DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF ATORVASTATIN AND IRBESARTAN IN COMBINED DOSAGE FORM

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ABSTRACT: ***A specific, precise and accurate RP-HPLC method has been developed for simultaneous estimation of irbesartan and atorvastatin in synthetic mixture. The use of a 250 × 4.6 mm, 5 µm particle size, C18 column with 50: 10: 40 %, v/v/v acetonitrile: methanol: 0.1 % formic acid (pH adjusted to 3.5 with triethylamine) as isocratic mobile phase at flow rate of 1 ml/min enabled separation of drugs. UV detection was performed at 271 nm. The method was validated as per ICH Q2 (R1) guidelines. The retention time for irbesartan and atorvastatin were found to be 4.01 ± 0.03 and 7.03 ± 0.03 min respectively. The linearity of method was satisfactory over the range 300-1500 ng (correlation coefficient 0.9993) and 20-100 ng (correlation coefficient 0.9996) for irbesartan and atorvastatin respectively. The limits of detection for irbesartan and atorvastatin were 10.94 and 1.33 ng respectively. The limits of quantitation for irbesartan and atorvastatin were 33.14 and 4.03 ng respectively. Recovery of irbesartan and atorvastatin ranged from 99.84-99.98 % and 99.90-100.67 % respectively. The method was successfully applied to synthetic mixture for quantitative analysis of irbesartan and atorvastatin.***

Keywords- Irbesartan, Atorvastatin, RP-HPLC, analysis

I. INTRODUCTION

1.1 Hypertension[1-5] Definition:

High blood pressure, also known as hypertension, is elevated pressure of blood in the arteries. Hypertension is manifestation of two major factors as follows:

The heart pumps blood with unnecessary force.

Arterioles, smaller blood vessels will exact more pressure.

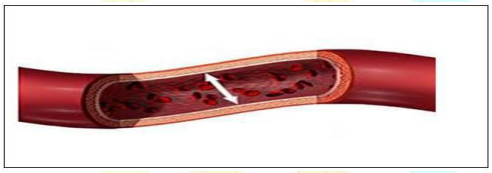

Figure 1.1 Cross section of artery

Hypertension is defined arbitrarily as sustained increases in systemic blood pressure (either systolic or diastolic), greater than 140/90 mm Hg.

Systolic Pressure:

It is the pressure when heart contracts and pushes the blood on its journey through the body and displays the activity of heart. It is considered high if it is consistently over 120.

Diastolic Pressure:

It is the pressure present in artery when the heart is relaxed and shows the condition of blood vessels. It is considered high if it is consistently over 80

Factors involved in Blood Pressure control: [1, 4-6]

Blood pressure is controlled by an integrated neuronal and hormonal control system that modulates- Blood Volume; Cardiac Output; Peripheral Vascular Resistance

Table 1.1 Categories for Blood Pressure levels in adults

Category	Systolic (mm Hg)	Diastolic
Normal	<120	and <80
Pre-Hypertension	120-139	or 80-89
High Blood Pressure		
Stage 1	140-159	or 90-99
Stage 2	≥160	or ≥100

Classification of Hypertension

1. Essential or Primary Hypertension: It is characterized by an elevation of diastolic blood pressure with no apparent cause. It accounts for 90 – 95% of all cases and usually occurs in childhood, typically at ages above 40 years.
2. Secondary Hypertension: It accounts for 5 – 10% of all cases and, by definition is due to identifiable causes like renovascular diseases, etc.

Mechanism of action of Angiotensin II receptor Antagonist

Angiotensin II is a potent Vasoconstrictor. They block the Vasoconstrictor and aldosteron secreating effects of Anginotensin II by selectively blocking the binding of Anginotensin II to the AT1 receptor. The blockage of AT1 receptors directly causes Vasodilation, reduces secretion of Vasopressin and also reduces the production and excretion of aldosterone. The combined effect reduces blood pressure.

1.2 Hyperlipidemia [2, 6-12]

Hyperlipidemia, a broad term also called hyperlipoproteinemia, is a common disorder in developed countries and is the major cause of coronary heart disease. It results from abnormalities in lipid metabolism or plasma lipid transport or a disorder in the synthesis and degradation of plasma lipoproteins. The lipids that are involved in hypercholesterolemia are cholesterol, an essential component of cell membrane and a precursor of steroid hormone synthesis and triglycerides, an important energy source. They are transported in blood as
Lipoproteins. An increase in the plasma concentration of this substance is termed as hyperlipidemia.
Mostly hyperlipidemia is caused by lifestyle habits or treatable medical conditions. Lifestyle habits include obesity, not exercising, and smoking. Medical diseases that may result in Hyperlipidemia are diabetes, kidney disease, pregnancy, and an under active thyroid gland. One can also inherit hyperlipidemia.

Hyperlipidemia in general has no apparent symptoms and it is discovered and diagnosed during routine examination or evaluation for atherosclerotic cardiovascular disease. However, deposits of cholesterol may be formed under the skin in individuals with familial forms of the disorder or in persons with very high levels of cholesterol in the blood.
For diagnosis of hyperlipidemia, levels of total cholesterol, low density lipoprotein cholesterol, high density lipoprotein cholesterol, and triglycerides are measured in blood sample. The pharmacological agents, which reduce the concentration of plasma lipids, are called hypercholesterolemic agents. Lipoproteins consist of central core of hydrophobic lipid (triglycerides or cholesterylesters) enhanced in a more hydrophilic coat of polar substance phospholipids, free cholesterol and associated protein.
Lipid lowering drugs act either by reducing the production of lipoprotein or by which increasing their removal from blood. The main aim is to decrease plasma cholesterol. The risk of atherosclerosis is associated with increased plasma cholesterol and a high LDL: HDL ratio. There are several mechanisms by which pharmacological agents can affect the metabolism of cholesterol and relative levels of various cholesterol carrying lipoproteins in the plasma.

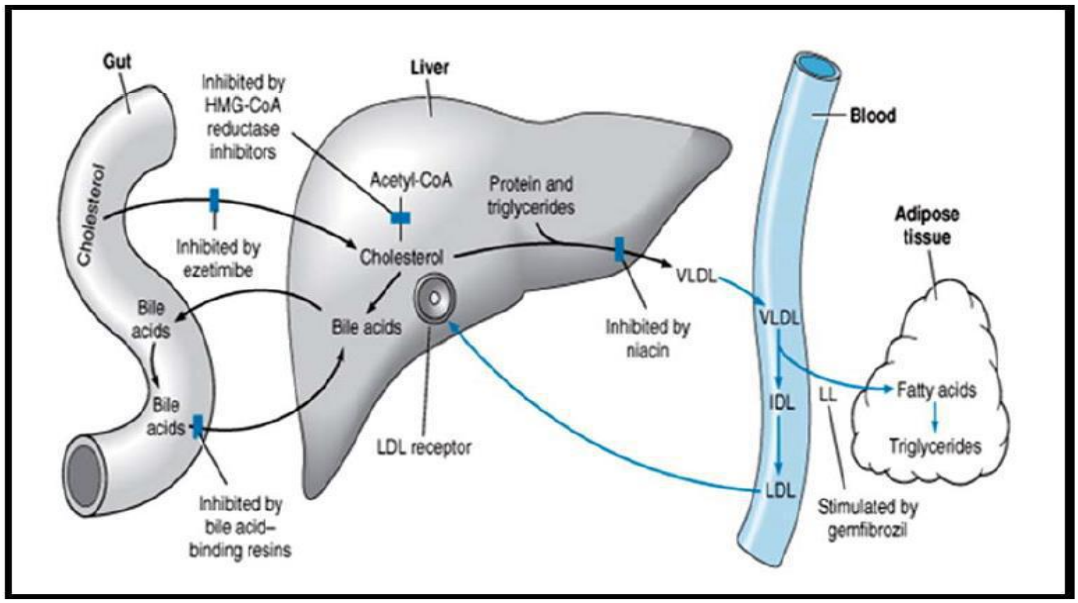

Figure 1.2 Sites and mechanism of drugs for Hyperlipidemia

1.3 High Performance Liquid Chromatography (HPLC) [13-23]

Chromatography has been defined in the classical manners as follow, "A separation process that is achieved by the distribution of substances between two phases, a stationary phase and a mobile phase, those solutes distributed preferentially in the mobile phase will move more rapidly through the system than those distributed preferentially in the stationary phase. Thus the solutes will elute in order of their increasing distribution coefficient with respect to the stationary phase".
Chromatography is probably the most powerful and versatile analytical technique available to the modern chemist. Its power arises from its capacity to determine quantitatively many individual components present in a mixture in one, single analytical procedure. Its versatility comes from its capacity to handle a very wide variety of samples that may be gaseous, liquid or solid in nature. Another aspect of the versatility of the technique is that the analysis can be carried out, at one extreme, on a very costly and complex instrument and at the other, on a simple, inexpensive thin layer plate.
HPLC is fastest growing analytical technique for analysis of drugs. Its simplicity, high specificity and wide range of sensitivity makes it ideal for analysis of many drugs in both dosage forms and biological fluids. HPLC is a form of liquid chromatography to separate compounds that are dissolved in solution. HPLC is able to separate macromolecules and ionic species, labile natural products, polymeric materials, and a wide variety of other high molecular weight polyfunctional groups.
HPLC is presently used in pharmaceutical research and development in following area:

To purify synthetic or natural products.
To characterize metabolites.
To assay active ingredients, impurities, degradation products and in dissolution studies.

In pharmacokinetic and pharmacodynamic studies. Characteristics of HPLC method
Efficient, highly selective, widely applicable
Only small sample required
Readily adapted to quantitative analysis
High resolving power
Speed of separation

The HPLC system is often classified by separation mechanism or by the type of stationary phase. These include:

Partition or liquid- liquid chromatography
Adsorption or liquid- solid chromatography
Ion exchange chromatography
Size exclusion chromatography
Affinity chromatography
Chiral chromatography

1.3.1 Basic principle of HPLC

High Performance Liquid Chromatography (HPLC) is a separation technique utilizing differences in distribution of compounds to two phases; called stationary phase and mobile phase. The stationary phase designates a thin layer created on the surface of fine particles; a mobile phase designates the liquid flowing over particles. Under a certain dynamic condition, each component in a sample has different distribution equilibrium depending on solubility in the phases and/or molecular size. As a result, the components move at different speed over the stationary phase and thereby separated from each other.

The column is a stainless steel tube, which is packed with spherical particles. Mobile phase is constantly fed into the column inlet at a constant rate by a liquid pump. A sample is injected from a sample injector, located near the column inlet. The injected sample enters the column with the mobile phase and the components in the sample migrate through it, passing between the stationary and mobile phase. Compound move in the column only when is in the mobile phase.

Compound that tend to be distributed in the stationary phase migrate slower. In this way, each component is separated on the column and sequentially eluted from the outlet. Each compound eluting from the column is detected by a detector connected to the outlet of the column.

The time required for a compound to elute (retention time) and the relationship between compound concentration (amount) and peak area depend on the characteristics of the compound. Retention time is therefore used as an index for qualitative determination and peak surface are (or height) as an index for quantitative determination.

There are two mode of elution process; Isocratic elution and Gradient elution.

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 substances, the rates of individual band migrations must be changed during elution.

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 components in the eluent (acetonitrile) then strongly retained components will move faster and faster, because of the increase of the competition for the adsorption sites.

1.3.2 Modes of HPLC

Normal phase chromatography: In normal phase mode, the nature of stationary phase is polar and the mobile phase is non polar. In this technique, non-polar compounds travel faster and are eluted first because of the lower affinity between the non-polar compounds and stationary phase. Polar compounds are retained for longer time and take more time to elute because of their higher affinity with the stationary 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. Separations of oil-soluble vitamins, essential oils, nitro phenols, or more polar homologous series have been performed using alcohol/heptanes as the mobile phase.

Reversed phase chromatography: Reverse phase mode is the most popular mode for analytical and preparative separation of compounds of interest in chemical, biological, pharmaceutical, food and biomedical sciences. Hydrocarbons are retained more strongly than alcohols. Thus water is the weakest eluent. Methanol and acetonitrile are popular solvents because they have low viscosity and are readily available with excellent purity. An aqueous mobile phase allows the use of secondary solute chemical equilibrium (such as ionization control, ion suppression, ion pairing and complexation) to control retention and selectivity. The polar compound gets eluted first in this mode and non-polar compounds are retained for loner time. As most of the drugs and pharmaceuticals are polar in nature, they are not retained for longer times and hence elute faster.

Ion exchange chromatography: In ion-exchange chromatography, the separation mode is based on the exchange of ionic analytes with the counter-ions of the ionic groups attached to the solid support. Typical stationary phases are cationic exchange (sulfonate) or anionic exchange (quaternary ammonium) groups bonded to polymeric or silica materials. Mobile phases consist of buffers, often with increasing ionic strength, to force the migration of the analytes. Common applications are the analysis of ions and biological components such as amino acids, proteins/peptides and polynucleotides.

Size exclusion chromatography: Size-exclusion chromatography is a separation mode based solely on the analyte's molecular size. Large molecule is excluded from the pores and migrates quickly, whereas a small molecule can penetrate the pores and migrates more slowly down the column. It is often called gel permeation chromatography (GPC) when used for the determination of molecular weights of organic polymers and gel-filtration chromatography (GFC) when used in the separation of water-soluble biological materials.

Affinity chromatography: Affinity chromatography uses highly specific biochemical interactions for separation. The stationary phase contains specific groups of molecules which can adsorb the sample if certain steric and charge related conditions are satisfied. This technique can be used to isolate proteins, enzymes as well as antibodies from complex mixtures.

Table 1.2 Guideline for chromatographic mode selection

Sample Type	Analyte Type	Common Mode
Macromolecules	Organic polymers	GPC
(MW > 2,000)	Bio molecules	SEC, RPC, IEC, HILIC, HIC
Organics (MW < 2,000)	Polar	RPC, NP, HILIC
	Medium polarity	RPC
	Nonpolar	RPC, NARP, NP
	Ions, Ionisable compounds	RPC, RPC-IP, IEC, HILIC

Preparative	All	NP, RPC, GPC, IEC

Basic chromatographic descriptors

Four major descriptors are commonly used to report characteristics of the Chromatographic column, system and particular separation:

1. Retention factor (k)
2. Efficiency (N)
3. Selectivity (α)
4. Resolution (R)

Retention factor (k)

Retention factor (k) is the unit less measure of the retention of the particular compound on a particular chromatographic system at given condition defined as:

$k = VR-V0 / V0$

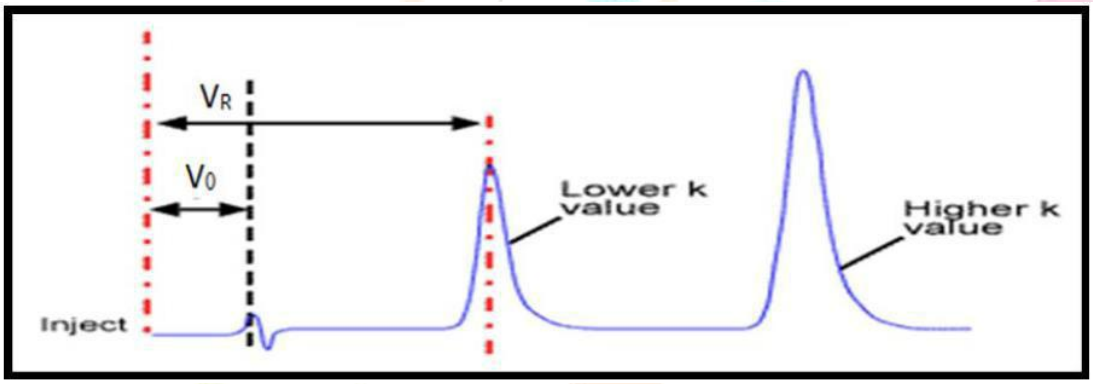

Figure 1.3 Schematics for calculating retention factor

Where, VR is the analyte retention volume and,
V0 is the volume of the liquid phase in the chromatographic system. Retention factor is convenient because it is independent on the column dimensions and mobile phase flow rate.

Note: all other chromatographic conditions significantly affect retention factor.

Efficiency (N)

Efficiency is the measure of the degree of the peak dispersion in a particular column; as such, it is essentially the characteristic of the column.

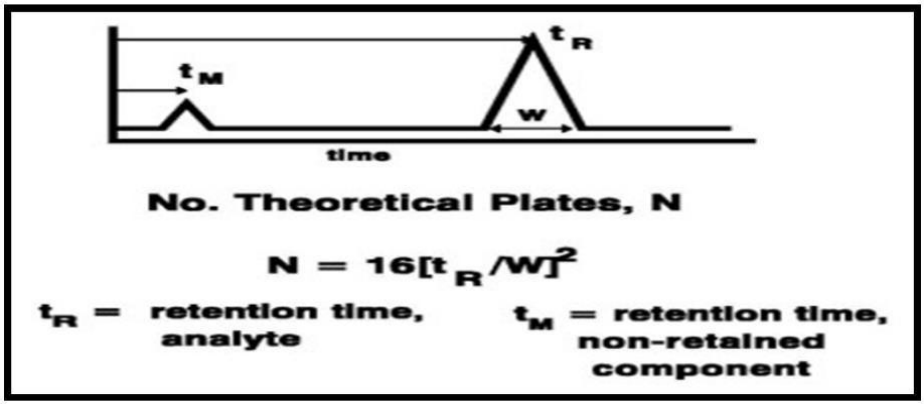

Figure 1.4 Schematics for calculating efficiency

Efficiency is expressed in the Number of theoretical plates (N) calculated as:
$N=16\ [t_R / W]^2$
Where tR is the analyte retention time, W is the peak width at the baseline.

Selectivity (α)
Selectivity is the ability of chromatographic system to discriminate two different analyte. It is the ratio of the capacity factors of both peaks, and the ratio of its adjusted retention times. The higher the value of α better is the separation. It can be calculated by using formula.
A = k 2 /k 1

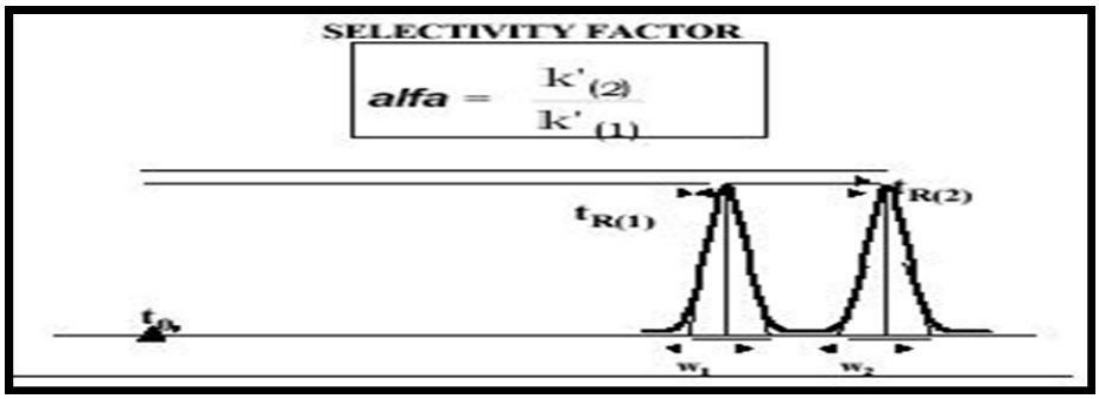

Figure 1.5 Schematics for calculating selectivity

Resolution (R)
Resolution is a combined measure of the separation of two compounds which include peak dispersion and selectivity. It characterizes the ability of a chromatographic column to separate two analyte. It is the difference between the retention times of two solutes divided by their average peak width. It is defined as:

R = 2 (t2 - t1)/ w1 + w2

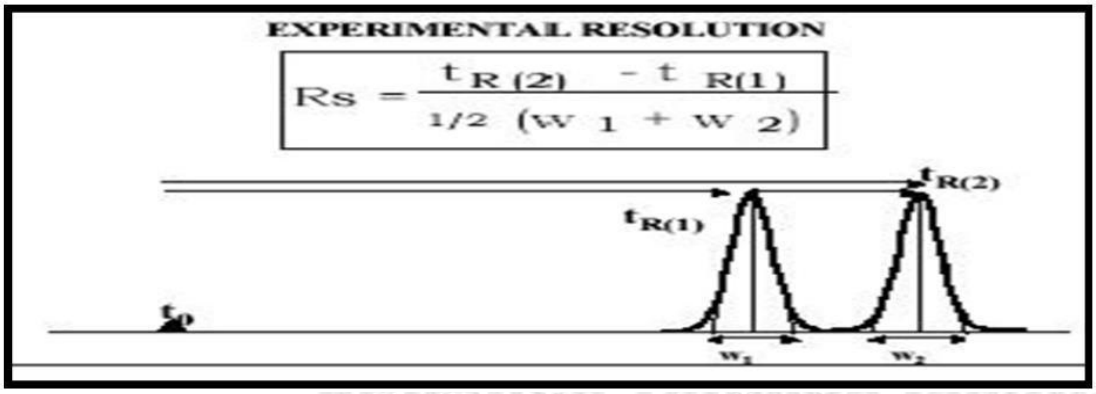

Figure 1.6 Schematics for calculating resolution

1.3.4 Various components of HPLC

- **a)** A solvent delivery system, including pump
- **b)** Sample injection system
- **c)** A chromatographic column
- **d)** A detector
- **e)** A strip chart recorder

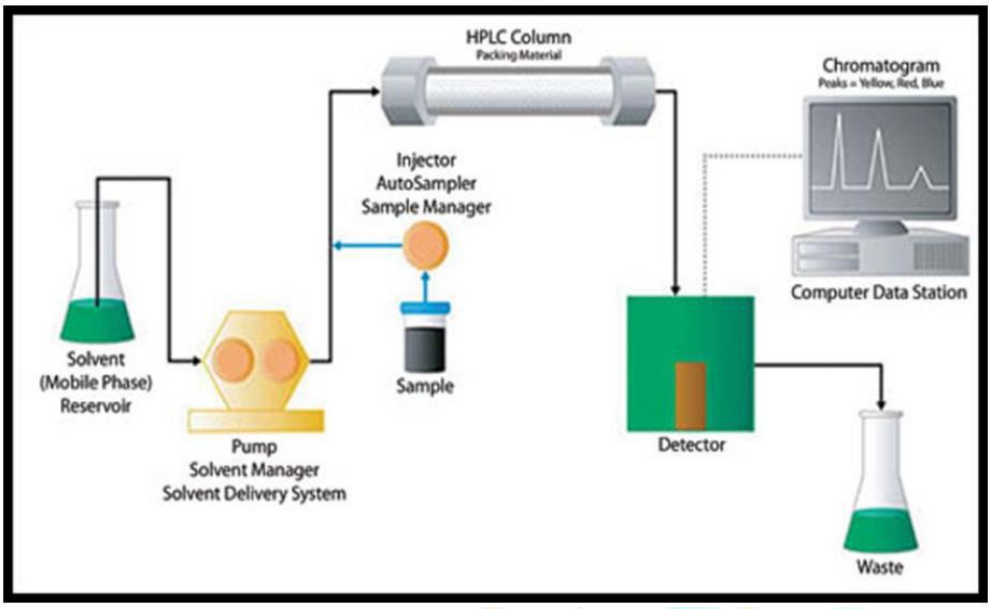

Figure 1.7 Schematic diagram of HPLC equipment

1.3.4.1 Solvent delivery system

A mobile phase is pumped under pressure from one or several reservoir and flows through the column at a constant rate. For normal phase separation eluting power increases with increasing polarity of the solvent but for reversed phase separation, eluting power decreases with increasing polarity.

A degasser is needed to remove dissolved air and other gases from the solvent. Special grades of solvents are available for HPLC and these have been purified carefully in order to remove absorbing impurities and particulate matter to prevent these particles from damaging the pumping or injection system or clogging the column.

Pumps

The high-pressure pumping system is at the heart of modern liquid chromatography. Pump is an essential part of HPLC which generates pressure for the flow of mobile phase through the column. They must be capable to generate a pressure of at least 5000 psi.

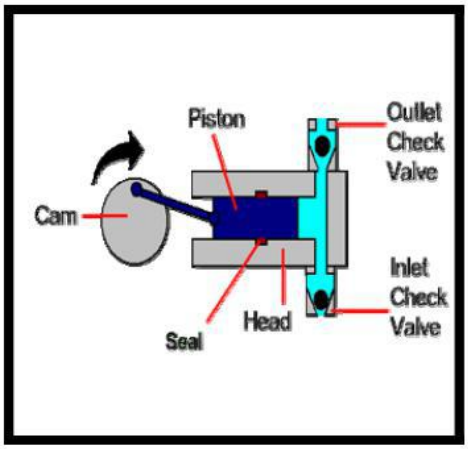

Figure 1.8 HPLC pump

1.3.4.2 Sample injection system

This allows an introduction (injection) of the analytes mixture into the stream of the mobile phase before it enters the column. Insertion of the sample onto the pressurized column must be as a narrow plug so that the peak broadening

attributable to this step is negligible. The injection system itself should have no dead (void) volume. The sample can be introduced into column either manually or with use of auto sampler. Most modern injectors are autosampler, which allow programmed injections.

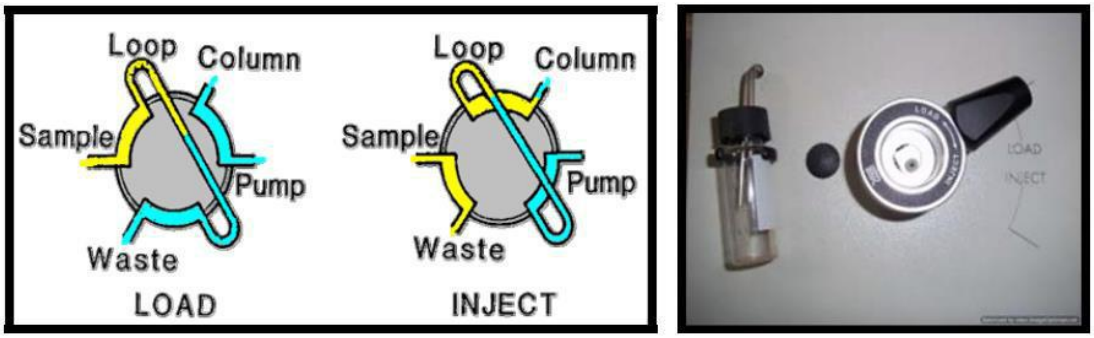

Figure 1.9 Injector with inject and load position

There are three important ways of introducing the sample into injection port.

Loop injection: In which, a fixed amount of volume is introduced by making use of fixed volume loop injector

Valve injection: In which, a variable volume is introduced by making use of an injection valve

On column injection: In which, a variable volume is introduced by means of a syringe through a septum

1.3.4.3 Chromatographic column

The column is usually made up of heavy glass or stainless steel tubing to withstand high pressure. The columns are usually 10-30 cm long and 4-10 mm inside diameter containing stationary phase at particle diameter of 5 to 30 microns. Columns with an internal diameter of 5 mm give good results because of compromise between efficiency, sample capacity, and the amount of packing and solvent required.

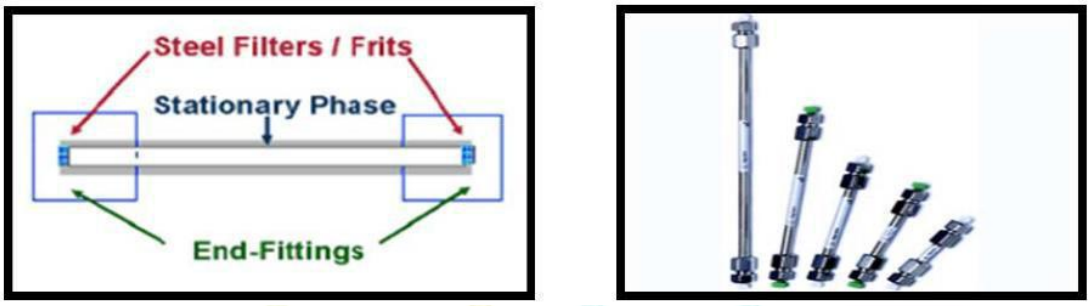

Figure 1.10 Columns and its construction

Column packing: The packing used in modern HPLC consist of small, rigid particles having a narrow particle size distribution.

There are three main types of column packing in HPLC.

i. Porous, polymeric beds: Porous, polymeric beds based on styrene divinyl benzene co-polymers used doe ion exchange and size exclusion chromatography. For analytical purpose these have now been replaced by silica based, packing which are more efficient and more stable.

ii. Porous layer beds: Consisting of a thin shell (1-3 μm) of silica or modified silica on a spherical inert core (e.g. Glass). After the development of totally porous micro particulate packing, these have not been used in HPLC.

iii. Totally Porous silica particles (dia. <10 μm): These packing have widely been used for analytical HPLC in recent years. Particles of diameter >20 μm are usually dry packed. While particles of diameter <20 μm are slurry packed in which particles are suspended on a suitable solvent and the slurry so obtained is driven into the column under pressure.

1.3.4.4 Detectors

This part of HPLC helps in detection and identification of compounds in the sample. Great consideration should be encountered in selecting the detector of choice. Detectors can be broadly classified into bulk property and solute property detectors. Bulk property detectors continuously monitor some property of the mobile phase, such as refractive index, conductance, or dielectric constant. These detectors generally have poor limits of detection and are in general not suitable for trace analysis. They also respond to the mobile phase, the signal changes with changes in mobile-phase conditions, and these detectors are largely incompatible with gradient elution techniques.

Solute property detectors respond to some specific property of certain compounds, such as the ultraviolet absorbance detector. These detectors generally have much lower limits of detection, but are applicable only to those compounds showing that specific property.

UV-visible detectors are the most popular as they can detect a broad range of compounds and have a fair degree of selectivity for some analytes. Unfortunately UVvisible detectors are not universal detectors so it is worthwhile to look at the chemical structure of the analyte to see if it has suitable chormophores, such as aromatic rings, for UV-visible detection.

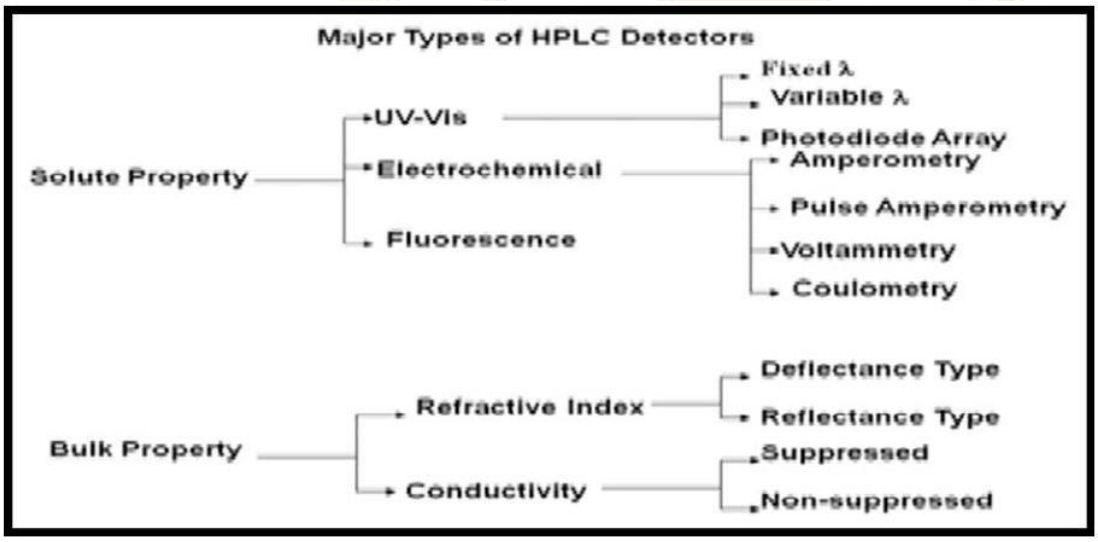

Figure 1.11 Major types of HPLC detectors

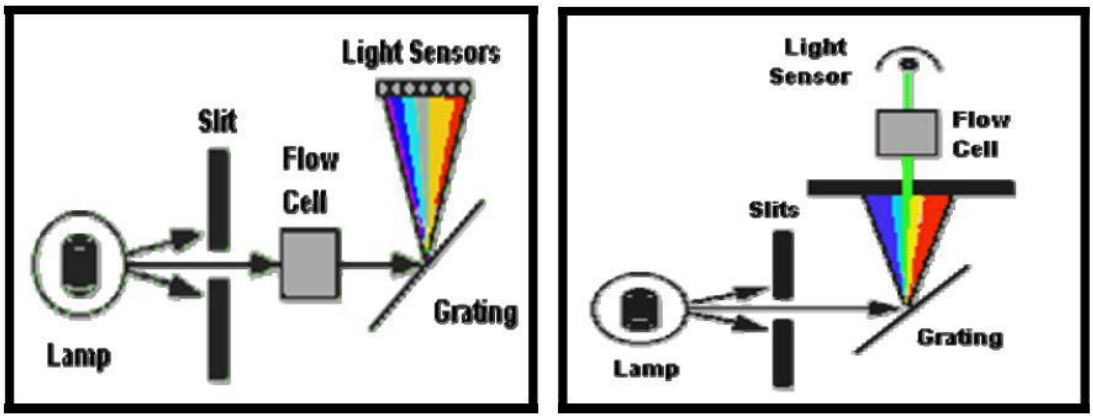

Figure 1.12 HPLC detector (UV and PDA)

1.3.5 Strategy for method development in HPLC

Many factors should be considered while development of method goes on. The initial steps include collecting as much information about the analyte in regard to the physicochemical properties (pKa, log P, solubility) and determining which mode of detection would be suitable for analysis (i.e., suitable wavelength in case of UV detection).

Sample preparation, which includes centrifugation, filtration and/or sonication and type of diluent, plays an integral role in method development because this may affect the chromatography and the recovery of the analytes. Determination of the solution stability in the diluent is also important during early method development. Choice of the mobile-phase and gradient conditions is dependent on the ionogenic nature of the analyte and the hydrophobicity of the analytes in the mixture respectively. This is a crucial step in the method development process because these two factors will probably have the most impact on the change in the analyte selectivity, especially for ionizable compounds.

HPLC method development follows series of step which are summarized as below :

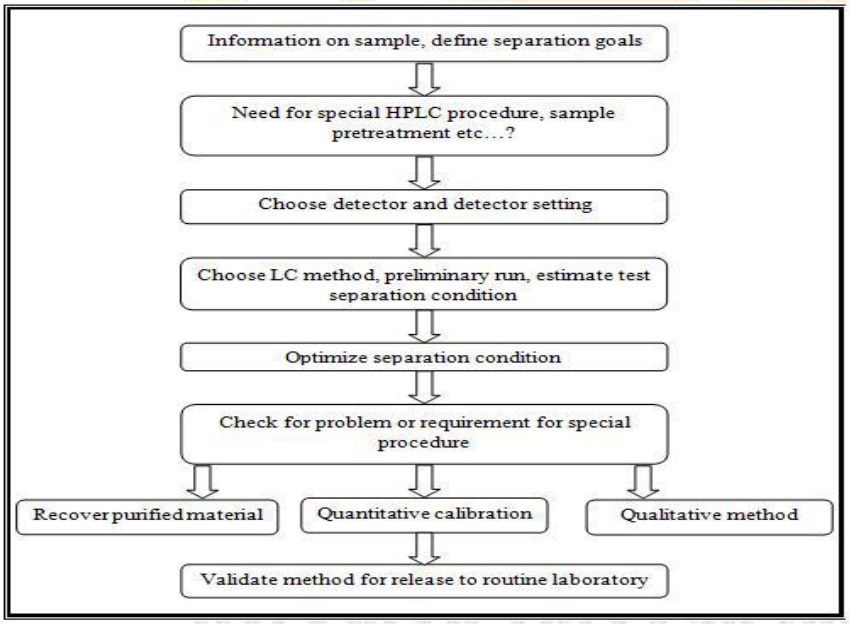

Figure 1.13 Steps in HPLC method development

1) Nature of sample

Before proceeding with development of method for a particular sample, it is absolutely essential to have detailed information about sample. What are the components present, excipients present should be identified. Impurity present in sample must be identified. Some important information concerning sample are:

- Number of components present
- Chemical structures (functionality) of compounds
- Molecular weights of compounds
- pKa value of compounds
- UV spectra of compounds

- Concentration range of compounds in samples of interest
- Sample solubility

2) Separation goal

The goals of HPLC separation need to be specified clearly. Some related questions that should be asked at the beginning of method development include:

- Is the primary goal quantitative analysis, the detection of a substance, the characterization of unknown sample components or the isolation of purified material?
- Is it necessary to resolve all sample components?
- If quantitative analysis is requested, what levels of accuracy and precision are required?
- For how many different sample matrices should the method be designed?
- How many samples will be analyzed at one time?

3) Sample pre-treatment

Sample pre-treatment is very important in development of a new method.
Most of sample required dilution before injection. Samples come in various forms:

- Solution ready for injection.
- Solution requires dilutions, buffering, and addition of an internal standard.
- Solid that must be dissolved or extracted.
- Samples that require sample pretreatment to remove interference and/or to protect the column or equipment from damage.

4) Detector and detector settings

Before the first sample is injected during HPLC method development, we must be reasonably sure that the detector selected will sense all sample components of interest. Variable-wavelength ultraviolet (UV) detectors normally are the first choice, because of their convenience and applicability for most samples. For this reason, information on UV spectra can be important aid for method development. UV spectra can found in the literature, estimated from chemical structures of sample components of interest, measured directly (if pure compounds are available), or obtained during HPLC separation by means of photodiode- array (PDA) detector.

5) Developing the separation

Selecting an HPLC method and initial conditions.
An exact recipe for HPLC method development cannot be provided because method development involves considerable trial and error procedures. The strategy recommended for choosing the experimental conditions for the first separation is explained below. Based on knowledge of sample composition and the goals of separation, the first question is: which chromatographic method is most promising for this particular sample? We assume that HPLC has been chosen but this decision should not be made before considering the alternatives.

a) The best mobile phase:

In reverse phase chromatography, the mobile phase is more polar than the stationary phase. Mobile phase in these systems is usually mixtures of two or more individual solvents with or without additives or organic solvent modifiers. The usual approach is to choose what appears to be the most appropriate column, and then to design the mobile phase that will optimize the retention and selectivity of the system.
Separation in these systems is considered to be due to different degree of hydrophobicity of the solutes. The polarity of organic modifier and its proportion control the rate of elution of the components in the mobile phase. The rate of elution is increased by reducing the polarity. The simple alteration of composition of the mobile phase or of the flow rate allows the rate of the elution of the solutes to be adjusted to an optimum value and permits the separation of wide range of the chemical types. First isocratic run followed by gradient run is preferred. Since the mobile phase governs solute-stationary phase interaction, its choice is critical. Practical considerations dictate that it should not degrade the equipment or the column packing. For this reason, strong acids, bases and halide solutions should be avoided. Chemical purity of solvents is an important factor. Since large volumes of solvent are pumped through the

column, trace impurities can easily concentrate in column and eventually be detrimental to the results. HPLC grade solvents are recommended. Volatility should be considered if sample recovery is required. Viscosity should be less than 0.5 centipoises, otherwise higher pump pressures are required and mass transfer between solvent and stationary phase will be reduced.

b) The best detector

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

c) The best column length

Many chromatographers make the mistake of simply using what is available.
Often this is a 250 x 4.6 mm C18 column. These columns are able to resolve a wide
Variety of compounds (due to their selectivity and high pate counts) and are common to most laboratories. While many reverse phase separations can be carried out on such column, its high resolving capabilities are often unnecessary. Method development can be streamlined by starting with shorter columns; 150, 100 or even 50 mm long. This is simply because they have proportionally shorter run times. triethylamine, although this should not be necessary with modern phases like Wakosil. One point often forgotten is the effect of temperature changes on a separation. To, maximize the reproducibility of a method, it is best to use a column heater to control the temperature of the separation. A temperature of 35º – 400º C is recommended.

d) Buffer selection

In reverse phase HPLC, the retention of analytes is related to their hydrophobicity. The more hydrophobic the analyte, the longer it is retained. When an analyte is ionized, it becomes less hydrophobic and, therefore, its retention decreases. When separating mixtures containing acid and/or bases by reversed phase HPLC, it is necessary to control the pH of mobile phase using appropriate buffer in order to achieve reproducible results.
When separating acids and bases a buffered mobile phase, by definition, resists changes in pH so that the analytes and silica will be consistently ionized, resulting in reproducible chromatography. If the sample is neutral, buffers or additives are generally not required in the mobile phase. For basic or cationic samples, "less acidic" reverse-phase columns are recommended and amine additives for the mobilephase may be beneficial. Optimum buffering capacity occurs at a pH equal to the pKa of the buffer. Beyond that, buffering capacity will be inadequate.
Buffers play an additional role in the reproducibility of a separation. The buffer salts reduce peak tailing for basic compounds by effectively masking silanols. They also reduce potential ion-exchange interactions with unprotonated silanols. To be most effective, a buffer concentration range of 10 – 50 mM is recommended for most basic compound.

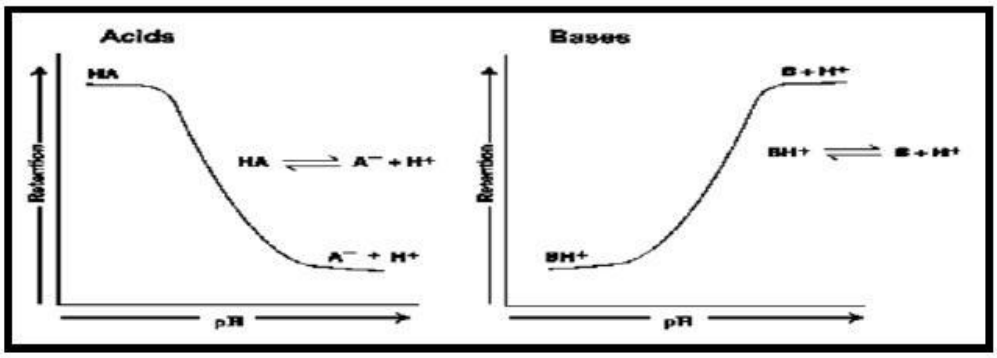

Figure 1.17 Effect of pH on ionization of acid and base

Table 1.4 commonly used buffers for reversed phase HPLC

Buffer	pKa (25°C)	Maximum Buffer range	UV Cut off (nm)
TFA	0.3		210
Phosphate, pK1H2PO4	2.1	1.1 - 3.1	< 200
Phosphate, pK2HPO4^{-2}	7.2	6.2– 8.2	< 200
Phosphate, pK3PO4^{-3}	12.3	11.3– 13.3	< 200
Citrate pK1 C3H5O(COOH)2 (CO)$^{-1}$	3.1	2.1– 4.1	230
Citrate pK2 C3H5O(COOH)2 (COO)$^{-2}$	4.7	3.7– 5.7	230
Citrate pK3 C3H5O (COO)$^{-3}$	6.4	4.4– 6.4	230
Carbonate, pK1HCO3^{-1}	6.1	5.1– 7.1	< 200
Carbonate, pK2CO3^{-2}	10.3	9.3 -11.3	> 200
Acetate	4.8	3.8– 5.8	210
Ammonia	9.3	8.3 - 10.3	200
Borate	9.2	8.2 - 10.2	NA
TEA	10.8	9.8 - 11.8	< 200

e) Selection of pH

The pH range most often used for reversed-phase HPLC is 1-8 and can be divided into low pH (1-4) and intermediate pH (4-8) ranges. Each range has a number of advantages. Low pH has the advantage of creating an environment in which peak tailing is minimized and method ruggedness is maximized. For this reason, operating at low pH is recommended. At a mobile phase pH greater than 5, dissolution of silica can severely shorten the lifetime of columns packed with silica-based stationary phases.

The pKa value (acid dissociation [ionization] constant) for a compound is the pH at which equal concentrations of the acidic and basic forms of the molecule are present in aqueous solutions. Analytes may sometimes appear as broad or tailing peaks when the mobile phase pH is at, or near, their pKa values. If analytes are ionizable, the proper mobile-phase pH must be chosen based on the analyte pKa so the target analyte is in one predominate ionization state ionized or neutral.

Dramatic changes in the retention and selectivity (peak spacing) of basic and acidic compounds can occur when the pH of the mobile phase is changed. His is often a result of different interactions between the column and the analytes when the ionization of these compounds changes. It is important to evaluate these changes when a method is developed in order to select the mobile phase pH that provides the most reproducible results.

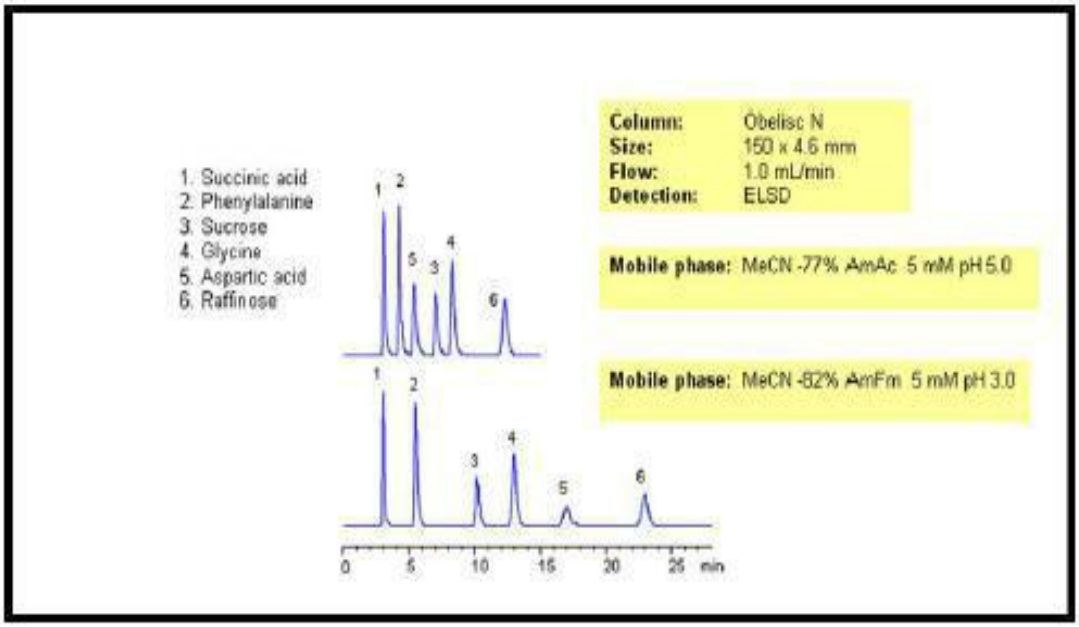

Figure 1.18 Effect of mobile phase pH

1.4 Analytical method validation [24]

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

- Specificity
- Linearity
- Range
- Accuracy
- Precision
  - Repeatability
  - Intermediate Precision
- Detection Limit
- Quantitation Limit
- Robustness

Table 1.5 lists those validation characteristics regarded as the most important for the validation of different types of analytical procedures. This list should be considered typical for the analytical procedures cited but occasional exceptions should be dealt with on a case-by-case basis. Robustness is not listed in the table but should be considered at an appropriate stage in the development of the analytical procedure.

Revalidation may be necessary in the following circumstances:

- changes in the synthesis of the drug substance
- changes in the composition of the finished product
- changes in the analytical procedure

The degree of revalidation required depends on the nature of the changes. Certain other changes may require validation as well.

Table 1.5 Validation characteristics for different analytical procedures

Type of analytical procedure characteristics	Identification	Testing for Impurities		Assay- dissolution (measurement only)- content/potency
		Quantitation	Limit	
Accuracy	-	+	-	+
Precision				
Repeatability	-	+	-	+
Interm. Precision	-	+ (1)	-	+ (1)
Specificity(2)	+	+	+	+
Detection Limit	-	- (3)	+	-
Quantitation Limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

-signifies that this characteristic is not normally evaluated
+signifies that this characteristic is normally evaluated

(1) In cases where reproducibility has been performed, intermediate precision is not needed
(2) Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s)
(3) May be needed in some cases

1.4.1 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.
Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s).
This definition has the following implications:

- Identification: to ensure the identity of an analyte.
- Purity Tests: to ensure that all the analytical procedures performed allow an accurate statement of content of impurities of an analyte, i.e. related substances test, heavy metals, residual solvents content.
- Assay (content or potency): to provide an exact result which allows an accurate statement on the content or potency of the analyte in a sample.

An investigation of specificity should be conducted during the validation of identification tests, the determination of impurities and the assay. The procedures used to demonstrate specificity will depend on the intended objective of the analytical procedure. It is not always possible to demonstrate that an analytical procedure is specific for a particular

analyte (complete discrimination). In this case a combination of two or more analytical procedures is recommended to achieve the necessary level of discrimination.

1.4.1.1 Identification

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

1.4.1.2 Assay and Impurity Test(s)

For chromatographic procedures, representative chromatograms should be used to demonstrate specificity and individual components should be appropriately labelled. Similar considerations should be given to other separation techniques.

Critical separations in chromatography should be investigated at an appropriate level. For critical separations, specificity can be demonstrated by the resolution of the two components which elute closest to each other. In cases where a non-specific assay is used, other supporting analytical procedures should be used to demonstrate overall specificity. For example, where a titration is adopted to assay the drug substance for release, the combination of the assay and a suitable test for impurities can be used.

The approach is similar for both assay and impurity tests:

Impurities are available

For the assay, this should involve demonstration of the discrimination of the analyte in the presence of impurities and/or excipients; practically, this can be done by spiking pure substances (drug substance or drug product) with appropriate levels of impurities and/or excipients and demonstrating that the assay result is unaffected by the presence of these materials (by comparison with the assay result obtained on unspiked samples).

For the impurity test, the discrimination may be established by spiking drug substance or drug product with appropriate levels of impurities and demonstrating the separation of these impurities individually and/or from other components in the sample matrix.

Impurities are not available

If impurity or degradation product standards are unavailable, specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to a second well-characterized procedure e.g.: pharmacopoeial method or other validated analytical procedure (independent procedure). As appropriate, this should include samples stored under relevant stress conditions: light, heat, humidity, acid/base hydrolysis aand oxidation.

- for the assay, the two results should be compared;
- for the impurity tests, the impurity profiles should be compared.

Peak purity tests may be useful to show that the analyte chromatographic peak is not attributable to more than one component (e.g., diode array, mass spectrometry).

1.4.2 Linearity

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

A linear relationship should be evaluated across the range (see section 1.5.3) of the analytical procedure. It may be demonstrated directly on the drug substance (by dilution of a standard stock solution) and/or separate weighings of synthetic mixtures of the drug product components, using the proposed procedure. The latter aspect can be studied during investigation of the range.

Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares. In some cases, to obtain linearity between

assays and sample concentrations, the test data may need to be subjected to a mathematical transformation prior to the regression analysis. Data from the regression line itself may be helpful to provide mathematical estimates of the degree of linearity.
The correlation coefficient, y-intercept, slope of the regression line and residual sum of squares should be submitted. A plot of the data should be included. In addition, an analysis of the deviation of the actual data points from the regression line may also be helpful for evaluating linearity.
Some analytical procedures, such as immunoassays, do not demonstrate linearity after any transformation. In this case, the analytical response should described by an appropriate function of the concentration (amount) of an analyte in a sample. For the establishment of linearity, a minimum of 5 concentrations is recommended. Other approaches should be justified.

1.4.3 Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.
If assay and purity are performed together as one test and only a 100% standard is used, linearity should cover the range from the reporting level of the impurities to 120% of the assay specification.

1.4.4 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.

1.4.4.1 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 (independent procedure, see 1.5.1.2)
- **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 analysed 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.

1.4.4.2 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 (see 1.5.1.2). 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.

1.4.4.3 Recommended Data

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.

1.4.5 Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution.

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

1.4.5.1 Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

Repeatability should be assessed using:

- **a)** A minimum of 9 determinations covering the specified range for the procedure (e.g., 3 concentrations/3 replicates each); or
- **b)** A minimum of 6 determinations at 100% of the test concentration.

1.4.5.2 Intermediate precision

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. The applicant should establish the effects of random events on the precision of the analytical procedure. Typical variations to be studied include days, analysts, equipment, etc. It is not considered necessary to study these effects individually. The use of an experimental design (matrix) is encouraged.

1.4.5.3 Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

Reproducibility is assessed by means of an inter-laboratory trial. Reproducibility should be considered in case of the standardization of an analytical procedure, for instance, for inclusion of procedures in pharmacopoeias. These data are not part of the marketing authorization dossier.

Recommended Data

The standard deviation, relative standard deviation (coefficient of variation) and confidence interval should be reported for each type of precision investigated.

Detection limit

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

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: $\frac{\sigma}{S}$

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 σ may be carried out in a variety of ways, for example:

Based on the Standard Deviation of the Blank

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

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

Recommended Data

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.

Quantitation limit

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

Several approaches for determining the quantitation limit are possible, depending on whether the procedure is non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

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

1.4.6 System Suitability Test

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated.

AIM AND OBJECTIVES

3.1 Aim of work

The number of drugs introduced into the market is increasing every year.
These drugs may be either new entities or partial structural modification of the existing one. The pharmaceutical dosage forms are widely presented with multiple active components i.e. in combined dosage forms. These combination products present challenge to the analytical chemist responsible for the development and validation of analytical methods. This has opened new task for analyst for simultaneous estimation of different drugs in such combined dosage form.

Atorvastatin is an antihyperlipidemic agent, used for reducing high cholesterol level. Irbesartan is an antihypertensive agent, used in treatment of hypertension. In August 2013, Hanmi pharmaceutical (Korea) has applied for patent that includes combination of irbesartan and atorvastatin which is used in treatment of coronary artery disease.

Various analytical methods have been reported for estimation of irbesartan as well as atorvastatin and in alone and in combination with other drugs but no method has been reported for their simultaneous estimation in combined dosage form. Therefore, it was thought of interest to develop RP-HPLC method for simultaneous estimation of irbesartan and atorvastatin in combined dosage form.

3.2 Objective of work

To develop reversed phase high performance liquid chromatographic method for simultaneous estimation of irbesartan and atorvastatin in synthetic mixture.
To validate developed method as per ICH Q2 (R1) guidelines.

REVIEW OF LITERATURE [27, 31-32]

An extensive survey was carried out for the simultaneous estimation of Irbesartan and Atorvastatin in bulk and marketed dosage forms. Some of the methods reported are present below.

1) **ParasVirani1, Rajanit Sojitra , Hasumati Raj and Vineet Jain-**
   A simple, accurate and precise spectroscopic method was developed for simultaneous estimation of Irbesartan and atorvastatin in synthetic mixture using simultaneous equation Method. In this spectroscopic method, 226.00 nm and 246.00 nm wavelengths were selected for measurement of absorptivity. Both the drugs show linearity in a concentration range of 05-30 µg/ml at their respective λmax. Accuracy, precision and recovery studies were done by QC samples covering lower, medium and high concentrations of the linearity range. The relative standard deviation for accuracy, precision studies were found to be within the acceptance range (<2%). The limit of determination was 0.033µg/ml and 0.125 µg/ml for Irbesartan and atorvastatin, respectively. The limit of quantification was 0.1008 µg/ml and 0.3792 µg/ml for Irbesartan and atorvastatin, respectively. Recovery of Irbesartan and atorvastatin were found to be 99.75 % and 99.52% respectively confirming the accuracy of the proposed method. The proposed method is recommended for routine analysis since they are rapid, simple, accurate and also sensitive and specific by no heating and no organic solvent extraction.

2) **Majdi Mohammad Bkhaitan-**
   The present study describes a convenient stability-indicating HPLC-DAD method for the separation and simultaneous determination of the drugs atorvastatin, irbesartan, and amlodipine in bulk and formulations. The method proposed demonstrated good separation between atorvastatin, irbesartan, amlodipine and their main degradation products. Separation was executed on a Waters XBrigde C18 column (5 µm, 25 × 0.46 cm) using a gradient mobile phase system consisting of acetonitrile and orthophosphoric acid buffer (pH 2.2) at a flow rate of 1 mL/min and UV detection at 240 nm. The drugs were subjected to acidic and basic hydrolysis, oxidation, exposure to UV light, and exposure to temperature (dry heat) to apply stress conditions. Linearity of the method was evaluated in the range 5–30 µg/mL for each drug with correlation coefficient values (r 2) of 0.9982, 0.9973, and 0.9986 for atorvastatin, irbesartan, and amlodipine, respectively. The limits of detection were 0.05, 0.06, and 0.08 µg/mL for the three compounds, respectively. No interference was observed with the detection of the compounds in presence of degradable products; hence, it is confirmed that the analysis is stability-indicating.

3) **Charles G. Smith-**
   From the literature survey no method available for fix dose combination of Irbesartan and atorvastatin. Recently Hanmi pharmaceutical take patent on bilayer tablet and solid oral dosage form of Irbesartan and atorvastatin so the formulation of this two drug is require for single therapy of hypertension and hyperlipidaemia. The development of new dosage form in pharmaceutical industry requires analytical method for estimation, assay, impurity, stability, etc. In development of new dosage form for Irbesartan and atorvastatin primary requirement is development of new analytical method and it's apply for various purpose of identification, impurity profiling, assay estimation and other application.

MATERIALS AND METHODS

Apparatus and instruments

- **Instrument:**

Sr. No.	Name of Instrument	Company Name
1	HPLC Instrument	System: HPLC Binary Gradient System Model no.: HPLC 3000 Series Software: HPLC Workstation
2	UV-Spectrophotometer	Analytical Technologies Limited
3	Column (C18)	Column: Grace C18 (250mm x 4.6ID, Particle size: 5 micron)
4	pH meter	VSI pH meter (VSI 1-B)
5	Balance	WENSARTM High Resolution Balance

Reagents and materials

Irbesartan (Gift sample recieved from Alembic pharmaceuticals, Vadodara) (99.8%, w/w)
Atorvastatin (Gift sample received from Vapicare pharma, Vapi) (99.8%, w/w)

Sr. No.	Chemical/Solvent/Reagent	Make	Grade
1	Water	MI	HPLC
2	Methanol	Honeywell	HPLC
3	Acetonitrile	Fisher Scientific	HPLC
4	Formic acid	Fisher Scientific	Extra Pure
5	Triethylamine	Fisher Scientific	Extra Pure

Characterization of drugs

4.3.1 Melting point

Melting point was determined by capillary method. Observed values were compared with reported values.

4.3.2 IR spectra

Infrared spectra of irbesartan and atorvastatin were recorded and compared with reference spectra.

4.4 Preparation of standard solutions

4.4.1 Stock solution of IRB

Accurately weighed quantity of irbesartan 150 mg was transferred in to 100 ml volumetric flask, dissolved and diluted to mark with methanol (1500 µg/ml).

4.4.2 Working standard solution of IRB

One ml aliquot from irbesartan stock solution was diluted to 10 ml with mobile phase (150 µg/ml).

4.4.3 Stock solution of ATR

Accurately weighed quantity of atorvastatin 25 mg was transferred into 25 ml volumetric flask, dissolved and diluted to mark with methanol (1000 µg/ml).

4.4.4 Working standard solution of ATR

One ml aliquot from atorvastatin stock solution was diluted to 10 ml with mobile phase (100 µg/ml). Further, 1 ml was diluted to 10 ml with mobile phase (10 µg/ml).

4.4.5 Mixture solution (IRB + ATR)

Five ml aliquot from irbesartan working standard solution and five ml aliquot from atorvastatin working standard solution were transferred into 10 ml volumetric flask (75 µg/ml IRB and 5 µg/ml ATR).

4.5 Preparation of synthetic mixture

The formula for preparation of synthetic mixture was obtained from patent.[78] The ingredients are listed in Table 4.1.

Table 4.1 List of ingredients for preparation of synthetic mixture

Sr. No.	Ingredients	Quantity
1	Irbesartan	150 mg
2	Atorvastatin	10 mg
3	Pre- gelatinized starch	23 mg
4	Croscarmellose sodium	12 mg
5	Povidone	8 mg
6	Poloxamer188	9 mg
7	Lactose	120 mg
8	Microcrystalline cellulose	65.6 mg
9	Crospovidone	36 mg
10	Magnesium carbonate	45 mg
11	Hydroxy propyl cellulose	3 mg
12	Polysorbate 80	1.2 mg
13	Magnesium stearate	7 mg

14	Water	380 mL

4.6 Development of HPLC method

4.6.1 Selection of wavelength.

The solutions of irbesartan (25 µg/ml) and atorvastatin (25 µg/ml) were scanned individually in the range of 200-400 nm against mobile phase as blank in UV-visible spectrophotometer. Wavelength was selected from the overlain UV spectrum.

4.6.2 Mobile phase optimization

Initial trials were conducted with a view to select a suitable solvent system.

The suitability of the mobile phase was decided on the basis of the sensitivity of the assay, time required for the analysis, ease of preparation, and use of readily available cost-effective solvents. These include methanol, acetonitrile, water and formic acid in different proportions with different pH. Trials are depicted in Table 4.2

- **Preparation:**

The solvents were mixed, pH was adjusted, filtered through Milipore filtration assembly (0.45 µ pore size) and sonicated for 15 minutes for degassing.

Table 4.2 Mobile phase optimization

MOBILE PHASE	FLOW RATE	REMARK
Methanol: Water (70: 30, v/v)	1.0 ml/min	Splited peaks
ACN: 0.1% formic acid (50: 50, v/v)	1.0 ml/min	Tailing
ACN: 0.1% formic acid (50: 50, v/v) (pH 3.5 with triethylamine)	1.0 ml/min	Higher retention time
ACN: Methanol: 0.1% formic acid (50: 10: 40, v/v) (pH 3.5 with triethylamine)	1.0 ml/min	Sharp and resolved peaks, Total run time < 8 min

4.6.3 Optimized chromatographic conditions

The optimized chromatographic conditions are described in Table 4.3

Stationary phase	C18, Thermo (250 mm × 4.6 mm, 5µm)
Mobile phase	Acetonitrile: methanol: 0.1% formic acid (50: 10: 40 %, v/v/v)
pH	adjusted to 3.5 with trimethylamine
Flow rate	1 mL/min
Temperature	25 ± 2˚C
Wavelength	271 nm
Total run time	10 min

Table 4.3 Optimized chromatographic conditions

4.6.4 System suitability test

The resolution, column efficiency and peak symmetry were calculated for the standard solution mixture (Section 4.4.5).

4.6.5 Solution stability study

Stability of solutions was established by using standard and test solution. The solutions were stored at room temperature for 24 hours and re-analyzed. Results were compared against freshly prepared solutions and % variation was calculated.

4.7 Validation of proposed method

4.7.1 Specificity

The lack of chromatographic interference from endogenous matrix components was investigated using blank sample and as well as placebo as per the procedure given in section 4.6.3. Ingredients for preparation of placebo are listed in Table 4.4.

Table 4.4 Ingredients for preparation of placebo

Sr. No.	Ingredient	Quantity
1	Pre- gelatinized starch	23 mg
2	Croscarmellose sodium	12 mg
3	Povidone	8 mg

4	Poloxamer188	9 mg
5	Lactose	120 mg
6	Microcrystalline cellulose	65.6 mg
7	Crospovidone	36 mg
8	Magnesium carbonate	45 mg
9	Hydroxy propyl cellulose	3 mg
10	Polysorbate 80	1.2 mg
11	Magnesium stearate	7 mg
12	Water	380 mL

4.7.2 Linearity

The linearity was evaluated by linear regression analysis. The linear responses of irbesartan and atorvastatin was determined by analysing five independent levels (4, 8, 12, 16 and 20 µl of mixture solution, section 4.4.5) as per the procedure given in section 4.6.3.

4.7.3 Precision

4.7.3.1 Repeatability

It was determined by analysing standard mixture solution (section 4.4.5) seven times as per the procedure given in section 4.6.3.

4.7.3.2 Intraday precision

It was determined by analyzing three concentrations of standard mixture solution (section 4.4.5) three times in a day as per the procedure given in section 4.6.3.

4.7.3.3 Interday precision

It was determined by analyzing three concentrations of standard mixture solution (section 4.4.5) on three different days as per the procedure given in section 4.6.3.

4.7.4 Accuracy

The accuracy in terms of extraction efficiency of the method was determined by standard addition. The known amount of irbesartan and atorvastatin were added to prequantified synthetic mixture sample at three levels (i.e 80%, 100% and 120%) and analysed.

Procedure:

Synthetic mixture powder equivalent to 150 mg of irbesartan and 10 mg of atorvastatin was accurately weighed and transferred into four individual 100 ml volumetric flasks.

Standard irbesartan 120mg, 150 mg and 180 mg as well as standard atorvastatin 8 mg, 10 mg and 12 mg were spiked in first, second and third volumetric flask respectively.

The flasks were filled to about 80 % with methanol, sonicated for 10 minutes and diluted with methanol to mark.

These solutions were filtered through Whatman filter (paper no. 42) individually; the first few ml was discarded.

One ml aliquot from filtrate was diluted to 10 ml with mobile phase individually.

Further, 5 ml was diluted to 10 ml with mobile phase individually. (80% level: 135 µg/ml IRB + 9 µg/ml ATR, 100% level: 150 µg/ml IRB + 10 µg/ml ATR, 120% level: 165 µg/ml IRB + 11 µg/ml ATR).

Thus obtained mixture solutions were analyzed by injecting 6 µl using chromatographic conditions given in section 4.6.3.

4.7.5 Limit of detection

The LOD was estimated from the set of 5 calibration curves used to determine linearity. The LOD may be calculated as

$$LOD = 3.3 \times (SD/Slope)$$

Where, SD = Standard deviation of the intercepts of the 5 calibration curves. Slope = Mean slope of the 5 calibration curves.

4.7.6 Limit of quantitation

The LOQ was estimated from the set of 5 calibration curves used to determine linearity. The LOQ may be calculated as LOQ = 10 × (SD/Slope)

Where, SD = Standard deviation of the intercepts of the 5 calibration curves. Slope = Mean slope of the 5 calibration curves.

4.7.7 Robustness

To evaluate HPLC method robustness a few parameters were deliberately varied. The parameters included variation of flow rate, Wavelength, Temperature.

4.8 Analysis of synthetic mixture

Synthetic mixture powder equivalent to 150 mg of irbesartan and 10 mg of atorvastatin was accurately weighed and transferred into 100 ml volumetric flask.

The flask was filled to about 80 % with methanol, sonicated for 10 minutes and diluted with methanol to mark.

The solution was filtered through Whatman filter (paper no. 42); the first few ml was discarded.

One ml aliquot from filtrate was diluted to 10 ml with mobile phase.

Further, 5 ml was diluted to 10 ml with mobile phase (75 µg/ml irbesartan + 5 µg/ml atorvastatin).

Thus obtained mixture solution was analyzed by injecting 12 µl using chromatographic conditions given in section 4.6.3.

RESULTS AND DISCUSSION

5.1 Characterization of drugs

5.1.1 Melting point

Data of melting point is given in Table 5.1

Table 5.1 Melting point of ATR and IRB

Drugs	Reported M.P. [28, 31]	Observed M.P.
Irbesartan	180- 182^{0}C	180.5- 182.3^{0}C
Atorvastatin	159.2- 160.7^{0}C	158.2- 159.5^{0}C

5.1.2 IR Spectrum

Recorded IR spectra of irbesartan and atorvastatin are shown in Figure 5.1 and 5.3 respectively.

These were compared with the reference IR spectra of irbesartan and atorvastatin, shown in Figure 5.2 and 5.4 respectively. The spectra were found to be identical.

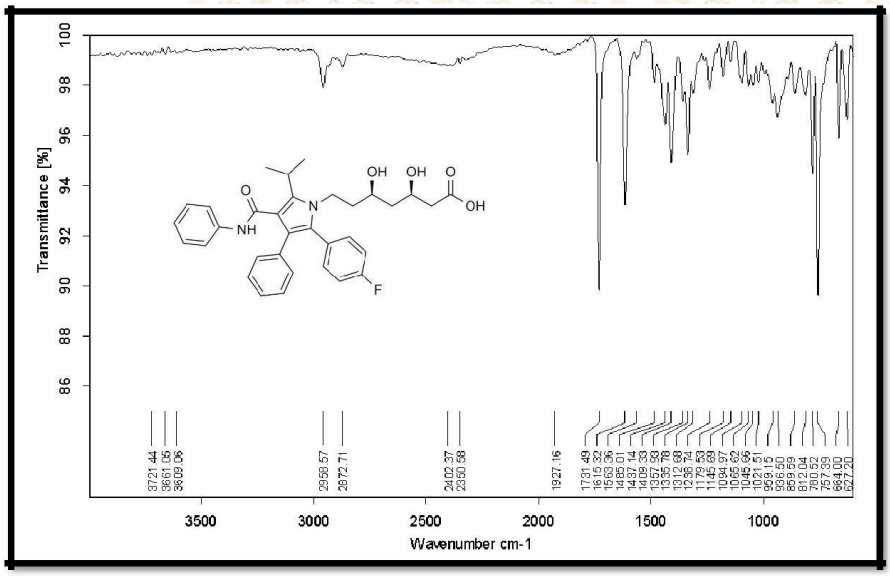

Figure 5.1 Recorded IR Spectra of irbesartan

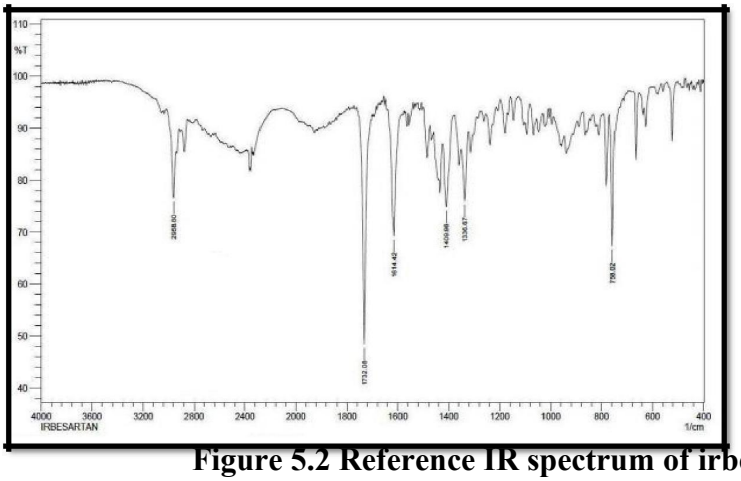

Figure 5.2 Reference IR spectrum of irbesartan[79]

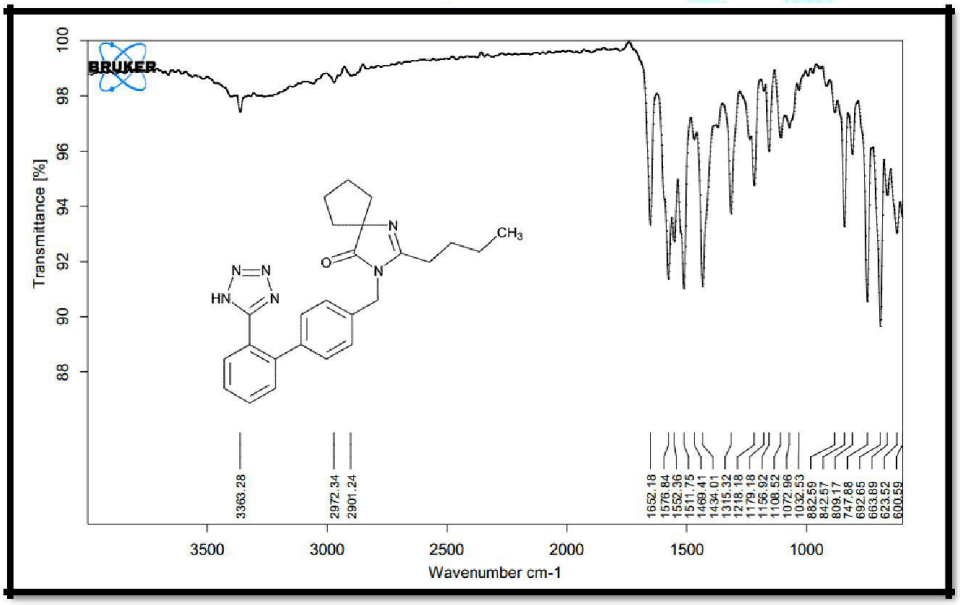

Figure 5.3 Recorded IR Spectra of atorvastatin

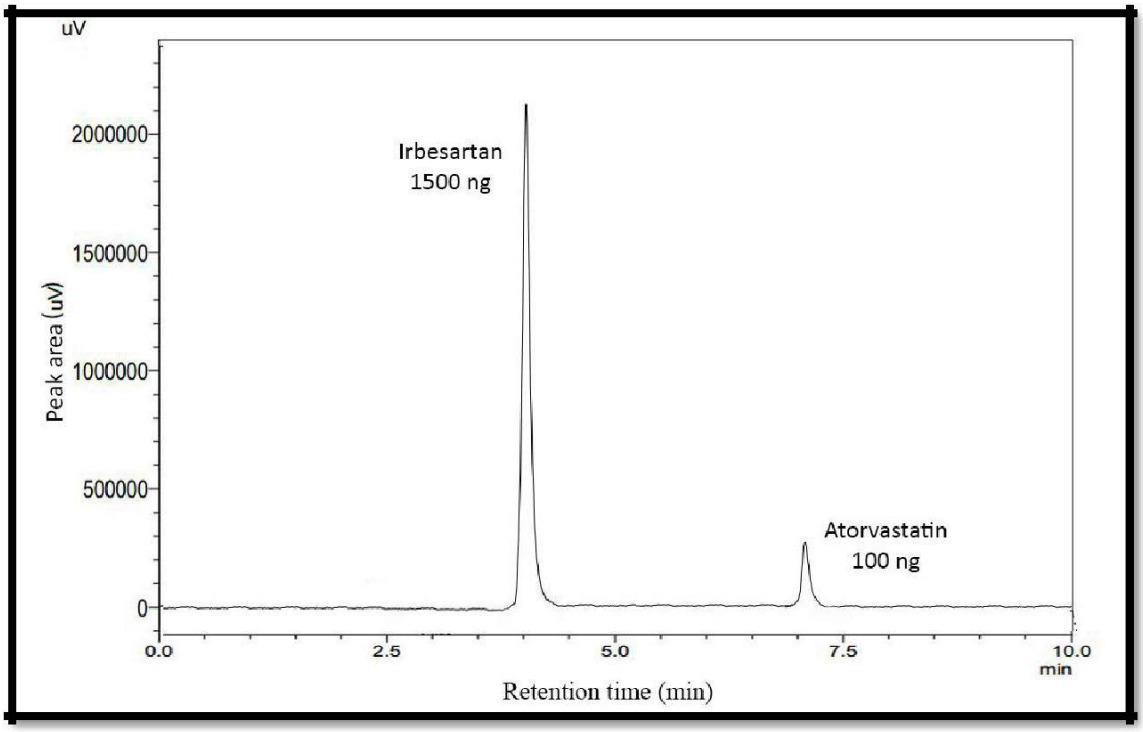

Figure 5.6 Chromatogram of system suitability test (injection volume 20 µl)

5.5 Solution stability study

Results are shown in table 5.3

Table 5.3 Solution stability study data

Sample	Mean area of peak Fresh solution (n=3)	Mean area of peak After 24 hours (n=3)	% variation
IRB standard (900 ng)	5420.95	5406.70	0.26
ATR standard (60 ng)	439.94	442.54	0.59
IRB test (900 ng)	5405.72	5415.65	0.18
ATR test (60 ng)	440.71	441.49	0.17

The results indicated no significant degradation within indicated period. So the solutions are stable for 24 hours at room temperature.

5.6 Validation of method

5.6.1 Specificity

Chromatograms of blank, placebo and mixture solution are shown in Figure 5.7, 5.8 and 5.9 respectively.The blank and placebo solution did not show any peaks and both drug peak were found to appear at retention time different from each other

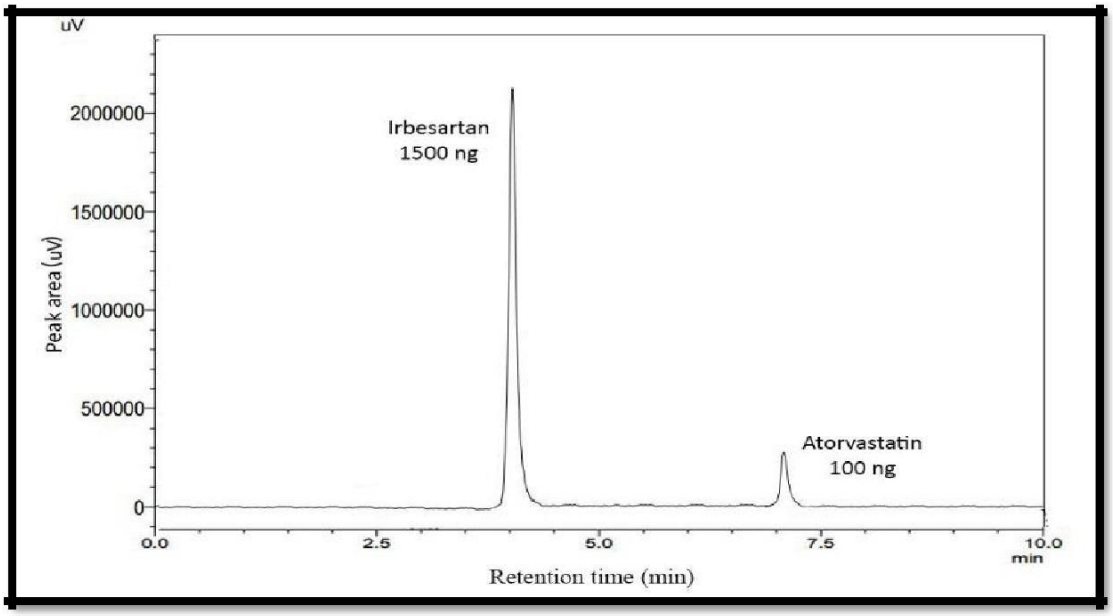

Figure 5.9 Chromatogram of mixture solution (injection volume 20 µl)

As there was no interference of impurities and excipients, the method can be considered specific.

5.6.2 Linearity

The calibration curve was prepared by plotting peak area against respective concentration. The peak areas of irbesartan and atorvastatin were linear with respect to concentrations over the range of 300-1500 ng and 20-100 ng respectively. Results are expressed in terms of correlation coefficient which were found to be 0.9993 and 0.9996 for irbesartan and atorvastatin respectively. Data is shown in Table 5.4. The overlain chromatogram is shown in Figure 5.10. Calibaration curve plot for irbesartan and atorvastatin is shown in Figure 5.11 and 5.12 respectively.

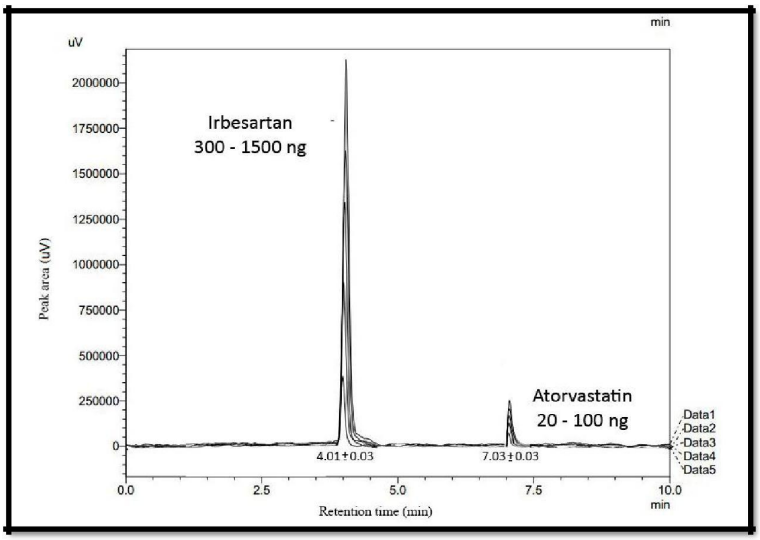

Table 5.4 Linearity data for IRB and ATR

Irbesartan			Atorvastatin		
Amount of IRB injected (ng)	**Peak area (mV) Mean ± S.D. (n = 5)**	**R.S.D.**	**Amount of IRB injected (ng)**	**Peak area (mV) Mean ± S.D. (n = 5)**	**R.S.D.**
300	1237.84 ± 04.94	0.40	20	235.03 ± 0.51	0.22
600	3200.38 ± 08.31	0.26	40	332.03 ± 1.23	0.37
900	5417.82 ± 17.33	0.32	60	441.33 ± 1.97	0.45
1200	7733.22 ± 20.02	0.26	80	544.72 ± 1.22	0.22
1500	9886.11 ± 35.02	0.35	100	654.16 ± 3.08	0.47

The results show excellent correlation between peak area and concentrations.

Irbesartan			Atorvastatin		
Amount of IRB injected (ng)	Peak area (mV) Mean ± S.D. (n = 3)	R.S.D.	Amount of ATR injected (ng)	Peak area (mV) Mean ± S.D. (n = 3)	R.S.D
600	3205.39 ± 06.15	0.19	40	331.05 ± 1.18	0.36
900	5427.47 ± 12.79	0.24	60	441.98 ± 1.73	0.39
1200	7727.95 ± 23.79	0.31	80	544.05 ± 1.06	0.20

Table 5.6 Intraday precision data for IRB and ATR

The method was found to be precise.

5.6.3.3 Interday precision

Interday precision data for irbesartan and atorvastatin is shown in Table 5.7.

Irbesartan			Atorvastatin		
Amount of IRB injected (ng)	Peak area (mV) Mean ± S.D. (n = 3)	R.S.D.	Amount of ATR injected (ng)	Peak area (mV) Mean ± S.D. (n = 3)	R.S.D.
600	3199.66 ± 09.95	0.31	40	332.28 ± 1.58	0.48
900	5415.82 ± 22.80	0.42	60	440.36 ± 1.78	0.40
1200	7733.79 ± 28.16	0.36	80	544.80 ± 1.65	0.30

Table 5.7 Interday precision data for IRB and ATR

The method was found to be precise.

5.6.4 Accuracy

Percent recovery data for irbesartan and atorvastatin is shown in Table 5.8 and 5.9 respectively.

% Recovery Level	Amount of IRB in synthetic mixture (mg)	Amount of std IRB spiked (mg)	Amount of IRB Recovered (mg)	% Recovery	Mean % Recovery
80%	150	120	119.93	99.94	99.84
	150	120	119.71	99.71	
	150	120	119.85	99.87	
100%	150	150	150.13	100.09	99.98
	150	150	149.85	99.90	
	150	150	149.93	99.95	
120%	150	180	179.91	99.95	99.98
	150	180	179.91	99.94	
	150	180	180.09	100.05	

5.6.5 Robustness

Robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variation in method parameters. The robustness of a method is evaluated by varying method parameters such as wavelength, temperature, flow rate, etc. and determining the effect (if any) on the results of the method. The parameters were evaluated in the robustness studies of the estimation of related substances. System suitability was checked at each variable condition and data found within the acceptance criteria. Relative retention time of ideal conditions data was compared with data at each variable condition.

Change in detector wavelength

Table: 5.11 System suitability data

System suitability parameter	Ideal condition (256nm)	Wavelength decrease (255nm)	Wavelength increase (257nm)
Resolution Between Atorvastatin & Irbesartan	3.95	3.96	3.95
USP tailing	1.05	1.05	1.05
Theoretical plates	6129	6118	6198

Table: 5.12 Robustness- Change in wavelength

DRUG	RRT(SST)			RRT(spiked sample)		
	Ideal (256 nm)	Wavelength (255 nm)	Wavelength (255 nm)	Ideal (256 nm)	Wavelength (255 nm)	Wavelength (255 nm)
Atorvastatin	1.00	1.00	1.00	1.00	1.00	1.00
Irbesartan	1.21	1.21	1.21	1.21	1.21	1.21

Change in column oven temperature

Table: 5.13 System suitability data

System suitability parameter	Ideal condition (35°C)	Temp. decrease (30°C)	Temp. increase (40°C)
Cc	3.95	3.95	3.95
USP tailing	1.05	1.05	1.05
Theoretical plates	6129	6187	6092

Table: 5.14 Robustness - Change in column oven temperature

Name	RRT(SST)			RRT(spiked sample)		
	Ideal(35°C)	Temp. dec. (30°C)	Temp. inc. (40°C)	Ideal(35°C)	Temp. dec. (30°C)	Temp. inc. (40°C)
Atorvastatin	1.00	1.00	1.00	1.00	1.00	1.00
Irbesartan	1.21	1.21	1.21	1.21	1.21	1.21

Change in flow rate of mobile phase

Table: 5.15 System suitability data

System suitability parameter	Ideal condition (1 ml/min)	Flow decrease (0.9 ml/min)	Flow increase (1.1 ml/min)
Resolution Between ATORVASTATIN &	3.95	4.00	3.91

IRBESARTAN			
USP tailing	1.05	1.06	1.05
Theoretical plates	6129	6383	5889

Table: 5.16 Robustness - change in flow rate

Name	RRT(SST)			RRT(spiked sample)		
	Ideal (1 ml/min)	Flow dec. (0.9 ml/min)	Flow inc. (1.1 ml/min)	Ideal (1 ml/min)	Flow dec. (0.9 ml/min)	Flow inc. (1.1 ml/min)
Atorvastatin	1.00	1.00	1.00	1.00	1.00	1.00
Irbesartan	1.21	1.20	1.21	1.21	1.20	1.21

5.7 Summary of validation

Table 5.17 Summary of validation results

Parameters	**Results**	
	Irbesartan	**Atorvastatin**
Specificity	Specific	Specific
Linearity (ng) (n=5)	300 – 1500	20 – 100
Straight Line Equation	Y= 7.2765x - 1053.7	Y= 5.2547x + 126.17
Correlation coefficient (R^2)	0.9993	0.9996
Repeatability (R.S.D.) (n=7)	0.16	0.20
Intraday precision (R.S.D.) (n=3*3)	0.19 – 0.31	0.20 – 0.39
Interday precision (R.S.D.) (n=3*3)	0.31 – 0.42	0.30 – 0.48
% Recovery (n=3*3)	99.84 – 99.98	99.90 – 100.67
LOD (ng)	10.94	1.33
LOQ (ng)	33.14	4.03

The method represents a fast analytical procedure for the simultaneous estimation of irbesartan and atorvastatin. The sample preparation is simple and the elution is isocratic. The method is amenable to the large number of samples with excellent precision and accuracy.

5.8 Analysis of synthetic mixture

The method was successfully applied to synthetic mixture for the quantitative Analysis of irbesartan and atorvastatin. The chromatogram is shown in Figure 5.14. Results are shown in Table 5.12.

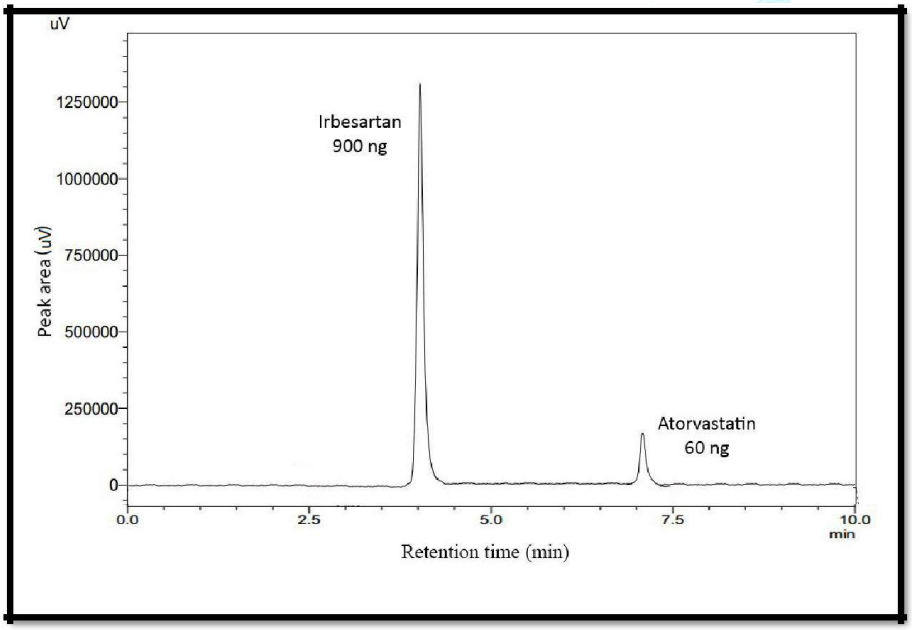

Figure 5.14 Chromatogram of synthetic mixture

Table 5.18 Analysis of synthetic mixture

Drugs	Amount of drug in synthetic mixture (mg)	Amount of drug found in synthetic mixture (mg) (n=3)	Assay (%) Mean ± SD
Irbesartan	150	149.34	99.56 ± 0.24
Atorvastatin	10	9.83	98.30 ± 0.32

CONCLUSION

The present work involves the development and validation of RP-HPLC method for simultaneous estimation of irbesartan and atorvastatin in synthetic mixture. The method was validated as per ICH Q2 (R1) guidelines. The

method was found to be sensitive, specific, accurate and precise. The proposed method can be successfully applied for routine analysis of combined dosage forms.

SUMMARY

Irbesartan is an antihypertensive agent. It is an angiotensin II type I receptor antagonist and therefore blocks the vasoconstricting and aldosterone-secreting effects of angiotensin II. The combined effect reduces blood pressure. Atorvastatin is a lipid lowering agent. It competitively inhibits HMG-CoA reductase, the enzyme that catalyses the conversion of HMG-CoA to mevalonic acid. This results in the induction of the LDL receptors, leading to lowered LDL-cholesterol concentration.

In august 2013, Hanmi Pharmaceuticals (Korea) filed a patent for a dosage form that includes combination of irbesartan and atorvastatin for the treatment of coronary artery disease.

Irbesartan is official in EP 7.0 and USP 32, while atorvastatin is official in IP 2010. Several methods have been described in literature for estimation of irbesartan and atorvastatin in alone and in combination with other drugs. But to best of our knowledge, no scientific literature has been published for simultaneous estimation of irbesartan and atorvastatin in combined dosage forms. So the aim was to develop a liquid chromatographic method for the same. Since formulation is not yet available in the Indian market (till April 2015), we formulated a synthetic mixture and applied the proposed method.

The proposed RP-HPLC method was used for simultaneous estimation of irbesartan and atorvastatin in synthetic mixture. The chromatographic separation was achieved on C18 column (250 x 4.6 mm, 5 µm) using acetonitrile: methanol: 0.1% formic acid (pH adjusted to 3.5 with triethylamine) in the proportion of 50: 10: 40 %, v/v/v as isocratic mobile phase at flow rate 1 ml/min. UV detection was carried out at 271 nm. The retention time for irbesartan and atorvastatin were found to be 4.01 ± 0.03 and 7.03 ± 0.03 min respectively. The resolution was found to be 12.51. The theoretical plates were found to be 7339.6 and 8916.2 for irbesartan and atorvastatin respectively. The tailing factor was found to be 1.404 and 1.311 for irbesartan and atorvastatin respectively.

The method was validated as per ICH Q2 (R1) guidelines. The linearity of method was satisfactory over the range 300-1500 ng (correlation coefficient 0.9993) and 20-100 ng (correlation coefficient 0.9996) for irbesartan and atorvastatin respectively. The Solutions were found to be stable at room temperature for 24 hours. The RSD for repeatability was found to be 0.16 % and 0.20 % for irbesartan and atorvastatin respectively. The RSD for intraday precision was found to be 0.19-0.31 % and 0.20-0.39 % for irbesartan and atorvastatin respectively. The RSD for interday precision was found to be 0.31-0.42 % and 0.30-0.48 % for irbesartan and atorvastatin respectively. The limits of detection for irbesartan and atorvastatin were 10.94 and 1.33 ng respectively. The limits of quantitation for irbesartan and atorvastatin were 33.14 and 4.03 ng respectively. Recovery of irbesartan and atorvastatin ranged from 99.84 – 99.98 % and 99.90 – 100.67 % respectively. The method was successfully applied to synthetic mixture for quantitative analysis of of irbesartan and atorvastatin. The assay results were found to be 99.56 ± 0.24 % and 98.30 ± 0.32 % for irbesartan and atorvastatin respectively.

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