METHOD DEVELOPMENT AND METHOD VALIDATION FOR RAMIPRIL AND TELMISARTAN BY RP-HPLC IN PHARMACENTICAL DOSAGE FORM.

PHARMACEUTICAL ANALYSIS

26116628

Dept. of pharmaceutical analysis *JKKMMRF college of pharmacy*

Telmisartan

INTRODUCTION

Analytical chemistry has played a major role in the changes facing the pharmaceutical Industry today. Traditionally viewed as a service organization, the analytical department has become a significant parameter in the drug development process. Indeed, the demand for analytical data has become a critical path activity for selection of molecule for full development. The pharmaceutical analysis plays a major role in assuring, identity, safety, efficacy, purity and quality of drug product the need for pharmaceutical analysis is driven largely by regulatory requirements. (Satinder Ahuja, 2005; Takeru Higuchi, 2002)

The discipline of analytical chemistry consists of,

- Qualitatitive analysis: - Which reveals the identity of element and compound in a sample. (Alexeyev, 1994)
- Quantitative analysis: - Indicate the amount of individual element or compound present in sample.
- Method of Quantitative Analysis (Devaraj Rao, 2004)

Under the Quantitative Analysis, the Procedures of quantitative analytical chemistry are applied to the analysis of material used in pharmaceuticals.

There are various methods for quantitative analysis of pharmaceuticals. In general the components are related characteristically to some physical property and on the basis of that, the quantity is estimated. They are classified into the following types:

- Chemical Methods
- Volumetric or Titrimetric

* Acid-base or Neutralization Titrations
* Oxidation –Reduction or Redox Titrations

* Precipitation Titration
* Complex metric Titrations
* Non-aqueous Titrations
* Gravimetric
* Geometric

Instrumental or Physio-chemical methods

Instrumental methods are based on the relation between the content and corresponding physical or physio-chemical Properties of the system being analyzed.

- Chromatographic Methods
  * Thin Layer Chromatography (TLC)
  * Paper Chromatography
  * Column Chromatography
  * Gas Chromatography
  * High Performance Liquid Chromatography (HPLC)
- Hyphenated Techniques
  - GC-MS (Gas Chromatography – Mass Spectrometry)
  - LC-MS (Liquid Chromatography – Infrared Spectroscopy)
  - ICP-MS (Inductive Coupled Plasma – Mass Spectrometry)
- Microbiological Methods
- Biological Methods

❖ Factors effecting the choice of analytical methods (Mendham, 2002)
❖ The type of analysis required.
❖ Problem arising from the nature of the material
❖ Possible interference from components of the material other than those of Interest.
❖ The Concentration range which needs to be investigated.
❖ The accuracy required
❖ The facilities available
❖ The time required for complete analysis
❖ Similar type of analysis performed

CHROMATOGRAHY

Chromatography is family of Analytical chemistry techniques for the separation of mixtures. It involves passing the sample, mixture which contains the analyte, in the "mobile phase" often in a steam of solvent, through "stationary phase". The stationary phase retards the passage of the components of the sample. When components pass through the system at different rates they become separated in time, like runners in a marathon. Ideally, each component has characteristic time of passage through the system. This is called its "retention time". (Clark's 2004).

Physical separation method in which the components of a mixture are separated by differences in their distribution between two phases, one of which is stationary (stationary phase) while the other (mobile phase) moves through it in a definite direction. The substances must interact with the stationary phase to be retained and separated by it.

A chromatograph takes a chemical mixture carried by liquid gas and separates it into its component parts as a result of differential distributions of the solute as they flow around or over a stationary liquid or solid phase. Various techniques for separation of complex mixtures relay on the differential affinities of substances for a gas or liquid mobile medium and for a stationary absorbing medium through which they pass; such as paper, gelatin, or magnesium silicate gel.

Analytical chromatography is used to determine the identity and concentration of the molecules in mixture. Preparative chromatography is used to purify large quantities of a molecular species.

DEFINITIONS FOR CHROMOTOGRAPHY

Chromatography is a physical method of separation in which the compound to be separated or distributed between two phase. One of which is stationary (stationary phase) while the other mobile (Mobile phase) moves in definite direction (IUPAC, 1993).

THE DIFFERENT TYPES OF CHROMATOGRAHY

Adsorption chromatography

Adsorption chromatography probably one of the oldest types of the chromatography around. It utilizes a mobile liquid or a gaseous phase that is adsorbed on to the surface of a stationary solid phase. The equilibration between mobile and stationary phase accounts for the separation of different solutes.

Partition chromatography

This form of chromatography is based on a thin film formed on the surface of the solid support by a liquid stationary phase. Solute equilibrates between the mobile phase and the stationary liquid.

Ion Exchange chromatography

In this type of chromatography the use of resin (the stationary solid phase) is used to a covalently attach anions or a cations on to it. Solute ions of the opposite charge in the mobile liquid phase are attracted to the resin by electro static forces.

Molecular Exclusion Chromatography

Also known as Gel Permeation or Gel Filtration, this type of chromatography lacks an attractive interaction between the stationary phase and solute. The liquid or gaseous phase passes through a porous gel, which separates the molecules according

to its size. The pores or normally small and exclude the larger solute molecules, but allows smaller molecules to enter the gel, causing them to flow through a larger volume. This causes the larger molecules to pass through the column at faster than the smaller once.

Affinity Chromatography

This is the most selective type of chromatography employed. It utilizes the specific interaction between one kind of solute molecule and a second molecule. That is immobilized on a stationary phase. For example, the immobilized molecule may be an antibody to some specific protein. When solute containing a mixture of proteins is passed by this molecule, only the specific protein is reacted to this antibody, binding it to the stationary phase.

This protein is later extracted by changing the ionic strength or PH.

Working principle

In all chromatography there is an mobile phase and stationary phase. The stationary phase is a phase that doesn't move and the mobile phase is the phase does moves. The mobile phase moves through the stationary phase picking up the compounds to tested. As the mobile phase continuous to travel through the stationary phase it takes the compounds with it. At different points in the stationary phase the different components of the compound are going to be absorbed and are going to stop moving within the mobile phase. This is how the results the chromatography are gotten, from the point at which the different components of the compound stop moving and separate from the other components.

In paper and thin-layer chromatography the mobile phase is the solvent. The stationary phase in paper chromatography is strip or piece of the paper that is placed in the solvent. In thin-layer chromatography the stationary phase is the thin-layer cell. Both these kinds of chromatography use capillary action to move the solvent through the stationary phase.

Retention Factor (R_f)

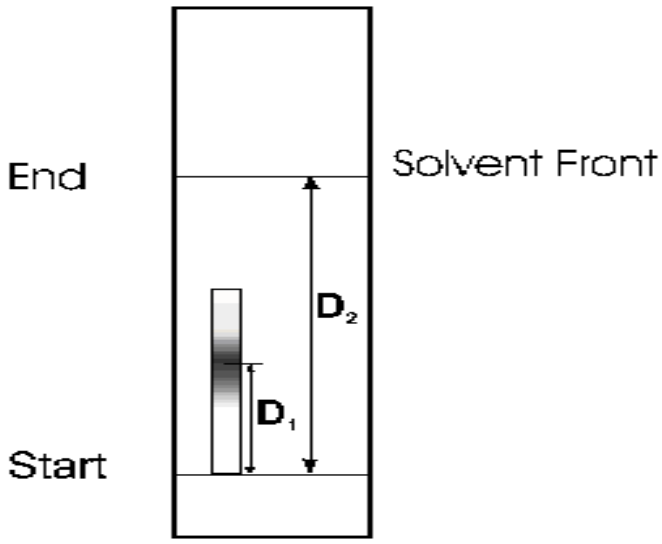
The retention factor, R_f , is the quantitative indication of how far a particular compound travels in a particular solvent. The R_f value is a good indicator of whether and unknown compound and a known compound are similar, if not identical. If the R_f Value for the unknown compound is close or the same as the R_f Value for the known compound then the two compounds are most likely similar or identical. The retention factor, R_f, is defined as R_f = Distance the solute (D1) moves divided by the distance traveled by the solvent front (D2).

$R_F = D_1/D_2$

Where,

D_1 = distance that spot travelled, measured from centre of the band of spot to the point where Food color was applied.

D_2 = total distance that solvent travelled.



HIGH PERFORMANCE LIQUID CHROMATOGRAHY

HPLC is able to separate macro molecules and ionic species. Labile, natural products polymeric materials and a wide variety of other high-molecular weight polyfunctional groups.

High performance liquid chromatography is a separation technique utilizing differences in distribution of compounds to two phases; called stationary phase and mobile phase.

Basic Principle of HPLC

High performance liquid chromatography is separation technique utilizing differences in distribution of compounds to two phase; called stationary phase and mobile phase. The stationary phase designates a thin layer created on the surface of fine particles and the mobile phase designates the liquid flowing over the particles. Under a certain dynamic condition each component in a sample has different distribution equilibrium depending on the solubility in the phases and or molecular size. As a result the components move at different speeds over the stationary phase

and are there by separated from each other the column is stainless steel (or resin) tube which is packed with spherical solid particles. Mobiles phase is constantly fed in to the column inlet at a constant rate by a liquid pump. A sample is injected from sample injector located near the column inlet. The injected sample enters the columns with the mobile phase and the components in the sample migrate through it passing between stationary and mobile phases. Compound move in the column only when it is in the mobile phase. Compounds that tend to be distributed in the mobile phase therefore migrate faster through the column while the compounds that tend to distributed in the stationary phase migrates slower. In this way each component is separated on the column and sequentially elutes from the outlets. Each compound eluting from the column is detected by a detector connected to the outlet of the column.

TYPES OF HIGH PERFORMANCE LIQUID CHROMATOGRAHY

Based on modes of chromatography
- Normal phase chromatography
- Reverse phase chromatography

Based on Principles of separation
- Adsorption chromatography
- Ion exchange chromatography
- Ion pair chromatography
- Size exclusion chromatography
- Affinity chromatography
- Chiral phase chromatography

Based on elusion technique
- Isocratic separation
- Gradient separation

Based on the scale of operation
- Analytical HPLC
- Preparative HPLC

Isocratic Elution

In Isocratic elution a sample is injected onto a given column and the mobile phase is unchanged through the time required for the sample components to elute from the column. The isocratic separation of samples widely varying with k' (partition ratio) values long elution times. To adequately handle samples that have both weakly retained and strongly retained and strongly retained substances, the rates of individual band migrations must be changed.

Gradient Elution

Steady changes of the mobile phase composition during the chromatographic run are called gradient elution. The main purpose of gradient elution is to move strongly retained components of the mixture faster, but having the least retained component well resolved.

Starting with the low content of the organic component in the eluent we allow the least retained components to be separated. Strongly retained components will sit on the adsorbent surface on the top of the column, or will move very slowly.

When we start to increase an amount of organic component in the eluent (Acetonitrile) then strongly retained components will move faster and faster, because of the steady increase of the competition for the adsorption sites.

Gradient elution also increases quasi-efficiency of the column. In the isocratic elution, the longer a component is retained the wider its peak. In gradient elution especially with the smooth gradient shape without flat regions the tail of the peak is always under the influence of the stronger mobile phase when compared to the peak front. Thus molecules on the tail of the chromatographic zone (peak) will move faster. This will tend to compress zone and narrow the resultant peak.

The optimum gradient for a particular separation is selected by trial and error.

Normal Phase High Performance Liquid Chromatography (NP-HPLC)

Normal – phase Liquid – liquid chromatography uses a polar stationary phase and less polar mobile phase. To select an optimum mobile phase, it is best to start with a pure hydrocarbon mobile phase such as heptane. If the sample is strongly retained the polarity of the mobile phase should be increased, perhaps by adding small amounts of methanol or dioxane.

Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

In the normal phase mode, separations of oil-soluble vitamins, essential oils, nitro phenols, or more polar homologous series has been performed using alcohol/heptane as the mobile phase. Column used in the normal phase chromatography for chiral separation

Reverse phase chromatography uses hydrophobic bonded packing, usually with an octadecyl or octyl functional group and a polar mobile phase, often a partially or fully aqueous mobile phase. Polar substances prefer the mobile phase and elute first. As the hydrophobic character of the solutes increases, retention increases. Generally, the lower the polarity of the mobile phase, the higher is its eluting strength. The elution order of the classes of the compounds in table is reversed (Thus, the name reverse-phase chromatography). Hydrocarbons are retained more strongly than alcohols. Also, the elute strength of the various solvents reverse-phase chromatography follows approximately the reverse order given in

table. Thus water is weakest elute. Methanol and Acetonitrile are popular solvents because they have low viscosity and are readily available with excellent purity.

Table-1

COMPARISION OF NP-HPLC AND RP-HPLC

Proprietors	Normal Phase	Reverse Phase
Polarity of Stationary Phase	High	Low
Polarity of Mobile Phase	Low to Medium	Low to High
Sample elution order	Leader polar first	Most polar first
Retention will be increased by	Increasing surface of stationary phase Increasing of n-alkyl chain length of stationary phase Decreasing polarity of mobile phase Increasing polarity of sample	Increasing surface of Stationary phase Increasing polarity of mobile phase Decreasing polarity of sample molecules

Ion exchange chromatography

Ion exchange chromatography uses a stationary phase support which has been derivatized to as to permanently bind charged groups to the surface the mobile phase is typically as aqueous buffer. This technique is used primarily for the analysis of ions such as strong acids or basis or for separation of large molecules such as nucleic acids, proteins, or large peptides.

Ion-pair chromatography

Ion-pair chromatography is a "hybrid" technique in which charges groups are temporarily bound to the surface of a (Reverse-phase) type of column packing. This technique is often used for the analysis of small, weak-acid or weak-base compounds.

Size-exclusion chromatography

This separates molecules according to their molecular mass. Larger molecules are eluted first and the smallest molecules elute last. This method is generally used when a mixture contains compounds with a molecular mass difference of at least 10%. This mode can be further subdivided into gel permeation chromatography (with organic solvents) and gel filtration chromatography (with aqueous solvents).

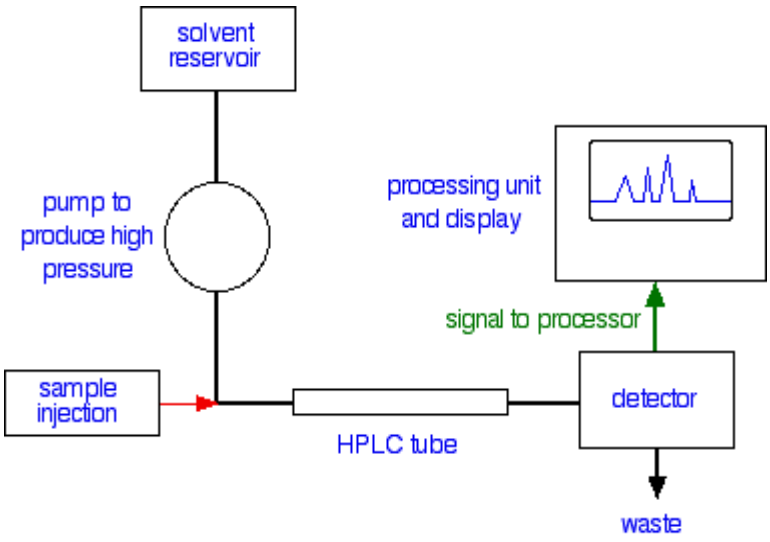
Affinity Chromatography

Affinity chromatography operates using immobilized biochemical's that have a specific affinity to the compound of interest. Separation occurs as the mobile phase and sample pass over the stationary phase. The sample compound or compounds of interest are retained as the rest of the impurities and mobile phase through. The compounds are then eluted by changing the mobile phase conditions

HPLC INSTURMENTATION

- The general instrumentation for HPLC incorporates the following components:
- There is a solvent reservoir for the mobile phase.
- Sampling valves or loops are used to inject the sample in the flowing mobile phase just at the head of the separation column. Samples should be dissolved in a portion of the mobile phase to eliminate unnecessary peak.
- Ahead of the separation column there may be a guard column or an in-line filter to prevent contamination of the main column by small particulate.
- To measure column inlet pressure a pressure gauge is inserted in front of the separation column.
- The separation column contains the packing needed to accomplish the desired HPLC Separation. These may be silica's for adsorption chromatography, bonded phases for liquid-liquid chromatography, exchange functional groups bonded to stationary support for exchange chromatography, gels of specific porosity for exclusion chromatography, or some unique packing for particular separation method

Figure 1: A flow Scheme for HPLC



Mobile-phase delivery system

The mobile phase must be delivered to the column over a wide range of flow rates and pressure. A de-gasser is needed to remove dissolved air and other gases from the solvent. Another desirable feature in the solvent-delivery system is the capability for generating a solvent gradient.

Pump

A pump should able to be operate to at least 1500 psi, a pressure suited to less expensive chromatographs. However, 6000 psi is a more desirable pressure limit. For many analytical columns only moderate flow rates of 0.5 – 2.0 ml / min need to be generated.

Separation Columns

Column is the important part of a HPLC instrument columns are constructed of heavy-wall, glass-lined metal tubing or stainless steel tubing to with stand high pressure (up to 7000 psi) and the chemical action of the mobile phase. Most column

lengths range from 10 – 30 cms; short, fast columns are 3 to 8 cms long. For exclusion chromatography, columns are 50 to 100 cms long.

Standard Columns

Many HPLC separations are done on columns with an internal diameter of 4 to 5 mm. Such columns provide a good compromise between efficiency sample capacity, and the amount of packing and solvent required. Column packing feature particles that is uniformly sized and mechanically stable. Particle diameters lie in the range 3 – 5 ml, occasionally up to 10 ml or higher for preparative chromatography. The columns are classified into various categories depending on their carbon loading.

Detectors

The sensitivity of universal detector for HPLC as not been devised yet. Thus it is necessary to select a detector on the basis of the problem.

UV Visible Photometers and Spectrometers

Optical detectors based on UV-Visible absorption are the workhorses of HPLC, constituting over 70% of all detections system of all in us. Basically three types of absorbance detectors are available: a fixed wavelength detector, a variable detector, and a scanning wavelength.

Fixed wave length detectors

A fixed wavelength detector uses a light source that emits maximum light intensity at one or several discrete wavelengths that are isolated by appropriate filters.

Variable Wavelength detector

A variable wave length detector is a relatively wide-band pass it offers a wide selection of UV and Visible wavelength, but at increased cost.

Photo Diode Array (PDA) Detector

To obtain a real time spectrum for each solute at it elutes, solid-state diode arrays are required. The diode arrays work in parallel simultaneously monitoring all wavelengths.

Digital electronic integrators

Were, widely used today in HPLC for measuring Peak areas. These devices automatically sense peaks and print out the areas in numerical forms. Computing integrators are even more sophisticated and offer a number of features in addition to basic digital integration because these devices have both memory and computing capabilities to upgrade integrating parameters to maintain accuracy as the separation progress and eluting peaks become broader. Many of these devices print out a complete report including names of the compounds, retention times, peak areas and area correction factors. With the help of peak area and height values, the peak width can be calculated (considering the peak as a triangle) and it can also be used for the calculation of number of theoretical plates.

BUFFERS IN REVERSED-PHASE LIQUID CHROMOTOGRAPHY

Selection of a buffered aqueous mobile phase for reversed-phase liquid chromatography (RPC) may seen intimidating, but with an understanding of the

fundamental effect of pH on retention of ionic analytes and checking some C. Properties of the buffer options, a logical and reasonable choice can be derived.

This selection may need to be revised by consideration of certain factors, such as solubility or limitations of compatibility with the means of detection. Practical aspects of buffer preparation should also be observed for reproducible and trouble-free operation.

The pH of the mobile phase will dramatically effect the retention of ionic analytes within 1.5 pH units of their pKa. Thus, it can be understood why control of mobile phase pH has great utility in method development and is critical for reproducible separations of ionic analytes. Obviously, if the analyte is not ionic, mobile phase pH won't directly effect its retention.

Table-2

Buffer	pKa (25°C)	Maximum buffer range	UV Cutoff (nm)
TFA	0.3	_	210 (0.1%)
Phosphate,H_2PO_4 pK_1	2.1	1.1-3.1	<200
Phosphate,$pK_2HPO_4^{2-}$	7.2	6.2-8.2	<200
Phosphate, pK_3 PO_4^{3-}	12.3	11.3-13.3	<200
Citrate,$pKC_3H_5O(CO_2H)_2$ $(CO_2-)_1$	3.1	2.1-4.1	230
Citrate, pK_2 $C_3H_5O(CO_2H)_1$ $(CO_2-)_2$	4.7	3.7-5.7	230
Citrate, pK_3 $C_3H_5O(CO_2-)_3$	6.4	4.4-6.4	230
Carbonate,$pK_1HCO_3^{2-}$	6.1	5.1-7.1	<200
Carbonate, pK_2 CO_3^{2-}	10.3	9.3-11.3	>200
Formate	3.8	2.8-4.8	210 (10 NM)
Acetate	4.8	3.8-5.8	210 (10 NM)
Ammonia	9.3	8.3-10.3	200 (10 NM)
Borate	9.2	8.2-10.2	N/A
TEA	10.8	9.8-11.8	<200

SELECTIVITY OF HPLC – METHOD DEVELOPMENT

Most of the drugs can be analyzed by HPLC method because of several advantages like rapidity, specificity, accuracy, precision, case of automation and eliminates tedious extraction and isolation procedures. Some of the advantages are:

* Speed (analysis can be accomplished in 20 or Less)
* Greater sensitivity (Various detectors can be employed))
* Improved Resolution (Wide variety of stationary phases)
* Reliable columns (Wide variety of stationary phases)
* Ideal for substances of low volatility
* Easy sample recovery, handling and maintenance
* Easy programming of the numerous functions in each module
* Time programmable operation sequence, such as initiating operation of detector lamp and pump to obtain a stable baseline and equilibrated column before the work day begins.
* Excellent reproducibility of retention times.
* An injection volume variable from 0.1 to 100 micro liters without any hardware modification.
* The flexibility of data analysis.
* Suitable to avoid any interference from impurity.

Suitable, for preparative liquid chromatography on a much larger scale.

METHOD DEVELOPMENT BY RP-HPLC

HPLC method development is not very difficult when a literature reference for the same or similar compounds to be analyzed can be found.

The first consideration when developing an HPLC method is to determine the solubility of the sample components. Knowing the nature of the analytes will allow the most appropriate mode of the HPLC to be selected. Summarizes appropriate separation modes and mobile phases to consider for method development. (Complex sample may require an initial clean – up step to remove possible interferences) 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 with some trial and error, but the key to efficient method development planning.

THE BEST DETECTORS

The next consideration should be choice of detector. There is a little use in running a separation if the detector one uses cannot "see" all the components of interest, or conversely, if it "see" too much. UV – Vis detectors are the most popular as they can detect a broad range of compounds and have a fair degree of selectivity for some analytes. Unfortunately UV – Vis detectors are not universal detectors so it is worthwhile to look at the chemical structure of the analyte to see if it has suitable chromophores, such as aromatic rings, for UV – Vis detection.

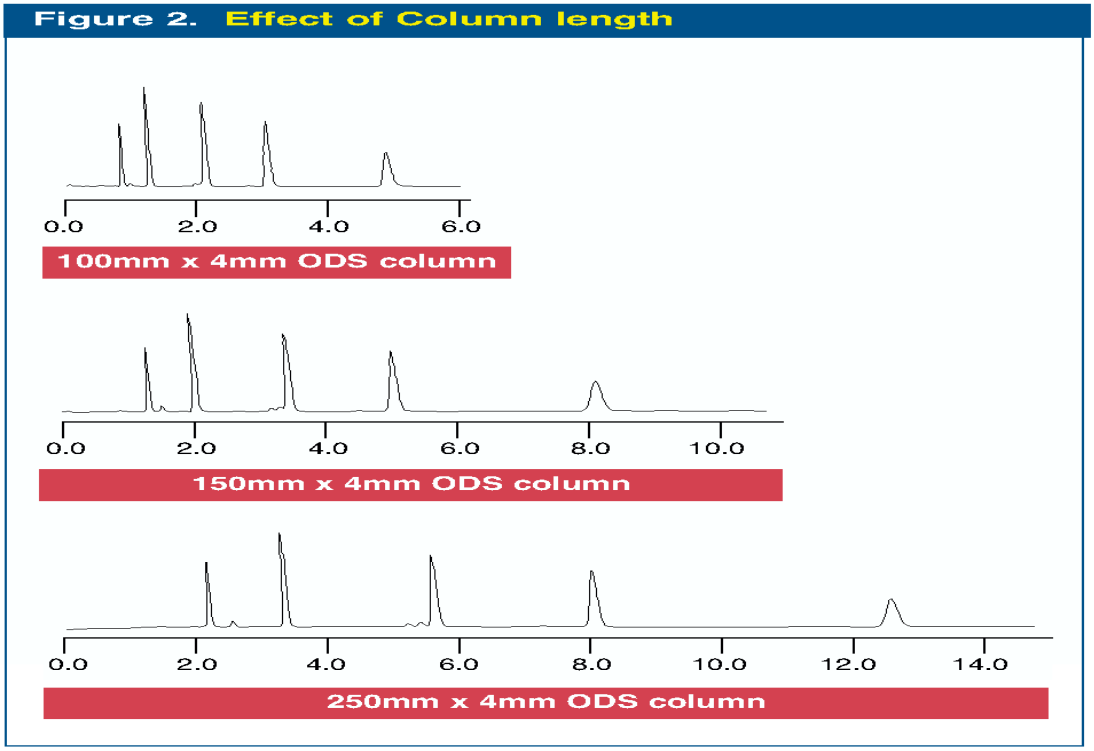
Table.3 summarizes some of the available options.

Table 1. Detector options

Detector	Analytes	Solvent Requirements	Comments
UV-vis	Any with chromophores	UV-grade non-UV absorbing solvents	Has a degree of selectivity and is useful for many HPLC 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
Conductivity	Charged or polar compounds	Mobile phase must be conducting	Excellent for Ion Exchange methods
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
Mass Spectrometer (MS)	Broad range of compounds	Must use volatile solvents and volatile buffers	Highly sensitive and is a powerful 2nd dimensional analytical tool. Many modes available. Needs trained operators

THE BEST COLUMN LENGTH

Many chromatograms make the mistake of simply using what is available. Often this a 250 x 4.6 mm c18 column. These columns are able to resolve a wide variety of compounds (due to their selectivity and plate counts) and more common to most laboratories. While many reverse phase separations can be carried out on such a column, its high resolving capabilities are often unnecessary, as illustrated fig. 2. Method development can be streamlined by starting with shorter column; 150, 100, or even 50 mm long. This simply because they have proportionally shorter run times.



THE BEST STATIONARY PHASE

Selecting an appropriate stationary phase can also help to improve the efficiency of method development. For example, a C8 phase (reverse phase) can provide a further time saving over a C18, as it does not retain analytes as strongly as the C18 phase. For normal phase applications, cyano (nitrile) phase are the most versatile.

THE BEST MOBILE PHASE

Since the mobile phase governs solute-stationary phase interactions, it 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 are pumped through the column, trace impurities can easily concentrate in the column and eventually be determined

to the results. AR and 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 pressure are required and mass transfer between solvent and stationary phase will be reduced LC/MS – only volatile buffers.

THE BEST INTERNAL DIAMETER

By selecting a shorter column with an appropriate phase, run times can be minimized so that an elution order and an optimum mobile phase can be quickly determined. It can also be advantageous to consider the column internal diameter. Many laboratories use 4.6 mm ID Columns as a standard, but it is worth considering the use of 4 mm ID Columns as an alternative. These require only 75% of solvent flow that a 4.6 mm column uses. This translates to a 25% solvent saving over the life of the column and can be even more significant if a routine method is developed for such a column.

POLARITY OF COMMON ORGANIC FUNCTIONAL GROUPS AND SOLVENT

Functional Groups	Non-polar	Solvent
Aliphatic hydrocarbons		Hexane
Olefins		Carbon tetrachloride
Aromatic hydrocarbons		Ether
Halides		Benzene
Sulphides		Methylchloride
Ethers		THF
Nitro components		Isopropanol
Esters, aldehydes, ketones		Chloroform
Alcohols, amines		Ethyl acetate
Sulphones		Acetonitrile
Sulphoxides	Polar	Methanol
Amides		Water

ANALYTICAL METHOD VALIDATION

Method validation can be defined as (ICH*)* "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 an integral part of the method development; it is the process by which a method is tested by the developer or user for reliability, accuracy and preciseness of its intended purpose and demonstrating that analytical procedures are suitable for their intended use that they support the identity, quality, purity, and potency of the drug substances and drug products Data thus generated become part of the methods validation package submitted to Center for Drug Evaluation and Research (CDER). Simply, method validation is the process of proving that an analytical method is acceptable for its intended purpose.

Methods should be reproducible when used by other analysts, on other equivalent equipment, on other days or locations, and throughout the life of the drug product. Data that are generated for acceptance, release, stability, or pharmacokinetic will only be trustworthy if the methods used to generate the data are reliable. The process of validation and method design also should be clearly in the development cycle before important data are generated. Validation should be on going in the form of re-validation with method changes. (United states of Pharmacopeia XX11)

Though many types of HPLC techniques are available, the most commonly used method, the reversed-phase HPLC with UV detection, is selected to illustrate the parameters for validation. The criteria for the validation of this technique can be

extrapolated to other detection methods and chromatographic techniques. For acceptance, release or stability testing, accuracy should be optimized since the need to show deviation from the actual or true value is of the greatest concern.

All the variables of the method should be considered, including sampling procedure, sample preparation, chromatographic separation, and detection and data evaluation. For chromatographic methods used in analytical applications there is more consistency in validation practice with key analytical parameters includes namely

- System suitability
- Specificity
- Accuracy
- Precision
- Linearity
- Limit of Detection
- Limit of Quantitation
- Ruggedness
- Robustness

System Suitability

According to the USP, system suitability tests are an integral part of chromatographic methods. These tests are used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. System suitability tests are based on the concept that the equipment, electronics, analytical operations, and samples constitute an integral system that can be evaluated as a whole. The purpose of the system suitability test is to ensure that the complete

testing system (including instrument, reagents, columns, analysts) is suitable for the intended application.

Similar to the analytical method development, the system suitability test Strategy should be revised as the analysts develop more experience with the assay. In general, consistency of system performance. (Eg: Replicate injections of the standard) and chromatographic suitability. (Eg: Tailing factor, column efficiency and resolution of the critical pair) are the main components of system suitability.

During the early stage of the method development process some of the more sophisticated system suitability tests may not be practical due to the lack of experience with the method. In this stage, usually a more "generic" approach is used. For example, evaluation of the tailing factor to check chromatographic suitability, and replicate injections of the system suitability solution to check injection precision may be sufficient for an HPLC impurities assay. As the method matures more experience is acquired for this method, a more sophisticated system suitability test may be necessary.

System suitability is the checking of a system to ensure system performance before or during the analysis of unknowns. Parameters such as plate count, tailing factors; resolution and reproducibility (%RSD retention time and area for six repetitions) are determined and compared against the specifications set for the method. These parameters are measured during the analysis of system suitability "sample" that is a mixture of main components and expected by-products.

Table 4: System suitability parameters and recommendations

Parameter	Recommendation
Capacity Factor (k')	The peak should be well-resolved from other peaks and the void volume, generally k' 1 to 20
Repeatability	RSD $\leq$ 1% for N $\geq$ 5 is desirable.
Relative retention	Not essential as long as the resolution is stated.
Resolution (R_s)	R_s of > 2 between the peak of interest and the closest eluting potential interfering (impurity, excipient, degradation product, internal standard, etc.
Tailing Factor (T)	T of >0.5 and $\leq$ 2
Theoretical Plates (N)	N > 3000

Specificity/Selectivity

The terms selectivity and specificity are often used interchangeably. According to ICH, the term specific generally refers to a method that produces a response for a single analyte only while the term selectivity refers to a method that provides responses for a number of chemical entities that may or may not be distinguished from each other. If the response is distinguished from all other responses, the method is said to be selective. Since there are very few methods that respond to only one analyte, the term selectivity is usually more appropriate.

Specificity is the ability of a method to discriminate between the analyte(s) of interest and other components that are present in the sample. Studies are designed

to evaluate the degree of interference, if any, which can be attributed to other analytes, impurities, degradation products, reagent "blanks" and excipients. This provides the analyst with a degree of certainty that the response observed is due to the single analyte of interest. The degree of specificity testing varies depending on the method type and the stage of validation. Specificity should be evaluated continually through the drug development process. Specificity is sometimes used interchangeably with the term "selectivity". The argument over which term is more correct is one of semantics. Although there is some dissention, the term "specificity" has been adopted by the regulatory guidance documents and should be used to prevent further confusion.

Non-Interference of Placebo

This portion of specificity evaluation applies to the finished drug product only. Excipients present in the formulation should be evaluated and must not interfere with the detection of the analyte. Individual solutions of each excipient prepared at several times the normal concentration of the component in the drug product ensure that any detector response from the excipient will be readily visible. Injecting individual solutions of each excipient into the HPLC system in comparison with a standard solution of the analyte is one means of performing this experiment. The absence of a peak eluting at the retention time of the active ingredient is sufficient to demonstrate specificity for excipients.

Challenge Study

Injecting solutions of known process impurities, degradation products, intermediates, homologues, dimers etc. further challenges the specificity of a method. Identification of these compounds may require an extensive search in order to identify all possible species that may be present in the sample. For new chemical entities (NCE), this information may not be readily available. Probable suspects should be identified by careful review of the synthetic route and manufacturing process to identify any likely species that may be present in the sample.

Degradation Studies

Degradation studies involve exposing the sample to a variety of stressed conditions to further evaluate the specificity of degradation products. In this study, the drug substance, drug product, and the combined recipients (or placebos) are each exposed to the stressed conditions. These may include, but are not limited to, heat, light, acidic media, alkaline media, and oxidative environments. Other conditions may be used depending on the nature and chemistry of the test subject. Forced degradation is usually evaluated with not more than 20% degradation of the drug substance, although more may be acceptable depending on the particular properties of the drug. A reasonable effort should be made to degrade samples in order to identify possible degradation products. If the planned experiments do not show any appreciable degradation, the strength and/or exposure time of the stress condition may be increased, but degradation is not required for every condition studied. There is a point beyond which the stress condition becomes extreme and unrealistic. Sound scientific judgment should be used to determine the extent and degree of degradation studies.

Accuracy

Accuracy is the measure of how close the experimental value is to the true value. Accuracy should be established across the specified range of the analytical procedure.

Assay

(Drug Substance)

Several methods of determining accuracy are available:

a) Application of an analytical procedure to an analyte of known purity (e.g. reference material);

b) Comparison of the results of the proposed analytical procedure with those of a second well-characterized procedure, the accuracy of which is stated and/or defined.

c) Accuracy may be inferred once precision, linearity and specificity have been established.

Drug Product

Several methods for determining accuracy are available:

a. Application of the analytical procedure to synthetic mixtures of the drug product components to which known quantities of the drug substance to be analyzed have been added.

b. In cases where it is impossible to obtain samples of all drug product components, it may be acceptable either to add known quantities of the analyte to the drug product or to compare the results obtained from a second, well Characterized procedure, the accuracy of which is stated and/or defined.

c. Accuracy may be inferred once precision, linearity and specificity have been established.

Impurities (Quantitation*)*

Accuracy should be assessed on samples (drug substance/drug product) spiked with known amounts of impurities. In cases where it is impossible to obtain samples of certain impurities and/or degradation products, it is considered acceptable to compare results obtained by an independent procedure. The response factor of the drug substance can be used. It should be clear how the individual or total impurities are to be determined e.g., weight/weight or area percent, in all cases with respect to the major analyte.

Recommendations

Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g. 3 concentrations / 3 replicates each of the total analytical procedure). Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals.

Precision

Precision is the measure of how close the data values are to each other for a number of measurements under the same analytical conditions. ICH has defined precision to contain three components: repeatability, intermediate precision and reproducibility. Ruggedness as defined in 1990 incorporates the concepts described under the terms "intermediate precision", "reproducibility" and "robustness" of this guide.(USP XXII)

Repeatability

Injection Repeatability

Sensitivity is the ability to detect small changes in the concentration of the analyte in the sample. Sensitivity can be partially controlled by monitoring the specification for injection reproducibility (system suitability testing).

The sensitivity or precision as measured by multiple injections of a homogeneous sample (prepared solution) indicates the performance of the HPLC instrument under the chromatographic conditions and day tested.

The information is provided as part of the validation data and as a system suitability test. The specification, as the coefficient of variation in % or relative standard deviation (RSD), set here will determine the variation limit of the analysis. The tighter the value, the more precise or sensitive to variation one can expect the results. This assumes that the chromatograph does not malfunction after the system suitability testing has been performed. Keep in mind, however, that it does not consider variations due to the drug product manufacturing and laboratory sample preparation procedures. The set of four duplicate samples were injected sequentially. Variations in peak area and drift of retention times are noted.

Precision refers to the reproducibility of measurement within a set, that is, to the scatter of dispersion of a set about its central value. The term 'set' is defined as referring to a number (N) of independent replicate measurements of some property. One of the most common statistical terms employed is the standard deviation of a population of observation. Standard deviation is the square root of the sum of

squares of deviations of individual results for the mean, divided by one less than the number of results in the set. The standard deviation S, is given by

$$s = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - \overline{x})^2},$$

Standard deviation has the same units as the property being measured.

The square of standard deviation is called variance (S^2). Relative standard deviation is the standard deviation expressed as a fraction of the mean, i.e., S/x. It is sometimes multiplied by 100 and expressed as a percent relative standard deviation. It becomes a more reliable expression of precision.

% Relative standard deviation = S x 100 / x

Recommendations

As part of methods validation, a minimum of 10 injections with an RSD of 2% is recommended. With the methods for release and stability studies, an RSD of 2% for precision of the system suitability tests for at least five injections (n=5) for the active drug either in drug substance or drug product is desirable. For low-level impurities, higher variations may be acceptable.

Analysis Repeatability

Determination, expressed as the RSD, consists of multiple measurements of a sample by the same analyst under the same analytical conditions.

For practical purpose, it is often combined with accuracy and carried out as a single study.

Intermediate Precision

Intermediate precision was previously known as part of ruggedness. The attribute evaluates the reliability of the method in a different environment other than that used during development of the method. The objective is to ensure that the method will provide the same results when similar samples are analyzed once the method development phase is over. Depending on time and resources, the method can be tested on multiple days, analysts, instruments, etc.

Intermediate precision in the test method can be partly assured by good system suitability specifications. Thus, it is important to set tight, but realistic, system suitability specifications.

Linearity

The linearity of a method is its ability to obtain test results that are directly proportional to the analyte concentration over a given range. For HPLC methods, the relationship between analyte concentration and detector response (peak area or height) is used to make this determination.

Concentration Ranges

The concentration range used for linearity should be large enough to encompass the desired range of the method. A minimum of five concentration ranges should be investigated and a plot of the detector response vs. the sample concentration should be generated. It is important that the concentration ranges selected for the linearity study are relatively equally spaced throughout the range of the method (e.g., 50%, 75%, 100%, 125% and 150%), and not clustered, as this will provide a skewed estimation of linearity.

Acceptance Criteria

Acceptance criteria should be evaluated to ensure that they are meaningful when compared with the performance of the method. Table 6 gives a list of suggested acceptance criteria for use in evaluating method linearity. The ranges in Table 6 are suggestions only and should be adjusted to ensure that all specification limits are within the validated linear range for any given method. Under most circumstances, regression coefficient (r) is 0.999. Intercept and slope should be indicated.

Statistical Analysis

Linearity data should be evaluated using appropriate statistical methods. A simple regression line of the detector response *vs* the analyte concentration is the most common means of evaluation. Regulatory agencies require the submission of the correlation coefficient, y-intercept, slope of the regression line, and the residual sum of squares for linearity evaluation. A graphical representation of the linearity data should also be generated. Additional analysis of the deviation of the actual values from the regression line is suggested, especially when the method uses a single-point calibration standard. The percent y-intercept is calculated by dividing the y-intercept by the detector response at the nominal concentration expressed as a percentage. For single-point calibration, this value should be less than 1-2% to ensure accurate results.

Table 5

Test	Level	Range	Acceptance criteria
Assay	5	50% to 150%	R> 0.999,
Dissolution	5-8	10% to 150%	R > 0.99,
Impurity	5	LOQ to 2%	R > 0.98

Limit of Detection

These limits are normally applied to related substances in the drug substance or drug product. Specifications on these limits are submitted with the regulatory impurities method relating to release and stability of both drug substance and drug product.

Limit of detection is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. With UV detectors, it is difficult to assure the detection precision of low-level compounds due to potential gradual loss of sensitivity of detector lamps with age, or noise level variation by detector manufacturer. At low levels, assurance is needed that the detection and quantitation limits are achievable with the test method each time. With no reference standard for a given impurity or means to assure detectability, extraneous peak(s) could "disappear/appear." A crude method to evaluate the feasibility of the extraneous peak detection is to use the percentage claimed for detection limit from the area counts of the analyte. Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental.

Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

Based on Signal-to-Noise

This approach can only be applied to analytical procedures which exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

Based on the Standard Deviation of the Response and the Slope

The detection limit (DL) may be expressed as:

$$DL = \frac{3.3\ \sigma}{S}$$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

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

Based on the Standard Deviation of the Blank

Analyzing an appropriate number of blank samples and calculating the standard deviation of these responses perform measurement of the magnitude of analytical background response.

Based on the Calibration Curve

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

Recommendations

The detection limit and the method used for determining the detection limit should be presented. If DL is determined based on visual evaluation or based on signal to noise ratio, the presentation of the relevant chromatograms is considered acceptable for justification.

In cases where an estimated value for the detection limit is obtained by calculation or extrapolation, this estimate may subsequently be validated by the independent analysis of a suitable number of samples known to be near or prepared at the detection limit.

Limit of Quantification

Limit of quantitation is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. Several approaches for determining the quantitation limit

are possible, depending on whether the procedure is a non-instrumental or instrumental.

Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. The quantitation limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

Based on Signal-to-Noise Approach

This approach can only be applied to analytical procedures that exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

Based on the Standard Deviation of the Response and the Slope

The quantitation limit (QL) may be expressed as:

$$QL = \frac{10\,\sigma}{S}$$

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of s may be carried out in a variety of ways.

Based on Standard Deviation of the Blank

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

Based on the Calibration Curve

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

Recommendations

The quantitation limit and the method used for determining the quantitation limit should be presented. The limit should be subsequently validated by the analysis of a suitable number of samples known to be near or prepared at the quantitation limit. Otherwise the information that is expressed as % area or height of the drug substance peak from the same HPLC chromatogram will be biased. It should also be noted that the extraneous peak using area count does not consider the detection response that depends on the UV extinction coefficient or absorptivity of the compound.

Ruggedness

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as different laboratories, analysts, instruments, reagents, elapsed assay times, assay temperatures, or days. It is normally expressed as the lack of influence on test results of operational and environmental variables of the analytical method. Method Ruggedness is defined as the reproducibility of results when the method is performed under actual use conditions. Method ruggedness may not be known when a method is first developed, but insight is obtained during subsequent use of that method.

Recommendations

The ruggedness of an analytical method is determined by analysis of aliquots from homogeneous lots in different laboratories, by different analysts, using operational and environmental conditions that may differ but are still within the specified parameters of the assay. The degree of reproducibility of test results is then determined as a function of the assay variables. This reproducibility may be compared to the precision of the assay under normal conditions to obtain a measure of the ruggedness of the method.

Robustness

ICH defines robustness as a measure of the method's capability to remain unaffected by small, but deliberate variations in method parameters. Robustness can be partly assured by good system suitability specifications. The evaluation of robustness should be considered during the development phase and depends on the

type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters. If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used.

Examples of typical variations are:

- Stability of analytical solutions
- Extraction time

In the 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.

In the case of gas chromatography, examples of typical variations are

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

Recommendations

Data obtained from studies for robustness, though not usually submitted, are recommended to be included as part of method validation.

General Recommendation:

System suitability testing is essential for the assurance of the quality performance of the chromatographic system. The amount of testing required will depend on the purpose of the test method. For dissolution or release profile test methods using an external standard method, k', T and RSD are minimum recommended system suitability tests. For acceptance, release, stability, or impurities/degradation methods using external or internal standards, k', T, R_S and RSD are recommended as minimum system suitability testing parameters. In practice, each method submitted for validation should include an appropriate number of system suitability tests defining the necessary characteristics of that system. Additional tests may be selected at the discretion of the applicant or the reviewer.

HPLC methods for drug substance and drug product, methods should not be validated as a one-time situation, but methods should be validated and designed by the developer or user to ensure ruggedness or robustness throughout the life of the method. The variations due to the drug product manufacturing process, the laboratory sample preparation procedure and the instrument performance contribute to the accuracy of the data obtained from the analysis. With proper validation and tight chromatographic performance (system suitability) criteria, an improvement in the reliability of the data can be obtained. Variations except from the drug product-manufacturing process will be minimized only with good reliable validated methods can data that are generated for release, stability, and pharmacokinetic is trust-worthy.

LITERATURE REVIEW

RP-HPLC estimation of ramipril and telmisartan in tablets, *Kurade .V.P et at.,* (2009),

A rapid high performance liquid chromatographic method has been developed and validated for the estimation of ramipril and telmisartan simultaneously in combined dosage form. A Genesis C18 column having dimensions of 4.6×250 mm and particle size of 5 µm in isocratic mode, with mobile phase containing a mixture of 0.01 M potassium dihydrogen phosphate buffer (adjusted to pH 3.4 using orthophosphoric acid): methanol:acetonitrile (15:15:70 v/v/v) was used. The mobile phase was pumped at a flow rate of 1.0 ml/min and the eluents were monitored at 210 nm. The selected chromatographic conditions were found to effectively separate ramipril (R_t : 3.68 min) and telmisartan (R_t : 4.98 min) having a resolution of 3.84. The method was validated in terms of linearity, accuracy, precision, specificity, limit of detection and limit of quantitation. Linearity for ramipril and telmisartan were found in the range of 3.5-6.5 µg/ml and 28.0-52.0 µg/ml, respectively. The percentage recoveries for ramipril and telmisartan ranged from 99.09-101.64% and 99.45-100.99%, respectively. The limit of detection and the limit of quantitation for ramipril was found to be 0.5 µg/ml and 1.5 µg/ml respectively and for telmisartan was found to be 1.5 µg/ml and 3.0 µg/ml, respectively. The method was found to be robust and can be successfully used to determine the drug content of marketed formulations.

Development and Validation of RP-HPLC Method for Simultaneous Estimation of Ramipril, Telmisartan and Hydrochlorothiazide in Pharmaceutical Dosage Forms, *Kalyan Kumar .B et al.,* (2011)

A new simple, accurate, rapid and precise isocratic High performance liquid chromatographic (HPLC) method was developed and validated for the determination of Hydrochlorothiazide (HCTZ), Ramipril (RAM) and Telmisartan (TEL) in tablet formulation. The Method employs Waters HPLC system on XTerra RP8 Column (4.6 x 150 mm and 3.5 Âµm) and flow rate of 0.8 ml/min with a load of 20Âµl. Acetonitrile and Phosphate buffer was used as mobile phase in the composition of 45:55. The Detection was carried out at 215 nm. Linearity ranges for Hydrochlorothiazide, Ramipril and Telmisartan were 12.5-22.5 Âµg/ml, 5-9Âµg/ml and 40-72Âµg/ml respectively. Retention Time of Hydrochlorothiazide, Ramipril and Telmisartan were found to be 2.83 min, 3.65 min and 5.03 min respectively. Percent Recovery study values of HCTZ, RMP and TEL were found to be within 98-102 %.This newly developed method was successfully utilized for the Quantitative estimation of Hydrochlorothiazide, Ramipril and Telmisartan in pharmaceutical dosage forms. This method was validated for accuracy, precision, linearity and Robustness as per ICH guidelines.

RP-HPLC Method for Simultaneous Estimation of Telmisartan and Ramipril in Tablet Dosage Form, *Harikridhnan .N et al* .,(2011)

A simple, selective, rapid, precise and economical reverse phase HPLC method has been developed for the simultaneous estimation of telmisartan and Rampiril in tablet formulation. Chromatography was performed on a ODS Hypersil C18 (25cm x 4.6 mm i.d., 5µ) column from thermo in isocratic mode with a mobile phase consisting of acetonitrile: 10mM KH2PO4 PH 3.0 (40:60). The flow rate was 1.0 ml/min and eluent was monitored at 245 nm; the selected chromatographic conditions were found to be effectively separate telmisartan (RT- 5.35 min) and

ramipril (RT-10.30 min). Linearity for telmisartan and ramipril were found in the range of 16-24 µg/ml and 2-3 µg/ml, repectively. The proposed method can be used forthe simultaneous estimation of these drugs in tablet formulation.

D.Isocratic Rp-Hplc Method Development And Validation For The Simultaneous Estmation Of Ramipril And Telmisartan In Tablet Dosage Form ***Manju Latha et al*** **.,(2012)**

An RP-HPLC method has been developed for the simultaneous estimation of ramipril and telmisartan in tablet dosage forms. The developed method was validated as per ICH guidelines and specificity, linearity & range, accuracy, precision and robustness was performed. Specificity was determined by comparing the results obtained by running the placebo solution with that of standard and method was found to be specific due to no interference between placebo peaks and drugs peaks. Linearity range was found to be 4 to16 µg/ml and 32 to 128 µg/ml of ramipril and telmisartan respectively. The method was found to be linear in the range of4 to 16µg/ml and 32 to128 µg/ml for ramipril and telmisartan respectively. In the linearity study, regression equation and coefficient of correlation for ramipril and telmisartan were found to be (y = 924480x - 151831, r =0.9997) and (y = 2901878.3558x + 3803877, r = 0.9996) respectively. This newly developed method was successfully utilized for the simultaneous estimation of ramipril and telmisartan in pharmaceutical tablet dosage forms.

Simultaneous Estimation of Ramipril and Telmisartan in Tablet Dosage Form by Spectrophotometry, ***Popat B.Mohite et al*** **.,** ***(2010)*****.**

Two simple, accurate, sensitive and specific methods are described for the simultaneousdetermination of Ramipril and Telmisartan in binary mixture. The method based on UVspectrophotometricdetermination of two drugs, Method A is by using multicomponant method. Itinvolves absorbance measurement at 205.0 nm(λmax of Ramipril) and 291.0 nm (λmax ofTelmisartan) in 0.2M H2SO4;. Beer's law is obeyed in the concentration range of 5-40 µg mL-1 forRamipril and 2-20 µg mL-1 for Telmisartan. Method B is graphical absorbance method which isbased on measurement of absorbance of Ramipril and Telmisartan at 222.0nm (iso-absorptivepoint of Ramipril and Telmisartan) and 291.0 nm (λmax of Telmisartan) Both these methods havebeen successively applied to pharmaceutical formulation and were validated according to ICHguidelines.

Simultaneous Determination of Ramipril, Hydrochlorothizide and Telmisartan by Spectrophotometry, *Tapadiya.G.G et. al*., (*2009).*

A simple, fast and precise multicomponent mode analysis method has been developed for simultaneous determination of Ramipril (RMP), Hydrochlorothiazide (HCT) and Telmisartan (TEL) in tablet formulation. The wavelengths selected for these drugs were 218nm, 271nm and 296nm respectively using methanol as solvent. The linearity for these drugs at all the selected wavelengths lies between 0.5-3.5 _gml-1for RP, 1.25-8.75 _gml-1for HCT and 4-28 _gml-1for TEL. Theconcentrations of these drugs were evaluated in laboratory mixture and marketed formulation. Accuracy was determined byrecovery studies from tablet dosages forms and ranges from 99.09-99.52%. Precision of method was find out as repeatability,day to day and analyst to analyst variation and shows the values within acceptable limit (R.S.D. _ 2 percent).

RP-HPLC Method for Simultaneous Estimation of Hydrochlorothiazide,Ramipril and Telmisartan in Tablet Dosage Form, ***Anandkumar R. Tengli et al*** **.,*(2012).***

A simple, sensitive and specific liquid chromatographic method with UV detection was developed for the simultaneous estimation of hydrochlorothiazide, ramipril and telmisartan in tablet dosage form and bisoprolol as internal standard. Separation was achieved with an phenomenexluna 5µ C18(2) 100R, 250X4.60 mm 5 micron size column, ambient temperature with a low pressure gradient mode with mobile phase containing acetonitril, 0.5% of potassium dihydrogen phosphate buffer pH 3.5 adjusted with orthophosphoric acid (50:50). The flow rate was 1 mL min-1 and eluent was monitored at 220 nm. The selected chromatographic conditions were found to effectively separate hydrochlorothiazide, ramipril and telmisartan with retention time of 3.1, 5.0 and 13.8 min respectively. The linearity range of hydrochlorothiazide, ramipril and telmisartan found in the range of 2-10 µgml-1, 5-25 µgml-1 and 10-50 µgml-1 respectively. The proposed method was found to be accurate, precise, reproducible and specific and it can also be used for routine quality control analysis of these drugs in combination tablets.

Development and Validation of HPTLC Method for the Simultaneous Estimation of Telmisartan and Ramipril in Combined Dosage Form, ***Patel*** **.V .A** ***et al*** **.,*(2010).***

Developed and validated thin layer liquid chromatography (TLC) method for the Simultaneous estimation of telmisartan and ramipril in a combined dosage form. Procedure does not require prior separation of components from the sample. Telmisartan and Ramipril were determined by High Performance Thin Layer

chromatography method (HPTLC) in tablet dosage form. The method was carried out in TLC Precoated silica gel on aluminum plate 60 F 254, (10 cm ×10 cm, prewashed by methanol and activated at 60° C for 5 min prior to chromatography). The solvent system was Acetone: Benzene: Ethyl acetate: Glacial acetic acid in the proportion of 5:3:2:0.03, (v/v/v/v) with Rf Value for telmisartan and ramipril was 0.673 and 0.353 respectively. The linearity regression analysis for calibration showed 0.996 and 0.998 for telmisartan and ramipril with respect to peak area and height in the concentration range of 100- 1800 ng/spot and 300-1800 ng/spot respectively. The method developed can be used for routine analysis of drugs content in tablet dosage form

Simultaneous estimation of telmisartan and ramipril in combined dosage form by using HPTLC*, Sunil Singh et al*., *(2012).*

In this paper describes developed and validated thin layer liquid chromatography (TLC) method for the simultaneous estimation of telmisartan and ramipril in a combined dosage form. Telmisartan and Ramipril were determined by High Performance Thin Layer chromatography method (HPTLC) in tablet dosage form. The method was carried out in TLC Precoated silica gel on aluminum plate 60 F 254, (10 cm ×10 cm, prewashed by methanol and activated at 60° C for 5 min prior to chromatography). The solvent system was Acetone: Benzene: Ethyl acetate: Glacial acetic acid in the proportion of 6:4:1:0.05, (v/v/v/v) with Rf Value for telmisartan and ramipril was 0.673 and 0.353 respectively. The linearity regression analysis for calibration showed 0.999 and 0.998 for telmisartan and ramipril with respect to peak area and height in the concentration range of 150- 1700 ng/spot and

300-1900 ng/spot respectively. The method developed can be used for routine analysis of drugs content in tablet dosage form.

simultaneous determination of Ramipril (RMP), Hydrochlorothiazide (HCT) and Telmisartan (TEL) in tablet formulation, *Bankey.S et. al.,* (*2011).*

A simple, fast and precise multicomponent mode analysis method has been developed for simultaneous determination of Ramipril (RMP), Hydrochlorothiazide (HCT) and Telmisartan (TEL) in tablet formulation. The wavelengths selected for these drugs were 218nm, 271nm and 296nm respectively using methanol as solvent. The linearity for these drugs at all the selected wavelengths lies between 0.5-3.5 gml-1 for RP, 1.25-8.75 gml-1 for HCT and 4-28 gml -1 for TEL. The concentrations of these drugs were evaluated in laboratory mixture and marketed formulation. Accuracy was determined by recovery studies from tablet dosages forms and ranges from 99.09-99.52%. Precision of method was find out as repeatability, day to day and analyst to analyst variation and shows the values within acceptable limit (R.S.D. 2 percent).

stability-indicating HPTLC method has been developed for the quantitative simultaneous estimation of Telmisartan and Ramipril in combined pharmaceutical dosage form and validation. *Sivasubrahmanian, et al.,(2010),*

A simple, selective, rapid, precise and stability-indicating HPTLC method has been developed for the quantitative simultaneous estimation of Telmisartan and Rampril in combined pharmaceutical dosage form and validation was done. The proposed HPTLC method involves the use of HPTLC plates (Merck) precoated with silica gel 60F254 on aluminium sheets and a mobile phase comprising of toluene:

acetonitrile: formic acid: water (5:5:0.3:1) Densiometric analysis of both the drugs was carried out in the absorbance mode at 212 nm. This method has been successfully applied for estimation of Telmisartan and Ramipril in combined tablets formation. Both the drugs were subjected to acid-alkali hydrolysis, oxidation and photolytic degradation and both of them were found to be susceptible to acid-alkali hydrolysis, oxidation and photolytic degradation. Linearity of Telmisartan was found to be within the range of 500-2500 ng/spot and for Ramipril the range was found to be 250-1250 ng/spot, with significant high values of correlation coefficient for both the drugs. The method was validated for the precision, robustness and recovery. As method could effectively separate the drug from its degradation products, it can be employed as a stability indicating one.

Isocratic rp-hplc-uv method development and validation forthe simultaneous estmation of ramipril and telmisartan in tablet dosage form, *Yogesh et al* .,(2009),

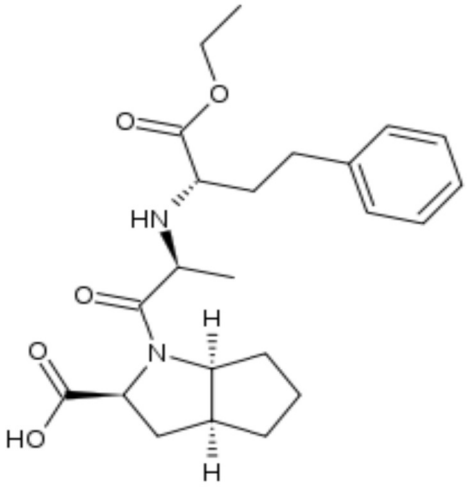
An RP-HPLC method has been developed for the simultaneous estimation of ramipril and telmisartan in tablet dosage forms, using UV-detector. The developed method was validated as per ICH guidelines and specificity, linearity& range, accuracy, precision and robustness was performed. Specificity was determined by comparing the results obtained by running the placebo solution with that of standard and method was found to be specific due to no interference between placebo peaks and drugs peaks. Linearity range was found to be 4 to 16 µg/mland 32 to 128 µg/ml of ramipril and telmisartan respectively. The method was found to be linear in the

range of 4 to 16 µg/ml and 32 to 128 µg/ml for ramipril and telmisartan respectively. In the linearity study, regression equation and coefficient of correlation for ramipril and telmisartan were found to be (y = 924480x - 151831, r = 0.9997) and (y = 2901878.3558x + 3803877, r = 0.9996) respectively. This newly developed method was successfully utilized for the simultaneous estimation of ramipril and telmisartan in pharmaceutical tablet dosage.

DRUG PROFILE

Drug Name : Ramipril

Molecular structure :



IUPACNAME : (2*S*, 3*aS*, 6*aS*) - 1 - [(2*S*) - 2 - {[(2*S*) - 1 - ethoxy - 1 - oxo - 4 - phenylbutan - 2 - yl]amino} propanoyl] - octahydrocyclopenta [*b*] pyrrole - 2 - carboxylic acid

Molecular formula **:** $C_{23}H_{32}N_2O_5$

Molecular weight **:** 416.511 g/mol

Category **:** **Anti-hypertensive**

Description **:** A white powder

Solubility **:** It is practically insoluble in water and freely soluble In methanol

Melting point **:** 109ºc

Purity **:** 99.90%

Mechanism of action: ACE inhibitors, as the name suggests, inhibit the actions of angiotensin converting enzyme (ACE), thereby lowering the production of angiotensin II and also decreasing the breakdown of bradykinin. The decrease in angiotensin II results in relaxation of arteriole smooth muscle leading to a decrease in total peripheral resistance, reducing blood pressure as the blood is pumped through larger diameter vessels. Its effect on bradykinin is responsible for the dry cough side effect. Ramipril, a prodrug, is converted to the active metabolite ramipril at by liver esterase enzymes Ramipril is mostly excreted by the kidneys. The half-life of ramiprilat is variable (3–16 hours), and is prolonged by heart and liver failure, as well as kidney failure.

Dosage: Doses in the ranges of 1.25, 2.5, 5.0 and 10 mg are available.

Pharmacokinetic data:

Absorption: oral

Distribution: Protein binding 56% -73% (ramipril)

Bioavailability: 28%

Metabolism: Hepatic

Half Life: 2 to 4 hours

Route of elimination: Renal (60%) and fecal (40%)

Therapeutic Uses: Indications for its use include:

- Hypertension;

- Congestive heart failure,
- Following heart attack in patients with clinical evidence of heart failure;
- Susceptible patients over 55 years: prevention of heart attack, stroke, cardiovascular death or need of revascularization procedures.
- Diabetic nephropathy with microalbuminuria
- **Adverse effects:** low blood sugar (in patients taking medication for diabetes), causing sweating or shakiness
- dry cough
- dizziness and light-headedness due to low blood pressure
- tiredness and fatigue, especially in the early stages
- mouth dryness in the early stages
- nausea, vomiting, diarrhea (persistent in rare cases)
- fainting
- change in amount of urine
- signs of infection (e.g., fever, chills, persistent sore throat)
- yellowing of eyes or skin, dark urine
- stomach or abdominal pain

- neutropenia (low white blood cells)
- impotence (erectile dysfunction)

Serious allergic reactions to this drug are unlikely, but immediate medical attention must be sought if they occur. Symptoms of a serious allergic reaction include, but are not limited to a rash or swelling of the face, mouth, tongue, or throat.

In extreme cases, ramipril may lead to potentially fatal liver problems.

Contraindicated Renovascular disease, severe renal impairment (especially in patients with one kidney or with bilateral renal artery stenosis), volume-depleted patients, history of angioedema while on an ACE inhibitor, pregnancy, hypotension

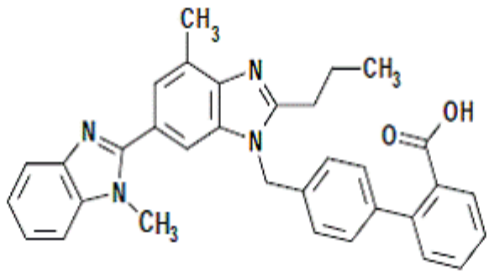
Drug interactions: esomeprazole, ramipril alcohol/food Interactions, warfarin, rosuvastatin, Drospirenone, Lithium, Potassium, Spironolactone, Tizanidine, Tobramycin, Treprostinil Triamterene

Storage: Keep this medication in the blister pack it came in, tightly closed, and out of reach of children. Store it at room temperature and away from excess heat and moisture (not in the bathroom). Throw away any medication that is outdated or no longer needed. Talk to your pharmacist about the proper disposal of your medication.

DRUG PROFILE

Drug Name : **Telmisartan**

Molecular structure :



IUPAC NAME : 4' - [(1,4' – dimethyl - 2' - propyl [2, 6' – bi - 1H -benzimidazol] - 1' - yl) methyl] - [1, 1' - biphenyl] - 2 - carboxylic acid

Molecular formula : $C_{33}H_{30}N_4O_2$

Molecular weight : 514.63 g/mol

Category : Anti-hypertensive

Description : A white to slightly yellowish solid

Solubility: It is practically insoluble in water and in the pH range of 3 to 9, sparingly soluble in strong acid (except insoluble in hydrochloric acid), and soluble in strong base

Melting point: 261-263ºc

Purity: 99.80%

Mechanism of action: Telmisartan is an angiotensin II receptor blocker that shows high affinity for the angiotensin I receptor type II (AT_1), with a binding affinity 3000 times greater for AT_1 than AT_2. It has the longest half-life of any ARB (24 hours) and the largest volume of distribution

In addition to blocking the RAs, telmisartan acts as a selective modulator of peroxisome proliferator-activated receptor gamma (PPAR-γ), a central regulator of insulin and glucose metabolism. It is believed that telmisartan's dual mode of action may provide protective benefits against the vascular and renal damage caused by diabetes and cardiovascular disease (CVD).

Dosage: 20-80 mg

Pharmacokinetic data:

Absorption: oral

Distribution: Protein binding ≥99.5%

Bioavailability: 42–100%

Metabolism: Minimal hepatic

Half Life: 24 hours

Route of elimination: Faecal 97%

Therapeutic Uses: Telmisartan is indicated in the treatment of essential hypertension.

Adverse effects: Side effects are similar to other angiotensin II receptor antagonists and include tachycardia and bradycardia (fast or slow heartbeat), hypotension (low blood pressure), edema (swelling of arms, legs, lips, tongue, or throat, the latter leading to breathing problems), and allergic reactions.

Contraindicated: Telmisartan is contraindicated during pregnancy. Like other drugs affecting the renin-angiotensin system (RAS), telmisartan can cause birth defects, stillbirths, and neonatal deaths. It should not be taken by breastfeeding women since it is not known whether the drug passes into the breast milk.

Drug interactions: digoxin, amiloride, ketoprofen

Storage: Keep this medication in the blister pack it came in, tightly closed, and out of reach of children. Store it at room temperature and away from excess heat and moisture (not in the bathroom). Throw away any medication that is outdated or no longer needed. Talk to your pharmacist about the proper disposal of your medication.

AIM AND PLAN OF WORK

From the literature survey conducted, it was found that there are few analytical methods reported for Ramipril and Telmisartan by reverse phase HPLC method alone or in combination with other drugs. Presently the new formulation of Ramipril and Telmisartan is available in market. There are only few methods reported for the determination of Ramipril and Telmisartan in combined pharmaceutical dosage form. So it was felt that there is a need to develop a sensitive analytical method for the simultaneous estimation of Ramipril and Telmisartan in Tablet dosage form available in market.

AIM:

To develop a sensitive, simple, rapid and accurate analytical method for the simultaneous estimation of **Ramipril and Telmisartan** in formulations and validation of developed method by using RP-HPLC technique.

OBJECTIVE:

1. The scope of developing and validating method is to ensure a suitable strategy for a particular analyte which is more specific, accurate and precise. Here the main focus is drawn to achieve improvement in conditions and standard operating procedures to be followed.
2. To develop a method that is rapid, sensitive and at the same time cost effective.

PLAN OF WORK:

The plan of proposed work includes the following steps:

a) Method development by RP-HPLC

- Selection of suitable wave length.
- Selection of chromatographic technique.
- Method development trails
- Optimization of chromatographic condition.

b) Quantitative Estimation of Ramipril and Telmisartan by the developed method.

c) Validation of developed method using following parameters as per the ICH guidelines.

- System suitability
- Linearity
- Precision
- Accuracy
- Robustness
- Limit of detection and Limit of quantification

MATERIALS AND METHOD DEVELOPMENT

MATERIALS AND INSTRUMENTS

Instruments	Name of the company
HPLC	Waters 2695 (isocratic system)
UV-VIS Spectrophotometer	Lab India UV 3000^{+}
Analytical Balance	Shimadzu electronic balance (0.1mg-220g)
Ultra Sonicator	Bandelin sonorex
Vacuum pump	Gelman science vacuum pump
pH meter	Elico

Table-6

Data Handling System: Empower-2 Software

REAGENTS AND CHEMICALS

Name of the Chemical	**Name of the company**
Methanol ,HPLC grade, Merck	Merck
Acetonitrile ,HPLC grade, Merck	Merck
Water (HPLC grade)	Merck
Potassium dihydrogen Ortho phosphate (HPLC grade)	Merck
Working standards of Ramipril and Telmesartan	Hetero Labs

Table-7

Drug samples

Ramipril and Telmisartan pure drugs were obtained as gift samples from Sipra labs, Hyderabad.

Reference standards

1. Ramipril - % purity – 99.9

2 .**Telmisartan -** % purity – 99.8

Formulation used

TAZLOC-R ™ tablets (Hetero Labs Ltd) containing 40mg of **Telmisartan** and 5 mg of **Ramipril** was procured from local market.

5.1 RP-HPLC Technique for the estimation of Ramipril and Telmisartan

The high performance liquid chromatography technique is broadly divided in to two categories namely normal phase and reverse phase. When the stationary phase used for chromatography is more polar than the mobile phase the technique in called as normal phase chromatography and when the stationary phase used is non-polar compared to the solvent used for mobile phase preparation the technique is called as reverse phase chromatography. The sample elution order in normal phase is least polar first whereas it is most polar first in reverse phase. The effect of increasing solvent polarity in normal phase reduces elution time whereas in reverse phase it increases elution time. HPLC has been most useful and versatile among all chromatographic techniques because of its wider applicability to different classes of compounds and the sensitivity levels provided. There has been a considerable amount of improvement in the hardware used since its innovation. Major improvement in the field has been due to change over to column with a 3-5µ particle size instead of 30µ column used in the earlier days. HPLC has found application in various sector of pharmaceutical industry. Various types of pharmaceutical analysis such as drug assay and dissolution testing of formulation and biological studies require a very versatile, sensitive and reliable technique like HPLC. Whereas ultra high resolution is the requirement for assay and stability studies, faster analysis with moderate resolution is the requirement for biostudies in a faster analysis with the higher resolution and better sensitivity. Various research teams have studied the effect of using different stationary phases, solvent strength effect and temperature has also been studied. Original developments carried out using normal phase chromatography on silica columns have been gradually replaced by reverse phase and separation due to the versatility associated with the latter technique.

The most column stationary phase has been used is the octadecylsilane chemically bonded to the silica using different bonding and end capping techniques, almost 50% of the total reverse phase separations carried out today are reported using this stationary phase. Although these stationary phases have a great potential in terms of the three most sought after parameters in chromatography namely separation, selectivity and speed, they have been hardly exploited for the purpose. Some research team have worked and reported various developed method for separation of different mixture, by controlling different parameters which help in achieving these goals. A practical approach for method development and method validation are considered greatly with respect to separation selectivity and speed.

Selection of column:

Initially different C_8 and C_{18} columns were tried for selected composition of mobile phase and quality of peaks were observed for the drugs. Finally the column was fixed upon the satisfactory results of various system suitability parameters such as column efficiency, retention time, tailing factor, peak, and asymmetry of the peaks.

Selection of Wavelength:

The wavelength for the analysis was selected from the UV spectrum of ramipril and telmisartan by scanning in the range of 200-400nm. From this, the wavelength of 230nm was selected for the RP-HPLC method as these drugs has shown good absorbances.**Fig.1**

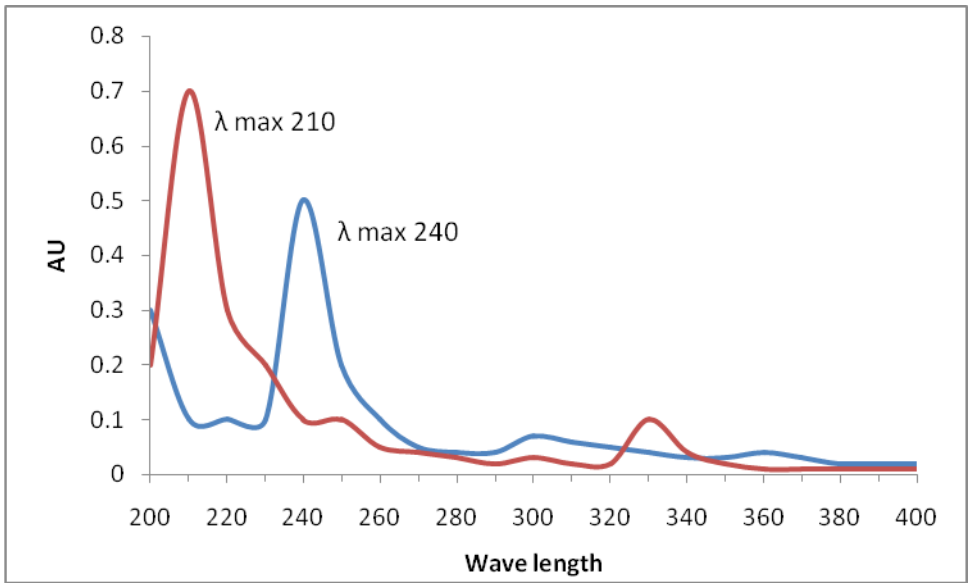


Fig.1. Overlain absorption spectra of Ramipril and Telmisartan in Methanol

Selection of mobile phase:

To develop a precise and robust HPLC method for simultaneous determination of Ramipril and telmisartan, its standard solution were injected in the HPLC system. After literature survey and solubility data different composition of mobile phase of different flow rates were employed in order to determine the best condition for effective separation of both drugs.

Selection of chromatographic technique:

The choice of the method is based on the nature of the sample. Ramipril and Telmisartan are freely soluble in solvents like methanol and acetonitrile and practically insoluble in water. So the reverse phase chromatographic technique was selected for the present study.

In RP-HPLC technique, generally C_{18} and C_8 columns are used as stationary phase. For the present study C_8 column is used as stationary phase.

Method development trails:

Preparation of Phosphate buffer:

Weighed 2.68 grams of KH_2PO_4 into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC water. Adjusted the pH to 5 with Orthophosphoric acid

Preparation of mobile phase

Mix a mixture of above buffer 700 ml (70%) and Acetonitrile 300 ml (30%) and degas in ultrasonic water bath for 5 minutes. Filter through 0.45 µ filter under vacuum filtration.

Method Development Trails

Different chromatographic conditions were tried to optimize the method, which include the following:

Trial 1:

Mobile phase : 20 mM phosphate buffer :Methanol: Acetonitrile (40:5:55%v/v)

Column : Zorbax SB C_8, 150 X 4.6mm, particle size 3.5µ

Flow rate : 0.8 ml/min

Temparature : a) column tempature:30^0c

b)sample temperature:25^0c

Detection : 230 nm

Retention time : 2.568 min

Result: Fronting of Ramipril and no resolution for Telmisartan was observed.

Trial 2:

Mobile phase : 20 mM phosphate buffer: Methanol: Acetonitrile (40:5:55%v/v)

Column : Zorbax SB C_8, 150 X 4.6mm, particle size 3.5µ

Flow rate : 1.0 ml/min

Temparature : a) column tempature:30^0c

b)sample temperature:25^0c

Detection : 230 nm

Retention time : 3.148 and 4.324 min

Result: Fronting of Ramipril peak and assymetric peak of Telmisartan was observed

Trial 3:

Mobile phase : 20 mM phosphate buffer: Methanol: Acetonitrile (45:25:30%v/v)

Column : Zorbax SB C_8, 150 X 4.6mm, particle size 3.5µ

Flow rate : 1.0 ml/min

Temparature : a) column tempature:30^0c

b)sample temperature:25^0c

Detection : 230 nm

Retention time : 3.215 and 5.073 min

Result: Fronting of Ramipril peak and asymmetric peak of Telmisartan was observed.

Trial 4:

Mobile phase : 20 mM phosphate buffer: Acetonitrile (55:45%v/v)

Column : Zorbax SB C_8, 150 X 4.6mm, particle size 3.5µ

Flow rate : 1.0 ml/min

Temparature : a) column tempature:30^0c

b)sample temperature:25^0c

Detection : 230 nm

Retention time : 2.057 and 2.253 min

Result: Peak broadening of Ramipril and Tailing of Telmisartan was observed.

Trial 5:

Mobile phase : 20 mM phosphate buffer: Acetonitrile (70:30%v/v)

Column : Zorbax SB C_8, 150 X 4.6mm, particle size 3.5µ

Flow rate : 1.0 ml/min

Temparature : a) column tempature:30^0c

b)sample temperature:25^0c

Detection : 230 nm

Retention time : 2.285 and 4.288

Result: Both Ramipril and Telmisartan peaks show good symmetry and with a resolution of 2.5.

5.2.2 OPTIMIZED CHROMATOGRAPHIC CONDITIONS

Stationary phase : Zorbax SB C_8, 150 X 4.6mm, particle size 3.5µ

Flow rate : 1.0ml/min

Temparature : a) column tempature:30^0c

b)sample temperature:25^0c

Selected wave length : 230 nm.

Mobile phase ratio : 20 mM phosphate buffer: Acetonitrile (70:30%v/v)

Diluent : mobile phase

Injection volume : 10 µl

Run time : 6min.

Preparation of standard solution

Weight accurately about 10 mg of ramipril 80 mg of telmisartan and transferred in to a clean 100 ml volumetric flask dissolved in few ml of methanol and make up to the volume with methanol. Sonicate for 10 minutes and filtered through membrane filter and marked as standard stock solution.

Preparation of sample solution

Weigh and powder 20 tablets, weight accurately a quantity of powder equivalent to 10 mg of ramipril and 80 mg of telmisartan and transfered it into a clean 100 ml standard flask. Add few ml of methanol and dissolved, make up the volume with methanol. The solution is sonicated for 10 minutes and filtered through membrane filter, and marked as sample stock solution.

Validation of the Method

Validation Report

Validation studies were carried out as per the protocol. The data is compiled and discussed in the subsequent part of this report under the relevant parameters.

System suitability parameters and System precision

The standard solution, prepared using ramipril and telmisartan working standard as per test method was injected six times into HPLC system.

The system suitability parameters and % relative standard deviation (RSD) for peak areas for six replicate injections was found to be with in limits.

System suitability parameters	Ramipril	Telmisartan
Tailing factor	1.427	1.831
Resolution	9.248	
No. of theoretical plates	5882	3045

Table: 8 System Suitability

Table: 9 System Precision

Linearity of Detector Response

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

Different Approaches for linearity determination are;

The first approach is to weigh different amounts of authentic sample directly to prepare linearity solutions of different concentrations.Another approach is to prepare a stock solution of high concentration, then perform serial dilution from the stock solution to obtain solutions of lower concentrations for linearity determination.Linearity was assessed by performing single measurement at several analyte concentrations. Varying quantities of the mixed standard stock solutions was diluted with the mobile phase to give concentration of 20 – 60 µg/ml of ramipril and 160 – 480 µg/ml of telmisartan.

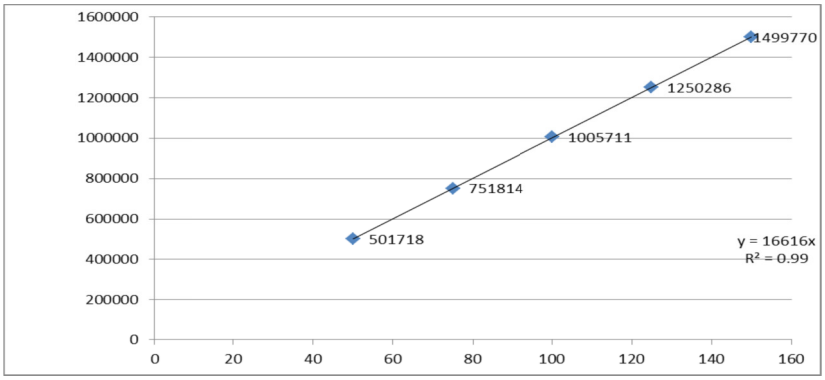


Fig.2.1. Ramipril

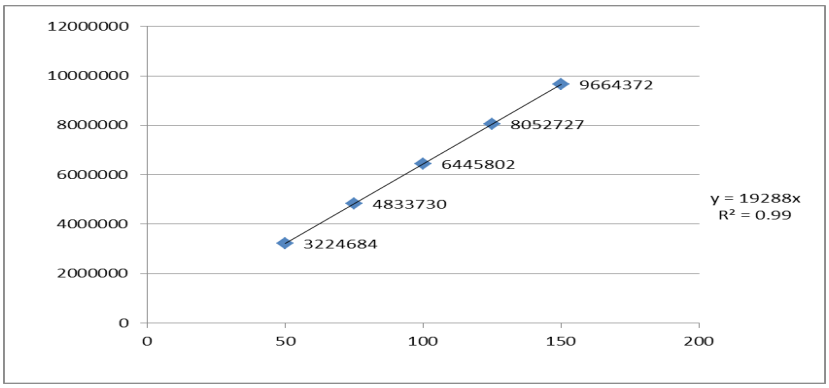


Fig.2.2. Telmisartan

S.No	Ramipril		Telmisartan	
	Concentration (µg / ml)	**Peak Area**	**Concentration (µg / ml)**	**Peak Area**
1	20 501718	160	3224684	
2	30	751814	240	4833730
3	40	1005711	320	6445802
4	50	1250286	400	8052727
5	60	1499770	480	9664372
	Slop	25003	**Slop**	20132
	Intercept	1439.1	**Intercept**	1754.9
	Correlation coefficient	0.9999	**Correlation coefficient**	1.0000

Table: 10 Linearity Test

Acceptance Criteria

The correlation coefficient should be not less than 0.999.

Precision of test method

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements from multiple sampling of the same homogenous sample under prescribed conditions. Precision may be considered at three levels.

- Repeatability
- Intermediate precision
- Reproducibility

Repeatability

Repeatability expresses the precision under the same operating conditions over a short internal of time.

Repeatability of a method can be determined by multiple replicate preparations of the same sample. This is can be done either by multiple sample preparation in the same experiment or by preparing 3 replicates at 3 different concentrations.

Intermediate precision

Intermediate precision expresses within laboratories variations, different days, different analysts, different equipments etc.

Reproducibility

Reproducibility expresses the precision between laboratories.

This is optional validation parameter that requires demonstration of laboratory-to-laboratory variation only if multiple laboratories use the same procedure.

Repeatability

The precision of test method was evaluated by assaying six samples of a single batch of ramipril and telmisartan tablet formulations.

Sample no.	**Area for Ramipril**	**Area for Telmisartan**	**Ramipril assay**		**Telmisartan assay**	
1	1000248	6441837	9.98	99	79.97	99.96
2	1006401	6439339	10.04	100.48	79.94	99.92
3	1002225	6468698	10.0	100.06	80.30	100.38
4	1004116	6442674	10.02	100.20	79.98	99.97
5	1003631	6416405	10.02	100.17	79.65	99.57
6	1003324	6441791	10.01	100.17	79.97	99.96
Average	1004843	6447719	10.01	100.15	79.97	99.96
Std. Deviation	0.827	0.712	0.655	0.2042	0.404	0.2574
%RSD	0.801	0.709	0.66	0.2038	0.4	0.2575

Table: 11 Method Precision

Drug	Amount (mg/tablet)		% Drug content	SD	% RSD*
	Label	Estimated			
Ramipril	10	10.01	100.15	0.2042	0.2038
Telmisartan	80	79.97	99.96	0.2574	0.2575

Table: 12 Estimation of formulation.

Acceptance criteria

The % RSD (Relative Standard Deviation) of assay result should be not more than 2.0%.

Accuracy

The accuracy of an 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.

Accuracy is often determined by recovery studies in which the analytes are spiked into a solution containing the matrix. The matrix should be found not to interfere with the assay of the compound of interest.

A recovery study of ramipril and telmisartan was conducted by mixing a known quantity of standard drug with the preanalyzed sample formulation and the contents were reanalysed by the proposed methods.

The preanalyzed formulation solution (10 µg / ml equivalent to ramipril) was spiked with different concentration of the standard solution of ramipril 5, 10, 15 µg / ml. The amount recovered was calculated.

The preanalyzed formulation solution (80 µg / ml equivalent to telmisartan) was spiked with different concentration of the standard solution of telmisartan 40, 80, 120 µg / ml. The amount recovered was calculated.

Samples solutions were placed in triplicate for each spike level and assayed as per the test method. The % recovery was found to be within the limits.

Drug	Amount added in (µg / ml)	Amount Found in (µg / ml)	Percentage recovery	SD	% RSD
Ramipril	5	4.99	99.80	0.2314	0.2306
	10	10.02	100.20	0.0545	0.0545
	15	15.02	100.10	0.4894	0.4223
Telmisartan	40	40.04	100.10	0.5627	0.5349
	80	80.09	100.11	0.4164	0.5382
	120	120.13	100.10	0.4089	0.5347

Table: 13 ACCURACY

Acceptance Criteria

% Recovery should be within 97.0 to 103.0% of the added amount.

ROUBUSTNESS

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

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature variation was made to evaluate the impact on the method.

	SampleName	**Inj**	**RT**	**Area**
Temperature1	Ramipril	1	2.273	1002292
Temperature2	Ramipril	1	2.282	996330
Flow rate 1	Ramipril	1	1.830	805697
Flow rate 2	Ramipril	1	3.030	1319197

Table-14

Component summary table

RAMIPRIL

	SampleName	**Inj**	**RT**	**Area**
Temperature1	Telmisartan	1	4.231	6329892
Temperature2	Telmisartan	1	4.262	6282947
Flow rate 1	Telmisartan	1	5.604	8354170
Flow rate 2	Telmisartan	1	3.490	5051669

Table-15

Component summary table

TELMISARTAN

Temparature-1: 25^0c , Temparature-2: 35^0c

Flowrate-1: 0.8 ,Flowrate-2: 1.2

Limit of Detection (L.O.D)

The LOD is the smallest concentration of the analyte that gives a measurable response (signal to noise ratio of 3). The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantified as an exact value. Detection limit corresponds to the concentration that will give a signal-to-noise ratio of 3:1.

The lowest amount of analyte in sample that can be detected, but not necessary quantified was determined by comparison of measured signal with 2.727 µg/ml and 2.990 µg/ml of ramipril and telmisartan standard solutions respectively with those of blank (Mobile phase).

s.no	Parameter	Name	Inj	RT	Area
1	LOD	Ramipril	1	2.280	427694
2	LOD Telmisartan	1	4.320	3141789	

TABLE-16
Component Summary Table for LOD
RAMIPRIL& TELMISARTAN

Limit of Quantitation (L.O.Q)

The LOQ is the smallest concentration of the analyte, which gives response that can be accurately quantified (Signal to noise ratio of 10). The quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be determined quantitatively with suitable precision and accuracy.

The Quantification limit is the concentration of related substances in the sample that will give a signal to noise ratio of 10:1.

The lowest amount of analyte in the sample that can be determined with acceptable precision and accuracy was determined by the comparison of measured signal with 9.091 µg/ml and 9.968 µg/ml of ramipril and telmisartan standard solution respectively with those of blank (mobile phase).

s.no	Parameter	Name	Inj	RT	Area
1	LOQ	Ramipril	1	2.279	540073
2	LOQ	Telmisartan	1	4.310	3868301

TABLE-17
Component Summary Table for LOQ
RAMIPRIL& TELMISARTAN

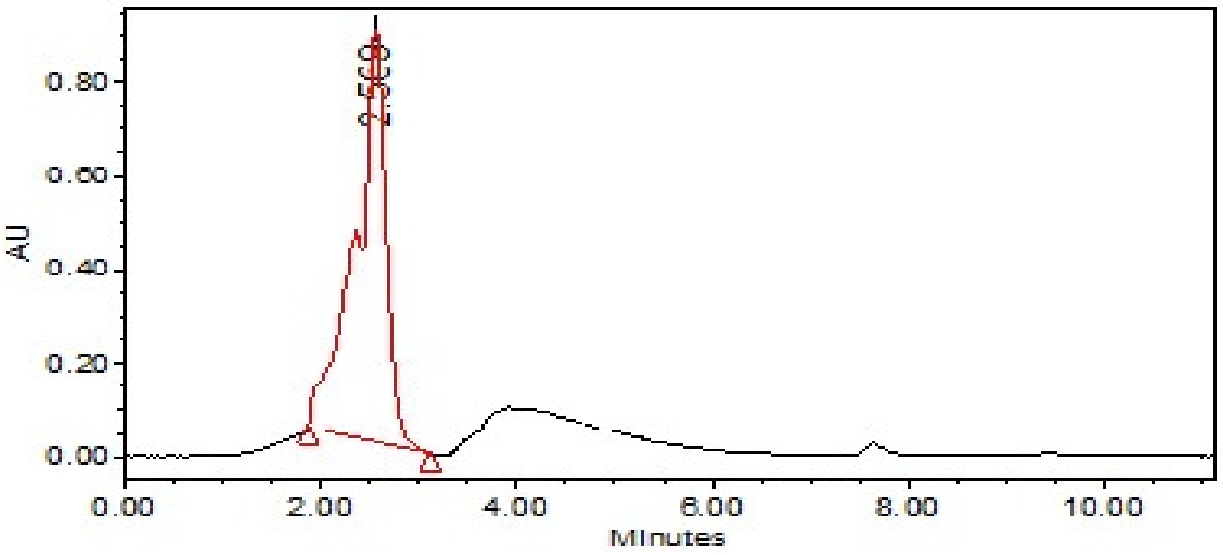


Fig: 3.1 Trail - 1

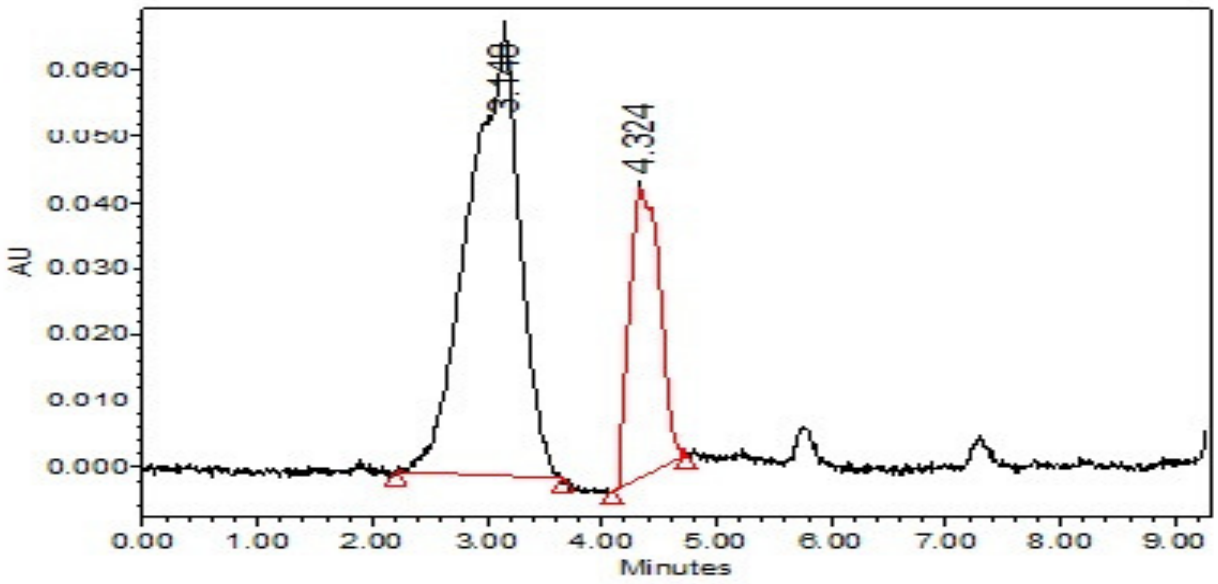


Fig: 3.2 Trail - 2

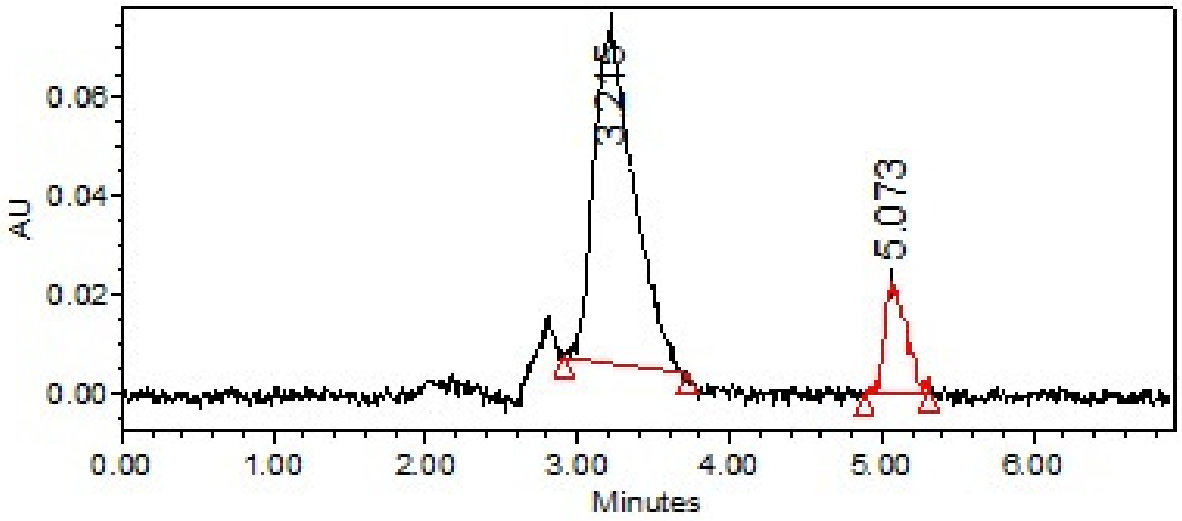


Fig: 3.3 Trail – 3

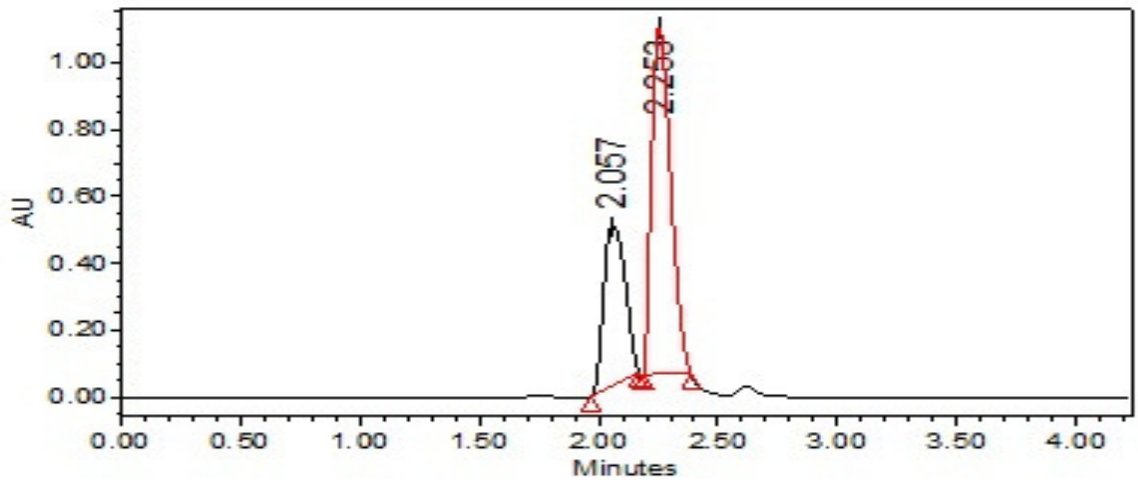


Fig: 3.4 Trail - 4

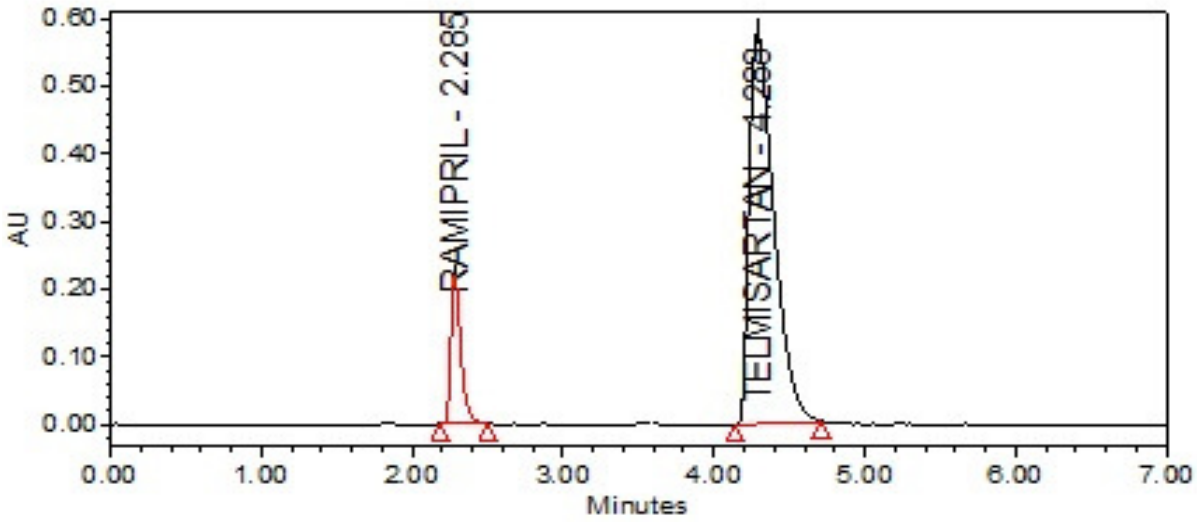


Fig: 3.1 Trail – 5

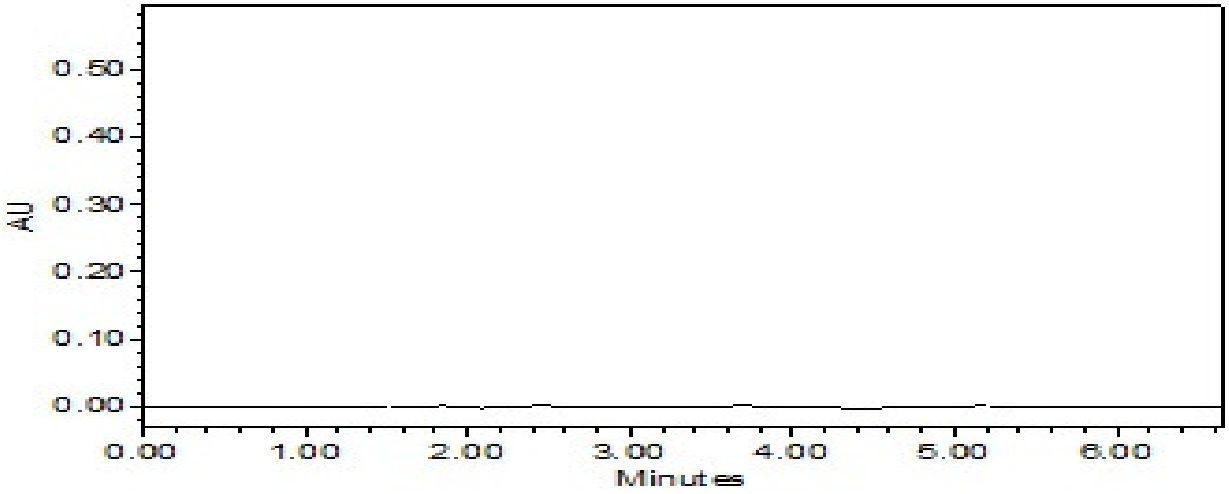


Fig: 4 Blank Mobile Phase

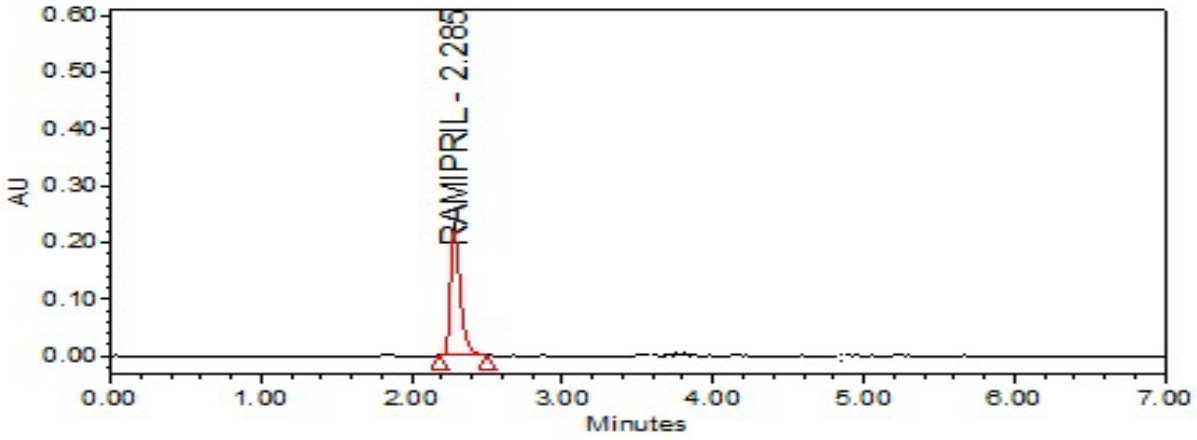


Fig: 5 Chromatogram of Ramipril standard

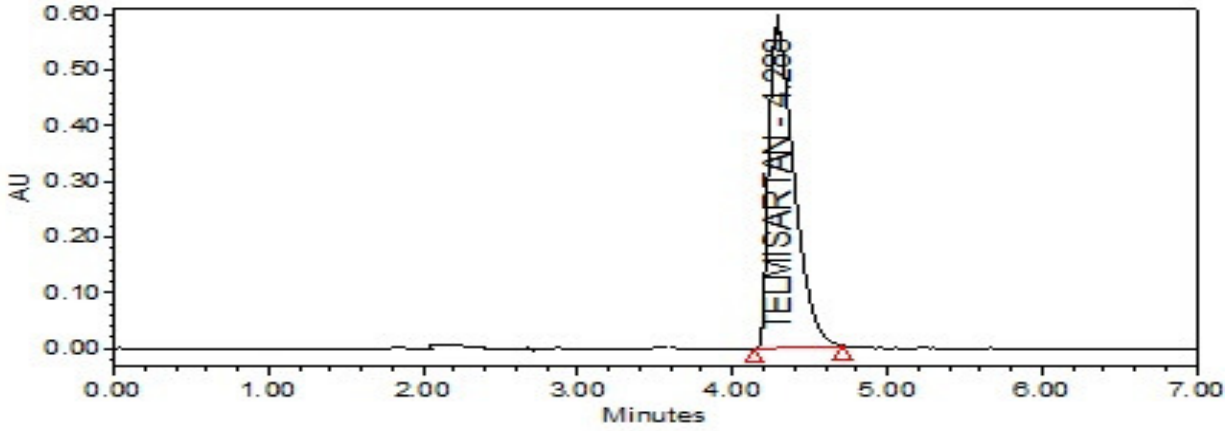


Fig: 6 Chromatogram of Telmisartan standard

SYSTEM SUITABILITY& SYSTEM PRECIESION

Fig: 7.1

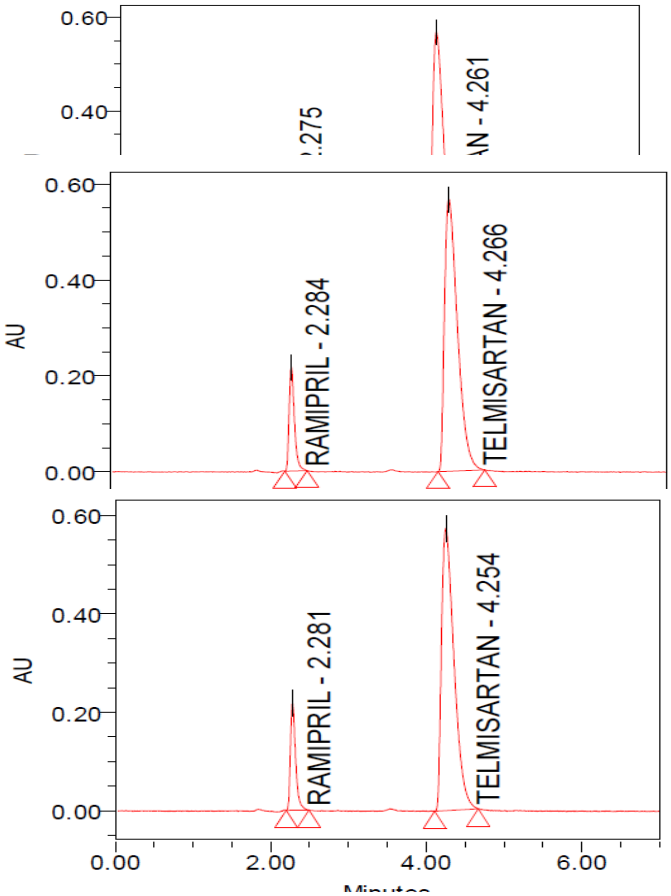


Fig: 7.3

Fig: 7.2

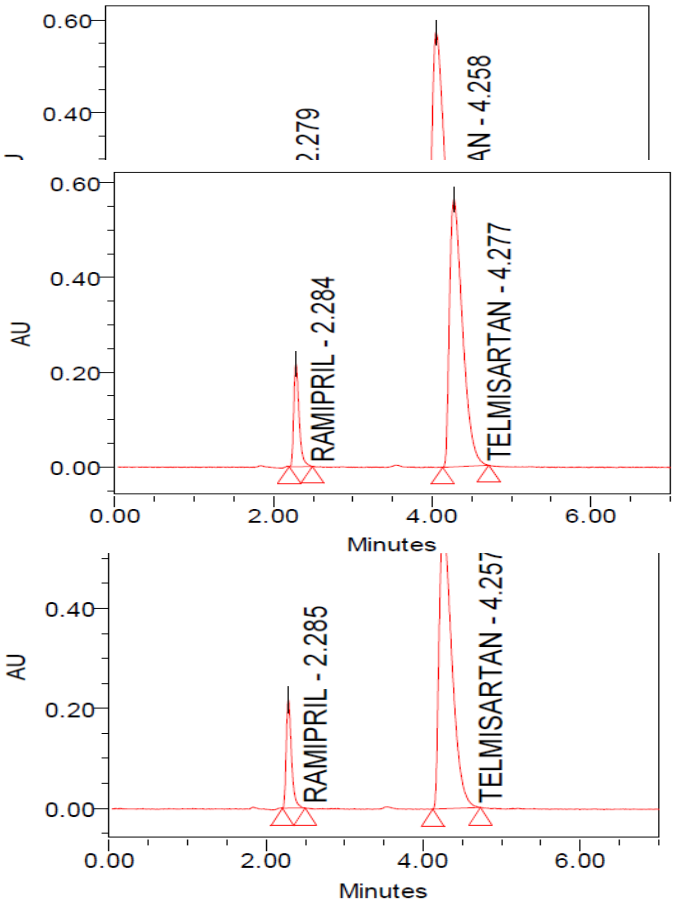


Fig: 7.4

Fig: 7.5

Fig: 7.6

LINEARITY

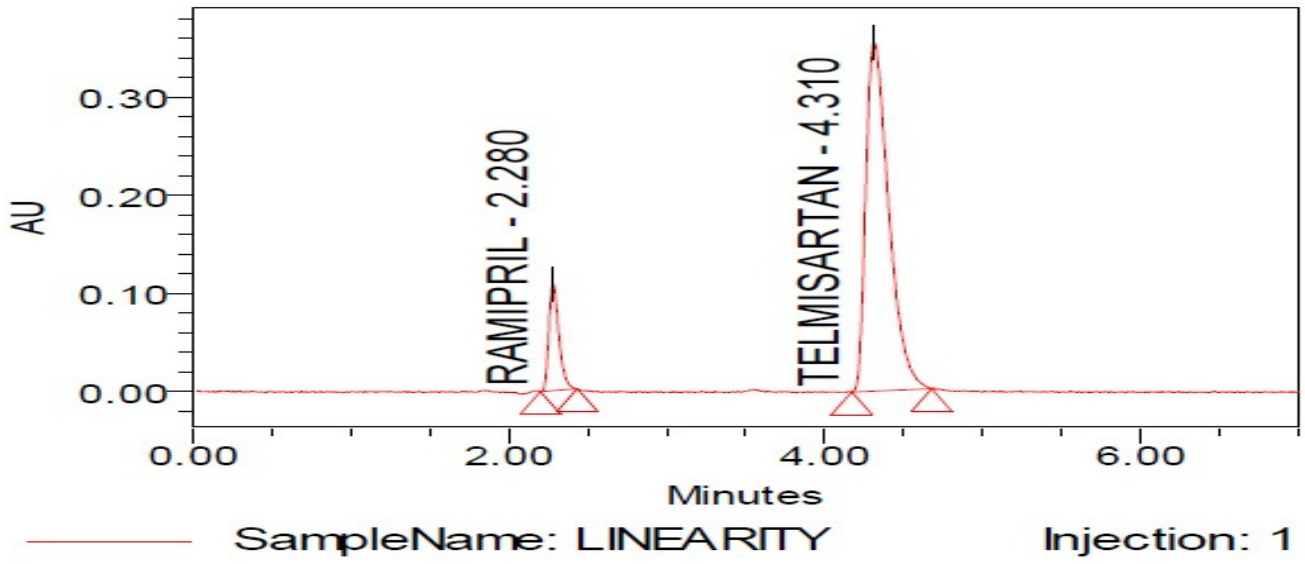


Fig: 8.1 Linearity -1

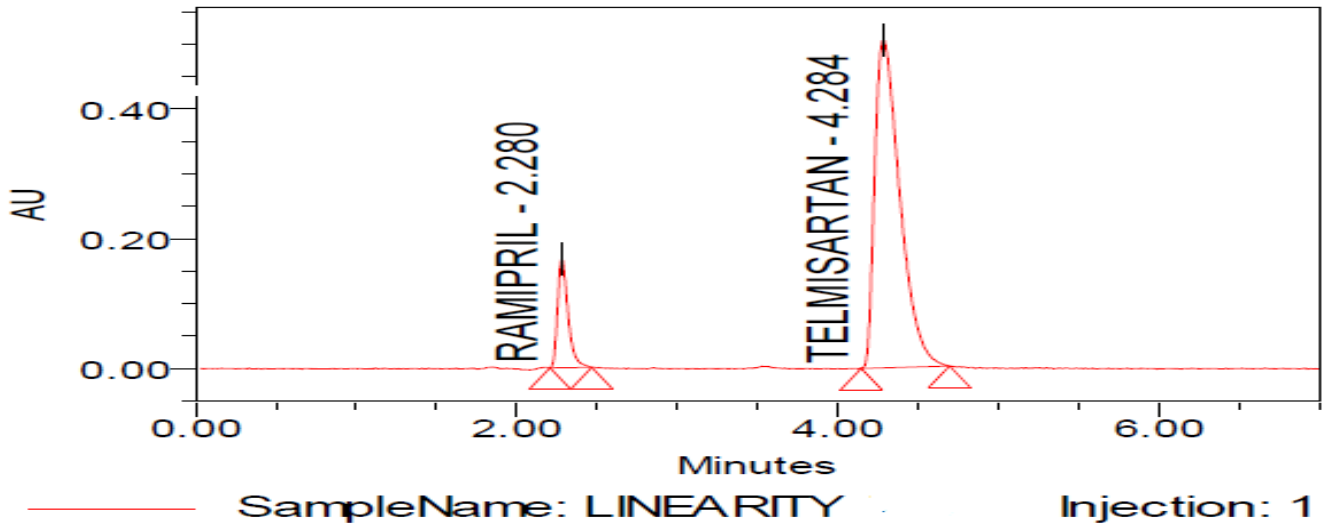


Fig: 8.2 Linearity -2

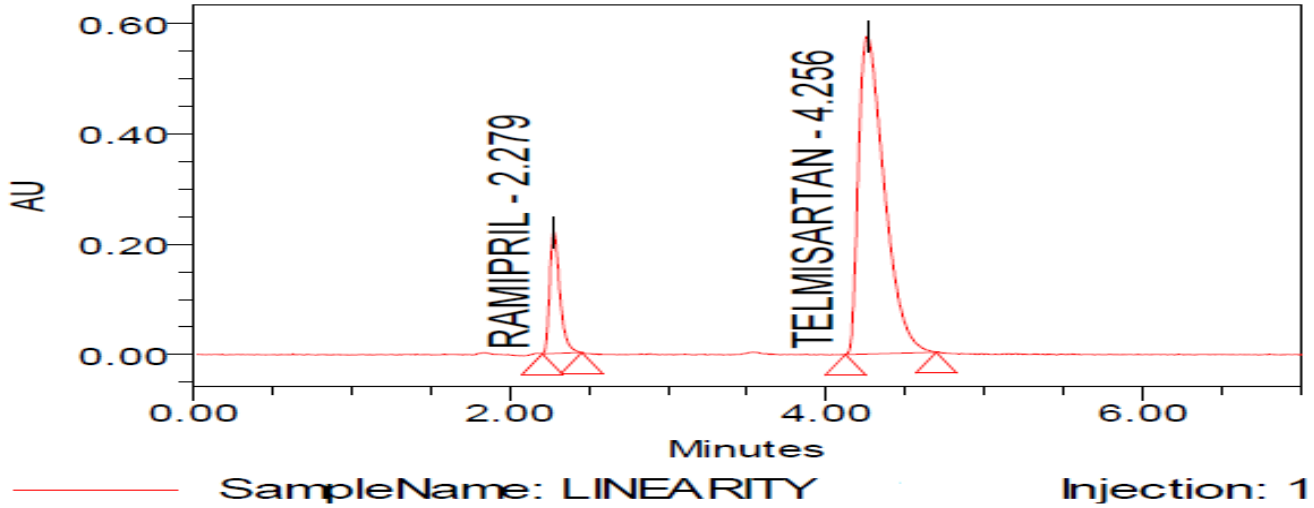


Fig: 8.3 Linearity -3

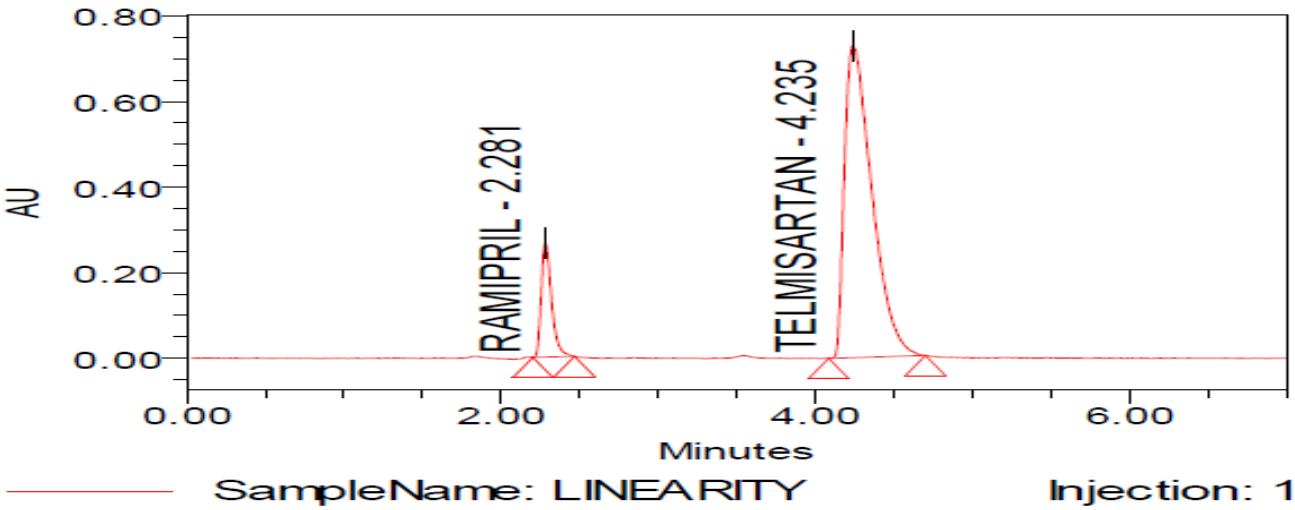


Fig: 8.4 Linearity - 4

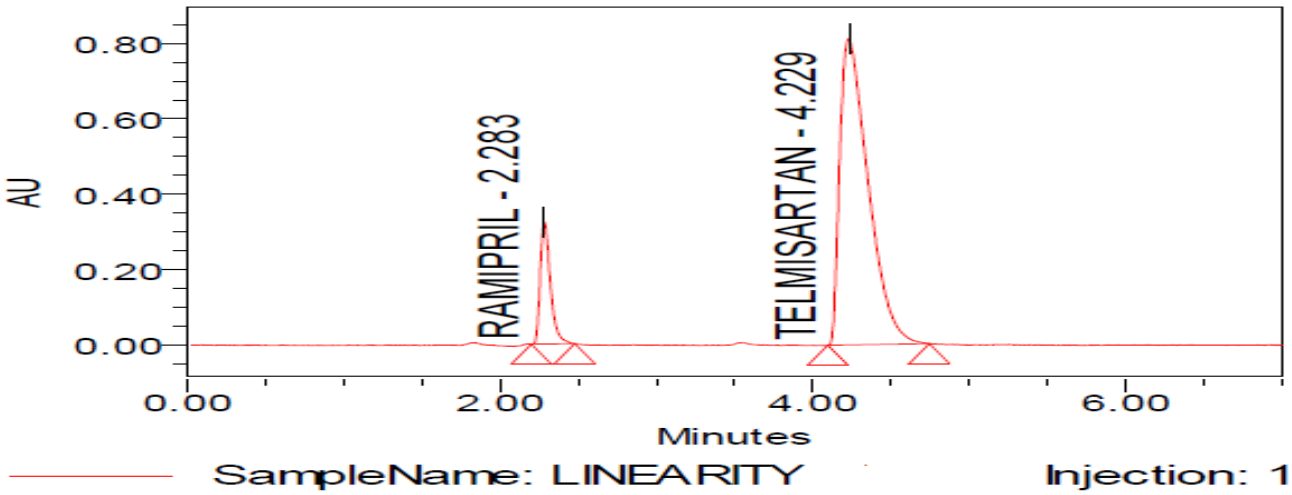
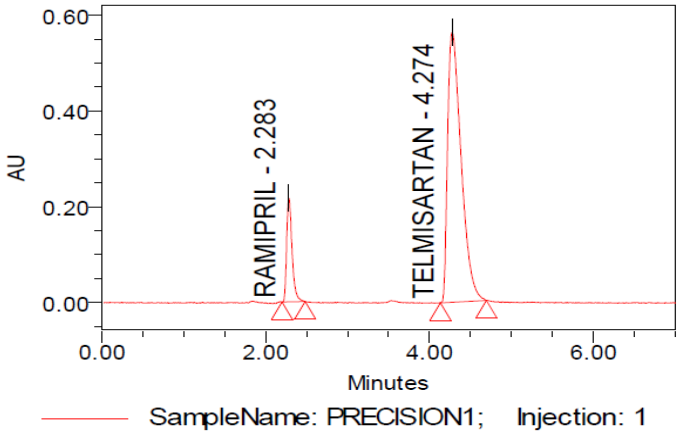
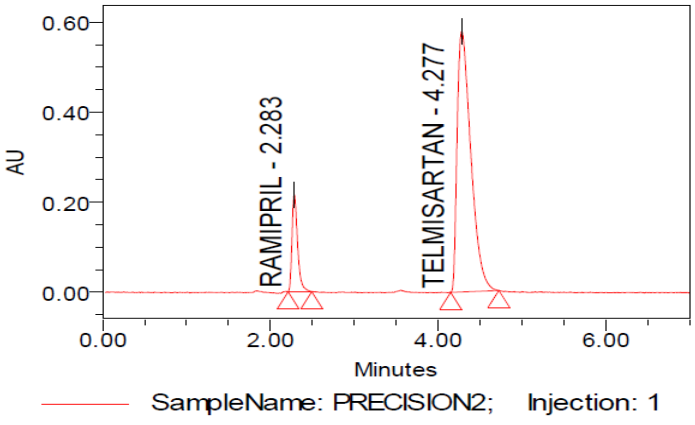
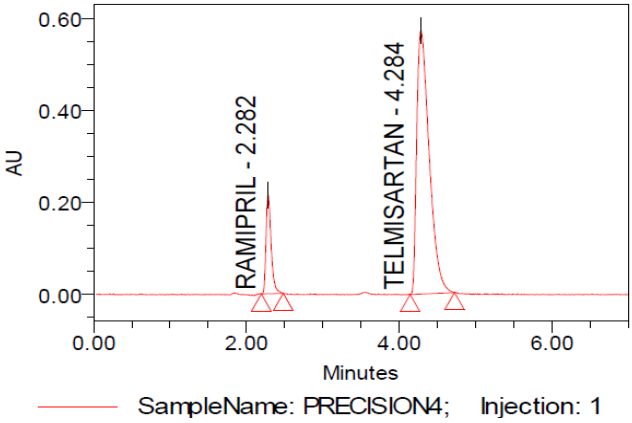


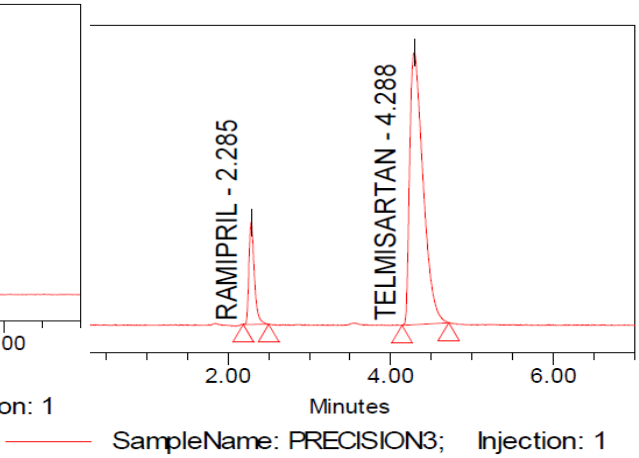
Fig: 8.5 Linearity -5

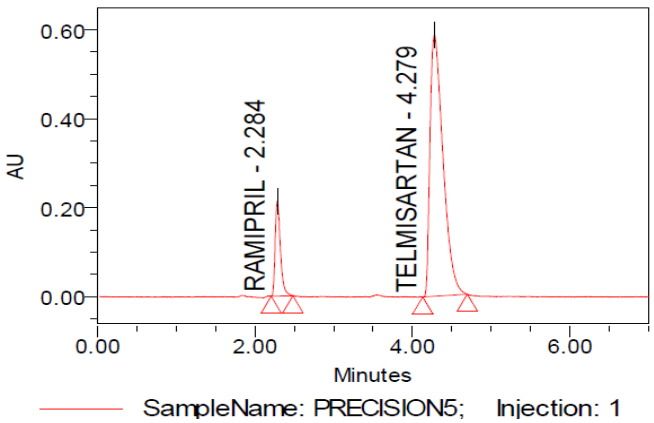
Fig: 9.1- 9.6 METHOD PRECISION











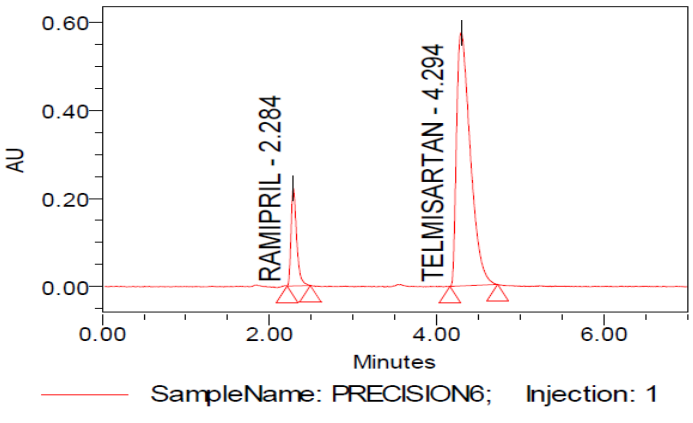
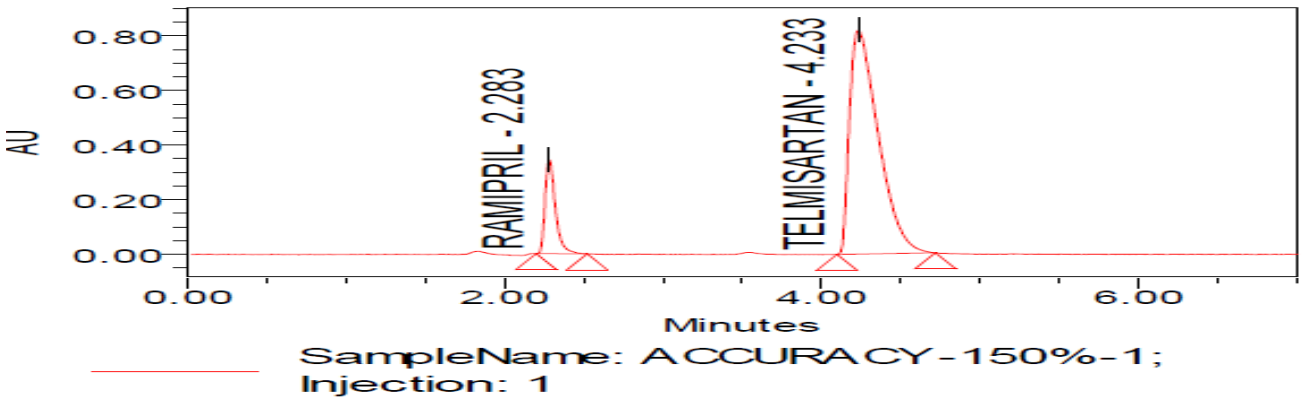
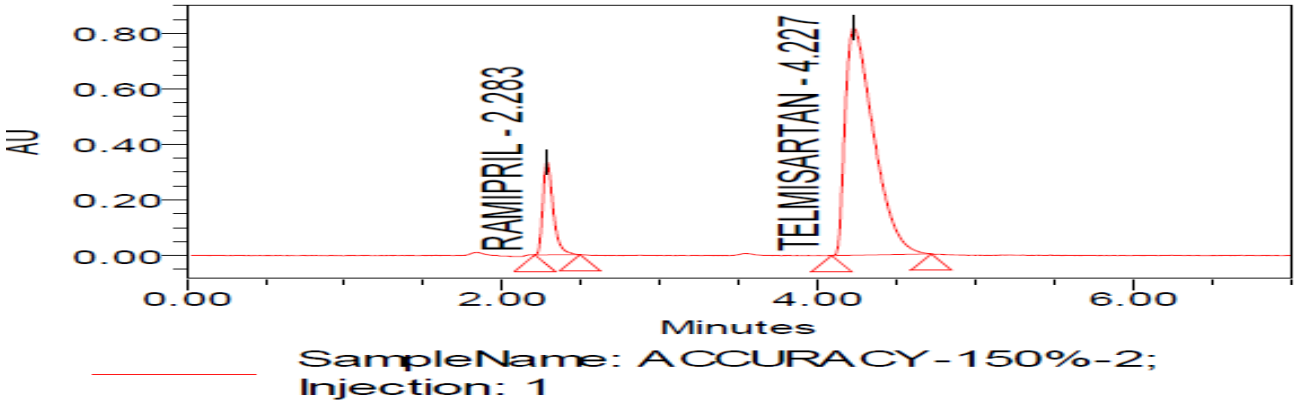


Fig: 10.7- 10.9 ACCURACY STUDIES





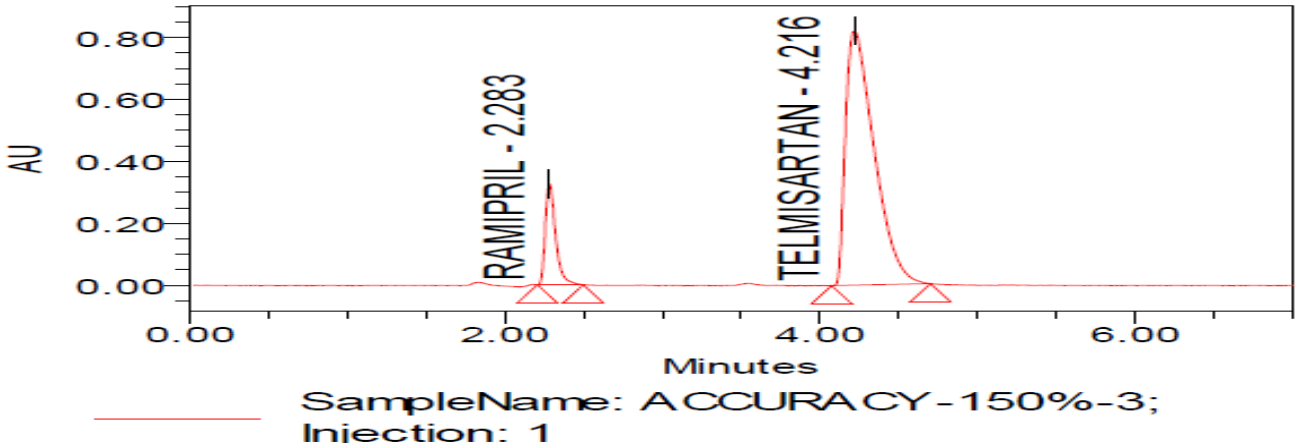
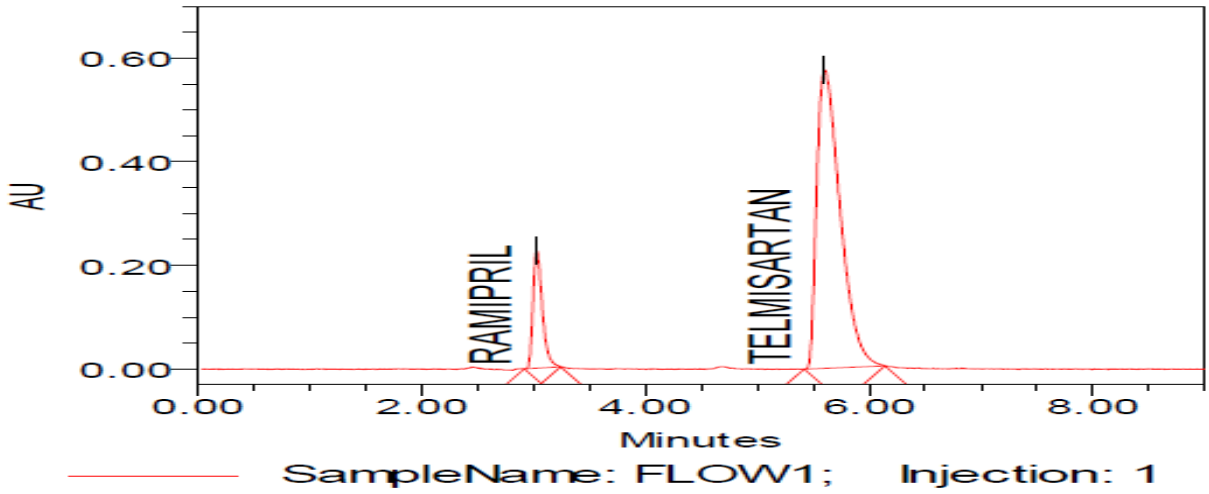


Fig: 11.1 – 11.2 ROBUSTNES STUDIES



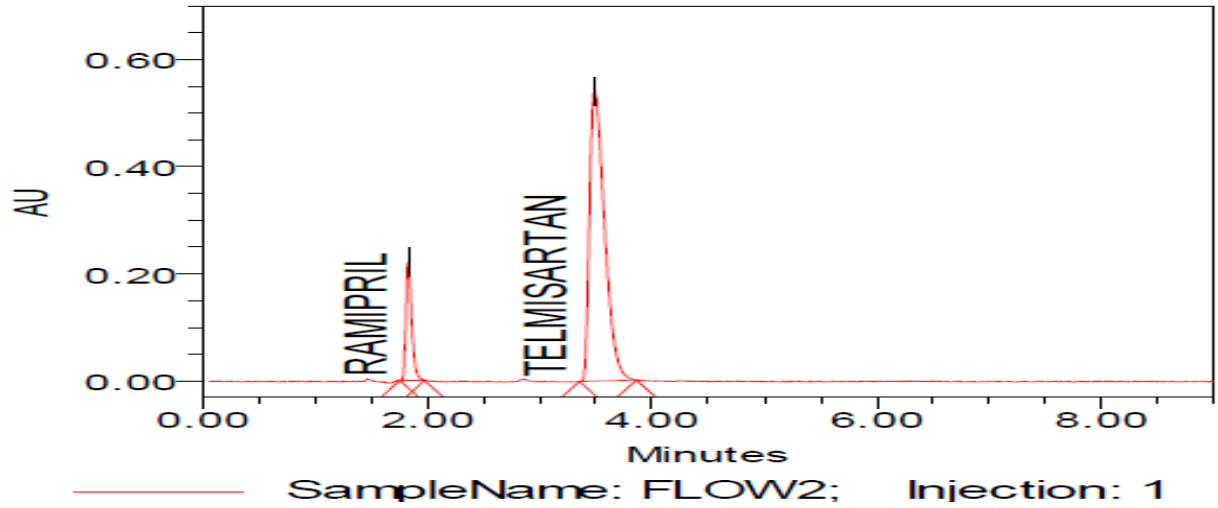
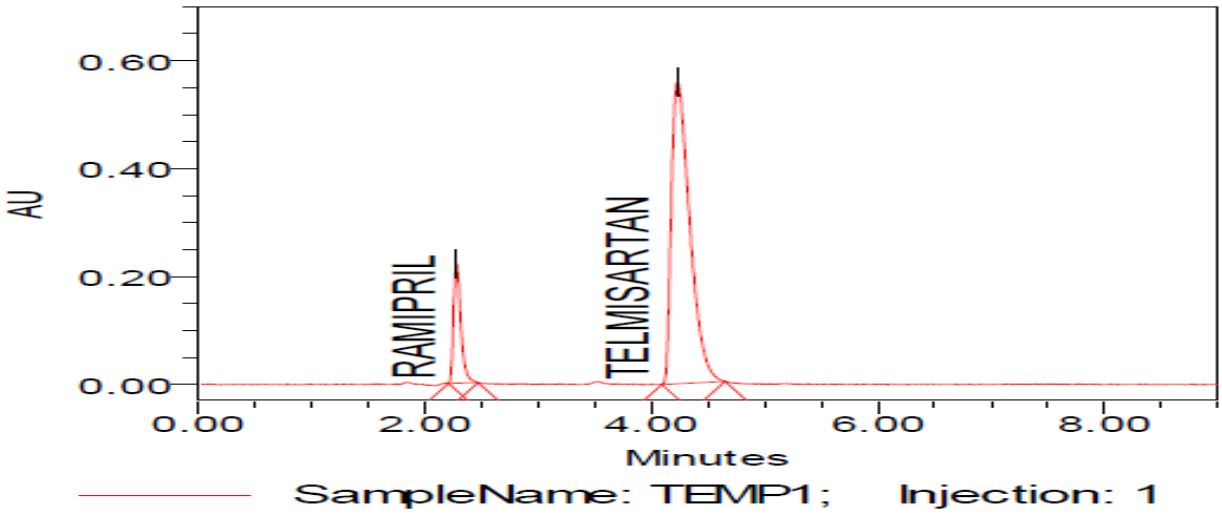


Fig: 11.3 – 11.4 ROBUSTNES STUDIES



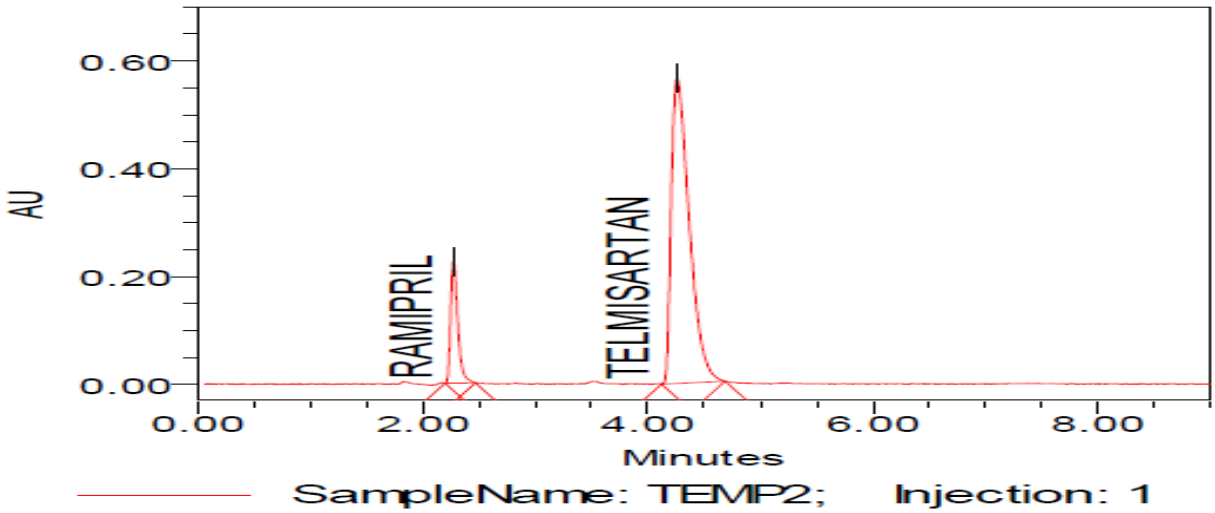


Fig: 12.1 LOD

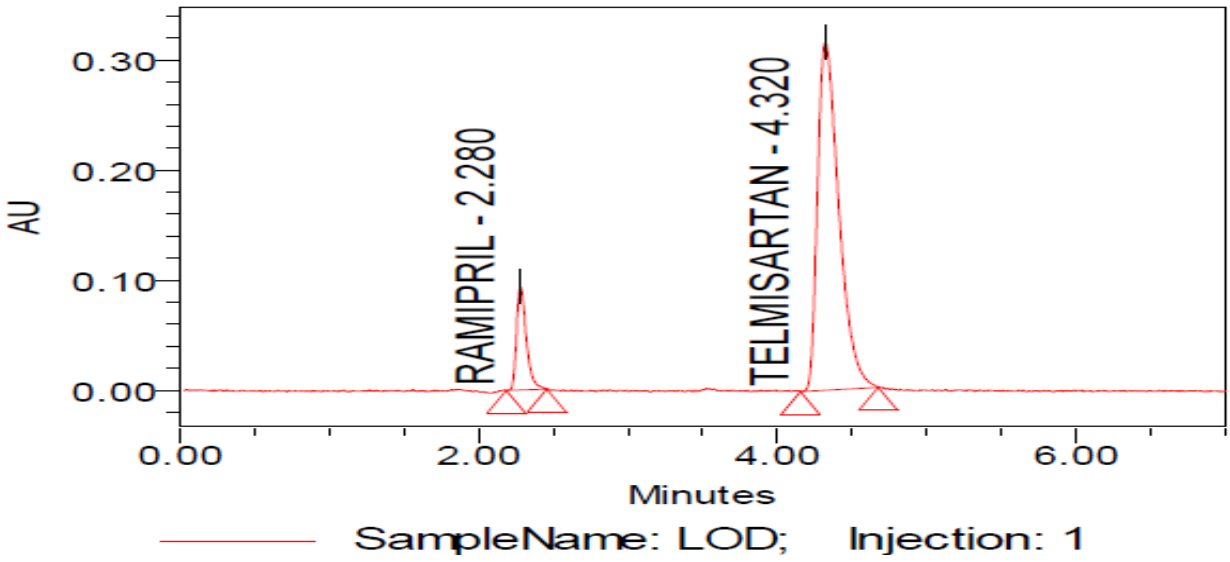
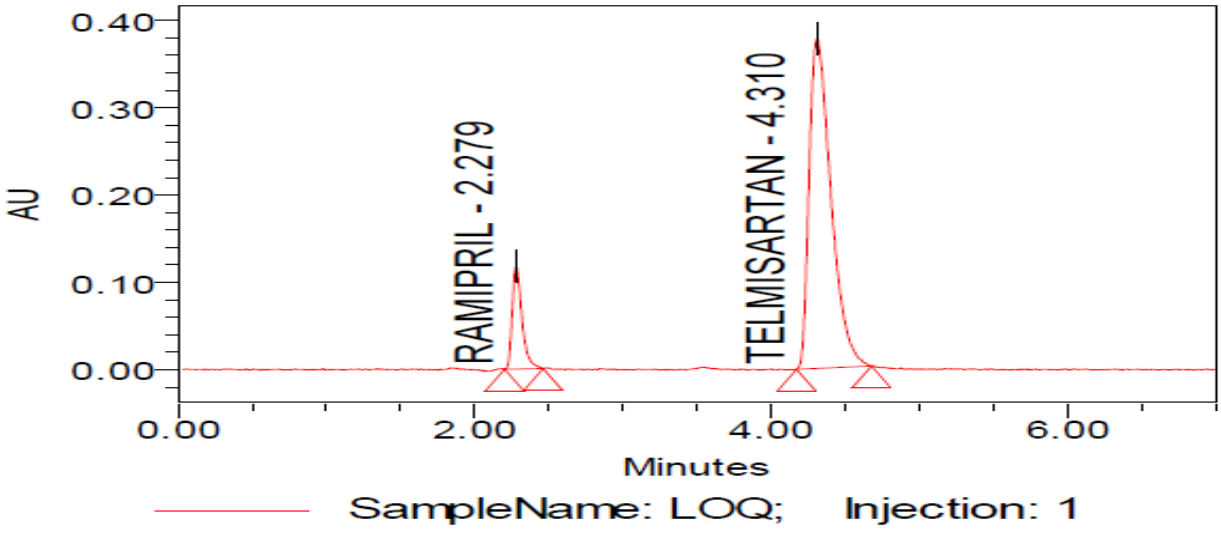


Fig: 12.2 LOQ



RESULTS AND DISCUSSION

Selected drug combination ramipril and telmisartan is available in fixed dosage combination mainly in tablet dosage forms. Many methods are available for estimation of the same as single components or in combination with other drugs.

Hence an involvement was made in this project to device a simple, accurate, less expensive, and sensitive RP-HPLC method for the simultaneous estimation of ramipril and telmisartan was developed and validated.

A simple reverse phase liquid chromatographic method has been developedand subsequently validated for simultaneous determination of ramipril and telmisartan in combined tablet dosage form. The separation was carried out by using a mobile phase consisting of 20 mM phosphate buffer of pH 5.0 : Acetonitrile in the ratio of 70:30%v/v. The column used was Zorbax SB C_8, 150 X 4.6mm, particle size 3.5µ with flow rate of 1.0ml/min using PDA detection at 230 nm.

The chromatogram of ramipril and telmisartan reference standard are presented in Figure 5 & 6 respectively.For quantitative estimation 230 nm was selected as suitable wavelength. The individual peaks of ramipril and telmisartan was identified by knowing the retention time 2.275 and 4.261 minutes respectively.

Linearity was evaluated by visual inspection of the plot of peak area as a function of analyte concentration for ramipril and telmisartan. The data regarding linearity for both the drugs were given in Table 10 and corresponding calibration graphs were shown in Figure2.1&2.2. The linearity of the method was determined at concentration levels ranging from 20-60 µg/ml for ramipril and 160-480 µg/ml for

telmisartan. The correlation co-efficient of ramipril was found to be 0.9999 and the correlation co-efficient of telmisartan was found to be 1.0000, these are within limit.

System suitability parameters such as resolution, tailing factor and number of theoretical plates are presented in Table 8

System precision was carried out, the % RSD for peak area of ramipril and telmisartan for six replicated injections was not more than 2.0% and the data was presented in Table 9.

Estimation of ramipril and telmisartan tablet dosage forms by RP-HPLCmethod was carried out using optimized chromatographic conditions. The standard and sample solutions were prepared. The chromatograms were recorded. The peak area ratio of standard and sample solutions was calculated. The results of a analysis shows that the amount of drugs was in good agreement with the label claim of the formulation. The quantitative estimation was carried out on tablet by taking the same concentration as for standard solution. The data regarding quantitative estimation is depicted in Table 12. The tablet shows percentage purity values ranging from 100.15 for ramipril and 99.96 for telmisartan respectively.

The acceptance criteria of method precision were found to be % RSD NMT 2.0% and the method shows precision of 0.20423 for ramipril and 0.25752 for telmisartan. Table.11

The accuracy of the method was determined by recovery experiments. The recovery study was carried out and the percentage recovery range found to be within the limit, 99.8-100.20 percentage for ramipril and 100.10-100.11 percentages for telmisartan. Table.13

The Limit of Detection (LOD) and Limit of Quantification (LOQ) of the developed method were determined by injecting progressively low concentrations of the standard solutions using the developed RP-HPLC method. The LOD is the smallest concentration of the analyte that gives a measurable response (signal to noise ratio of 3). The Detection Limit (LOD) was found to be 2.727 µg/ml and 2.990 µg/ml of ramipril and telmisartan respectively.

The LOQ is the smallest concentration of the analyte, which gives response that can be accurately quantified (signal to noise ratio of 10). The quantitation limit (LOQ) was found to be 9.091 µg/ml and 9.968 µg/ml of ramipril and telmisartan respectively.

The method was validated as per ICH guidelines in terms of linearity, accuracy, specificity, precision, repeatability of measurement of peak area as well as repeatability of sample application and the results are shown**.** Since this developed method can be used for routine analysis of two components in formulation.

Validation parameters	Ramipril	Telmisartan
Linearity range (µg/ml)	20-60 µg/ml	160-480 µg/ml
Correlation co-efficient	0.9999	0.9999
Retention time (min)	2.275 min	4.261 min
LOD (µg/ml)	2.727	2.990
LOQ (µg/ml)	9.091	9.968
Accuracy	100.03	100.1
System precision (% RSD)	0.79	0.68
No. of theoretical plates 9.248 Resolution factor	5882	
3045Tailing factor	1.427	1.831
Slope	25003	20132
Intercept	1439.1	1754.9
Method precision (% RSD	0.2038	0.2575

VALIDATION PARAMETERS HPLC METHOD

Table -14

S. No	Parameters	Observation		Limit	Pass/fail
		Ramipril	**Telmisartan**		
1	Linearity range (µg/ml)	20-60 µg/ml	160-480 µg/ml	No limit	passed
2	Correlation co-efficient	0.9999	0.9999	NLT-0.999	passed
3	System precision (% RSD)	0.79	0.68	NMT 2	passed
4	Accuracy 50% level	99.80	100.10	97-103	passed
5	Accuracy 100% level	100.20	100.11	97-103	passed
6	Accuracy 150% level	100.10	100.10	97-103	passed
7	Method precision (% RSD)	0.2038	0.2575	NMT 2	passed

Table-15

CONCLUSION

From the reported literature, there were few methods established for the determination of Ramipril and Telmisartan in individual and in combination with other drug.

It was concluded that there were only few methods reported for the simultaneous estimation of the above selected multi component dosage form, which promote to pursue the present work. The scope and objective of the present work is to develop and validate a new simple RP-HPLC method for simultaneous estimation of Ramipril and Telmisartan in combined dosage form.

In simultaneous RP-HPLC method development, Waters 2695 Separations Module with PDA Detector and column used is C_8 SB ZORBAX (150 X 4.6mm) column with3.5-micron particle size. Injection volume of 10 µL is injected and eluted with the mobile phase selected after optimization was Phosphate buffer and Acetonitrile in the ratio of 70:30 was found to be ideal. The flow rate was found to be optimized at 1.0 mL/min. Detection was carried out at 230 nm. This system produced symmetric peak shape, good resolution and reasonable retention times of Ramipril and Telmisartan were found to be 2.275 and 4.261 minutes respectively.

The Ramipril and Telmisartan showed linearity in the range of 20-60 µg/mL and 160-480 µg/mL respectively.

Precision of the developed method was studied under system precision and method precision. The %RSD values for precision was found to be within the acceptable limit, which revealed that the developed method was precise. The developed method was found to be robust. The %RSD value for percentage recovery

of Ramipril and Telmisartan was found to be within the acceptance criteria. The results indicate satisfactory accuracy of method for simultaneous estimation of the Ramipril and Telmisartan.

From the above experimental data and results, the developed HPLC method is having the following advantages:

- The standard and sample preparation requires less time.
- No tedious extraction procedure was involved in the analysis of formulation.
- Run time required for recording chromatograms were less than 10 minutes.
- Suitable for the analysis of raw materials, applicable to dissolution studies and can be used for the content uniformity studies.

Hence, the chromatographic method developed for the Ramipril and Telmisartan said to be rapid, simple, specific, sensitive, precise, accurate and reliable that can be effectively applied for routine analysis in research institutions, quality control department in industries, approved testing laboratories, bio-pharmaceutics and bio-equivalence studies and in clinical pharmacokinetic studies.

BIBLIOGRAPHY

1) Anonymous, *Instruction Manual*. Shimadzu High Performance Liquid Chromatography SPD-10AVP, Shimadzu Corporation, Koyato, Japan, 2001, 11.1-11.3.

2) Anonymous, *Instruction Manual.* Shimadzu Solvent Delivery Module LC-10ATvp. Shimadzu Corporation, Koyato, Japan, 2001,11.1-11.3.

3) Bankey, S, G. G Tapadiya*, S. S Saboo, S. Bindaiya, Deepti Jain, S. S. Khadbadi. simultaneous determination of Ramipril (RMP), Hydrochlorothiazide (HCT) and Telmisartan (TEL) in tablet formulation, *International Journal of PharmTech Research*, July-Sept 2011, Vol.3, No.3,pp 1737-1749,.

4) Beckett A. H. and Stanlake J. B. *Practical Pharmaceutical Chemistry,* 4th Edn, Part 2, CBS Publishers and Distributors, 2002, 1.

5) Beckett, A. H. and stenlake, J. B. *Practical pharmaceutical chemistry*, 4thedn., CBS Publishers and Distributors, New Delhi, 2007, II, 262-337.

6) Clark's Isolation and Identification of Drug, II nd Edition, Page No. 944.

7) David C. Lee, Micheal Webb., Pharmaceutical Analysis, 1, 32, 44.

8) Devaraj Rao, G. A. *Text Book of Pharmaceutical Analysis*. 5th edn., Birla Publication, New Delhi, 2007, 1-2.

9) Garry D. Chritian, Analytical Chemistry, 4th edn., University of Wellington, A.W. Sons, London, 1992, 1-4, 469-475.

10) Kalyan Kumar. B, T. Santhosh Kumar , A. Shravan Kumar, P. Venkateshwar Rao Development and Validation of RP-HPLC Method for Simultaneous Estimation of Ramipril, Telmisartan and Hydrochlorothiazide in Pharmaceutical Dosage Forms, *Journal of Pharmacy Research*, 2011, Vol 4,Pg No 10.

11) Kurade. VP, MG Pai, R Gude. RP-HPLC estimation of ramipril and telmisartan in tablets, *Indian Journal of Pharmaceutical Sciences*, 2009,Volume : 71,Issue : 2, Page : 148-151.

12) Manju Latha YB**,** Gowri Sankar D.Isocratic Rp-Hplc Method Development And Validation For The Simultaneous Estmation Of Ramipril And Telmisartan In Tablet Dosage Form, Inventi Rapid: Pharm Analysis & Quality Assurance , Vol. 2012 , Article ID- *" Inventi:ppaqa*/270/12 " , 2012.

13) Patel. V .A , P. G. Patel*2, B. G. Chaudhary, N. B. Rajgor2, S. G. Rathi2. Development and Validation of HPTLCMethod for the Simultaneous Estimation of Telmisartan and Ramipril in CombinedDosage Form, *International Journal on Pharmaceutical and Biological Research,*2010,*Vol*. 1(1), , 18-24.

14) Popat B.Mohite, Ramdas B.Pandharea, Vaidhun H.Bhaskarb. Simultaneous Estimation of Ramipril and Telmisartan in Tablet DosageForm by Spectrophotometry, *Eurasian J. Anal. Chem.* 2010,5(1): 89-94.

15) Sharma B.K., Instrumental Methods of Chemical Analysis, 24th edition, GOEL Publishers House, Meerut, 2005, 46, 68.

16) Sivasubramanian, Lakshmi.k.s, Krishnu pal.stability indicating HPTLC method for simultaneous determination of Telmisartan and Ramipril in tablets, *international journal of pharmacy and pharmaceutical sciences,vol.2,suppl 4,2010.*

17) Sunil Singh. Simultaneous estimation of telmisartan and ramipril in combined dosage form by using HPTLC*, Der Pharmacia Lettre*, 2012, 4 (2):509-514 (http://scholarsresearchlibrary.com/archive.html)

18) Takeru Higuchi and Einar Brochmann Hanssen. *Pharmaceutical Analysis.* 1st edn. CBS Publishers and Distributors, New Delhi, 1997, 1.

19) Tapadiya, G .G, S. S Saboo, S. Bindaiya, Deepti Jain, S. S. Khadbadi. Simultaneous Determination of Ramipril, Hydrochlorothizide and Telmisartan by Spectrophotometry, *International Journal of ChemTech Research*, April-June 2009, Vol.1, No.2, pp 183-188.

20) Tripathi K D, Essential of medical pharmacology, Jaypee publishers 5 edition 2003.

21) Yogesh, Gupta, A Shrivastava. Isocratic rp-hplc-uv method development and validation forthe simultaneous estmation of ramipril and telmisartan in tablet dosage form, *Asian Journal of Pharmaceutical and Clinical Research* October- December 2009 ,Vol.2 Issue 4.