derivative spectroscopy. The method was validated with respect to linearity, precision, accuracy, specificity, solution stability. The response was linear in the drug concentration range of 25-75 $\mu g.ml^{-1}$ of Atenolol and 5-15 $\mu g.ml^{-1}$ of Lercanidipine hydrochloride. The mean values (+/-RSD) of slope, intercept and correlation coefficient were 0.0302 (+/- 1.25), 0.0092 and 0.9993 (+/- 0.035) for Lercanidipine hydrochloride and 0.0149 (+/- 1.77), 0.0155 and 0.9994 (+/- 0.041) for Atenolol respectively. The RSD values for intra- and inter-day precision studies were +/- 0.468 and +/- 1.124 for Lercanidipine hydrochloride and +/- 0.276 and +/- 0.712 for Atenolol respectively. The recovery of the drug ranged between 98-102 % assessed by standard addition method. Lercanidipine hydrochloride and Atenolol in tablet was found to be 99.13 and 98. 34 respectively. The method was specific to drug and also selective to degradation products.

7.2 STABILITY INDICATING RP-HPLC ASSAY METHOD:

The present study describes degradation of Lercanidipine hydrochloride and Atenolol Combination in bulk drug and Formulation under different ICH prescribed stress conditions (hydrolysis, oxidation and photolysis), and establishment of a stability-indicating reversed-phase HPLC assay. Significant degradation of both Lercanidipine hydrochloride and Atenolol was found to occur in alkaline conditions, while Lercanidipine hydrochloride amongst both the drugs was found to be degraded in presence of light and oxidative stress condition. Mild degradation of Atenolol was found to be occurred in acidic condition, while no degradation was found to be occurred in neutral and thermal degradation conditions. Separation of both the drugs and the degradation products under various conditions was successfully achieved on an Inertsil ODS 3V

(250*4.6 mm, 5 µ) column utilizing 0.1% Ortho-Phosphoric Acid-acetonitrile the ratio of 55:45 having flow rate of 0.5 $ml.min^{-1}$. The detection wavelength was 239 nm. The method was validated with respect to linearity, precision, accuracy, solution stability, selectivity, specificity and robustness. The response was linear in the drug concentration range of 25-75 $\mu g.ml^{-1}$ of Atenolol and 5-15 $\mu g.ml^{-1}$ of Lercanidipine hydrochloride. The mean values (+/-RSD) of slope, intercept and correlation coefficient were 45806.67(+/- 0.54), 2490.967 and 0.9990 (+/- 0.04) for Lercanidipine hydrochloride and 10163.23(+/- 2.14), 7548.86 and 0.99876 (+/- 0.047) for Atenolol respectively. The RSD values for intra- and inter-day precision studies were +/- 0.536 and +/- 0.265 for Lercanidipine hydrochloride and +/- 0.314 and +/- 0.225 for Atenolol respectively. The recovery of the drug ranged between 98-102 % from a placebo. % assay of Lercanidipine hydrochloride and Atenolol in tablet was found to be 99.31 and 98.82 respectively. The method was specific to drug and also selective to degradation products.

FUTURE SCOPE:

- The related substance or impurity profiling study can be possible for the combination of Lercanidipine hydrochloride and Atenolol.
- The characteristic photodegradation product at retention time of 11.5 and Alkaline degradation product at retention time of ~8 (7.9) of Lercanidipine hydrochloride be isolate, identify and analyze for its biological action.

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