METHOD DEVELOPMENT AND VALIDATION OF RELATED SUBSTANCES FOR THE SIMULTANEOUS ESTIMATION OF ATORVASTATIN AND EZETIMIBE IN TABLET DOSAGE FORM BY RP-HPLC

A Dissertation submitted to

THE TAMILNADU Dr. M.G.R MEDICAL UNIVERSITY
CHENNAI – 600 032

In partial fulfillment of the requirements for the award of the Degree of

MASTER OF PHARMACY
IN
BRANCH – III→ PHARMACEUTICAL ANALYSIS

Submitted by

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CERTIFICATE

This is to certify that the project entitled **"METHOD DEVELOPMENT AND VALIDATION OF RELATED SUBSTANCES FOR THE SIMULTANEOUS ESTIMATION OF ATORVASTATIN AND EZETIMIBE IN TABLET DOSAGE FORM"** was submitted by **MOHAMED SAMEER .M.H (261930004)** in partial fulfillment for the award of the Degree of **Master of Pharmacy (Pharmaceutical Analysis).** The project was carried out at The Madras Pharmaceutical (P) Ltd., Karapakkam, Chennai - 600 097 and at C.L. Baid Metha College of Pharmacy, Chennai - 600 097 under my supervision in the Department of Pharmaceutical Analysis during the academic year 2019 – 2021.

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We found him conduct was good during the period. We wish him success in all his endeavors.

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DECLARATION

I hereby declare that this dissertation entitled, **"METHOD DEVELOPMENT AND VALIDATION OF RELATED SUBSTANCES FOR THE SIMULTANEOUS ESTIMATION OF ATORVASTATIN AND EZETIMIBE IN TABLET DOSAGE FORM"** has been originally carried out by me at The Madras Pharmaceutical (P) Ltd., Karapakkam, Chennai - 600 097 and under the guidance and supervision of **Dr. C. N. Nalini, M.Pharm., Ph.D.,** Head of the Department of Pharmaceutical Analysis, C.L.Baid Metha College of Pharmacy, Chennai- 600 097., during the academic year 2019 – 2021. This work has not been submitted in any other degree at any other university and that all the sources have used or quoted have been indicated and acknowledged by complete reference.

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INDEX

LIST OF ABBREVIATIONS

UV	:	Ultra-Violet
IR	:	Infrared
MS	:	Mass Spectrometry
GC	:	Gas Chromatography
TLC	:	Thin Layer Chromatography
LC–MS	:	Liquid Chromatography and Mass Spectrometry
UPLC	:	Ultra Performance Liquid Chromatography
HPLC	:	High Performance Liquid Chromatography
NP	:	Normal Phase
RP	:	Reverse Phase
nm	:	nanometer
psi	:	pounds per square inch
mL	:	milliliter
USP	:	United States Pharmacopeia
NF	:	National Formulary
SOP	:	Standard Operating Procedure
e.g.	:	example
etc	:	Etcetera
%	:	percentage
LOQ	:	Limit of Quantification
LOD	:	Limit of Detection
pKa	:	Dissociation constant
pH	:	Hydrogen ion concentration
mg	:	milligram
PDA	:	Photo Diode Array

min	:	minutes
ICH	:	International Conference on Harmonization
µm	:	micrometer
λ_{max}	:	Absorbance maximum
API	:	Active Pharmaceutical Ingredient
RS	:	Reference Standard
µg	:	microgram
ppm	:	parts per million
°C	:	Degrees Centigrade
µl	:	microliter
RT	:	Retention Time
Inj.	:	Injection
ACN	:	Acetonitrile
NMT	:	Not More Than
NLT	:	Not Less Than
g.	:	grams
AU	:	Absorbance Units
RSD	:	Relative Standard Deviation
rpm	:	revolutions per minute
PVDF	:	Polyvinylidenefluoride
Std.area	:	Standard area
Sam.area	:	Sample area
w/w	:	weight by weight
v/v	:	volume by volum

INTRODUCTION

1. INTRODUCTION

1.1 Introduction to Pharmaceutical Analysis

Pharmaceutical analysis plays a vital role in the Quality Assurance and Quality control of bulk drugs. Analytical chemistry involves separation, identification, and determining the relative amounts of components in a sample matrix; Pharmaceutical analysis is a specialized branch of analytical chemistry that derives its principles from various branches of sciences like physics, microbiology, nuclear science, and electronics etc. Qualitative analysis is required before a quantitative analysis can be undertaken.

Analytical method development and validation is a good research in the field of pharmaceutical analysis, utilized to determine the drug content in bulk and pharmaceutical dosage forms and in biological fluids like blood, serum, urine etc. In view of the industrial scenario and literature, it was noted that chromatographic techniques like HPLC, LC–MS/MS methods have created revolutionary precision and accuracy in quantification of drugs in Formulation and in biological fluids even at low concentration.

Need for pharmaceutical Analysis

- New drug development.
- Method Validation as for ICH Guidelines
- Research in Pharmaceutical Sciences
- Clinical Pharmacokinetic Studies

When promising results are obtained from explorative validation performed during the method development phase, then only full validation should be stared. The process of validating a method cannot be separated from the actual development of method conditions.

A diversity of analytical techniques such as spectroscopy (UV-Visible), gas chromatography (GC), high performance liquid chromatography (HPLC), Ultra performance liquid chromatography (UPLC), Supercritical fluid chromatography (SFC), capillary electrophoresis (CE) coupled with selective detectors, diode array detector (DAD) and mass spectroscopy (MS) are normally used to accomplish the above requirements.

In spite of various techniques existing, HPLC has become a universal tool for pharmaceutical and biomedical research, as well as product analysis. The accessibility of fully automated systems, excellent quantitative precision, accuracy, sensitivity, selectivity and increased selection of column stationary phases, applicability to a lane variety of sample,

Matrices and ability to hyphenate with several spectroscopic detectors has made HPLC or UPLC the instrument of choice for the analysis of most categories of drugs [1, 2].

Correspondingly, HPLC methods are profusely used in the field of biomedical analysis, viz. therapeutic drug monitoring, pharmacokinetic and bioequivalence studies. The assay of drugs in blood, plasma and tissues presents analytical challenges. The drug substance is typically present at low concentrations, bound to proteinaceous material and 1endogenous compounds typically present in the samples can interfere with the analysis. For these reasons, the analytical methods usually be highly sensitive to detect analytes at low concentrations and required a sample pre-treatment procedure such as liquid-liquid extraction (LLE) or solid phase extraction (SPE), to isolate the analyte from the complex biological matrix. Hence, high sensitivity and automation of sample processing tools to deal with large number of samples are strong incentives for the consideration of HPLC or UPLC methods in biomedical analysis [3].

1.2 HPLC

High-performance liquid chromatography (or High-pressure liquid chromatography, HPLC) is a specific form of column chromatography generally used in biochemistry and analysis to separate, identify, and quantify the active compounds [4]. HPLC mainly utilizes a column that holds packing material (stationary phase), a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times of the molecules. Retention time varies depending on the interactions between the stationary phase, the molecules being analyzed, and the solvent(s) used [5].

The sample to be analyzed is introduced in small volume to the stream of mobile phase and is retarded by specific chemical or physical interactions with the stationary phase. The amount of retardation depends on the nature of the analyte and composition of both stationary and mobile phase. The time at which a specific analyte elutes (comes out of the end of the column) is called the retention time. Common solvents used include any miscible combinations of water or organic liquids (the most common are methanol and acetonitrile) [5, 6].

Sepration has been done to vary the mobile phase composition during the analysis; this is known as gradient elution. The gradient separates the analyte mixtures as a function of the affinity of the analyte for the current mobile phase. The choice of solvents, additives and gradient depend on the nature of the stationary phase and the analyte.

Fig. 1: HPLC System

High-performance liquid chromatography used to separate and quantify compounds or a mixture of compounds that have been dissolved in solution. It is a specific form of column chromatography generally used in biochemistry and analysis to separate, identify, and quantify the active compounds.

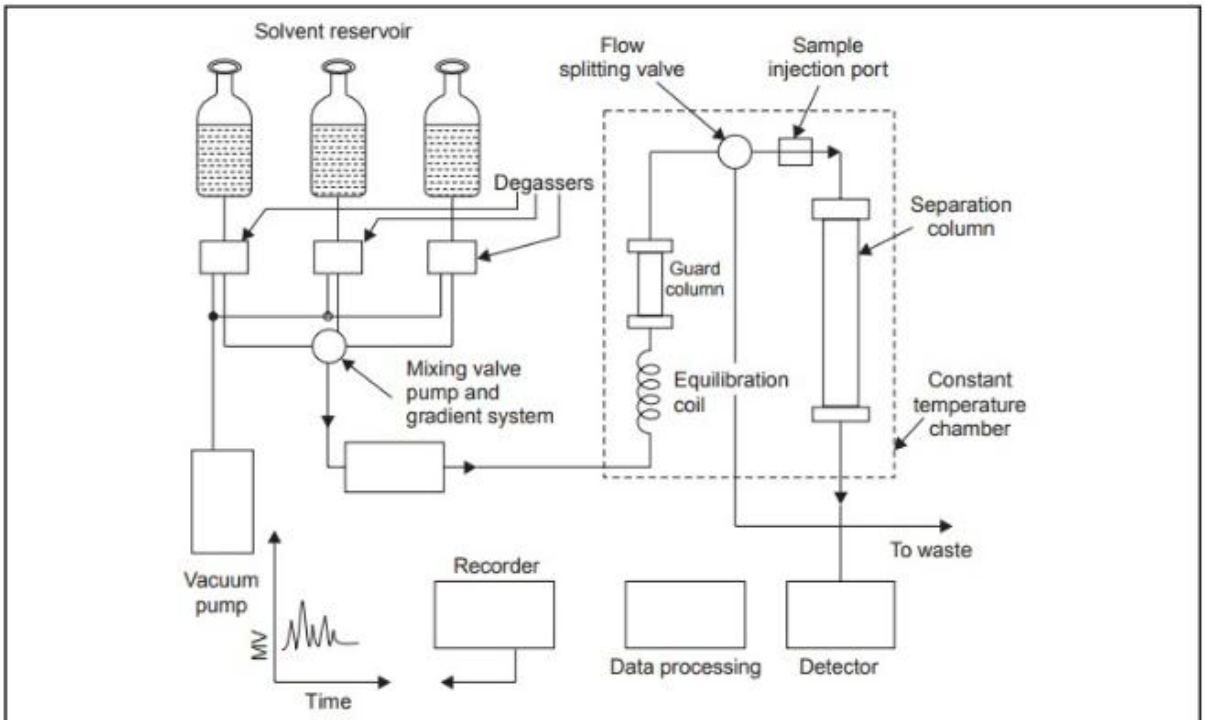


Fig. 2: Flow diagram of HPLC

It's a physical separation technique in which a sample dissolved in a liquid is injected into a column packed with small particles and it is separated into its constituent components.

HPLC utilizes a liquid mobile phase to separate the components of a mixture. The stationary phase can be a liquid or a solid phase. These components are first dissolved in a solvent, and then forced to flow through a chromatographic column under a high pressure. In the column, the mixture separates into its components. The amount of resolution is important, and is dependent upon the extent of interaction between the solute components and the stationary phase. The stationary phase is defined as the immobile packing material in the column. The interaction of the solute with mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases. As a result, HPLC acquires a high degree of versatility not found in other chromatographic systems and it has the ability to easily separate a wide variety of chemical mixtures.

1.2.1 Theories of HPLC

Two theories have been put forward regarding the rate of migration of solute and development of peaks in the chromatogram.

a) Plate Theory

According to plate theory developed by Martin and Synge, a chromatographic column consists of a series of discrete yet continuous horizontal layers, which are termed as the theoretical plates. The efficiency of separation in chromatographic column gets increased as the number of theoretical plate increases. If the length the column is L and the height equivalent of a theoretical plate is H, then N is given by

$$N = L / H$$

The height equivalent of a theoretical plate (HETP) refers to the height of a layer of the column, such that the solution leaving the layer is in equilibrium with the average concentration of the solute in the stationary phase throughout the layer.

b) Rate Theory

The rate theory is able to explain the effect of variables, such as mobile phase velocity and adsorb abilities, which determine the width of an elution band. It also relates the effects of these variables on the time taken by a solute to make its appearance at the end of the column. Migration of solute particles in a column occurs in a state of confusion, each solute molecule progressing in a stop and go sequence independent of any other molecule.

1.3 Types of HPLC Techniques

1.3.1. Based on modes of chromatography

1.3.1.1 Normal phase chromatography: In Normal phase mode the stationary phase is polar in nature & the mobile phase is non-polar in nature. In this technique, non-polar compounds eluted first because of less affinity b/w solute and stationary phase & polar compounds are retained for longer time. E.g. Stationary phase-Silica, Alumina. Mobile phase-Hexane, Dichloromethane, methanol, Isopropanol.

1.3.1.2. Reverse phase chromatography In Reverse phase mode the stationary phase is non-polar in nature & the mobile phase is polar in nature. In these technique polar compounds eluted first &non-polar compounds are retained for longer time.

E.g. Stationary phase -ODS silica gel, C18, C8. Mobile phase- water, methanol, tetrahydrofuran (THF).

1.3.2. Based on Principle of Separation

1.3.2.1 Adsorption chromatography

The principle of separation is adsorption. Separation of components takes place because of the difference in affinity of compounds towards stationary phase. This principle seen in normal phase as well as reverse phase mode, where adsorption takes place.

1.3.2.2. Ion exchange chromatography

The principle of separation is ion exchange, which is reversible exchange of functional groups. In ion exchange chromatography, an ion exchange resin is used to separate a mixture of similar charged ions.

1.3.2.3. Ion pair chromatography

In ion pair chromatography, a reverse phase column is converted temporarily into ion exchange column by using ion pairing agents like pentane or hexane or octane sulphonic acid sodium salt, tetramethyl or tetraethyl aluminium hydroxide etc.[7]

1.3.2.4. Size exclusion chromatography

In this type of chromatography, mixture of components with different molecular sizes is separated by using gels. The gel used acts as molecular sieve.

1.3.2.5. Affinity chromatography

Affinity chromatography uses the affinity of sample with specific stationary phases. This technique is mostly used in the field of biotechnology, microbiology, biochemistry etc.

1.3.2.6. Chiral phase chromatography

Separation of optical isomers can be done by using chiral stationary phases. The stationary phases used for this type of chromatography are mostly chemically bonded silica gel.

1.3.3. Based on elution technique

1.3.3.1. Isocratic separation

In this technique the same mobile phase is used throughout the process of separation. The same polarity or elution is maintained throughout the process.

1.3.3.2. Gradient separation

In this technique, a mobile phase combination of lower polarity or elution strength is used followed by gradually increasing the polarity or elution strength.

1.3.4. Based on the scale of operation

1.3.4.1. Analytical HPLC

Where only analysis of the samples is done. Recovery of samples for reusing is normally since the sample used is very low.eg. µg quantities

1.3.4.2. Preparative HPLC

Where the individual fractions of pure compounds can be collected using fraction collector. The collected samples are reused. E.g. separation of few grams of mixture by HPLC

1.3.5. Based on the type of analysis

1.3.5.1. Qualitative analysis

It is used to identify the compound and detect the presence of impurities, to find out the number of components etc. This is done by using retention time values.

1.3.5.2. Quantitative analysis

It is done to determine the quantity of the individual or several components in a mixture. This is done by comparing the peak area of the standard and sample.

1.4 Principle of separation in HPLC

The principle of separation in normal phase mode and reverse mode is adsorption. When mixtures of components are introduced into a HPLC column, they travel according to their relative affinities towards the stationary phase. The component which has more affinity towards the adsorbent travels slower. The component which has less affinity towards the stationary phase travels faster. Since no two components have the same affinity towards the stationary phase, the components are separated.

1.5 Instrumentation

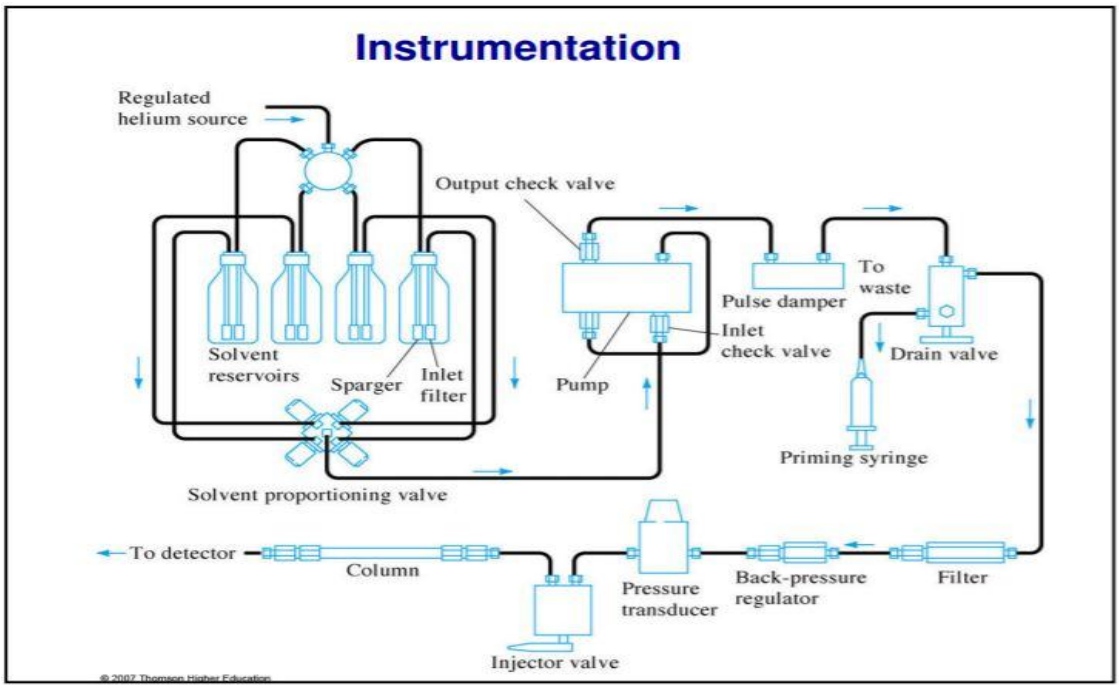


Fig. 3: Instrumentation of HPLC

The essential parts of the High-Performance Liquid Chromatography are:

1) Solvent Reservoir Systems & Delivery System

2) Pumps

3) Sample Injection System

4) Columns

5) Detectors

6) Recorders and Integrators

1.5.1 HPLC solvent reservoir systems

1.5.1.1 Mobile Phase Reservoir, Filtering

The most common type of solvent reservoir is a glass bottle. Most of the manufacturers supply these bottles with special caps, Teflon tubing and filters to connect to the pump inlet and to the purge gas (helium) used to remove dissolved air. Mobile phase is the phase which moves in a definite direction. The mobile phase consists of the sample being separated/ analyzed and the solvent that moves the sample through the column. In the case of HPLC the mobile phase consists of a non-polar solvent(s) such as hexane in normal phase or polar solvents in reverse phase chromatography and the sample being separated. The elutropic series lists solvents in order of their polarity which is useful when selecting a solvent for a particular separation where more polar compounds will normally require a more polar solvent.

High Polarity

Water > Acetic acid > Methanol > Ethanol > 1-Propanol > Acetonitrile > Ethyl acetate > Acetone > Dichloromethane > Chloroform > Diethyl ether > Toluene > Cyclohexane > n-Hexane

1.5.1.2 Degassing System

Mobile phase accounts for 70 % or more of all the problems in chromatography. Presence of particles in mobile phase may cause non-producible flow rate, decrease in selectivity, spurious peaks, increased back pressure, irreversible adsorption and decrease in life of column. Hence, 0-20-micron size filters are being used for filtration. Degassing is one of the most effective measures to eliminate these problems.

Benefits are:

- Stability in baseline, enhanced sensitivity
- Reproducible retention times for eluting peaks
- Reproducible injection volumes for quantitation.
- Stable pump operation

The methods for Degassing include: Sonication, Vacuum or offline degassing, Helium purge, Heat or offline degassing method, On-line membrane degassing.

Sonication is when pure solvents are mixed to make up the mobile phase, excess dissolved gas escapes to form bubbles. If the mobile phase reservoir is placed in an ultrasonic bath, the sound waves promote the coalescence of small bubbles which can escape more easily. Sonication alone will degas a gallon of solvent approximately in 20 minutes.

1.5.1.3 Solvent Delivery System:

The solvent or mobile phases used must be passed through the column at high pressure at about 1000 to 3000psi. This is because as the particle size of stationary phase the resistance to the flow of solvent is high. Hence such high pressure is recommended. Mixing unit is used to mix solvents in different proportions and pass through the column. There are two types of mixing units. They are low pressure chamber and high-pressure chamber. Mixing of solvents is done either with a static mixer or a dynamic mixer. [8]

1.5.2Pumps

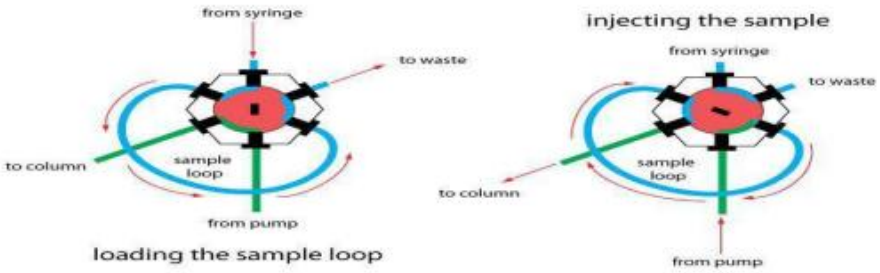


Fig 4: Schematic Diagram pumps

Pumps are used to produce an appropriate pressure to push solvent into the sample. A pump is capable of pumping solvent up to a pressure of 4000psi and flows up to 10ml/min. This is very important in HPLC, since its performance directly affects the retention time, reproducibility and detector sensitivity.

High pressure pumps are to force solvents through packed stationary phase beds. Smaller bed particles require higher pressures. Flow rate stability is another important pump feature that distinguishes pumps. The degree of flow control also varies with pump expense. More expensive pumps include such state-of-the-art technology as electronic feedback and multi headed configurations. The injection process plays an important role in determining total peak width, as it is one of several important sources of extra-column variance.

Types

1. Reciprocating pumps

2. Syringe pumps

3. Pneumatic Pumps

1.5.2.1. Reciprocating Pumps

Most widely used with Small internal volume (35 ~ 400 µL), high-pressure (105 psi), gradient elution, constant flow and need pulse damper.

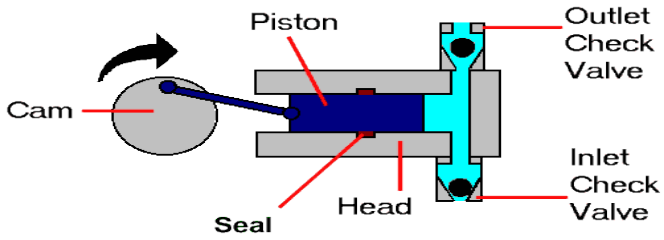


Fig. 5: Schematic Diagram of Reciprocating pump

1.5.2.2. Displacement Pump / Syringe Pump

This works on the principle of solvent displacement by a piston mechanically driven at constant rate in a piston chamber of about 250-500ml capacity with a generation of pulse less flow with high pressure capabilities (200-475 atm).

Syringe pumps are now mostly used for SFC and micro column chromatography. This type of pump is still occasionally used for the mobile phase supply to micro pore columns that require small volumes of mobile phase to develop the separation.

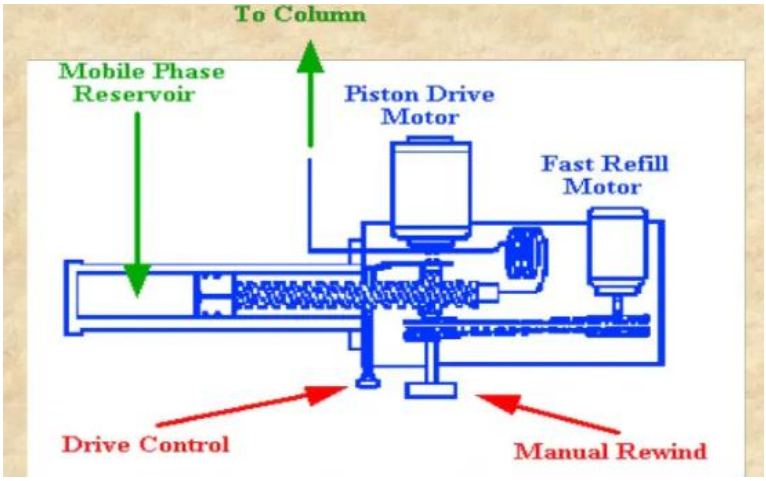


Fig. 6: Schematic Diagram of Displacement pump / Syringe pump

1.5.2.3. Pneumatic Pump

The pneumatic pump has a much larger flow capacity than the piston type pumps but, nowadays, is largely used for column packing and not for general analysis because this pump can deliver high pressures instantly by means of an on/off valve which drives the slurry or column packing material into the column. These pumps work by introduction of high-pressure gas into the pump and the gas in turn forces the solvent from the pump chamber into the column.

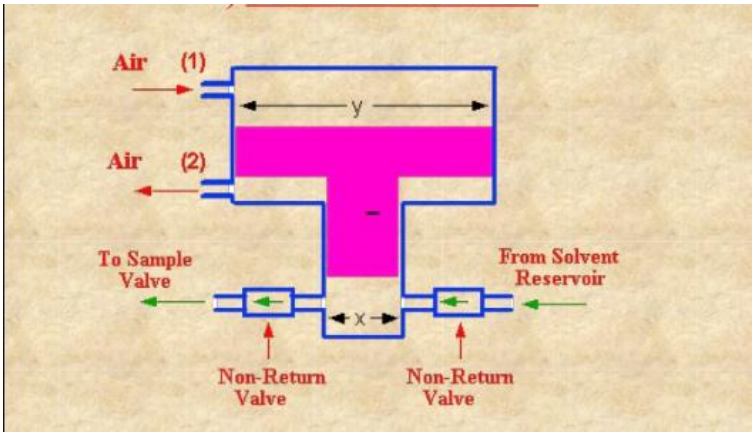


Fig. 7: Schematic Diagram Pneumatic Pump

1.5.3 Sample Injection

System Several devices are available either for manual or auto injection of the sample. Sample introduction can be accomplished in various ways. The simplest method is to use an injection valve. In more sophisticated LC systems, automatic sampling devices are incorporated where the sample is introduced with the help of auto samplers and microprocessors. In liquid chromatography, liquid samples may be injected directly and solid samples need to be dissolved in an appropriate solvent.

Different devices are:

1.5.3.1 Septum Injection Port:

Syringe is used to inject the sample through an inert septum directly into the mobile phase and Drawback is leaching effect of the mobile phase in contact with septum, which may give rise to ghost peaks.

1.5.3.2 Stop Flow SeptumLess Injection:

Flow of mobile phase through the column is stopped while Syringe is used to inject the sample and Drawback is formation of ghost peak.

1.5.3.3 Rheodyne Injectors (Loop Valve Type)

Rheodyne injector is the most popular injector. This has a fixed volume looplike 20µl or 50µl or more. Injector has 2 modes i.e., Load position and Inject mode.

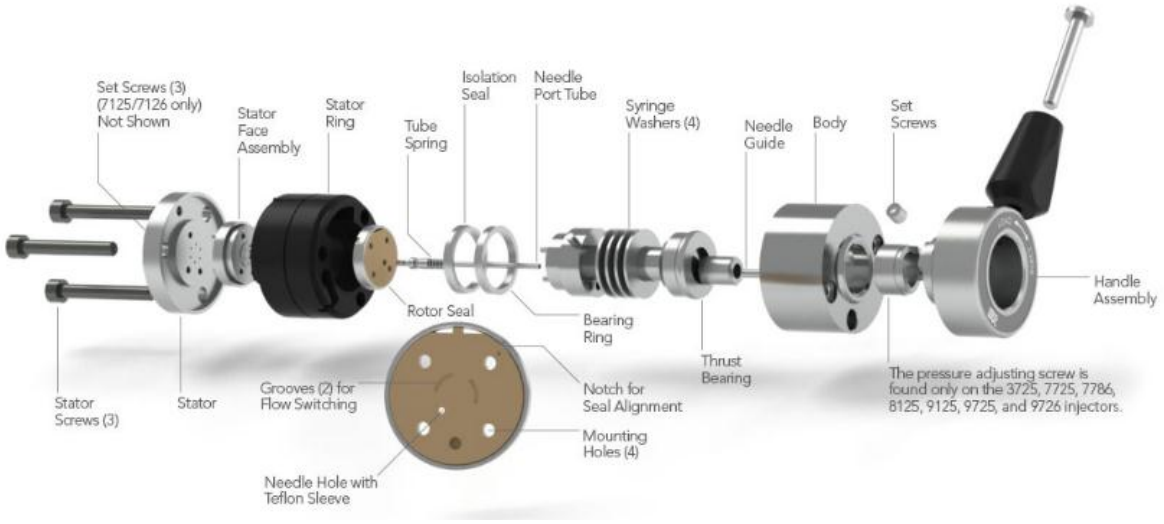


Fig. 8: Schematic Diagram of Rheodyne Injector

Because of their superior characteristics, valves are now used almost to the exclusion of syringe injection. With these sampling valves, samples can be introduced reproducibly into pressurized columns without significant interruption of flow, even at elevated temperatures. Position (A) shows the inject position. Injection in the front port causes the sample to flow into the sample loop. The tip of the needle passes through the rotor seal and, on injection, is in direct contact with the ceramic stator face. After injection, the valve is rotated to position (B) and the mobile phase flushes the sample directly onto the column. The sample is actually forced out of the beginning of the loop so it does not have to flow through the entire length of the loop.

1.5.4 Auto Samplers

Most modern HPLC systems use auto samplers. With the automatic sampling devices, large numbers of samples can be routinely analyzed by LC without operator intervention. The actual injection valve is the same as the manual valves described above, but the sample is

introduced by an automated syringe drawing samples from vials in a motorized tray. Most auto samplers work by penetrating the capped sample vial with a needle attached to a length of flexible tubing. Sample is then withdrawn from the vial into the loop of a sample injector by suction (or positive displacement in some cases). The injector then rotates into the inject position at the right time for the next sample injection into the LC. While this sample is being separated and analyzed, the needle and injector are washed out with rinse fluid, and the next sample vial on the turntable rotates into position for penetration by the needle. All of these operations (injection, rotation, etc.) are controlled by timers in either the LC or the auto sampler.

1.5.5. Columns

Fig. 9: Different Columns of HPLC

The columns most commonly used are made with 316-grade stainless steel (Cr-NiMo steel, relatively inert to chemical corrosion). The inside of the stainless-steel tube should be as smooth as possible, so the tubes are precision drilled or electro-polished after manufacture. Common dimensions are 6.35 mm external diameter, 4.6 mm internal diameter and up to 25 cm long. Standard size column: 4.0mm 0r 4.6 mm (id) ×15cm or 25 cm or 30cm.

Typical HPLC columns are 5, 10, 15 and 25 cm in length and are filled with small diameter (3, 5 or 10 µm) particles. The internal diameter of the columns is usually 4.6 mm; this is considered the best compromise for sample capacity, mobile phase consumption, speed and resolution. Pre-column is used to precondition the mobile phase in order to minimize the chemical attack by the mobile phase on the column packing of the analytical column.

The pre-column is packed loosely with ordinary silica, which does not have to be of HPLC-quality.

In-Line Filter is to catch particulate matter that could plug the column frit or column, causing poor separation or high column pressure - or both. These in-line filters consist of a low-volume frit-holder and a 0.5-micron or 2-micron frit.

The Guard Column is a short column packed with material similar to that contained in the analytical column. The job of the guard column is to pick up or retain sample impurities that could be irreversibly adsorbed onto the analytical column & also filters the mobile phase and sample that enters the analytical column, but the combination of an in-line filter plus guard column is even more effective. At the top of the column, there is a distributor for directing the injected sample to the center of the column and then a stainless-steel gauze or frit on top of the packing. At the lower end there is another frit to retain the packing the 4.6 mm type, a reducing union and a short length of 0.25 mm (0.01 in.) i.d. tubing to connect the column to the detector.

1.5.6 Detectors

The function of the detector in HPLC is to monitor the mobile phase emerging from the column. The output of the detector is an electrical signal that is proportional to some property of the mobile phase and/or the solutes. Detectors can be broadly classified into bulk property and solute property detectors.

Fig: 10: Different HPLC Detectors

Bulk property detectors continuously monitor some property of the mobile phase, such as refractive index, conductance, or dielectric constant, which changes as solute is added to the mobile phase. Bulk property detectors have a finite signal in the absence of a solute, and this result in two serious limitations of these detectors. First, the addition of a low

concentration of solute will add only a small increment to what may already be a large background signal; as a result, these detectors generally have poor limits of detection and are in general not suitable for trace analysis. Second, as 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[9].

1.5.7 Recorders and integrators

Recorders are used to record the responses obtained from detectors after amplification. They record the base line and all the peaks obtained, with respect to time. Retention time for all the peaks can be found out from such recordings, but the area of individual peaks cannot be known. Integrators are improved version of recorders with some data processing capabilities. They can record the individual peaks with retention time, height and width of peaks, peak area, percentage of area, etc. Integrators provide more information on peaks than recorders. Now a day's computers and printers are used for recording and processing the obtained data and for controlling several operations.

1.3 GENERAL INFORMATION ON HPLC METHOD DEVELOPMENT

A good method development strategy should require only as many experimental runs as are necessary to achieve the final desired result. Finally, method development should be as simple as possible, and it should allow the use of sophisticated tools such as computer modeling.

The important factors, which are to be taken into account to obtain reliable quantitative analysis, are

a) Careful sampling and sample preparation.
b) Precise sample injection.
c) Appropriate choice of the column.
d) Choice of the operating conditions to obtain the adequate resolution
e) Reliable performance of the recording and data handling systems.
f) Suitable integration/peak height measurement technique.
g) The mode of calculation best suited for the purpose
h) Validation of the developed method.

Separation goals:

The goals of HPLC separation need to be specified clearly include the use of HPLC to isolate purified sample components for spectral identification or quantitative analysis. It may be necessary to separate all degrades or impurities from a product for reliable content.

In quantitative analysis, the required levels of accuracy and precision should be known (a precision of 1 to 2% is usually achievable). Whether a single HPLC procedure is sufficient for raw material or one or more for mutations and/or different procedures are desired for formulations. When the number of samples for analysis at one time is greater than 10, a run time of less than 20 minutes often will be important.

Getting Started on Method Development

One approach is to use an isocratic mobile phase for this mebendazole analytical method. A better alternative is to use a mobile phase at (60:40) ratio then altering the mobile phase as necessary to optimize.

The initial separation with 40% B results in tailing in the sample peak, also causes rapid elution. Decreasing the solvent strength shows the rapid separation of component with a much longer run time, also reduced the peak tailing.

Table No: 1 Suitable parameter for HPLC method development

GOAL	COMMENTS
Resolution	Precise and rugged quantitative analysis requires that R_s be greater than 1.5.
Separation time	<5-10 min is desirable for routine procedures.
Quantitation	2% for assays; 5% for less-demanding analyses 15% for trace analyses.
Pressure	<150 bar is desirable, <200 bar is usually essential (new column assumed).
Peak height	Narrow peaks are desirable for large signal/noise ratios.
Solvent consumption	Minimum mobile-phase use per run is desirable.

1.3 VALIDATION

The word **"validation"** means "Assessment" of validity or action of validity or action of providing effectiveness'.

Definitions

FDA defines validation as "establish the documented evidence which provides a high degree of assurance that a specific process will consistently produce a product of predetermined Specifications and quantity attributes".

WHO action of providing that, any procedure, process, equipment, material, activity, or system leads to the expected results.

EUGMP define validation as "action of proving in accordance with the principle of Good manufacturing practice (GMP), that any material, activity or system actually lead to expected result".

AUSTRALIANGMP defines validation as "the action of proving that any material, process, activity, procedure, system, equipment or mechanism and intended results".

1.3.1 METHOD VALIDATION GUIDELINES

ICH guidelines

The ICH was initiated around 1990. It is an important regulatory initiative to standardize regulatory requirements between the European Community, Japan and the United States. Recognizing the benefits of having consistent international requirements, ICH developed a series of guidelines that have been recognized in these countries. The two main documents of ICH guidelines pertaining to analytical method validation are:

(i) Q2A: Text on Validation of Analytical Procedures [ICH-Q2A, 1995]

(ii) Q2B: Validation of Analytical Procedure Methodology [ICH-Q2B, 1997]

(iii) Analytical method is a process of proving that the method was acceptable for laboratory use to measure the sample concentration within GLP environment and acceptance criteria in ICH guidelines Q2(R1)**[10]**.

FDA guidelines

The FDA published the draft on "Guidance for Industry on Analytical Procedures and Method Validation" to aid pharmaceutical companies in meeting the code of federal regulations requirement [FDA, 2000]. This guidance, when approved, supersedes the FDA Guidance for Industry on Submitting Samples and Analytical Data for Method Validation [FDA, 1987]. According to the FDA, analytical methods are categorized into four tests: identification, testing for impurities (quantitative and limit), assay (dissolution, content, potency), and specific tests. Recently, the Centre for Drug Evaluation and Research (CDER), a division of the FDA, also issued guidance for validation of bio analytical methods [FDA-CDER, 2001].

USP guidelines

The USP [USP, 2006] categorizes analytical methods into four types of tests: quantitation of major components of drug product, testing for impurities (quantitative and limit), performance characteristics and identification tests. USP refers to the same definitions of ICH Q2A and Q2B recommendations for procedures on meeting validation requirements.

1.3.2 ANALYTICAL METHOD VALIDATION

Method validation is the process for establishing that performance characteristics of the analytical method are suitable for the intended application. Chromatographic methods need to be validation before first routine use. To obtain the most accurate results, all the variables of the method should be considered, including sampling procedure, sample preparation, chromatographic separation, detection and data evaluation, using the same matrix as that of the intended sample. The validity of an analytical method can only be verified by laboratory studies. All validation experiments used to make claims or conclusions about validity of the method should be documented in report.

Types of analytical procedures to be validated

- Identification test for impurities
- Quantitative test for impurities
- Limit test control of impurities
- Quantitative test for the active moiety in samples of drug substance or drug product, or other selected components (s) in the drug product.

Table 2: COMPARISON OF Validation Parameters Required for HPLC Related Substance Methods

ICH Guidelines	USP Guidelines	FDA Guidelines
Accuracy	Accuracy	Accuracy
Precision	Precision	Precision
Repeatability	--	Repeatability
Inter-day precision	--	Inter-day precision
Reproducibility	--	Reproducibility
Specificity	Specificity	Specificity
Limit of detection	Limit of detection	Limit of detection
Limit of quantification	Limit of quantification	Limit of quantification
Linearity	Linearity	Linearity
Range	Range	Range
--	Ruggedness	--
Robustness	Robustness	Robustness
System suitability	System suitability	System suitability

Validation parameters

The developed method was validated according to ICH guidelines [11, 12]

- Accuracy
- Precision (Method Precision & Intermediate Precision)
- Robustness
- Specificity
- Linearity
- Range
- Recovery

- Solution stability
- Filter integrity

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 should be established across the specified range of the analytical procedure**[15]**. In a method with high accuracy, a sample (whose "true value" is known) is analyzed and the measured value is identical to the true value. Typically, accuracy is represented and determined by recovery studies. There are three ways to determine accuracy:

- Comparison to a reference standard.
- Recovery of the analyte spiked into blank matrix.
- Standard addition of the analyte.

It should be clear how the individual or total impurities are to be determined.

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: precision under same operating conditions, same analyst over a short period of time.
- Intermediate precision: method is tested on multiple days, instruments, analysts etc.
- Reproducibility: inter-laboratory studies.

The ICH guidelines suggest that repeatability should be conformed duly utilizing at least 9 determinations with specified range for the procedure (e.g., three concentrations / three replicates each) or a minimum of 6 determinations at 100 % of the test concentration.

Linearity and Range

Linearity is the ability of the method to elicit test results that are directly proportional to the analyte concentration within the given range. Linearity is generally reported as a variance of the slope of the regression line. Range is the (inclusive) interval between the upper and lower levels of analyte that have been demonstrated to be determined with precision, accuracy, and linearity using the method. The range is normally expressed in the same units as the test results obtained by the method.

Specificity

One of the significant features of HPLC is its ability to generate signals free from interference. Specificity refers to the ability of the analytical method to differentiate and quantify the analyte in complex mixtures. An investigation of specificity is to be conducted during the determination of impurities and validation of identification tests. An ICH guideline defines specificity as ability to assess unequivocally the analyte in the presence of other compounds that may be likely to be present. Typically these might be impurities, degradants, matrix, etc. It is the measure of the degree of interference from such other things such as ingredients, excipients, impurities, and degradation products, ensuring that a peak response is due to single component only. The definition has the following implications:

- Identification test: Identification tests should be able to differentiate compounds of closely related structure which are expected to be present i.e., to assure identity of an analyte.
- Purity test: To ensure that the analytical procedure performed allows an accurate statement of content of the impurity of an analyte i.e. related substances, residual solvents content, heavy metals, etc.
- Assay: To arrive at an accurate result, this permits a correct report on the potency or content of analyte in a sample**.[15]**

Limit of Detection

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. LOD can be estimated based on visual evaluation or signal-to-noise ratio or standard deviation of the response and slope. Signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

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

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve

Limit of Quantitation

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. 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.

$$LOQ = \frac{10\sigma}{S}$$

Where,

σ = standard deviation of the response;

S = the slope of the calibration curve.

Robustness

Robustness is the capacity of a method to remain unaffected by small deliberate variations in method parameters. The robustness of a method is evaluated varying method parameters such as percent organic solvent, pH, ionic strength, or temperature and determining the effect (if any) on the results of the method. **(13,14,15)**

Solution Stability

The solution stability is stability of standard and extracted sample solution (ready to inject) from the sample or matrix and analyzed as per specified method, and it should be stored properly in room temperature and refrigerated condition depending upon the stability of the sample and standard solution. The stability of standard and sample solution should be

established in room temperature and refrigerated, if refrigerated before analyzing it should be thawing to room temperature. The analyzed solutions stored in necessary condition and the stability can be established for two days or solution stability can be established by an hour basis depending upon the nature of the product **[16]**.

Related substances Method

For dosage form monographs, the main purpose of a test for related substances is to control degradation impurities. However, the objective is to limit impurities arising during storage of the drug substance/product **[17]**.

Related substances

Related substances are structurally related to a drug substance. These substances may be identified or unidentified degradation products or impurities arising from a manufacturing process or during storage of a material **[18]**.

Impurity

As per ICH guideline Q3A impurity in a drug substance is ―any component of the drug substance that is not the chemical entity defined as the drug substance **[19]** and as per ICH guideline Q3B impurity in a drug product is ―any component of the drug product that is not the chemical entity defined as the drug substance or an excipient in the drug product **[20].**

Classification of Impurities

As per ICH guidelines Q3A and Q3B Impurities can be classified as:

- Organic impurities
- Inorganic impurities
- Residual solvents

Table 1: Thresholds for reporting Impurities [21]

Maximum daily dose	Reporting threshold	Identification threshold	Qualification threshold
Less or equal to 2 g/day	0.05%	0.10% or 1.0 mg/day (whichever is lower)	0.15% or 1.0 mg/day (whichever is lower)
>2 g/day	0.03%	0.05%	0.05%

Table 4: Acceptance criteria of validation for HPLC Assay method

S.NO.	Characteristics	Acceptance criteria
1	Accuracy	Recovery 98-102% with 50,100,150 spiked sample
2	Precision Repeatability Intermediate precision	 RSD < 2 RSD < 2
3	Specificity/Selectivity	No interference
6	Linearity	r2 > 0.995
7	Range	80 - 120%
8	Stability	>24hr or < 8hr

Parameters in Chromatography

A good resolution of components is achieved by optimization of various system suitability parameters such as Resolution (Rs), number of theoretical plates (N), capacity factor (k'), and peak asymmetry factor (AF), etc.

System Suitability

If measurements are susceptible to variations in analytical conditions, these should be suitably controlled, or a precautionary statement should be included in the method. One consequence of the evaluation of robustness and ruggedness should be that a series of system suitability parameters is established to ensure that the validity of the analytical method is maintained wherever used. Typical variations are the stability of analytical solutions, different equipment, and different analysts.

Acceptance criteria: The RSD variation of the results ± 2.5%.

System suitability criteria:

It is advisable to run system suitability tests in these robustness experiments. During the robustness testing of the method validation, critical method parameters such as mobile phase composition and column temperature are varied to mimic the day-to-day variability. Therefore, the system suitability results from these robustness experiments should reflect the expected range. Consequently, the limits for system suitability tests can be estimated from these experiments. The parameters used in system suitability test report are as follows:

- Number of theoretical plates or Efficiency (N)
- Capacity factor (K)
- Separation or Relative retention (α)
- Resolution (Rs)
- Tailing factor (T)
- Relative Standard Deviation (RSD)

These are measured on a peak or peaks of known retention time and peak width.

1.4.1 Plate number or number of theoretical plates (N)

This is a measure of the sharpness of the peaks and therefore the efficiency of the column. This can be calculated in various ways, for example the USP uses the peak width at the base and the BP uses the peak width at half the height as shown in figure2.

$$N = 5.545 [t/wh] 2 \quad (BP)$$

Or

$$N = 16 [t/wb] 2 \quad (USP)$$

Where,

Wh = peak width at 1/2 peak height

Wb = peak width at base (mL, sec or cm)

t = retention time of peak or elution volume (mL, sec or cm)

Therefore, plates number is high the column is more efficient. The plate number depends on column length - i.e. the longer the column the larger the plate number.

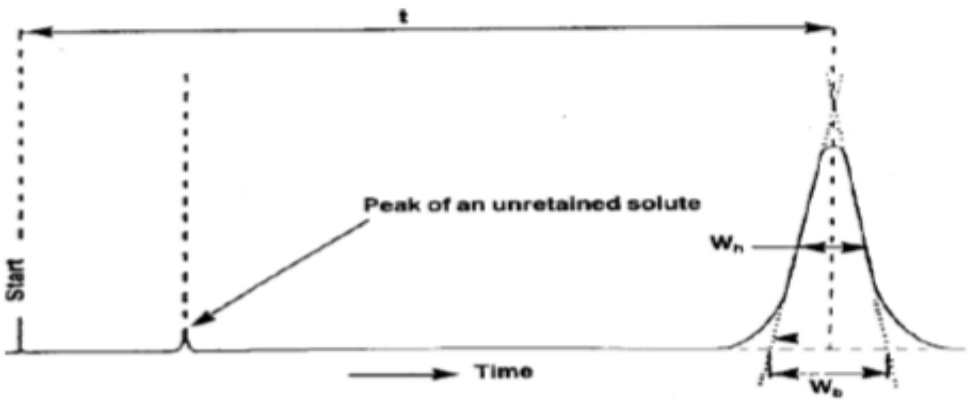


Fig 11: Plate number (N)

Therefore, the column's efficiency can also be quoted as the plate height (h) or the height equivalent to one theoretical plate (HETP).

$$h = L/N$$

Where,

L = length of column N or plates/meter

N = plate number.

1.4.2 Capacity factor or Capacity ratio (K)

This value gives an indication of how long each component is retained on the column (i.e., how many times longer the component is retarded by the stationary phase than it spends in the mobile phase) as shown in figure

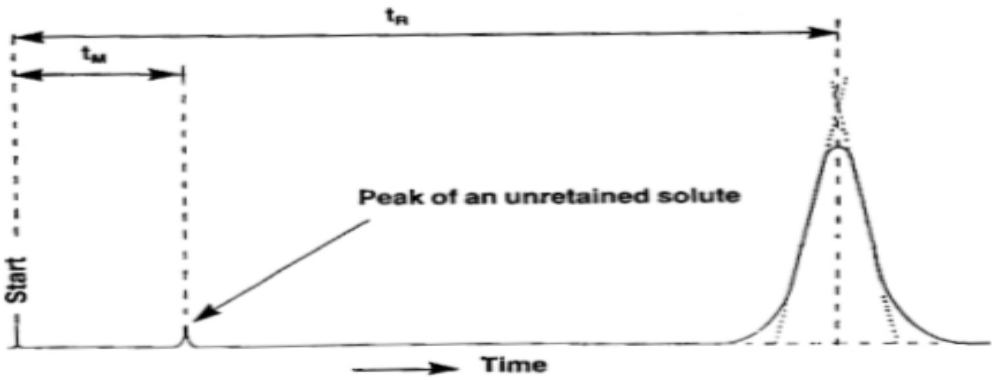


Fig 12: Capacity factor (K)

$$K = tR - tM / tM$$

Where,

tR = unretained peaks retention time,

tM = retention time of the peak of interest.

K is used in preference to retention time because it is less sensitive to fluctuations in

Chromatographic conditions (i.e., flow rate) and therefore ensures greater reproducibility from run to run. In practice the k value for the first peak of interest should be >l to assure that it is separated from the solvent.

1.4.3 Separation Factor or Relative retention (α)

This describes the relative position of two adjacent peaks. Ideally, it is calculated using the capacity factor because the peaks' separation depends on the components' interaction with the stationary phase as shown in figure

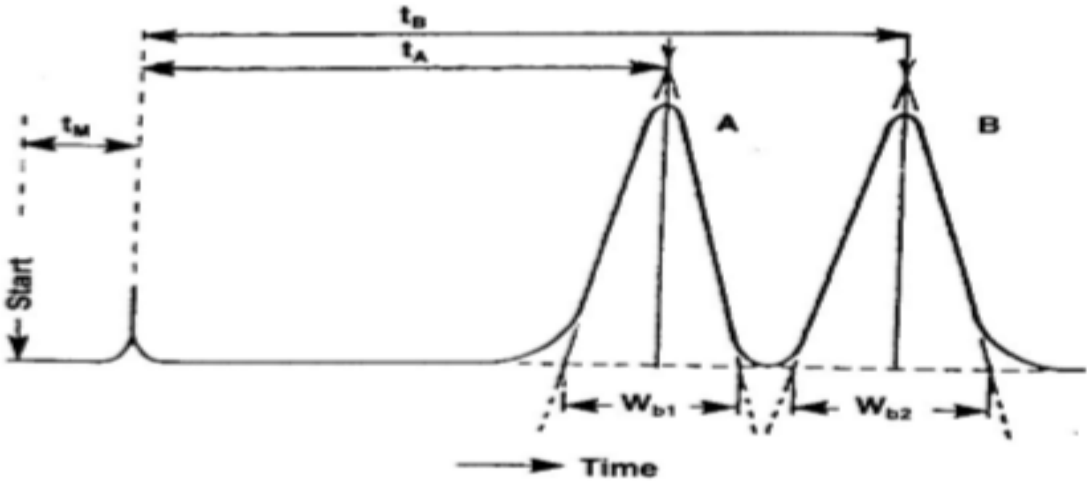


Fig 13: Separation Factor (α)

From the peaks A and B,

$\alpha = KB / KA$

Where,

KB = capacity factor for B

KA = capacity factor for A.

If the capacity factor is used then the separation factor should be consistent for a given column, mobile phase, composition, specified temperature and regardless of the instrument used

1.4.4 Resolution (Rs)

The resolution of two neighboring peaks is defined by the ratio of the distance between the two peak maxima. It is the difference between the retention times of two solutes divided by their average peak width. For baseline separation, the ideal value of resolution is 2.0. It is calculated by using the formula given along with figure 5.

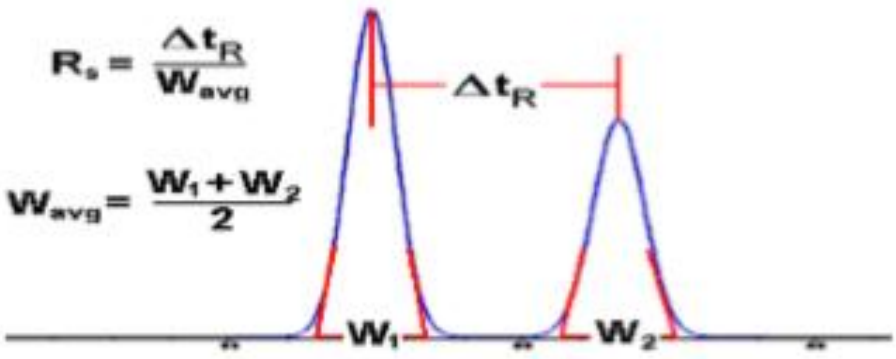


Fig 14: Resolution Between two peaks (Rs)

$$Rs = \quad Rt1 - Rt2$$

$$W_{avg} = 0.5\ (W1+W2)$$

Where,

Rt1 and Rt2 are the retention times of components 1 and 2

W1 and W2 are peak widths of components 1 and 2.

1.4.5 Tailing factor and Asymmetry factor

If the peak to be quantified is asymmetric, a calculation of the asymmetry would also be useful in controlling or characterizing the chromatographic system. Peak asymmetry arises from a number of factors. The increase in the peak asymmetry is responsible for a decrease in chromatographic resolution, detection limits, and precision. The peak asymmetry can be calculated by using formula,

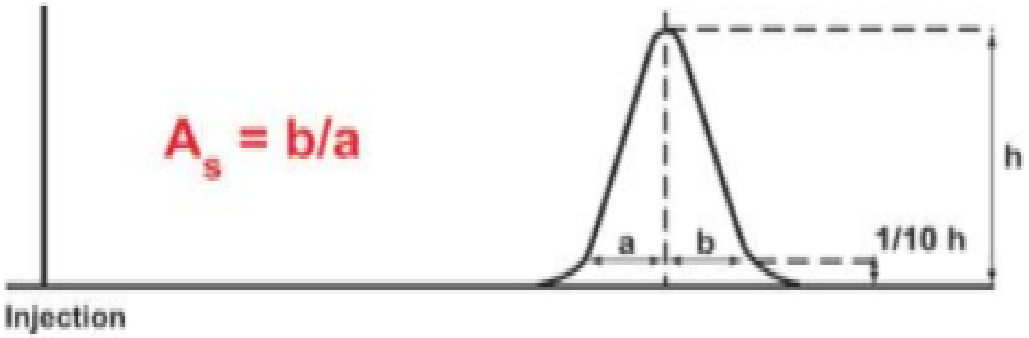


Fig15: Asymmetry Factor

Where,

As is peak asymmetry factor, figure

b is distance from the point at the peak midpoint to the trailing edge

a is distance from the leading edge of peak midpoint to the midpoint

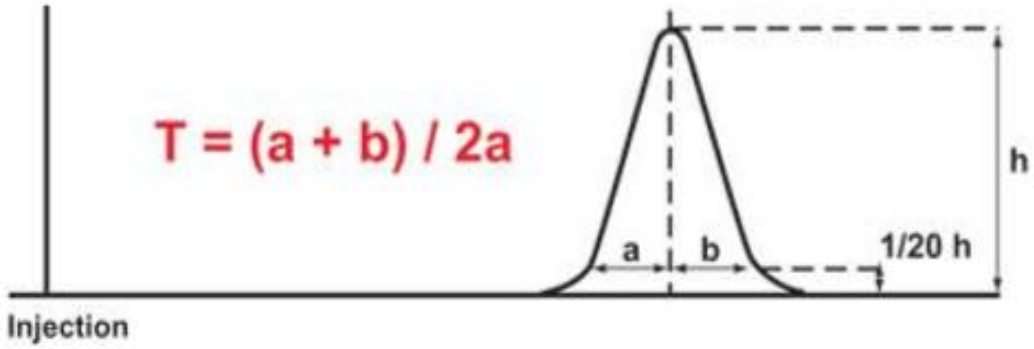


Fig 16: Tailing Factor

Where

T is tailing factor (measured at 5% peak height) as in figure

b is distance from the point at the peak midpoint to the trailing edge

a is distance from the leading edge of peak midpoint to the midpoint

1.4.6 Relative Standard Deviation or Precision

For an HPLC system this would involve the reproducibility of a number of replicate injections (i.e., 6) of an analytical solution.

The USP requires that unless otherwise specified by a method:

If a relative standard deviation of <2% is required, then five replicate injections should be used.

If a relative standard deviation of >2% is required, then six replicate injections should be used.

Limits of System Suitability Parameters (USP) are listed in table - 3.

Table 5: Limits of System Suitability Parameters (USP)

S.No	Parameter Name	Limit
1	Number of theoretical plates or Efficiency (N)	>2000
2	Capacity Factor (K)	>1
3	Separation or Relative retention (α)	≥1
4	Resolution (Rs)	≥5
5	Tailing factor or Asymmetry (T)	≤2
6	Relative Standard Deviation (RSD)	≤2

All the critical steps in method development have been summarized and prioritized. In order to develop a HPLC method effectively, most of the effort should be spent in method development and optimization, as this will improve the final method performance.

DRUG PROFILE

2. DRUG PROFILE

2.1. DRUG SUBSTANCE :

ATORVASTATIN CALCIUM [22]

IUPAC name : Calcium;(3*R*,5*R*)-7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-propan-2-ylpyrrol-1-yl]-3,5- dihydroxyheptanoate

Brand name : Lipitor (10mg), Atolva (10mg)

Molecular weight : 1155.36 g/mol

Molecular formula : C66H68CaF2N4O10

CAS Number : 134523-03-8

Structure:

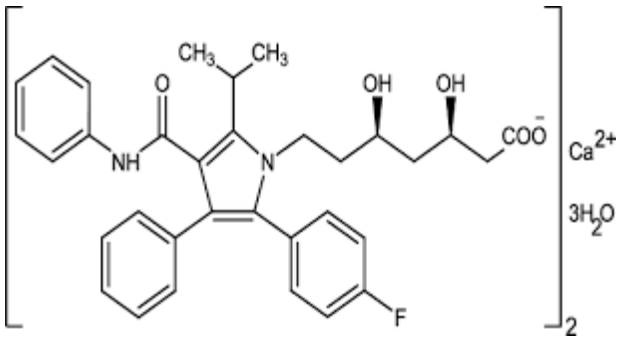


Fig 17: Structure of Atorvastatin Calcium

CHEMICAL CLASSIFICATION

Drug class : HMG-CoA reductase inhibitors (statins)

Therapeutic group : Antilipemic

Recommended dose : 10 mg/mL

2.1.1. PHYSICAL AND CHEMICAL PROPERTIES:

Appearance : Crystalline powder

Odor : Odourless

Colour : White to off-white

Solubility : Freely soluble in methanol, slightly soluble in ethanol, very slightly soluble in acetonitrile, distilled water and phosphate buffer pH 7.4; insoluble in aqueous solution of pH 4 and below.

Log P : 5.39

pKa : 4.46

Melting point : 164-180°C

2.1.2PHARMACOKINETICS:

Absorption : Rapidly absorbed; Tmax is 1 to 2 h. Bioavailability is approximately 14%; low bioavailability is because of presystemic C1 in G1 mucosa and/or hepatic first-pass metabolism. Food decreases rate and extent or absorbtion approximately 25% and 9% respectively, but does not alter efficacy.

Distribution : 98% bind to plasma protein albumin

Metabolism : Hepatic and extra hepatic metabolism, first-pass metabolism and CYP3A4.

Elimination : Eliminated primarily in bile. Less than 2% of dose is recovered in the urine. Plasma $t_{1/2}$ is approximately 14h.

Adverse Effects:

Atorvastatin Calcium are well tolerated: mild unwanted effects include gastrointestinal disturbance, increased plasma concentration of liver enzymes, insomnia and rash. More serious adverse effects are rare but include severe myositis (“rhabdomyolysis”) and angio edema.

Drug Interactions:

The risk of myopathy during treatment with statins is increased with concurrent administration of fibric acid derivatives, lipid-modifying doses of niacin, cyclosporine, or strong CYP 3A4 inhibitors (e.g., clarithromycin, HIV protease inhibitors, and itraconazole)

2.1.3 MECHANISM OF ACTION:

Atorvastatin Calcium a synthetic cholesterol-lowering agent, is a medicine called as HMG-CoA (3hydroxy-3-methylglutaryl-coenzyme A) reductase inhibitor. These enzymes are involved in cholesterol biosynthesis by catalyzing the conversion reaction of HMG-CoA to mevalonate. The function of lowering the amount of cholesterol leads to the result in clearing the LDP (low-density lipoprotein) cholesterol in the blood by increased LDL receptors. The Calcium salt of Atorvastatin is used in the treatment of primary hypercholesterolemia and dyslipidemia.

2.2. DRUG SUBSTANCE: EZETIMIBE [23]

IUPAC name : ((3R,4S)-1-(4-Fluorophenyl)-3-[(S)-3-(4-Fluorophenyl)-3-hydropropyl]-4-(4hydrophenyl) azetidine-2-one)

Brand name : Ezetrol(10mg), Zetica (10mg)

Molecular weight : 409.43 g/mol

Molecular formula : C24H21F2NO3

CAS Number : 163222-33-1

Structure:

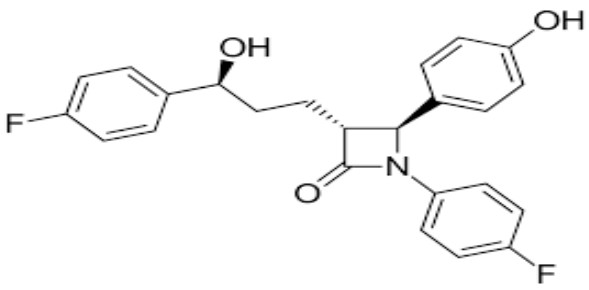


Fig 18: STRUCTURE OF EZETIMIBE

CHEMICAL CLASSIFICATION

Drug class : Cholesterol-lowering medications.

Therapeutic group : Anti-hyperlipidaemic

Recommended dose : 10 mg/mL

2.2.1. PHYSICAL AND CHEMICAL PROPERTIES:

Appearance : White Crystalline powder

Odor : Odorless

Colour : White to off-white

Solubility : Soluble in dilute acids, methanol, and chloroform and practically insoluble in water.

Log P : 4.52

pKa : 9.75

Melting point : 163°C

2.2.2 PHARMACOKINETICS:

Absorption : Rapidly absorbed and primarily metabolized in the small intestine and liver to its glucuronide

Distribution : Protein binding ranged from 99.5% to 99.8% and its Bioavailability is approximately 14%

Metabolism : Extensively metabolized in the small intestine and liver

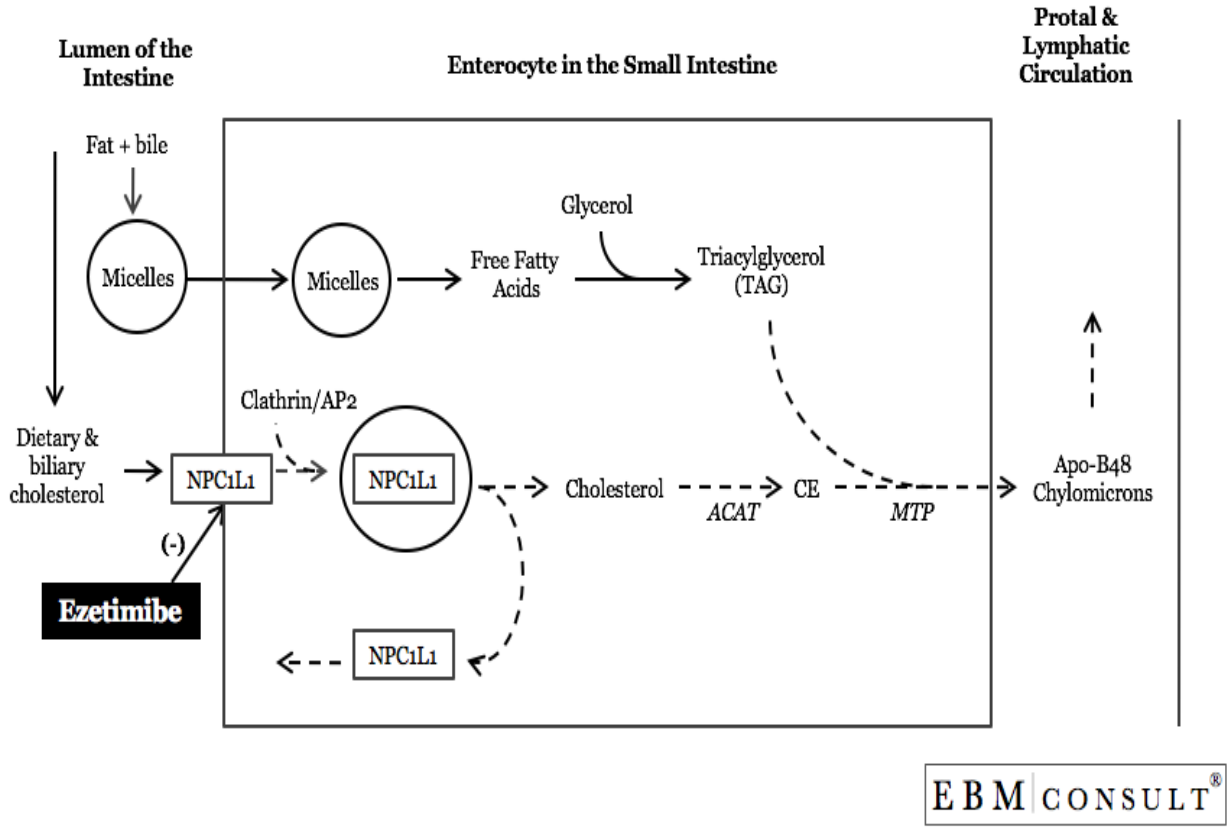
Excretion : Bilary secretion into the intestine and subsequent excretion in the feces (78%) and urine (11%). The estimated terminal elimination half-life is 16-31 hrs. Renal clearance is 5-10%.

Drug Interactions:

Ezetimibe, a novel drug that acts by inhibiting intestinal absorption of cholesterol and phytosterols. It interferes with a specific CH transport protein NPC1L1in the intestinal mucosa and reduce absorption of both dietary and biliray CH.

2.2.3 MECHANISM OF ACTION:

The mechanism of action is distinct from other potent lipid- lowering drug that acts in the gut. Ezetimibe is a potent inhibitor of cholesterol and phytosterols absorption in the small intestine, where both dietary and biliary cholesterol are available for absorption. However, its action is unique in that does not affect cholesterol micelle formation or increase bile acid secretion. It does not alter fat soluble vitamin and nutrient absorption.



EBM CONSULT®

LITERATURE REVIEW

3. LITERATURE REVIEW

Sandeep S. Sonawane et al., (2006) has developed two methods are described for the simultaneous determination of Atorvastatin calcium and Ezetimibe in binary mixture. The first method was based on UV-spectrophotometric determination of two drugs, using simultaneous equation method. It involves absorbance measurement at 232.5 nm (λmax of Ezetimibe) and 246.0 nm (λmax of Atorvastatin calcium) in methanol; linearity was obtained in the range of 5 – 25 µg.mL-1 for both the drugs. The second method was based on HPLC separation of the two drugs in reverse phase mode using Luna C18 column. Linearity was obtained in the concentration range of 8-22 µg.mL-1 for both the drugs. Both these methods have been successively applied to pharmaceutical formulation and were validated according to ICH guidelines.

U Seshachalamet al., (2008) reported a simple, isocratic, and sensitive reverse phase high performance liquid chromatographic (RP-LC) method has been developed, for the first time, for quantitative determination of atorvastatin and ezetimibe in pharmaceutical formulations. Atorvastatin and ezetimibe were chromatographed using 0.01 M ammonium acetate buffer (pH:3.0): Acetonitrile (50:50 v/v) as mobile phase. The detection was monitored at 254 nm. The retention times of ezetimibe and atorvastatin were 15.50 + 0.07 and 19.30 + 0.06, respectively. The linearity of the method was studied over the concentration range of 4 – 400 mg/mL for atorvastatin and 5 – 500 mg/mL for ezetimibe. The limit of detection for atorvastatin and ezetimibe were found as 1.25 mg/mL and 1.48 mg/mL, respectively. The proposed method was applied for the quantitative determination of atorvastatin and ezetimibe in commercial combination formulations.

Kumar SA et al., (***2015)*** describes a simple, precise and accurate RP-HPLC method for simultaneous estimation of atorvastatin and ezetimibe in plasma. The chromatographic separation of the drugs were performed on an X-Terra C8 (4.6 x 150 mm, 3.5 □m), with phosphate buffer [pH 3.5 with Ortho Phosphoric Acid] – acetonitrile 40:60 (v/v) as mobile phase. The detection was performed at 235 nm. The flow rate was maintained at 1.2 mL/min. The run time was 8.0 min. Results: The accuracy and reliability of the method was assessed by evaluation of linearity (5-25 µg/mL for both atorvastatin calcium and ezetimibe), precision (intra-day RSD 0.57 % and inter-day RSD 0.02 % for atorvastatin calcium and

intra-day RSD 0.56 % and inter-day RSD 0.1 % for ezetimibe), accuracy (100.08- 100.84 % for atorvastatin calcium and 100.56- 101.00 % for ezetimibe), and specificity, in accordance with ICH guidelines. The LLOQ obtained by the proposed method were 1.294 and 1.384 µg/mL for atorvastatin and ezetimibe respectively. Overall the proposed method was found to be suitable and accurate for the quantitative determination in plasma. The method was effectively separated the drug from plasma.

SS Qutab et al., (2007) proposed a simple, precise and sensitive reverse-phase high performance liquid chromatographic (RP-HPLC) method has been developed for the quantitation of atorvastatin calcium simultaneously with ezetimibe in pharmaceutical formulations. Chromatographic separation was achieved on a 250 × 4.6 mm, 5µ Hypersil® phenyl-2 column. Eluent was monitored by absorbance at 242 nm using a mixture of 0.1 M ammonium acetate (pH 6.5) and acetonitrile in the ratio of 28:72 (v/v). Calibration plots were linear in the concentration range of 12-52 µg mL-1 for both atorvastatin calcium and ezetimibe with correlation coefficient (R2) between 0.9966 and 0.9993. The total run time is less than 5 min. The proposed method was validated by testing its linearity, recovery, selectivity, repeatability and LOD/LOQ values and it was successfully employed for the determination of atorvastatin calcium and Ezetimibe in Pharmaceutical tablet formulations.

Baokar Shrikrishna et al., (2011) aimed at developing and validating an HPLC method for the assay of Ezetimibe in tablets' formulation A chromatographic system comprising ODS-3V 4.6 mm x 250mm column, a mobile phase of Buffer and acetonitrile, a flow rate of 1.5 ml/min and a UV detector set at 230 nm has shown good chromatographic separation for Ezetimibe. The degree of linearity of the calibration curves, the percent recoveries of Ezetimibe and related substances, the limit of detection (LOD), and limit of quantization (LOQ) for the HPLC method have been determined. The HPLC method under study was found to be specific, precise, accurate, reproducible indicating stability and robust.

RAiyaluet al., (2011) developed and validated a stability-indicating reversed-phase high performance liquid chromatographic (RP-HPLC) method for simultaneous estimation of atorvastatin calcium and ezetimibe for their multicomponent dosage form. The proposed RP-

HPLC method utilizes a 125 mm x 4.6 mm i.d 5 µm Phenomenex C-18 column at mbient temperature; the optimum mobile phase consists of acetonitrile and 0.4% v/v triethylamine (pH adjusted to 5.5 with ortho-phosphoric acid) in the ratio of 55:45, v/v respectively, flow rate of 1.0 ml/min. Measurements were made at a wavelength of 231 nm. Multicomponent dosage form was exposed to thermal, photolytic, hydrolytic and oxidative stress. No co eluting, interfering peaks from excipients, impurities were observed for the degradation products and hence the method was found to be specific. The method was linear in the range of 5-25 µg/ml for atorvastatin calcium and ezetimibe. The mean recoveries were 98.82% and 98.72% for atorvastatin calcium and ezetimibe respectively. The method was validated for linearity, range, precision, accuracy, specificity, selectivity, intermediate precision, ruggedness, robustness, solution stability and suitability.

Saeid Mezail Mawazi et al., (2014) developed and validated for the determination of Atorvastatin and Ezetimibe in tablet dosage form. The chromatographic separation was achieved on a Inspire C_{18}(4.6 x 250mm, 5mm) with a mobile phase combination of phosphate buffer, methanol and Acetonitrile (40:10:50) at a flow rate of 1.2 ml/min, and the detection was carried out by using UV detector at 233 nm. The total run time was 8minutes.The retention time of phosphate buffer, methanol and Acetonitrile were found to be 2.237 min. and 3.164 min. respectively. The performance of the method was validated according to the present ICH guidelines.

S Alam et al., (2018) Aimed at the development of a simple, precise, rapid and selective RP-HPLC method for the estimation of Atorvastatin as API in both- bulk and pharmaceutical formulation.The method was carried out on HPLC a C18 column (25 cm x 4.6 mm) with mobile phase consisting of methanol:water:acetonitrile: orthophosphoric acid (85:10:4, 1 v/v) and pH was adjusted to 3.2 with flow rate of 1.8 ml per min.Detection was carried out at 247 nm. Besides, all parameters were found to be under required limits including limits of detection and limits of quantification.Further,the method was developed and validated; linearity, accuracy, precision, ruggedness and robustness, through an efficient HPLC technique in accordance with the ICH validation guidelines. The results of all validated parameters - Linearity (r^2= 0.999), Accuracy (101.5%), Precision, Ruggedness, Robustness, LOD (0.0008µg/ml) and LOQ (0.0002µg/ml) were found to be within required limits.

AKK Gajjaret al., (2009) developed a simple, precise and rapid stability-indicating reversed-phase high performance liquid chromatographic (RP-HPLC) method has been developed and subsequently validated for the simultaneous estimation of Rosuvastatin (RSV) and Ezetimibe (EZE) from their combination drug product. The proposed method is based on the separation of the two drugs in reversed-phase mode using Hypersil C18 150 x 4.6 mm, 5µ column maintained at a temperature of 40°C. The optimum mobile phase consisted of 0.05 M phosphate buffer (pH 2.5)-Methanol (45+55, v/v), mobile phase flow rate of 1.0 mL min-1 and UV detection was set at 242 nm. Rosuvastatin, Ezetimibe and their combination drug product were exposed to thermal, photolytic, hydrolytic and oxidative stress conditions and the stressed samples were analyzed by the proposed method. There were no interfering peaks from excipients, impurities or degradation products due to variable stress conditions and theproposed method is specific for the simultaneous estimation of RSV and EZE in the presence of their degradation products. The method was validated according to ICH guidelines. It was found to be accurate and reproducible. Linearity was obtained in the concentration range of 5-80 µg mL-1 for both RSV and EZE with correlation coefficients of 0.99999 and 0.99994 respectively. Mean percent recovery of triplicate samples at each level for both drugs were found in the range of 98% to 102% with RSD of less than 2.0%. The proposed method can be successfully applied in the quality control of bulk manufacturing and pharmaceutical dosage forms.

Yehia Z. Baghdady et al., (2013) described a simple sensitive and specific method was developed for simultaneous determination of Ezetimibe (EZB) and atorvastatin calcium (ATVC) by high performance liquid chromatography without previous separation. Satisfactory resolution was achieved using a RP-C18 chromatographic column, Kromasil RP-18 column (250 mm* 4.6 mm i.d) and a mobile phase consisting of acetonitrile: water (6:4, v/v) at a flow rate 0.7 mL/min and the wavelength detection was 260.0 nm. The retention time for EZB and ATVC was 7.47 +- 0.02 and 4.67 +- 0.02 min, respectively. The described method was linear over a range of 10-10 g/ml for EZB and 5 ±100 g/ml for ATVC. The mean percent recoveries were 99.87 +- 0.769 and 100.04 +- 0.480 for EZB and ATVC, respectively. Method was validated according to ICH guidelines and successfully applied for analysis of bulk powder, pharmaceutical formulations and spiked human plasma.

Harsh Bhandari et al., (2021) Atorvastatin calcium is an HMG-CoA reductase inhibitor and Ezetimibe is a cholesterol inhibitor. Atorvastatin calcium is used to lower the LDL and triglycerides level. It also lower the chances of heart attack and stroke. Ezetimibe is used to reduce the hyperlipidemia, alone or in combination with other cholesterol lowering agents. The combination is used together with proper diet to treat high cholesterol and triglycerides levels in the blood. The combination was approved by US-FDA in May 2013 under the trade name Liptruzet, Atozet. A survey of literature and review published in various analytical and pharmaceutical journals has been conducted and the methods which were developed and used for determination as single and combination in bulk drugs, in pharmaceutical formulation, and in biological fluids have been reviewed. This review covers the analytical methods including spectrophotometric methods, chromatographic methods including HPLC, RP-HPLC and HPTLC, liquid chromatography tandem mass spectroscopy were reported

R Aiyaluet al., (2011) developed a simple, precise, rapid, selective, and economic high performance thin-layer chromatography method has been established for simultaneous analysis of atorvastatin (ATV) and ezetimibe (EZE) in tablet dosage forms. The chromatography separation was performed on precoated silica gel 60 GF_{254} plates with toluene–ethyl acetate–methanol 12:5:3 (v/v/v) as mobile phase. The plate was developed to a distance of 8.0 cm at ambient temperature. The retention factors for ATV and EZE were 0.31 and 0.57, respectively. The detection band was carried out at 254 nm. The calibration curve was linear in the concentration range of 200–1200 ng/spot for both ATV and EZE. For ATV, the recovery study results ranged from 99.44 to 99.54% with RSD value ranging from 0.067 to 0.107%. For EZE, the recovery results ranged from 98.88 to 99.00% with RSD value ranging from 0.154 to 1.756%. The assay was 99.89% for ATV and 99.84% for EZE. The calibration plots revealed a good linear relationship with $r^2 = 0.9991$ for ATV and 0.9992 for EZE. Both ATV and EZE were subjected to different stress conditions—acid, alkaline hydrolysis, oxidation, photo degradation, dry, and wet heat treatment—as prescribed by ICH. The degradation products were well resolved from the pure drug with significantly different R_f values. The method was validated for precision, accuracy, specificity, ruggedness, and robustness.

Y Shah et al., (2011) developed, optimized and validated for simultaneous determination of rosuvastatin and atorvastatin in human serum using naproxen sodium as an internal standard. Effect of different experimental parameters and various particulate columns on the analysis of these analytes was evaluated. The method showed adequate separation for rosuvastatin and atorvastatin and best resolution was achieved with Brownlee analytical C18 column (150 × 4.6 mm, 5 μm) using methanol–water (68:32, v/v; pH adjusted to 3.0 with trifluoroacetic acid) as a mobile phase at a flow rate of 1.5 ml/min and wavelength of 241 nm. The calibration curves were linear over the concentration ranges of 2.0–256 ng/ml for rosuvastatin and 3.0–384 ng/ml for atorvastatin. The lower limit of detection (LLOD) and lower limit of quantification (LLOQ) for rosuvastatin were 0.6 and 2.0 ng/ml while for atorvastatin were 1.0 and 3.0 ng/ml, respectively. All the analytes were separated in less than 7.0 min. The proposed method could be applied for routine laboratory analysis of rosuvastatin and atorvastatin in human serum samples, pharmaceutical formulations, drug–drug interaction studies and pharmacokinetics studies.

Ravi Teja.Tumburu et al., (2020) reported a simple, selective, robust and sensitive reversed phase high performance liquid chromatography method has been developed and validated for the simultaneous estimation of Atorvastatin and Ezetimibe in bulk drug and pharmaceutical formulations. The separation was achieved on a phenomenex C-18 (250 × 4.6 mm, packed with 5 μ) column by using an isocratic mobile phase mixture composed of Acetonitrile: ammonium acetate buffer pH 3.0 (50:50, v/v) with 1.1 mL/min as flow rate and the eluents were monitored at 247 nm. The retention times for Atorvastatin, Ezetimibe were 3.3, 4.5 min respectively, the linearity for both analytes was found to that r2 = 0.991 and 0.986 for Atorvastatin and Ezetimibe respectively. The method was validated for its system suitability, accuracy, precision and stability. The proposed method was successfully employed for the simultaneous quantification of Atorvastatin and Ezetimibe in their pharmaceutical formulation

Z Luo et al., (2015) reported a simple, sensitive and reproducible gradient reverse phase high performance liquid chromatographic (RP-HPLC) method for separation and determination of the related substances of ezetimibe was developed and validated. Eleven potential process- related impurities (starting materials, (3S,4S,3'S)-isomer, degradants and byproducts) were identified in the crude samples. Tentative structures for all the impurities

were assigned primarily based on comparison of their retention time and mass spectrometric data with that of available standards and references. This method can be applied to routine analysis in quality control of both bulk drugs and commercial tablets. Separation of all these compounds was performed on a Phenomenex Luna Phenyl-Hexyl (100 mm×4.6 mm, 5 μm) analytical column. The mobile phase-A consists of acetonitrile–water (pH adjusted to 4.0 with phosphoric acid)–methanol at 15:75:10 (v/v/v), and mobile phase-B contains acetonitrile. The eluted compounds were monitored at 210 nm. Ezetimibe was subjected to hydrolytic, acid, base, oxidative, photolytic and thermal stress conditions as per ICH serves to generate degradation products that can be used as a worst case to assess the analytical method performance. The drug showed extensive degradation in thermal, acid, oxidative, base and hydrolytic stress conditions, while it was stable to photolytic degradation conditions. The main degradation product formed under thermal, acid, oxidative, base and hydrolytic stress conditions corresponding to (2R,3R,6S)-N, 6-bis(4-fluorophenyl)-2-(4-hydroxyphenyl)-oxane-3-carboxamide(Ezetimibetetrahydropyran impurity) was characterized by LC- MS/MS analysis. The degradation products were well resolved from the main peak and its impurities, thus proved the stability-indicating power of the method. The developed method was validated as per international conference on harmonization (ICH) guidelines with respect to specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision and robustness.

Mary Ellen Sweeneyet al., (2007) Elevated serum cholesterol is a known risk factor for the development of coronary artery disease. Circulating cholesterol is a product of both cholesterol absorption from the gut and cellular cholesterol production. Ezetimibe is a novel cholesterol-lowering drug that acts at the brush border of the small intestine. Recent studies have further identified the molecular target as the Niemann-Pick C1-like transporter. Ezetimibe blocks the absorption of dietary and biliray sterols resulting in intracellular cholesterol depletion. Clinical studies have demonstrated beneficial improvements in the lipid profile with Ezetimibe as monotherapy, but dramatic effects are seen when Ezetimibe is combined with other lipid-lowering drugs, particularly 3-hydroxy-3-methylglutaryl coenzymeA reductase inhibitors (statins). Combination studies of Ezetimibe with statins, bile acid sequestrants, fenofibrate and niacin all demonstrate significant total and low density lipoprotein cholesterol lowering. An excellent safety and tolerability profile combined with once-daily dosing make this attractive adjunct therapy for hypercholesterolemia.

Stefan Ostwald et al., (2006) developed a selective and high-sensitive assay to measure serum concentration-time profiles, renal and fecal elimination of ezetimibe in pharmacokinetic studies. Ezetimibe glucuronide, the major metabolite of ezetimibe was demonstrated by enzymatic degradation to the parent compound. Ezetimibe was measured after extraction with tert-butyl ether using 4-hydroxychalcone as internal standard and liquid chromatography coupled with an APCI interface with tandem mass spectrometry for detection. The chromatography (column XTerra MS C18, 2.1 mm x 100mm, particle size 3.5 µm) was done isocratically with Acetonitrile: water: 60:40, flow rate 200µL/min. The MS/MS analysis was performed in the negative ion mode. The validation ranges for Ezetimibe were as follow serum 0.00001- 0.015µg/mL and 0.001-0.2 µg/mL; urine and fecal homogenate 0.025-10 µg/mL and 0.1-20 µg/mL, respectively. The assay was successfully applied to measure ezetimibe disposition in two subjects genotyped for the hepatic uptake transporter SLCO1B1.

Lincy Joseph et al., (2008) reported ‒Simultaneous Estimation of Atorvastatin and Ramipril by RP-HPLC and Spectroscopy‖. Separation was done by Intersil ODS column (250 × 4.6 mm) 5µ at 40ºC using 50% acetonitrile and 50% Sodium perchlorate buffer pH 2.5 with dilute phosphoric acid as mobile phase. With the flow rate was 1.2ml/ min at 215 nm. The simultaneous equation method has selected two wavelengths 247 nm and 208 nm the λmax of Atorvastatin Calcium and Ramipril, respectively.

SJ Varghese et al., (2014) developed aSimple, accurate, precise, sensitive, and validated high-performance liquid chromatography (HPLC) and high-performance thin-layer chromatography (HPTLC)-densitometric methods were developed for simultaneous determination of fenofibrate (FEN), atorvastatin (ATO), and ezetimibe (EZE) in combined tablet dosage form. In Method A, the gradient RP-HPLC analysis was performed on a Shim-pack C18 column (150 × 6 mm id), using a mobile phase consisting of 0.1% formic acid and acetonitrile in solvent gradient elution for 25 min at a flow rate of 1.5 mL min^{-1}. Quantification was carried out using a photodiode array UV detector at 245 nm. The employment of diode array detector allowed selectivity confirmation by peak purity evaluation. In Method B, the HPTLC analysis was carried out on an aluminum-backed sheet of silica gel $60F_{254}$ layer using toluene: methanol:triethylamine (8:1.5:0.1, v/v/v) as the mobile phase. Quantification was achieved with UV densitometry at 245 nm. The analytical methods were validated according to International Conference on Harmonization guidelines.

Low relative standard deviation values indicated good precision. Both the methods were successfully applied for the analysis of drugs in laboratory-prepared mixtures and commercial tablets. No chromatographic interference from tablet excipients was found, and hence these methods are applicable for simultaneous determination of FEN, ATO, and EZE in pharmaceutical formulations.

BG Chaudhari et al., (2006) developed asimple, precise, accurate and rapid high-performance thin-layer chromatographic method has been validated for the estimation of atorvastatin calcium and ezetimibe simultaneously in combined dosage forms. The stationary phase used was precoated silica gel 60_{F254}. The mobile phase used was a mixture of chloroform: benzene: methanol: acetic acid (6.0:3.0:1.0:0.1 v/v/v/v). The detection of spots was carried out at 250 nm. The method was validated in terms of linearity, accuracy, precision and specificity. The calibration curve was found to be linear between 0.8 and 4.0 µg/spot for atorvastatin calcium and 0.1 and 1.0 µg/spot for ezetimibe. The limit of detection and the limit of quantification for atorvastatin calcium were found to be 170 ng/spot and 570 ng/spot respectively, and for ezetimibe, 20 ng/spot and 70 ng/spot respectively. The proposed method can be successfully used to determine the drug content of marketed formulation.

Pavani Naidu S et al., (2016) develop a simple, selective and precise stability indicating reverse-phase high performance liquid chromatography method for the simultaneous estimation of atorvastatin calcium and ezetimibe hydrochloride in bulk and tablet dosage form. The chromatographic separation was performed by Agilent Zorbax column (250×4.6 mm, 5 µm) using methanol: 0.1 % v/v orthophosphoric acid in water (65:35) as mobile phase at flow rate of 1.0 ml/min with injection volume 20 µl and the detection was carried out using UV detector at 240 nm. The method was validated as per ICH guidelines.The retention time for atorvastatin calcium (ATV) and ezetimibe hydrochloride (EZT) was found to be 6.81 min and 4.96 min respectively. The linear regression analysis data for the calibration plots showed good linear relationship in the concentration range of 5-50 µg/ml for both ATV and EZT. The percentage recoveries of ATV and EZT in the marketed dosage form were found to be 100.82 and 94.27 respectively. The correlation coefficients for ATV and EZT were 0.9983 and 0.998 respectively. The percentage degradation at different stress conditions like acid, alkaline, oxidative and photolytic for atorvastatin calcium were found to be 14.91, 8.26, 8.02 and 2.65 respectively and for ezetimibe hydrochloride, found to be 9.70, 32.18, 2.51 and 0.16

respectively. The developed method was successfully validated as per ICH guidelines. This method is simple, selective, linear, precise, accurate and sensitive, and can be used for routine analysis of tablet dosage forms containing both the drugs.

Yogesh B. Zambare et al., (2009) reported Simultaneous Estimation of Atorvastatin and Ramipril by First Order Derivative Spectrophotometric Method. The first order derivative spectrophotometric method was developed using methanol as solvent. The wavelengths selected for the analysis were 246 nm and 208 nm for Atorvastatin Calcium and Ramipril, respectively. Beer's law obeyed the concentration range of 10 – 50 µg/ ml for both the drugs.

Nagaraj et al., (2007) reported Simultaneous Quantitative Resolution of Atorvastatin Calcium and Fenofibrate in Pharmaceutical Preparation by using Derivative Ratio Spectrophotometry and Chemometric Calibrations**.** In ratio spectra derivative spectrophotometry, analytical signals were measured at wavelengths corresponding to either maximums or minimums for both drugs in first derivative spectra of ratio spectra obtained by using either spectrum as divisor. For the remaining four methods using chemometric techniques, namely, classical least squares (CLS), inverse least squares (ILS), principal component regression (PCR) and partial least squares (PLS), the calibrations were constructed by using the absorption data matrix corresponding to the concentration data matrix, with measurements in the range of 231 - 310 nm (Deltalambda = 1 nm) in their zero-order spectra.

Khan M.R. et al., (2006) reported Simultaneous Spectrophotometric Determination of Atorvastatin Calcium and Amlodipine Besylate in Tablets**.** First method employs formation and solving of simultaneous equations using 245 nm and 363 nm as two analytical wavelengths. Second is dual wavelength method, which uses the difference of absorbance value at 259.9 nm and 354 nm for estimation of AtorvastatinCalcium and absorbance at 363 nm for Amlodipine besylate. Fifty percent methanol was used as solvent.

Lincy Joseph et al., (2008), reported Simultaneous Estimation of Atorvastatin and Ramipril by RP-HPLC and Spectroscopy. Separation was done by Intersil ODS column (250 × 4.6 mm) 5µ at 40ºC using 50% acetonitrile and 50% Sodium perchlorate buffer pH 2.5 with dilute phosphoric acid as mobile phase. With the flow rate was 1.2ml/ min at 215 nm. The

simultaneous equation method has selected two wavelengths 247 nm and 208 nm the λmax of Atorvastatin Calcium and Ramipril, respectively.

Shah D.A. et al., (2004), reported Development and Validation of a RP-HPLC Method for Determination Atorvastatin Calcium and Aspirin in a Capsule Dosage Form. The separation was achieved on a A Phenomenex Gemini C_{18}, 5 mm column having 250 x 4.6 mm i.d. in isocratic mode, with mobile phase containing 0.02 M potassium di hydrogen phosphate: methanol (20:80) adjusted to pH 4 using ortho phosphoric acid was used. The flow rate was 1.0 ml/ min and effluents were monitored at 240 nm. The retention times of Atorvastatin Calcium and Aspirin were 5.4 min and 3.4 min, respectively.

Shah D.A. et al., (2008), reported Stability Indicating RP-HPLC Estimation of Atorvastatin Calcium and Amlodipine Besylate in Pharmaceutical Formulations**.** The separation was carried out on a Phenomenex Gemini C_{18}, 5 µm column having 250×4.6 mm i.d. in isocratic mode, with mobile phase containing 0.02 M potassium dihydrogen phosphate: acetonitrile: methanol (30:10:60, v/v/v) adjusted to pH 4 using ortho phosphoric acid was used. The flow rate was 1.0 ml/ min and effluents were monitored at 240 nm. The retention times of Atorvastatin Calcium and Amlodipine besylate were 11.6 min and 4.5 min, respectively.

Jain N. et al., (2008), reported Development and Validation of RP-HPLC Method for Simultaneous Estimation of Atorvastatin Calcium and Fenofibrate in Tablet Dosage Forms**.** The separation was achieved by Luna C_{18} column and methanol: acetate buffer pH 3.7 (82:18 v/v) as mobile phase, at a flow rate of 1.5 ml/ min. Detection was carried out at 248 nm. Retention times of Atorvastatin Calcium and Fenofibrate was found to be 3.02+0.1 and 9.05+0.2 min, respectively.

Swapnil D. Jadhav et al., (2010), reported Spectrophotometric Methods for Estimation of Atorvastatin Calcium from Tablet Dosage Forms**.** In the present incestigation, hydrotropic solubilization is employed to enhance the aqueous solubilities of poorly water- soluble drugs like Atorvastatin Calcium in tablet dosage forms. This method utilizes 2.0 M urea solution as, hydrotropic solubilizing agent. In the urea solution Atorvastatin Calcium shows maximum absorbance at 240 nm. The 2.0 M urea solution does not show any interference with the sampling wavelength. Another method is formation of green color complex between the drug Atorvastatin Calcium and 0.3 % w/v ferric chloride and 0.02 % w/v potassium

ferricyanide. The green colored complex shows the maximum absorbance at 787 nm.

Anil kumar Sharma et al., (2010), reported Simultaneous Estimation of Atorvastatin Calcium, Ramipril and Aspirin in Capsule Dosage Form using HPTLC. The method was developed using precoated silica gel 60F254 as stationary phase. The mobile phase used was a mixture of benzene: ethyl acetate: toluene: methanol: glacial acetic acid (4.0:4.5:1.0:0.5:0.1 v/v). Detection was carried out with ultra- violet detection at 220 nm. The Rf values were about 0.45±0.02, 0.28±0.01 and 0.72±0.02 for Atorvastatin Calcium, Ramipril and Aspirin, respectively.

Nagavalli D. et al., (2010), reported Simultaneous Estimation of Atorvastatin Calcium, Ezetimibe and Fenofibrate in Pure and in Combined Dosage Form by RP-HPLC and HPTLC Methods**.** In method A, Atorvastatin Calcium, Ezetimibe and Fenofibrate were chromatographed on Phenomenax Luna ODS analytical column (150 mm x 4.6 mm i.d, 5µ) using Methanol: Acetonitrile: Water (50:30:20 v/v/v) as the mobile phase and scanned at 254 nm. Rt values of Atorvastatin Calcium, Ezetimibe and Fenofibrate were found to be 1.77 ± 0.02, 2.62 ± 0.01 and 9.26 ± 0.03 respectively. In method B, Atorvastatin Calcium, Ezetimibe and Fenofibrate were chromatographed on silica gel 60 F_{254} TLC plate using chloroform: Toluene: Methanol: Glacial acetic acid (6:3:1:0.5 v/v/v/v) as the mobile phase and scanned at 254 nm. The Rf value Atorvastatin Calcium, Ezetimibe and Fenofibrate were found to be 0.48 ± 0.02, 0.56 ± 0.03 and 0.87 ± 0.02 respectively.

Stephan Rathinaraj B. et al., (2010), reported Determination of Atorvastatin Calcium and Ezetimibe by using HPTLC Method**.** The stationary phase used was precoated silica gel 60F 254. The mobile phase used was a mixture of benzene, methanol, acetone and triethylamine (7:2:1:0.2 v/v/v/v). The detection spots were carried out at 266 nm.

Savitha S. Yadhav et al., (2005), reported a Simple and Sensitive HPTLC Method for the Determination of Content Uniformity of Atorvastatin Calcium Tablets**.** The stationary phase was precoated silica gel 60 F254. The mobile phase used was a mixture of benzene: methanol (7:3 v/v). Combination of benzene: methanol offered optimum migration (Rf = 0.46±0.02). Detection of the spot was carried out at 281nm.

Altuntas T.G., et al., (2004), reported Liquid Chromatographic Determination of Atorvastatin in Bulk Drug, Tablets and Human Plasma a simple, specific, and accurate high performance liquid chromatographic (HPLC) method for determination of atorvastatin in bulk drug, tablets, and human plasma have been developed. Liquid chromatography was performed on a RP-Supelcosil C_{18} (5 µm, 150 × 4.6 mm) column and the mobile phase consisted of an acetonitrile:methanol:water (45:45:10 v/v/v), and a flow rate of 1.0 mL/min. The effluent was monitored on a UV detector at 240 nm.

Oliveira P.R., et al (2006) developed a analytical method based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) was developed and validated for the determination of ezetimibe in human plasma. Ezetimibe and Etroricoxib (Internal Standard) were extracted from the plasma by liquid–liquid extraction and separated on a C18 analytical column (50 x 3.0 mm ID) with Acetonitrile: water: 85:15 as mobile phase. Detection was carried out by positive electro spray ionization in multiple-reaction monitoring (MRM) mode. The chromatographic separation was obtained within 2.0 min and was linear in the concentration range of 0.25-20ng/mL for free ezetimibe and of 1-300ng/mL for total ezetimibe. The mean extraction recoveries for free ezetimibe and total ezetimibe from plasma were 96.14 and 64.11% respectively. Method validation investigated parameters such as linearity, precision accuracy, specificity and stability, giving results within the acceptable range. The proposed method was successfully applied to the quantification of ezetimibe and its glucuronide in human plasma to support clinical and pharmacokinetics studies. Moreover, the method was used for the quality control analysis of pharmaceutical dosage forms.

Hossein Danafar.,et al (2013, developed a Selective and highly sensitive high performance liquid chromatography- electrospray ionization mass spectrometry method has been developed for determination of ezetimibe in human plasma. Ezetimibe was extracted from plasma with ethyl acetate followed by evaporation of the organic layer and then, reconstitution of the residue in mobile phase before injection to chromatograph. The mobile phase constituted of Acetonitrile-ammonium acetate (10mM. pH 3.0), 75:25. An aliquot of 10µ L was chromatographically analyzed on a pre-packed Zorabax XDB-ODS C18 column (2.1 x 100mm, 3.5 micron). Detection of analytes was achieved by mass spectrometry with atmospheric pressure chemical ionization (APCI) interface in the negative ion mode operated under the multiple-reaction monitoring mode (MRM). Standard curve were linear over the wide ezetimibe concentration range of 0.005-30.0ng/mL with acceptable accuracy and

precision. The limit of detection was 0.02ng/mL. The validated LC-APCI-MS method has been successfully throughout a bioequivalence study on an ezetimibe generic product in 24 healthy male volunteers.

Shuijun Li.,et al (2006) Simple, reliable and sensitive liquid-liquid chromatography-tandem spectrometry method (LC-MS/MS) was developed and validated for quantification of free and total ezetimibe in human plasma. The analyte and internal standard (Ezetimibe 13C6) were extracted by liquid-liquid extraction with methyl tert-butyl ether. The reversed-phase chromatographic separation was performed on a Capcell C18 column, and the plasma extract was eluted with a gradient consisting of Acetonitrile and 5mM ammonium acetate. The analyte was detected using negative ionization by multiple-reaction monitoring mode. The mass pair of 408.5 → 270.8 and m/z 414.5 →276.8 were used to detect ezetimibe and internal standard, respectively. The assay exhibited linear ranges from 0.02 to 20ng/mL for free ezetimibe and 0.25 to 250ng/mL for total ezetimibe in human plasma. Acceptable precision and accuracy were obtained for concentration of the calibration standard and quality control. The validated method was successfully used to analyze human plasma samples for application in a pharmacokinetic study.

Sistla R.,et al (2005) proposed a rapid, specific reversed-phase HPLC method has been developed for assaying ezetimibe in pharmaceutical dosage forms. Ezetimibe belongs to a group of selective and very effective 2-azetidone cholesterol absorption inhibitors that act on the level of cholesterol entry into enterocytes. The assay involved an isocratic elution of ezetimibe in Kromasil 100 C18 column using a mobile phase composition of water (pH 6.8, 0.05%, 1-heptane sulfonic acid) and Acetonitrile (30:70). The flowrate was 0.5mL/min and the analyte monitored at 232 nm. The method was successfully applied to estimate the amount of Ezetimibe in tablets.

AIM AND OBJECTIVE

4. AIM & OBJECTIVE

In pharmaceutical industries, quantitative chemical analysis is done to ensure that the raw material and the final product thus obtained meet certain specifications and to determine how much component is present in the final product. Development and validation of a method should be more specific, accurate and precise. The main objective for that is to improve the conditions and parameters, which should be followed in the development and validation.

From the literature survey, it was found that there is no effective method developed and validated on related substance for the simultaneous determination of Atorvastatin and Ezetimibe in RP-HPLC. So, it was decided to develop and validate a RP-HPLC method for the simultaneous determination of Atorvastatin and Ezetimibe and its related substance that is specific, accurate and precise.

Therefore, in the proposed project, a successful attempt has been made to develop a simple, accurate, economic and rapid method for the estimation and to validate the method. As a result, a simple, economical, precise and accurate method was developed and validated by **Reverse Phase High Performance Liquid Chromatography** (RP-HPLC).

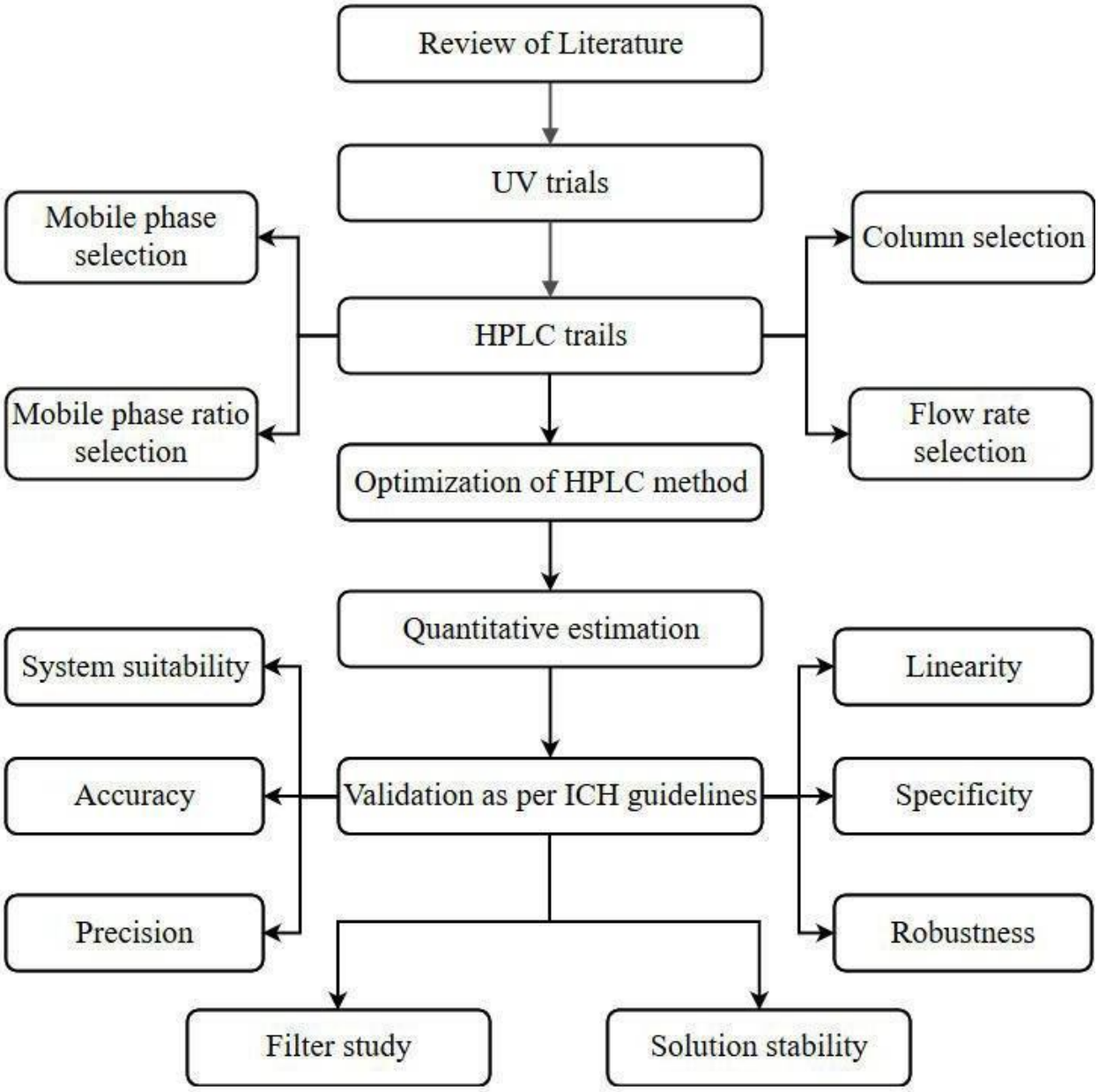
The method has been validated as per the guidelines given by **ICH requirements** to assure that the method consistently meets the predetermined specifications and quality attributes.

The present work therefore aims at the following ones:

- To develop a simple, specific, precise, and accurate RP-HPLC method for the simultaneous estimation of Atorvastatin and Ezetimibe using solid dosage form.
- To validate the developed RP-HPLC method for various parameters like Specificity, Linearity, Accuracy, Precision, Robustness etc., as per the ICH Q2 (R1)guidelines.
- To ensure that the methods can be used as quality control tool in routine analysis in pure and bulk pharmaceutical dosage forms.
- To ensure that the developed method can be utilized for routine analysis.

PLAN OF WORK

5. PLAN OF WORK



MATERIALS AND INSTRUMENTS

6. MATERIALS AND INSTRUMENTS

6.1. CHEMICALS AND REAGENTS

- Methanol and Acetonitrile (FINAR), HPLC grade of 99.9% purity.
- Ammonium acetate AR Grade.
- Milli-Q water was used for the study supplied by Milli Q Plus purification system.
- Nylon filter, Membrane filter, Poly tetrafluoroethylene filters, Polyvinylidine pyrolidine filters was used for filtration of mobile phase and sample supplied by Milllipores Ltd.
- Glasswares including volumetric flasks, Pipeetes, Vials, Measuring cylinders and Beaker was Class A Bororsil Glass.

6.2. INSTRUMENTS/ EQUIPMENT

Table 6: List of equipment used

S.NO	NAME OF THE INSTRUMENT	CODE NO	MAKE	MODEL
1	HPLC (PDA Detector)	MP/RAD/EQ/052	Agilent	Infinity-II
2	HPLC (PDA Detector)	MP/RAD/EQ/047	Shimadzu	LC-2030 3D
3	COLUMN	21/TMP/RAD/046	Inertsil	C18
4	COLUMN	20/TMP/RAD/043	Inertsil	C18
5	ANALYTICAL BALANCE	MP/RAD/EQ/039	RADWAG	AS 60/220.R2
6	SONICATOR	MP/RAD/EQ/026	Amekette	LUNA

6.3. LIST OF REFERENCE STANDARDS

Table 7: List of working standard

WORKING STANDARD	ATORVASTATIN	EZETIMIBE
LOT.NO:	21WS080020	21DS0032
POTENCY:	94.81%	99.59%
VALID UP TO:	APR-2023	JUN-2023

6.4 DOSAGE FORM (FORMULATION)

- Generic name : Atorvastatin calcium and Ezetimibe.
- Description : White to off white coloured, circular shape Biconvexed, plain on both sides and film coated tablets.
- Label claim : Each film coated tablet contains Atorvastatin calcium BP equ.to Atorvastatin 10 mg. Ezetimibe USP 10 mg.
- Manufacturing Company : THE MADRAS PHARMACEUTICALS, KARAPAKKAM, CHENNAI.
- Average weight : 288 mg
- Disintergation time : 30 minutes.

METHOD DEVELOPMENT

METHOD DEVELOPMENT:

Proper selection of HPLC method development depends upon the nature of the sample, its molecular weight and solubility. For successful method development various Chromatographic parameters such as pH, mobile phase, its composition and proportion, detection wavelength and other factors were exhaustively studied.

SELECTION OF CHROMATOGRAPHIC METHOD:

The drug selected for the present study was Polar. Polar compounds can be separated by either normal phase or reverse phase chromatography. Reverse phase chromatography was selected for initial separations from the knowledge of properties of the compounds**.**

SELECTION OF DILUENT:

The nature of the drug reveals certain information about the drug such as solubility, pKa. Based on the solubility of the drug, the diluent is selected. The solvent in which the drug has maximum solubility was selected as the diluent. Atorvastatin and Ezetimibe is soluble in Methanol. So, Methonal was used as the diluent.

SELECTION OF DETECTION WAVELENGTH:

Standard solution of Atorvastatin and Ezetimibe was injected into HPLC system then scanned over entire the UV range (200-400nm). The spectrum of Atorvastatin and Ezetimibe was recorded for determination of Z_{max}. The Z_{max} of Atorvastatin and Ezetimibe was detected at 240nm. So, the detection was carried out at 240nm.

SELECTION OF COLUMN:

In reverse phase chromatography, non-polar stationary phase is used for separation. C_8, C_{18} are the commonly used columns in reverse phase chromatography. Here, Inertsil ODS C_{18} column of dimensions 250 × 4.6mm with particle size 5µ; were used for the separation.

SELECTION OF MOBILE PHASE:

Several trials were made to find out the mobile phase for eluting the drug. The mobile phases containing Ammonium acetate was mixed with polar solvent acetonitrile in different ratios and used over different columns. Better peak shape and adequate retention time were aimed.

MODE OF SEPARATION:

Several trails were conducted by changing flow rate, injection volume and other parameters till satisfactory separation were achieved. The resulting chromatograms were recorded and the chromatographic parameters such as column efficiency, theoretical plates, tailing factor and peak purity were calculated. Finally, the condition that gives best result was selected for estimation.

7. METHOD DEVELOPMENT

7.1. Selection of mobile phase and column

Trial-1

Chromatographic conditions:

Mobile Phase : ACN: Ammonium acetate (45: 55)

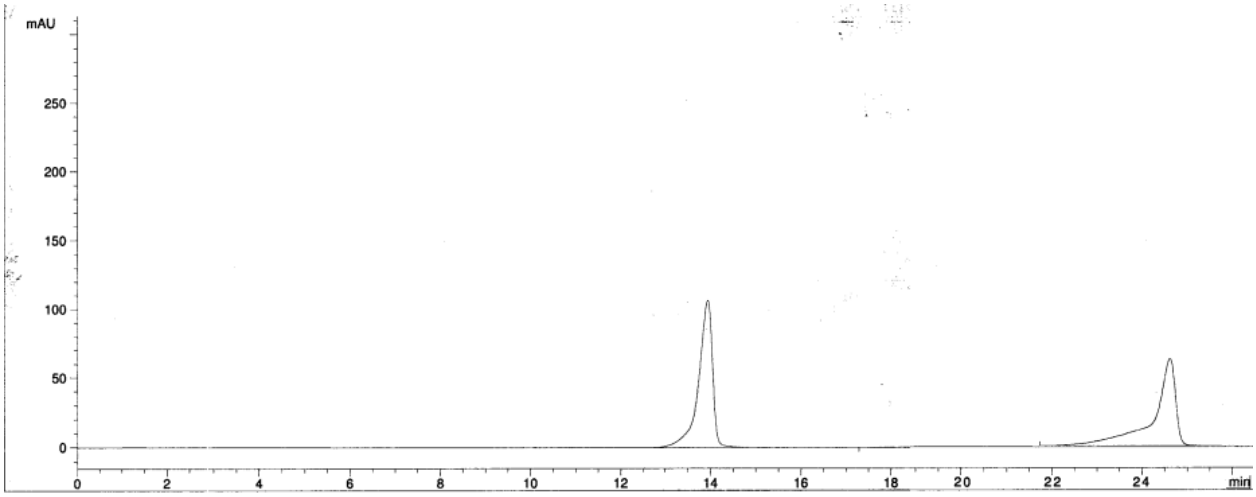
Column : C18, Inertsil ODS250 mm × 4.6 mm, 5µ

Flow rate : 0.5 ml / minute

Wavelength : 240 nm

Injection volume : 10 µL

Column Temperature : 25 ˚C



Peak#	Name	Ret. Time	Area	Tailing factor	Theoretical Plate
1.	Atorvastatin	13.8	261012	2.7	4837
2.	Ezetimibe	24.6	289087	2.7	3259

Observation:

Ezetimibe peak shows peak fronting and the retention time was very high. So, flow rate should be increased.

Trial-2

Chromatographic conditions:

Mobile Phase : ACN: Ammonium acetate (45: 55)

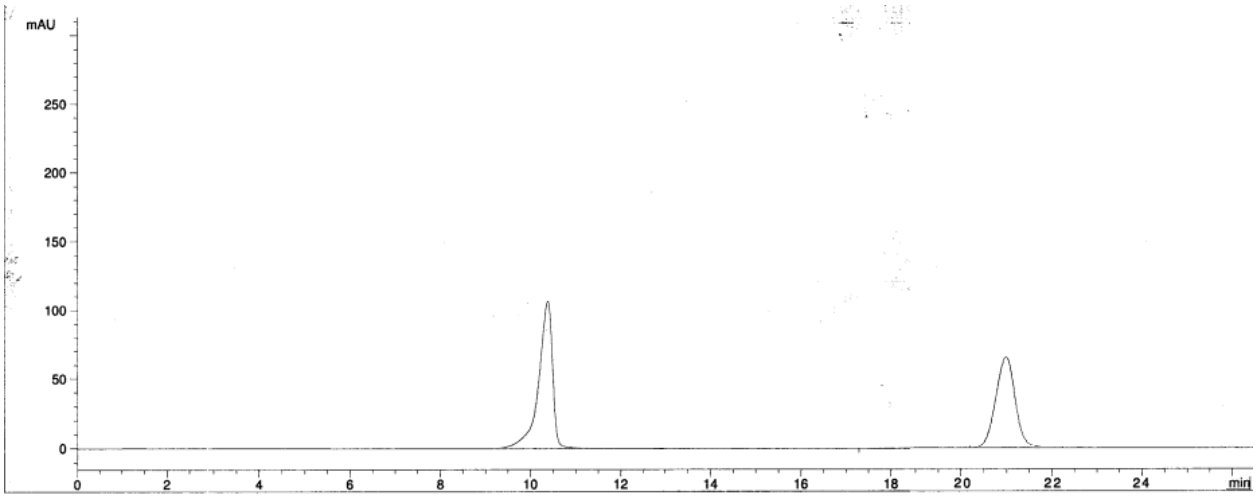
Column : C18, Inertsil ODS250 mm × 4.6 mm, 5µ

Flow rate : 1 ml / minute

Wavelength : 240 nm

Injection volume : 10 µL

Column Temperature : 25 ˚C



Peak#	Name	Ret. Time	Area	Tailing factor	Theoretical Plate
1.	Atorvastatin	10.4	281026	0.8	4903
2.	Ezetimibe	21.2	308931	0.9	5196

Observation:

Atorvastatin peak was good and the Ezetimibe peak also shows better peak but the peak was broad. Also the resolutions between two compounds were good.

Trial-3

Chromatographic conditions:

Mobile Phase : ACN: Ammonium acetate (45: 55)

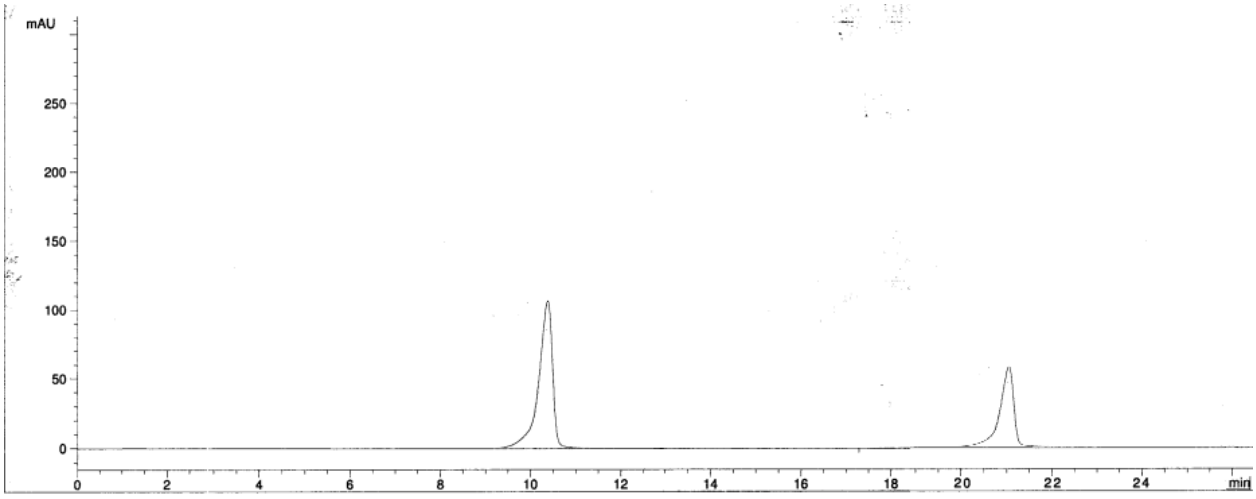
Column : C18, Inertsil ODS250 mm × 4.6 mm, 5µ

Flow rate : 0.5 ml / minute

Wavelength : 240 nm

Injection volume : 20 µL

Column Temperature : 25 ˚C



Peak#	Name	Ret. Time	Area	Tailing factor	Theoretical Plate
1.	Atorvastatin	10.3	302193	1.0	3994
2.	Ezetimibe	21.0	281774	0.9	4706

Observation:

Both the peaks were good and the resolutions of two drugs were also good, but the retention time was bit high. So, the flow rate has to be further increased to reduce the retention time.

Trial-4

Chromatographic conditions:

Mobile Phase : ACN: Ammonium acetate (45: 55)

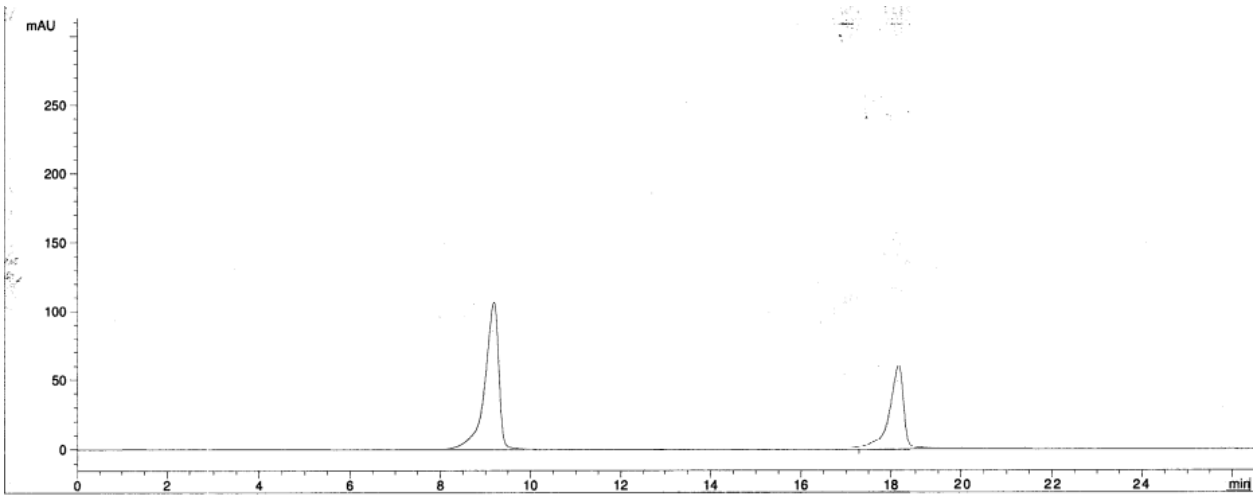
Column : C18, Inertsil ODS250 mm × 4.6 mm, 5µ

Flow rate : 1.5 ml / minute

Wavelength : 240 nm

Injection volume : 20 µL

Column Temperature : 25 ˚C



Peak#	Name	Ret. Time	Area	Tailing factor	Theoretical Plate
1.	Atorvastatin	9.2	322857	1.2	2964
2.	Ezetimibe	18.1	300717	1.3	4074

Observation:

.

Both the peaks passes the resolution, tailing factor and theoretical plates. Retention time also decreased.

7.2. QUANTITATIVE ESTIMATION

Optimized chromatographic condition

Mobile Phase : ACN: Ammonium acetate (45: 55)

Column : C18, Inertsil ODS250 mm × 4.6 mm, 5µ

Flow rate : 0.5 ml / minute

Wavelength : 240 nm

Injection volume : 10 µL

Column Temperature : 25 ˚C

Diluent : Methanol

7.2.1. SYSTEM SUITABILITY

Preparation of standard solution

Weigh accurately 52mg of Atorvastatin calcium WS and 50mg of Ezetimibe WS in 50 ml volumetric flask add 30ml of diluent sonicate to dissolve and makeup the volume with diluent. Pipette out 1ml of above solution in to 200 ml volumetric flask and make up the volume with diluent. Filter the solution through 0.45µ PVDF syringe filter. (5mcg/ml of Atorvastatin and 5mcg/ml of Ezetimibe).

Procedure:

System suitability was performed by injecting the standard solution in six replicates and the chromatograms were recorded.

Table 8: System suitability parameter and acceptance criteria

System Suitability Parameter	Acceptance Criteria
Tailing Factor (Standard Solution)	NMT 2.0
Theoretical Plates (Standard Solution)	NLT 2000
Relative Standard Deviation (Standard Solution)	NMT 5.0%
Resolution	NLT 5.0 between Atorvastatin and Ezetimibe

Table 9: Summary of system suitability results

S.NO	Peak Area		Theoretical Plates		Tailing Factor	
	ATOR	**EZET**	**ATOR**	**EZET**	**ATOR**	**EZET**
1	321925	300834	2951	4091	1.2	1.3
2	322897	301821	2953	4085	1.2	1.2
3	321903	301799	2948	4089	1.3	1.3
4	321889	300807	2955	4092	1.1	1.4
5	322916	301817	2958	4097	1.2	1.2
6	321909	301839	2945	4087	1.1	1.3
Average	322306	301416	2953	4091	1.2	1.2
% RSD	0.17	0.18				

OBSERVATION:

1. Resolution between Atorvastatin calcium and Ezetimibe = 9.9

2. Relative standard deviation of the areas of Atorvastatin calcium = 0.17%,
Ezetimibe = 0.18%

RESULT:

The system suitability parameter was performed for standard solution and the outcome results are within the acceptance limits. Hence the method found suitable for Atorvastatin and Ezetimibe Tablets- 10/10 mg.

METHOD VALIDATION

8. ANALYTICAL METHOD VALIDATION

8.1. SPECIFICITY

Specificity is the ability of the method to measure the analyte in presence of matrix components. The specificity of the method was demonstrated by injecting Blank, Placebo solution, standard solution, sample solution separately.

Preparation of Standard

Weigh accurately 52mg of Atorvastatin calcium WS and 50mg of Ezetimibe WS in 50 ml volumetric flask add 30ml of diluent sonicate to dissolve and makeup the volume with diluent. Pipette out 1ml of above solution in to 200 ml volumetric flask and make up the volume with diluent. Filter the solution through 0.45µ PVDF syringe filter. (5mcg/ml of Atorvastatin and 5mcg/ml of Ezetimibe).

Preparation of Placebo

Weigh and transfer the powder about 2676mg of placebo into 200ml volumetric flask, add 80ml of diluent and sonicate for 5 minutes dissolve and make up the volume with diluent, and allow to centrifuge for 15 minutes at 6000RPM. Filter the solution through a 0.45µ PVDF syringe filter.

Preparation of sample solution

Weigh and powder 20 tablets, Weigh accurately a quantity of the powder containing about 100mg equivalent of Atorvastatin in to 200 ml volumetric flask, add 80 ml of diluent and sonicate for 5 minutes, dissolve and make up the volume with diluent, and allow to centrifuge for 15 minutes at 6000RPM. Filter the solution through a 0.45µ PVDF syringe filter (500Mmcg/ml of Atorvastatin and 500mcg/ml of Ezetimibe).

Procedure

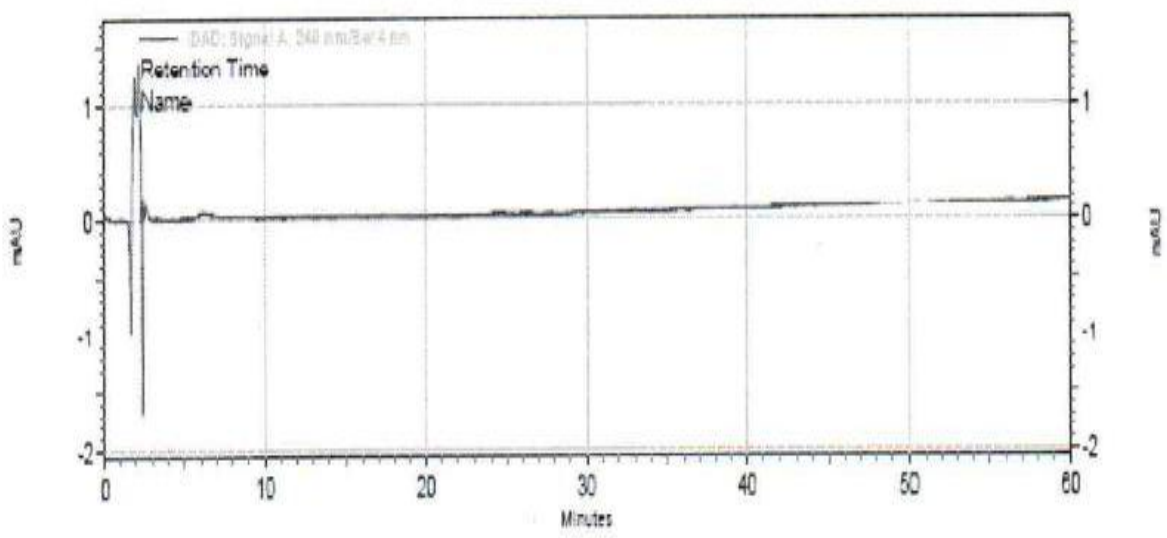
Prepared blank, standard, placebo and standard solution at specification level concentration. Injected all the solution as per the test method and the results were reported.

Acceptance Criteria

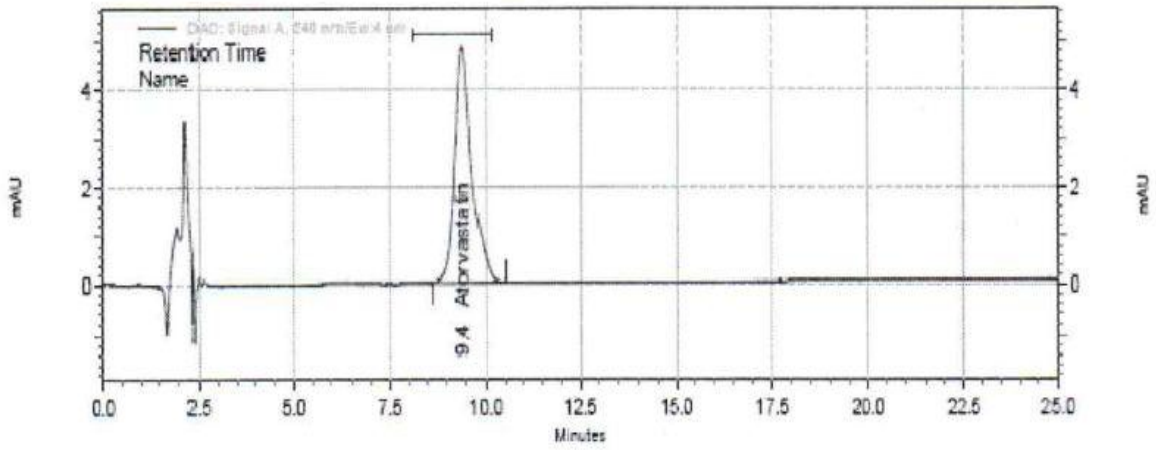
There should not be any interference of blank and placebo peaks at the Retention Time of Atorvastatin and Ezetimibe.

Result

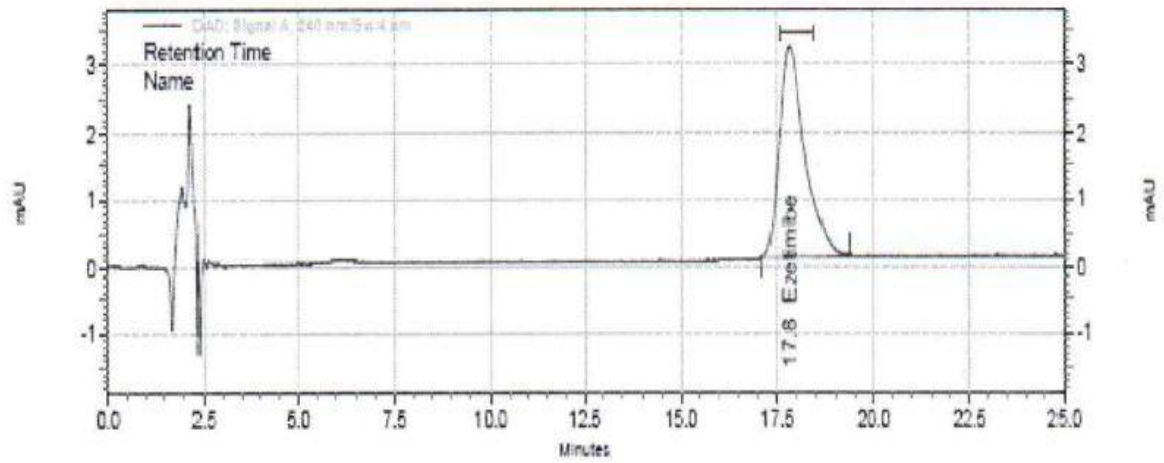
Chromatogram of Specificity-Blank



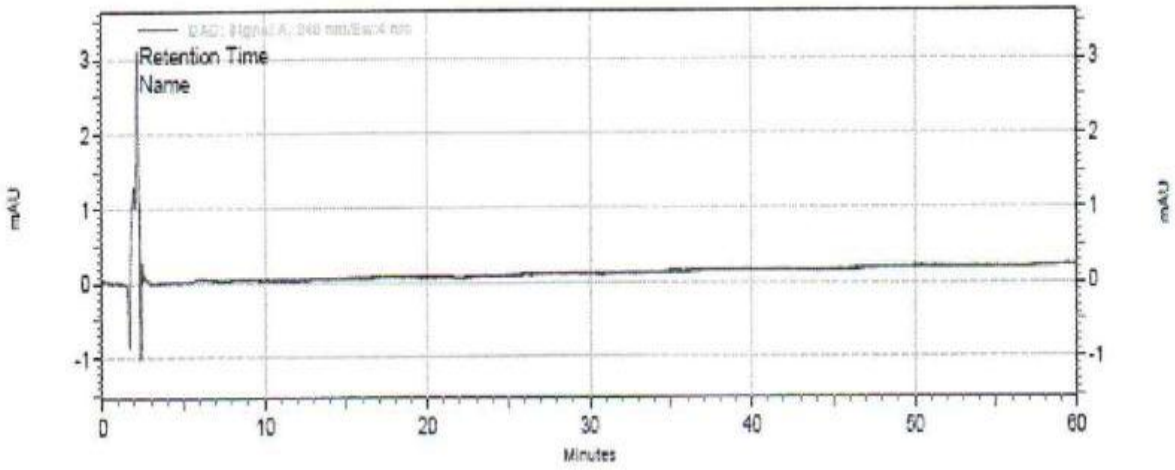
Chromatogram of Specificity-Standard I



Chromatogram of Specificity-Standard II



Chromatogram of Specificity-Placebo



Chromatogram of Specificity-Sample

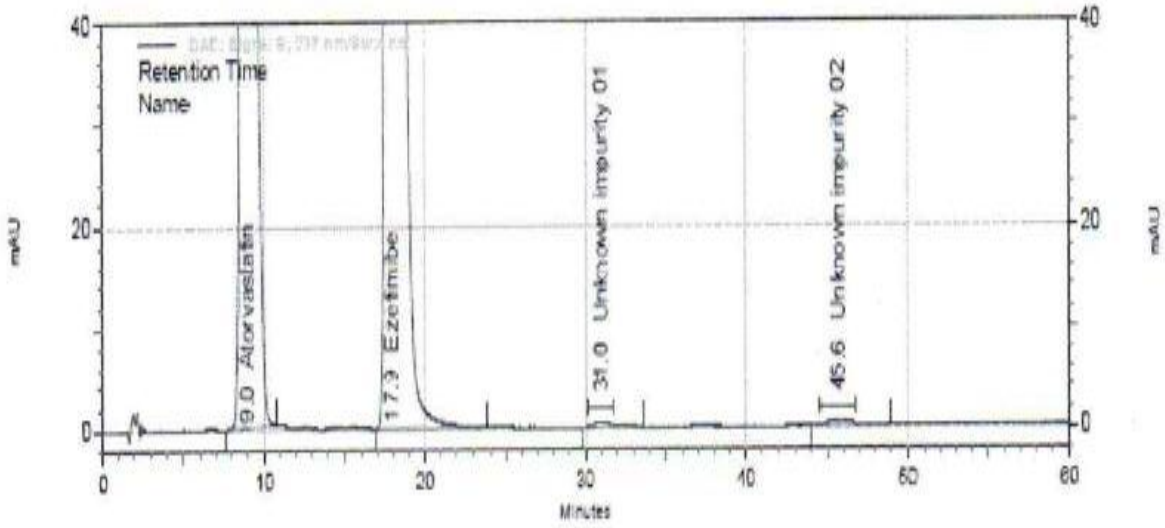


Table 10: Specificity of Atorvastatin and Ezetimibe

S.NO	Sample name		RT (minute)	Interference (YES/NO)
1	Blank		NA	NO
2	Placebo		NA	NO
3	Standard (Atorvastatin)		9.4	NO
4	Standard (Ezetimibe)		17.8	NO
5	Sample	Atorvastatin	9.0	NO
		Ezetimibe	17.9	

Discussion

There was no interference observed from blank, placebo peak at the retention time of Atorvastatin and Ezetimibe. The Peak Purity for standard, sample and its Unknown impurities met the acceptance criteria. Hence, the method found specific for Atorvastatin and Ezetimibe Tablets- 10/10 mg.

8.2. LINEARITY AND RANGE

The linearity of a method is a measure of how well a calibration plot of response vs concentration approximates a straight line. Linearity can be assessed by performing single measurement at several analyte concentrations. The data is then processed using a linear-square regression. The resulting plot slope, intercept and correlation coefficient provide the desired information on linearity.

Preparation of Standard Solutions

Preparation of Stock Solution I

52mg of Atorvastatin calcium and 50mg of Ezetimibe in 50ml volumetric flask and add 30 ml of diluent to dissolve and makeup the volume with diluent. Pipette out 1ml of above solution in to 200 ml volumetric flask and make up the volume with diluent.

Preparation of Stock Solution II

Take 10ml of solution from the stock solution I in 200ml volumetric flask and makeup the volume with diluent.

50% Concentration (2.6ppm)

Pipette out 5ml of solution from the stock solution II in 100ml volumetric flask and makeup the volume with diluent.

75% Concentration (3.9ppm)

Pipette out 7.5ml of solution from the stock solution II in 100ml volumetric flask and makeup the volume with diluent.

100% Concentration (5.2ppm)

Pipette out 10ml of solution from the stock solution II in 100ml volumetric flask and makeup the
volume with diluent.

125% Concentration (6.8ppm)

Pipette out 12.5ml of solution from the stock solution II in 100ml volumetric flask and makeup
the volume with diluent.

150% Concentration (7.8ppm)

Pipette out 15ml of solution from the stock solution II in 100ml volumetric flask and makeup the
volume with diluent.

Procedure

The above solutions ranging from 50% to 150% were prepared and each level was injected in duplicate. Recorded the area response at each level and calculated slope, intercept, correlation coefficient (R) and regression coefficient (R2). A graph plotted of concentration (mg/ml) on

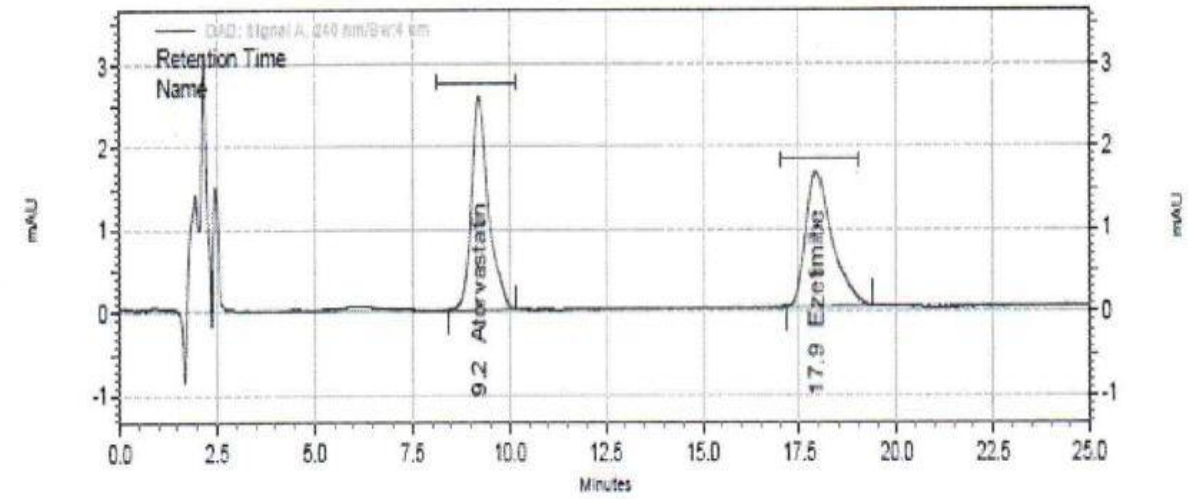
X-axis and area response under the curve on y-axis. The results are summarized in the table and Figure.

Acceptance Criteria

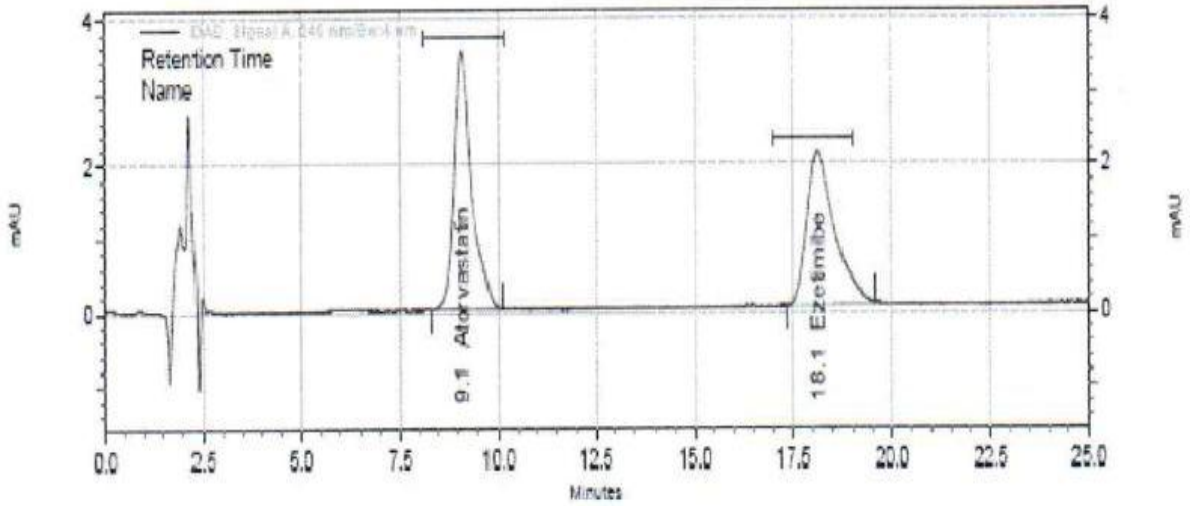
The plot of Concentration versus Peak Area should be linear with a Correlation Co-efficient of not less than 0.995.

Result

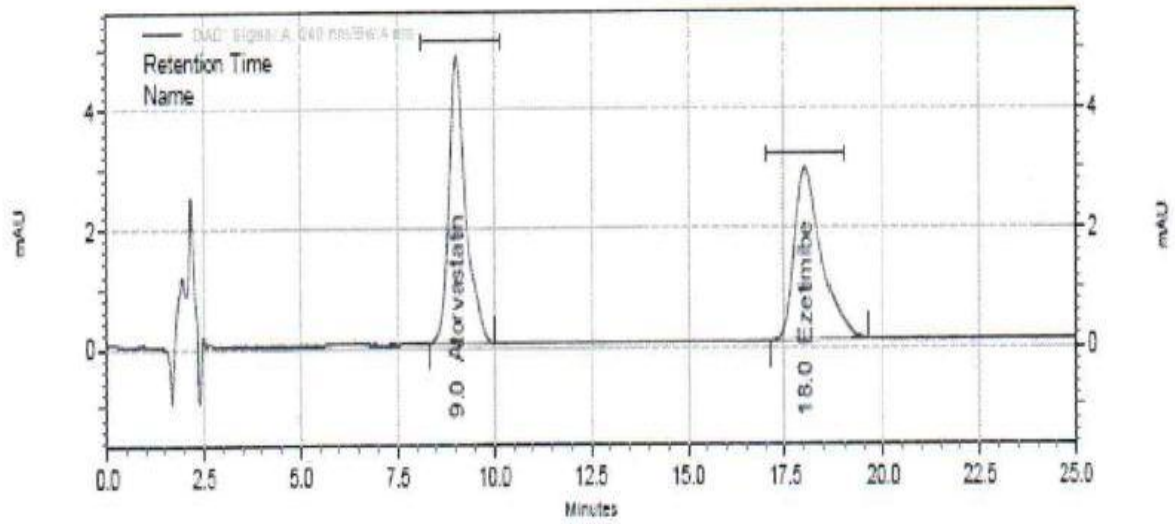
Chromatogram of linearity 50%



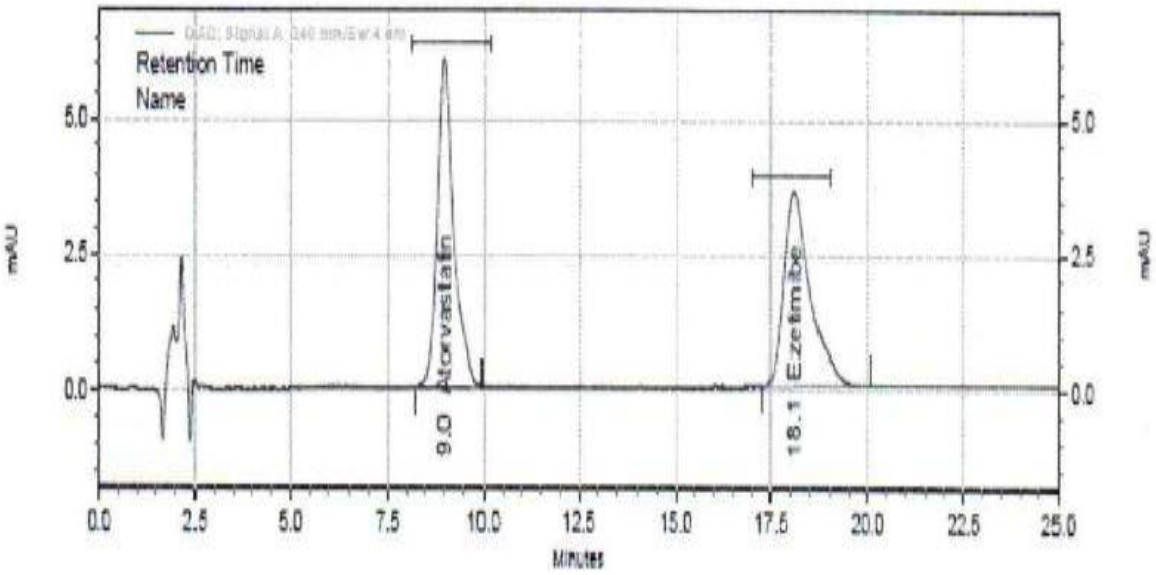
Chromatogram of linearity 75%



Chromatogram of linearity 100%



Chromatogram of linearity 125%



Chromatogram of linearity 150%

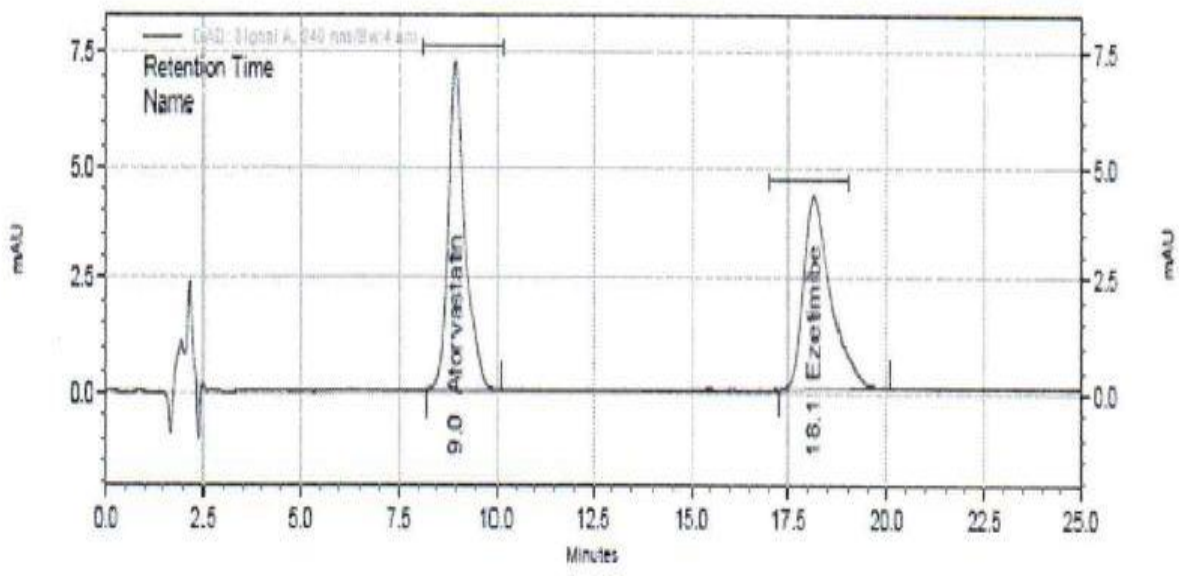


Table 11: Statistical Data for Atorvastatin Linearity

S. No.	Linearity Level in PPM	Conc in PPM	Peak Area
1	50%	2.6	163723
2	75%	3.9	225626
3	100%	5.2	303200
4	125%	6.5	380297
5	150%	7.8	452750
Slope			56363
Intercept			12029
Standard deviation			115933
Coefficient of correlation (r)			0.9992
Coefficient of regression (r^2)			0.9986

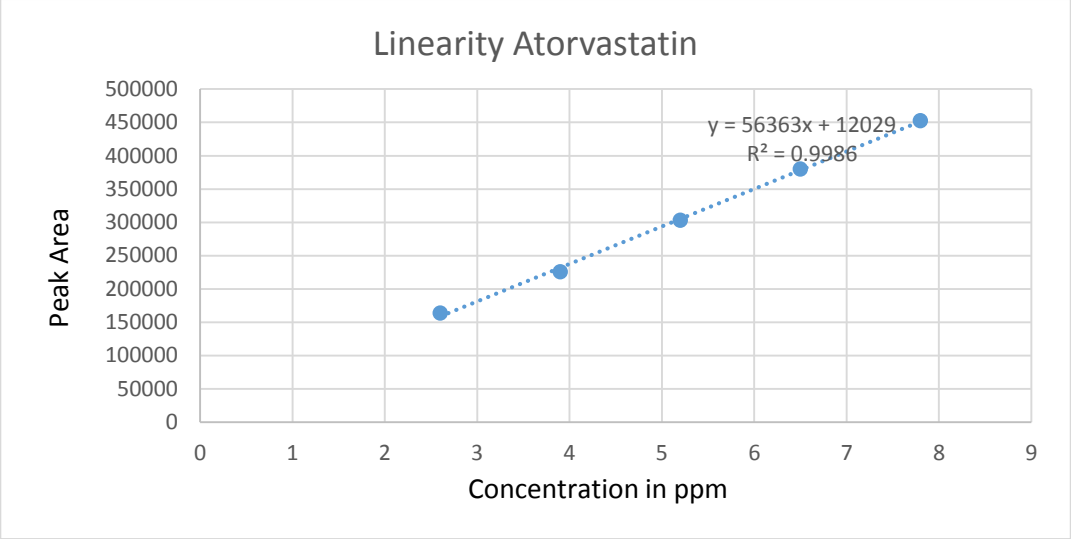


Fig. 17: Linearity Graph of Atorvastatin

Table 12: Statistical Data for Ezetimibe Linearity

S. No.	Linearity Level in PPM	Conc in PPM	Peak Area
1	50%	2.5	151979
2	75%	3.75	214491
3	100%	5.0	288339
4	125%	6.25	362984
5	150%	7.5	450066
Slope			59493
Intercept			12029
Standard deviation			117771
Coefficient of correlation (r)			0.9983
Coefficient of regression (r^2)			0.9968

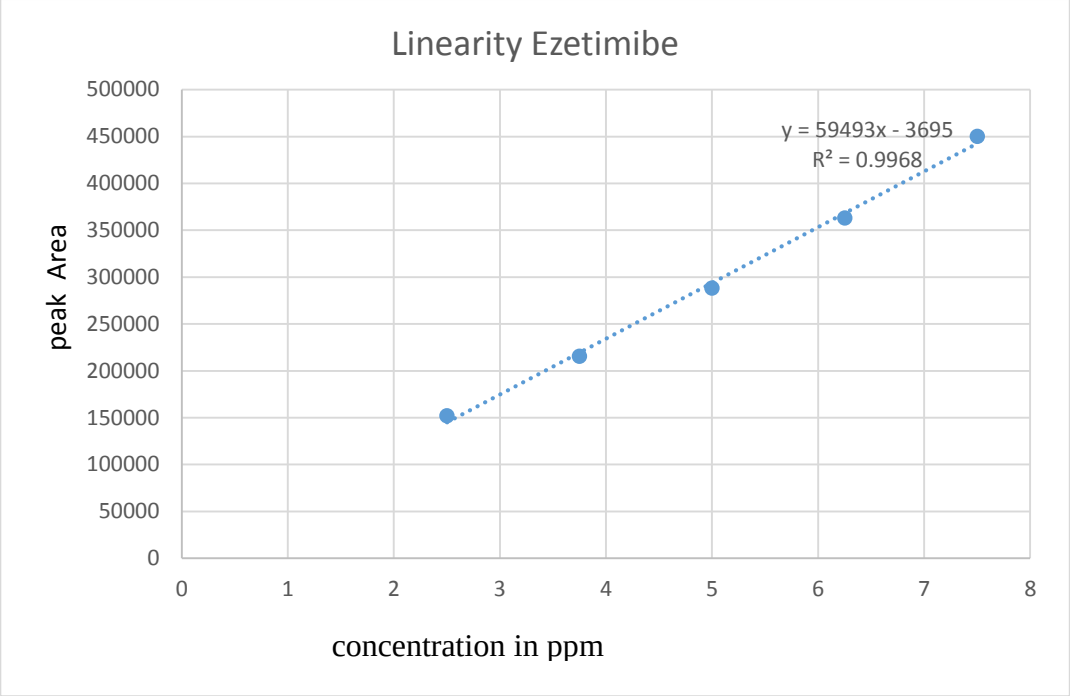


Fig. 18: Linearity Graph of Ezetimibe

Discussion

From the statistical linearity data of Atorvastatin and Ezetimibe it is clear that the response of Atorvastatin and Ezetimibe was found to be linear between LOQ to 150% of the related substances method specification level for Atorvastatin and Ezetimibe Tablets – 10/10 mg. The correlation coefficient for Atorvastatin and Ezetimibe was found to be 0.9992 and 0.9983.

The range of the HPLC method for determining the Atorvastatin and Ezetimibe in Atorvastatin and Ezetimibe Tablets – 10/10 mg is 50% to 150% of the working strength. The proposed method was found to be linear, accurate and precise within the range of 2.6 -7.8 ppm.

8.3. ACCURACY

The accuracy of a measurement is defined as the closeness of the measured value to the true value. In a method with high accuracy, a sample (whose “true value” is known) is analyzed and the measured value is identical to the true value.

Preparation of Standard solution

Weigh accurately 52mg of Atorvastatin calcium WS and 50mg of Ezetimibe WS in 50 ml volumetric flask add 30ml of diluent sonicate to dissolve and makeup the volume with diluent. Pipette out 1ml of above solution in to 200 ml volumetric flask and make up the volume with diluent. Filter the solution through 0.45µ PVDF syringe filter. (5mcg/ml of Atorvastatin and 5mcg/ml of Ezetimibe).

Preparation of 50% Accuracy solution:

Weigh accurately 26 mg of Atorvastatin calcium, 25 mg of Ezetimibe WS and 2676 mg Placebo in 50ml volumetric flask add 30 ml of diluent sonicate to dissolve and make up the volume with diluent. Pipette out 1 ml of above solution in to 200 ml volumetric flask and make up the volume with diluent. Filter the solution through 0.45µ PVDF syringe filter.(5mcg/ml of Atorvastatin and 5mcg/ml of Ezetimibe).

Preparation of 100% Accuracy solution:

Weigh accurately 52 mg of Atorvastatin calcium, 50 mg of Ezetimibe WS and 2676 mg Placebo in 50ml volumetric flask add 30 ml of diluent sonicate to dissolve and make up the volume with diluent. Pipette out 1 ml of above solution in to 200 ml volumetric flask and make up the volume with diluent. Filter the solution through 0.45µ PVDF syringe filter. (5mcg/ml of Atorvastatin and 5mcg/ml of Ezetimibe).

Preparation of 150% Accuracy solution:

Weigh accurately 78 mg of Atorvastatin calcium, 75 mg of Ezetimibe WS and 2676 mg Placebo in 50ml volumetric flask add 30 ml of diluent sonicate to dissolve and make up the volume with diluent. Pipette out 1 ml of above solution in to 200 ml volumetric flask and make up the volume with diluent. Filter the solution through 0.45µ PVDF syringe filter.(5mcg/ml of Atorvastatin and 5mcg/ml of Ezetimibe).

Procedure

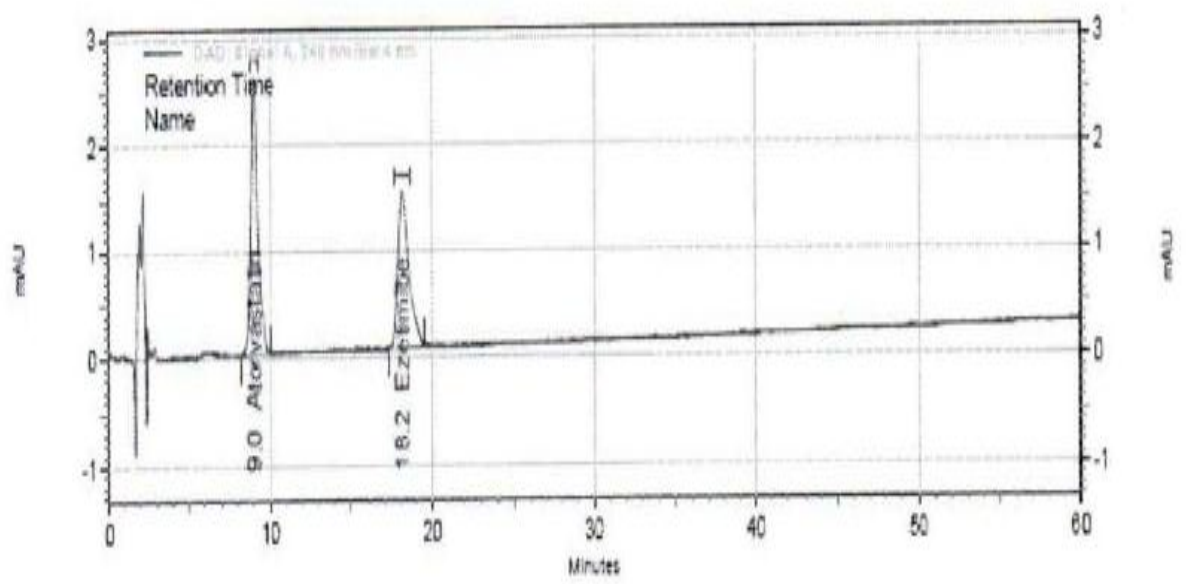
Standard solutions and recovery solutions in the range of 50%, 100% and 150% were prepared at three levels and each level was injected in duplicate and analysed as per the method. The recovery in each level has been calculated with the measured concentration against theoretical concentration. The results are summarized in the table.

Acceptance Criteria

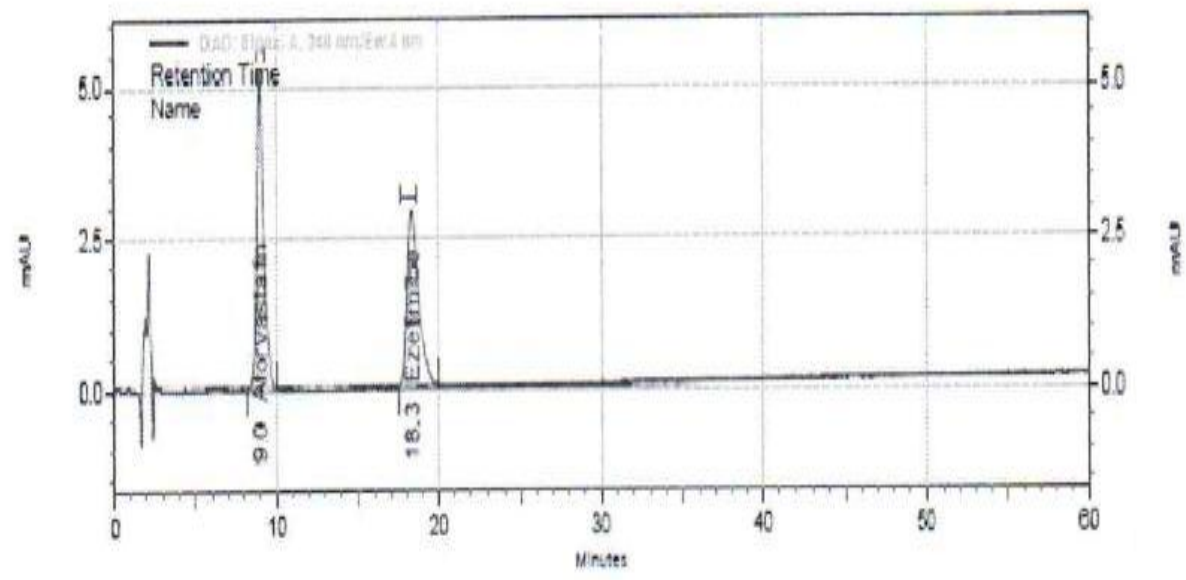
The recovery should be in the range of 98.0-102.0%

Result

Chromatogram of recovery 50%



Chromatogram of recovery 100%



Chromatogram of recovery 150%

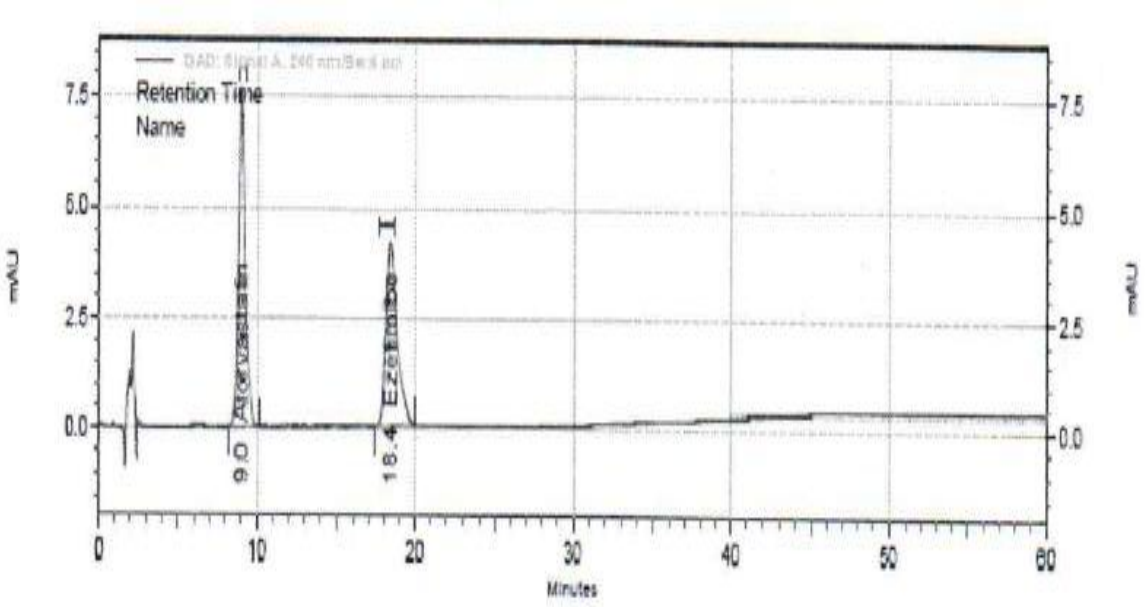


Table 13: Statistical Data of Accuracy for Atorvastatin

sample	Area	API added in (mg)	API Recovered in (mg)	% Recovery	Average	%RSD
50%	161043	2.5438	2.5278	99.4	99.9%	1.3
50%	159284	2.5254	2.5002	99.0		
50%	159281	2.5592	2.5986	101.5		
100%	328580	5.0924	5.1575	101.3	101.4%	0.3
100%	328892	5.1059	5.1624	101.1		
100%	319197	5.0991	5.1899	101.8		
150%	473331	7.6487	7.6960	100	99.7%	0.2
150%	474171	7.7423	7.7097	99.6		
150%	474563	7.7530	7.7161	99.5		
				average	**100.1%**	
				RSD%	**0.6**	

Discussion

The average percent recovery of 50%, 100%, 150% for Atorvastatin was about 99.9%, 101.4%, 99.7%. The percentage recovery values were in the range of 99.0%- 101.5% which is within the acceptance criteria.

Table 14: Statistical Data for Accuracy For Ezetimibe

sample	Area	API added in (mg)	API Recovered in (mg)	% Recovery	Average	%RSD
50%	149877	2.5050	2.5017	99.9	99.7	0.9
50%	148745	2.5150	2.4828	98.7		
50%	150852	2.5070	2.5180	100.4		
100%	302489	5.0120	5.0491	100.7	100.2	0.6
100%	302542	5.0350	5.0500	100.3		
100%	299876	5.0240	5.0050	99.6		
150%	457094	7.5580	7.6297	100.9	99.8	1.0
150%	442335	7.4350	7.3834	99.3		
150%	440642	7.4110	7.3551	99.2		
				average	**99.9**	
				RSD%	**0.2**	

Discussion

The average percent recovery of 50%, 100%, 150% for Ezetimibe was about 99.7%, 100.2%, 99.8%. The percentage recovery values were in the range of 99.2%- 100.9% which is within the acceptance criteria.

Conclusion:

The results met the acceptance criteria for all Accuracy level. The result shows that the method is Accurate for Atorvastatin and Ezetimibe Tablets – 10/10 mg.

8.4. PRECISION

The precision of an analytical procedure represents the nearness of agreement between a series of measurements got from multiple sampling of the same homogenous sample under the similar analytical conditions and it is divided into 3 categories.

• Repeatability: precision under same operating conditions, same analyst over a short period of time.

• Intermediate precision: method is tested on multiple days, instruments, analysts etc.

• Reproducibility: inter-laboratory studies.

8.4.1. System precision

Standard Solution:

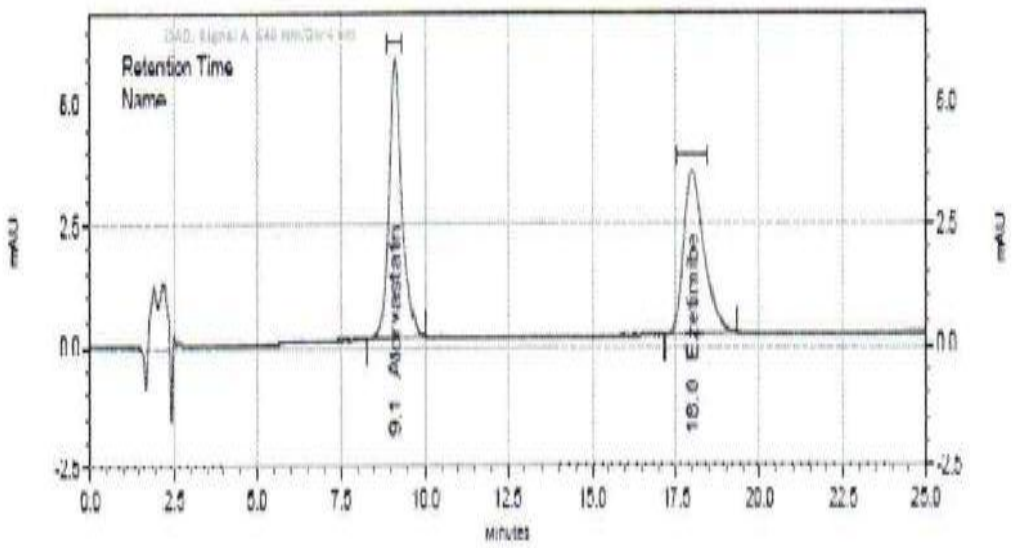
Weigh accurately 52mg of Atorvastatin calcium WS and 50mg of Ezetimibe WS in 50 ml volumetric flask add 30ml of diluent sonicate to dissolve and makeup the volume with diluent. Pipette out 1ml of above solution in to 200 ml volumetric flask and make up the volume with diluent. Filter the solution through 0.45µ PVDF syringe filter. (5mcg/ml of Atorvastatin and 5mcg/ml of Ezetimibe).

Procedure

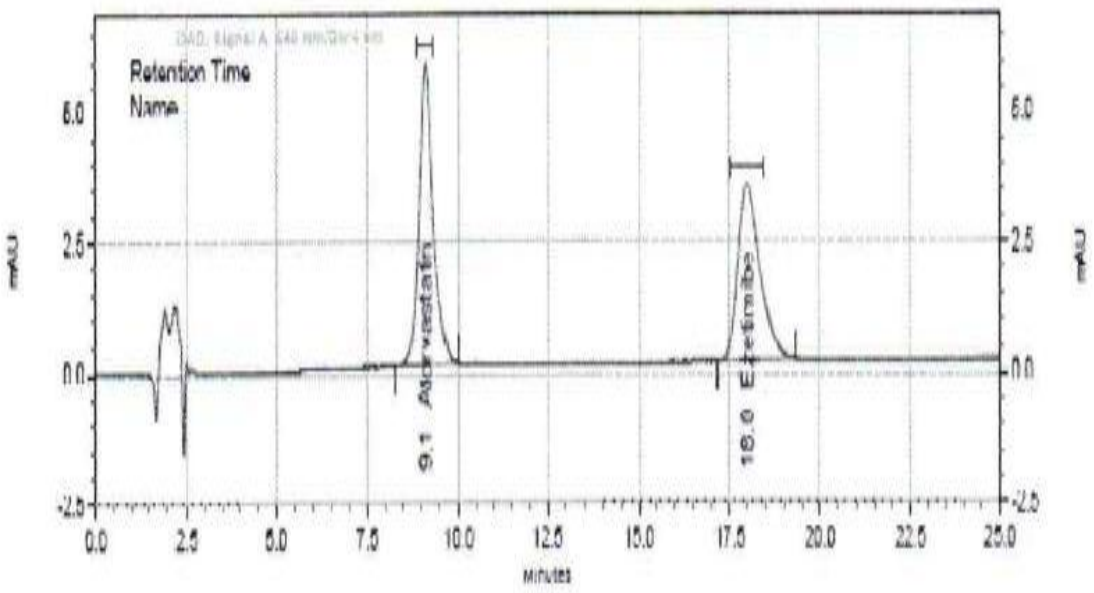
The system precision was checked by injecting six replicated injections of standard solution. The retention time and area of 6 standard determinations was measured and % RSD was calculated.

Result

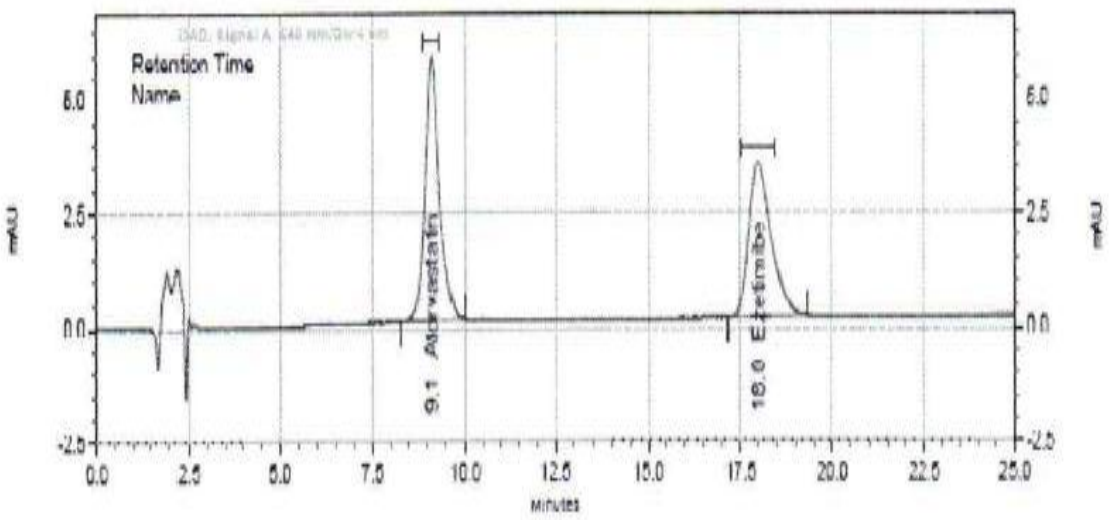
Chromatogram of system precision-1



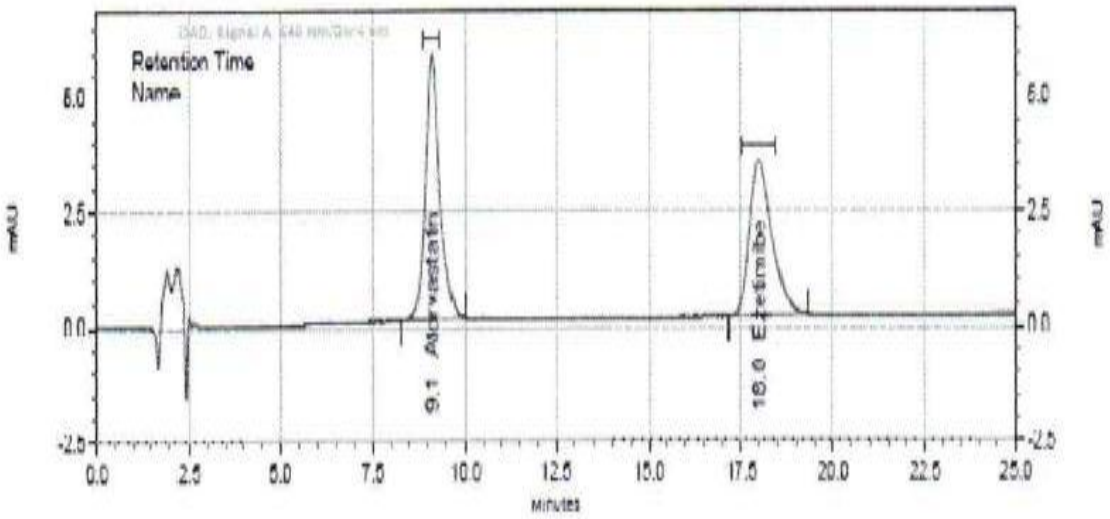
Chromatogram of system precision-2



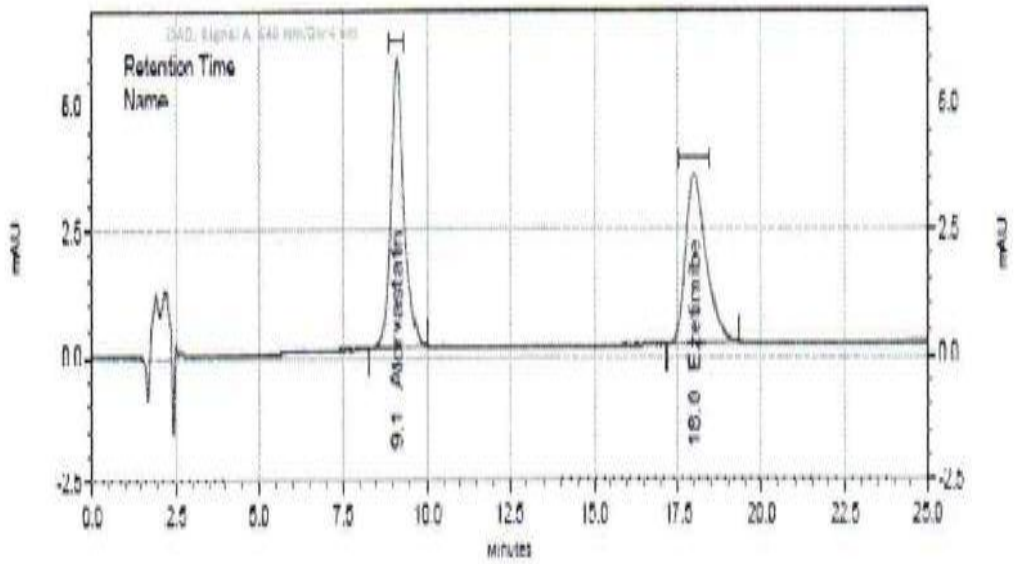
Chromatogram of system precision-3



Chromatogram of system precision-4



Chromatogram of system precision-5



Chromatogram of system precision-6

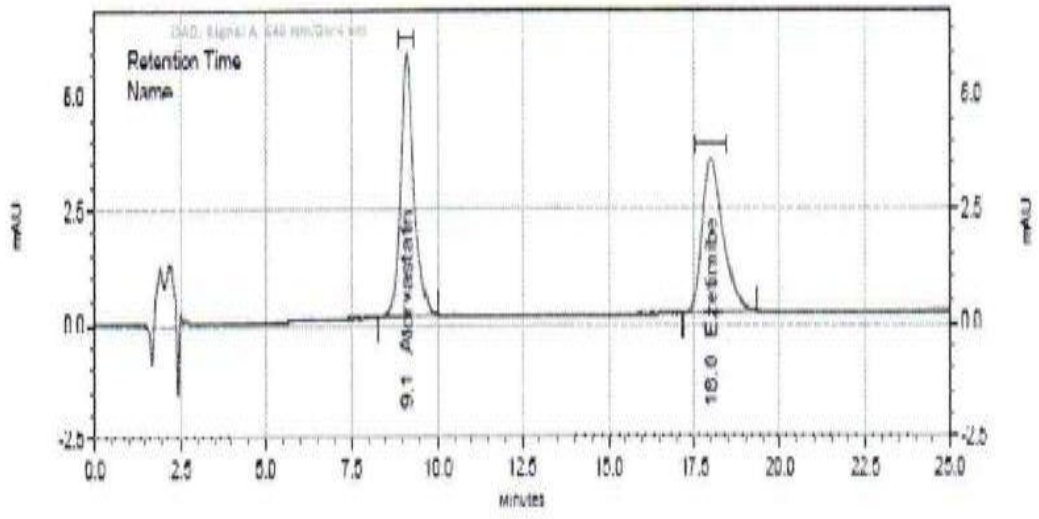


Table 15: Summary of System Precision Results

S.NO	Peak Area		Theoretical Plates		Tailing Factor	
	ATOR	EZET	ATOR	EZET	ATOR	EZET
1	321925	300834	2951	4091	1.2	1.3
2	322897	301821	2953	4085	1.2	1.2
3	321903	301799	2948	4089	1.3	1.3
4	321889	300807	2955	4092	1.1	1.4
5	322916	301817	2958	4097	1.2	1.2
6	321909	301839	2945	4087	1.1	1.3
Average	322306	301416	2953	4091	1.2	1.2
% RSD	0.17	0.18				

DISCUSSION:

The percentage relative standard deviation of peak areas of Atorvastatin and Ezetimibe was between 0.17 to 0.18%.

8.4.2. Method precision:

Standard solution:

Weigh accurately 52mg of Atorvastatin calcium WS and 50mg of Ezetimibe WS in 50 ml volumetric flask add 30ml of diluent sonicate to dissolve and makeup the volume with diluent. Pipette out 1ml of above solution in to 200 ml volumetric flask and make up the volume with diluent. Filter the solution through 0.45µ PVDF syringe filter. (5mcg/ml of Atorvastatin and 5mcg/ml of Ezetimibe).

Sample solution

Weigh and powder 20 tablets, Weigh accurately a quantity of the powder containing about 100mg equivalent of Atorvastatin in to 200 ml volumetric flask, add 80 ml of diluent and sonicate for 5 minutes, dissolve and make up the volume with diluent, and allow to centrifuge for 15 minutes at 6000RPM. Filter the solution through a 0.45µ PVDF syringe filter (500Mmcg/ml of Atorvastatin and 500mcg/ml of Ezetimibe).

Procedure:

The sample solution of Atorvastatin and Ezetimibe was prepared in six replicates as per the test method and each solution was injected six times to the LC system. The precision of the method was calculated by calculating % Impurities of each solution by Area Normalization Method. The % RSD of each impurity values were reported.

Acceptance Criteria

- The percentage of individual impurity should not be more than 1.0%
- The percentage of total impurities should not be more than 4.0%
- The % RSD of each impurities value should be less than 15.0.
- If the unknown impurities are below 0.1%, it should be NMT 15.0 and are above 0.1% it should be NMT 10.0.

Result

Chromatogram of method precision

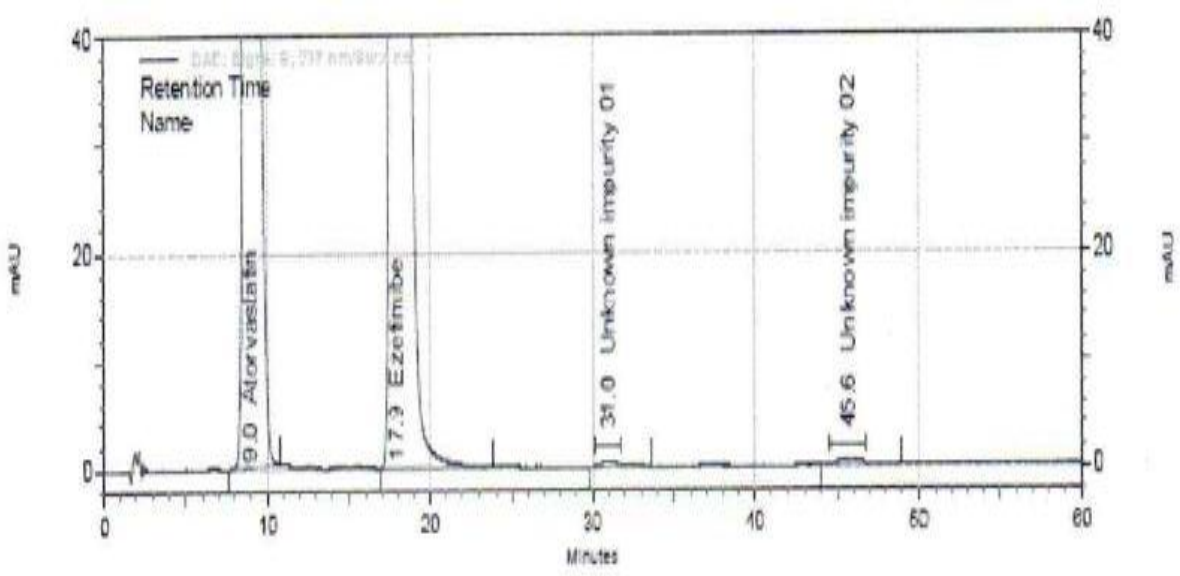


Table 16: Statistical Data for Method Precision

Sample	Assay %	Single maximum impurity 1 NMT 1%	Single maximum impurity 2 NMT 1%	Total impurity NMT 4%
1	99.87	0.27	0.17	0.44
2	99.53	0.27	0.17	0.44
3	100.25	0.28	0.17	0.45
4	100.79	0.27	0.18	0.45
5	99.94	0.27	0.18	0.45
6	100.12	0.28	0.17	0.45
AVERAGE	100.08	0.27	0.17	0.45
%RSD	0.42	1.89	2.98	1.16

DISCUSSION

- The % Individual Impurity I was in the range of 0.27% to 0.28% with RSD 1.89%
- The % Individual Impurity II was in the range of 0.17% to 0.18% with RSD 2.98%
- The % Total Impurities was in the range of 0.44% to 0.45% with RSD 1.16%

8.4.3. Ruggedness (Intermediate precision)

The ruggedness of the method was performed by analyzing the sample solution of Atorvastatin and Ezetimibe with the following varying parameters.

Preparation of Standard

Weigh accurately 52mg of Atorvastatin calcium WS and 50mg of Ezetimibe WS in 50 ml volumetric flask add 30ml of diluent sonicate to dissolve and makeup the volume with diluent. Pipette out 1ml of above solution in to 200 ml volumetric flask and make up the volume with diluent. Filter the solution through 0.45µ PVDF syringe filter. (5mcg/ml of Atorvastatin and 5mcg/ml of Ezetimibe).

Preparation of sample solution

Weigh and powder 20 tablets, Weigh accurately a quantity of the powder containing about 100mg equivalent of Atorvastatin in to 200 ml volumetric flask, add 80 ml of diluent and sonicate for 5 minutes, dissolve and make up the volume with diluent, and allow to centrifuge for 15 minutes at 6000RPM. Filter the solution through a 0.45µ PVDF syringe filter (500Mmcg/ml of Atorvastatin and 500mcg/ml of Ezetimibe).

Procedure

The sample solution was prepared by different analyst with different reagent on different day as per the test method. Each solution was injected with different instrument using different column. The Ruggedness of the method was reported by calculating % Impurities of each solution by Area Normalization Method. The overall % RSD of each impurities value in Set-I and Set-II was reported.

Acceptance criteria

- The percentage of individual impurity should not be more than 1.0%
- The percentage of total impurities should not be more than 4.0%
- The Overall % RSD should not be more than 15.0%.
- If the unknown impurities are below 0.1%, it should be NMT 15.0 and are above 0.1% it should be NMT 10.0.

Table 17: Statistical Data for Intermediate Precision

sample	% Assay		Single maximum impurity I NMT 1%		Single maximum impurity II NMT 1%		Total impurity NMT 4%	
	Set-I	Set-II	Set-I	Set-II	Set-I	Set-II	Set-I	Set-II
1	99.87	100.46	0.27	0.28	0.17	0.17	0.44	0.45
2	99.53	100.35	0.27	0.27	0.17	0.18	0.44	0.45
3	100.54	99.85	0.28	0.27	0.17	0.17	0.45	0.44
4	100.79	99.74	0.27	0.26	0.18	0.17	0.45	0.43
5	99.94	100.98	0.28	0.27	0.18	0.16	0.45	0.43
6	100.12	100.82	0.27	0.27	0.17	0.17	0.45	0.44
AVERAGE	100.13	100.37	0.27	0.27	0.17	0.17	0.45	0.44
%RSD	0.46	0.50	1.89	2.77	2.98	3.72	1.16	2.03
Overall average	100.25		0.27		0.17		0.45	
Overall % RSD	0.48		2.33		3.35		1.60	

RESULT AND DISCUSSION

The above result indicates that the test method is rugged for instrument to instrument, column to column, reagent to reagent, analyst to analyst and day to day variation.

- The % Individual Impurity I was in the range of 0.26% to 0.28% with overall RSD 2.33%.
- The % Individual Impurity II was in the range of 0.16% to 0.18% with overall RSD 3.35%.
- The % Total Impurities was in the range of 0.43% to 0.45% with overall RSD 1.60%

8.5. Robustness

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

Procedure

The robustness of the method was determined by analyzing the standard solution six times by means of varying HPLC conditions as determined below:

Parameter for robustness

Condition	Actual	Low	High
Flow rate	1.5ml/min	1.3ml/min	1.7ml/min
Column temperature	25℃	23℃	27℃
Wavelength	240nm	238nm	242nm

Standard solution

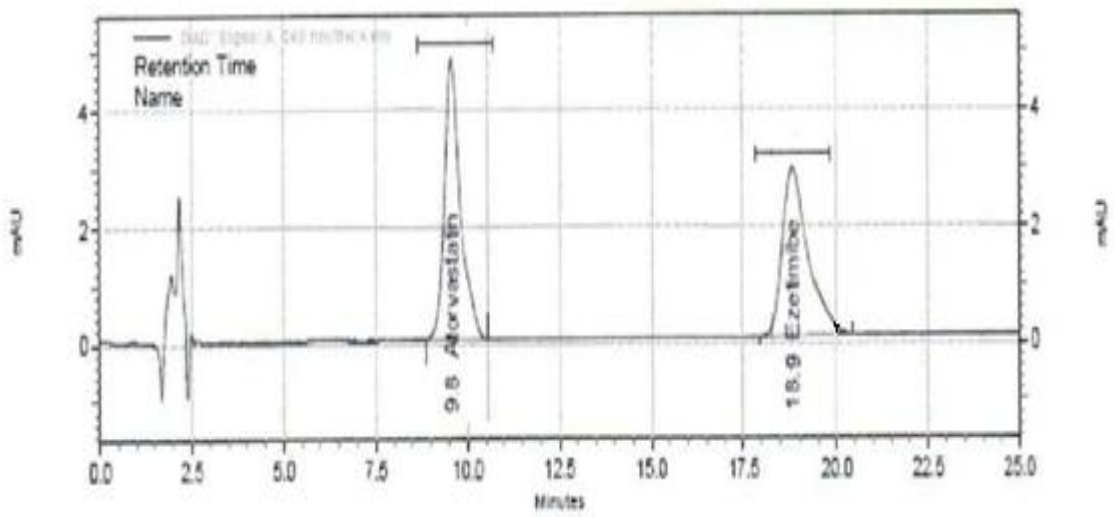
Weigh accurately 52mg of Atorvastatin calcium WS and 50mg of Ezetimibe WS in 50 ml volumetric flask add 30ml of diluent sonicate to dissolve and makeup the volume with diluent. Pipette out 1ml of above solution in to 200 ml volumetric flask and make up the volume with diluent. Filter the solution through 0.45µ PVDF syringe filter. (5mcg/ml of Atorvastatin and 5mcg/ml of Ezetimibe).

Test solution

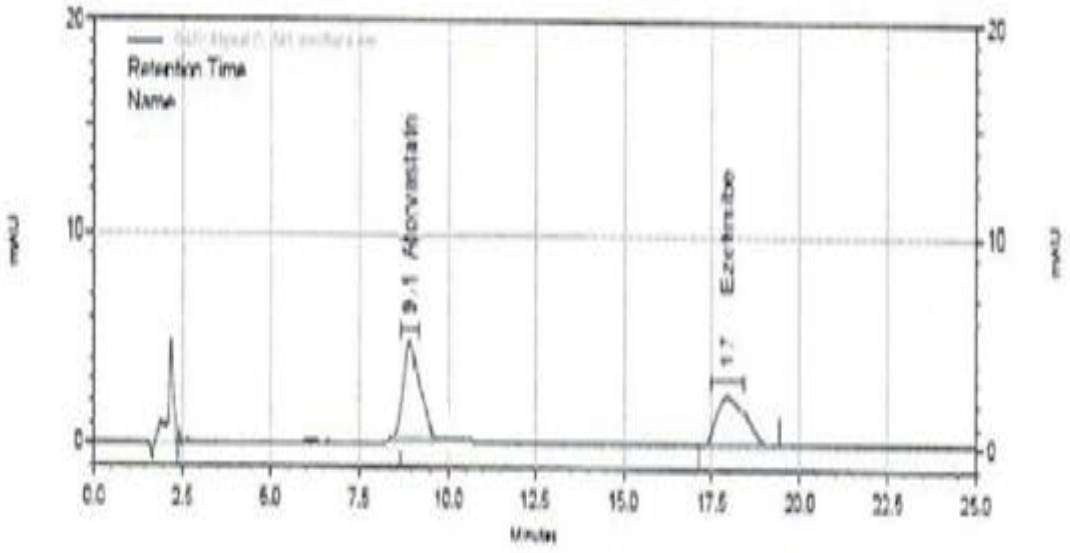
Weigh and powder 20 tablets, Weigh accurately a quantity of the powder containing about 100mg equivalent of Atorvastatin in to 200 ml volumetric flask, add 80 ml of diluent and sonicate for 5 minutes, dissolve and make up the volume with diluent, and allow to centrifuge for 15 minutes at 6000RPM. Filter the solution through a 0.45µ PVDF syringe filter (500Mmcg/ml of Atorvastatin and 500mcg/ml of Ezetimibe).

Result

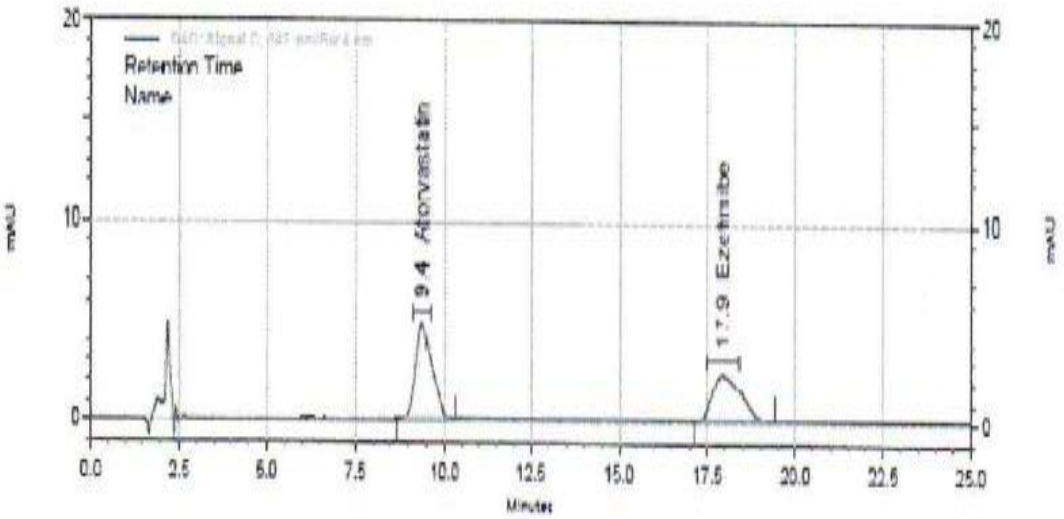
Chromatogram of flow rate low (1.3 ml)



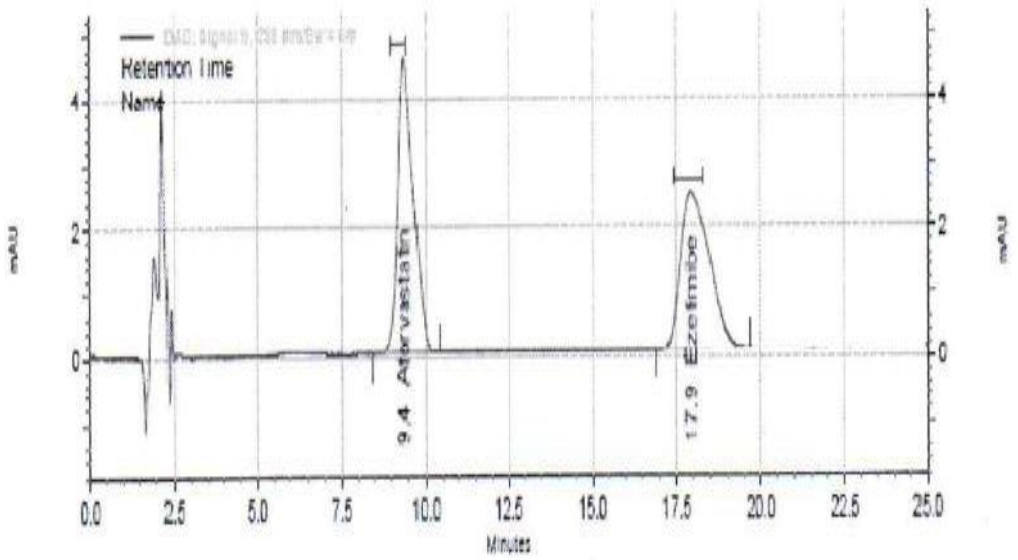
Chromatogram of flow rate high (1.7 ml)



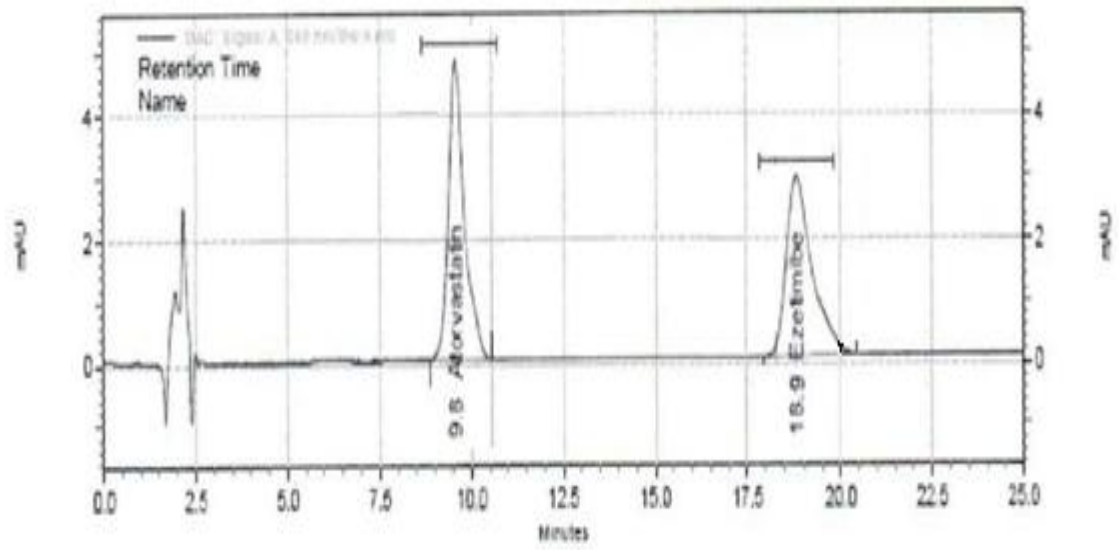
Chromatogram of wavelength low (238 nm)



Chromatogram of wavelength high (242 nm)



Chromatogram of column temperature Low (23 °C)



Chromatogram of column temperature high (27 °C)

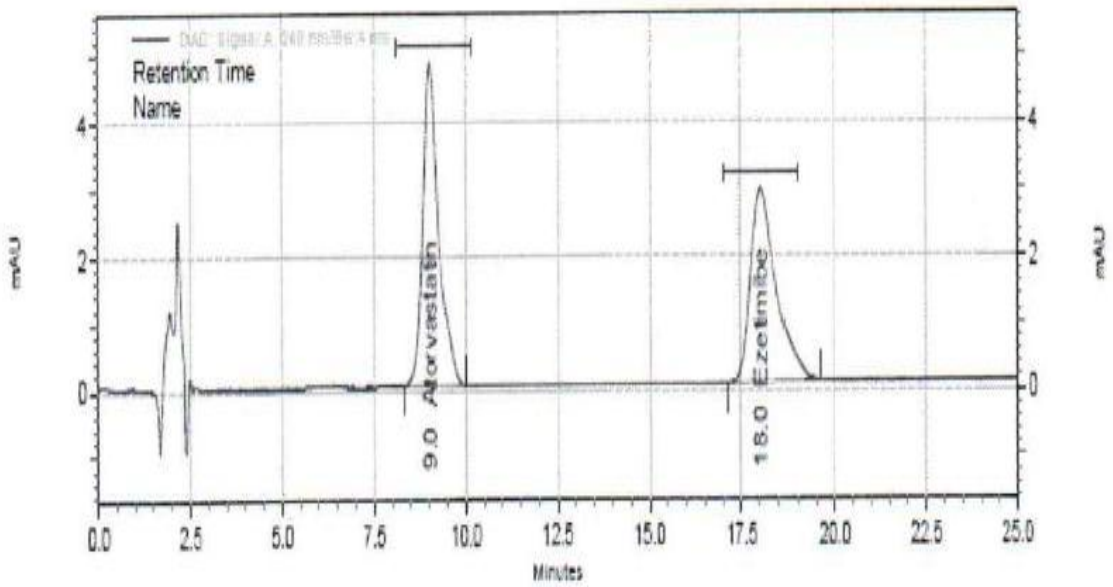


Table 18: Statistical Data for Flow Rate Change

S.NO	Area				Retention time (min)			
	ATORVASTATIN		EZETIMIBE		ATORVASTATIN		EZETIMIBE	
	1.3ml	1.7ml	1.3ml	1.7ml	1.3ml	1.7ml	1.3ml	1.7ml
1	344674	264267	318218	275136	9.8	9.1	18.9	17.0
2	345245	263324	318765	275876	9.9	9.1	18.9	17.1
3	344481	263476	319986	276989	9.8	9.2	18.9	17.0
4	344943	264854	318917	275816	9.8	9.2	18.9	17.1
5	344809	264120	318728	275691	9.8	9.1	19.0	17.1
6	345811	264303	319037	275150	9.8	9.1	18.9	17.0
Average	**344944**	**264057**	**318942**	**275776**	**9.8**	**9.1**	**19.0**	**17.1**
SD	**476**	**569**	**583**	**677**	**0.04**	**0.05**	**0.04**	**0.05**
%RSD	**0.14**	**0.22**	**0.18**	**0.25**	**0.42**	**0.57**	**0.22**	**0.32**

Table 19: Statistical Data for Wavelength Change

S.NO	Area				Retention time (min)			
	ATORVASTATIN		EZETIMIBE		ATORVASTATIN		EZETIMIBE	
	238nm	242nm	238nm	242nm	238nm	242nm	238nm	242nm
1	304674	314481	302218	288975	9.4	9.4	17.9	17.9
2	304637	315335	302455	289887	9.3	9.4	17.9	18.0
3	305376	314287	302476	288663	9.3	9.4	18.0	17.9
4	305872	314991	302654	288859	9.4	9.3	17.9	18.0
5	304273	315226	303228	289468	9.3	9.3	17.9	17.9
6	304374	314133	303387	288556	9.4	9.4	18.0	18.0
Average	304868	314742	302736	289068	9.4	9.4	18	18
SD	625.32	508.71	466.36	511.71	0.05	0.05	0.05	0.05
%RSD	0.21	0.16	0.15	0.18	0.59	0.55	0.29	0.31

Table 20: Statistical Data for Column Oven Temperature Change

S.NO	Area				Retention time (min)			
	ATORVASTATIN		EZETIMIBE		ATORVASTATIN		EZETIMIBE	
	23°C	27°C	23°C	27°C	23°C	27°C	23°C	27°C
1	322975	323481	300818	301975	9.1	9.2	17.9	17.9
2	323871	324335	301455	301887	9.1	9.1	17.9	17.9
3	322747	323287	301976	301663	9.1	9.2	17.9	17.9
4	322729	322991	301654	300859	9.1	9.1	17.9	17.9
5	323625	323622	300928	301468	9.1	9.1	17.9	17.9
6	322537	322153	301387	300556	9.1	9.1	17.9	17.9
Average	323081	323312	301370	301401	9.1	9.1	17.9	17.9
SD	540	723	437	573	0.02	0.02	0.02	0.02
%RSD	0.17	0.22	0.15	0.19	0.17	0.20	0.11	0.10

Table 21: Summary for Robustness results

Parameter	Variation	RSD for Atorvastatin	RSD for Ezetimibe
Change in Flow rate	**1.3mL/min**	**0.14**	**0.18**
	1.7mL/min	**0.22**	**0.25**
Change in Wavelength	**238nm**	**0.21**	**0.15**
	242nm	**0.16**	**0.18**
Change in Column temperature	**23˚C**	**0.17**	**0.15**
	27˚C	**0.22**	**0.19**

Discussion

The results are reported in Table 18-20 where the average % RSD of area for intentional changes in flow rate, wavelength and Temperature was between 0.14 % & 0.22 % for Atorvastatin and 0.15 % & 0.25 % for Ezetimibe. Which is well within the acceptance criteria. The results show that the intentional changes do not affect the method.

8.6. LIMIT OF DETECTION:

LOD is determined by injecting solution of known concentration of the analyte and establishing the minimum level at which the analyte can be detected with the signal to noise ratio should be at least 3.0

ESTABLISHMENT OF LOD:

Determination of LOD involves signal-to-noise ratio approach. The Atorvastatin & Ezetimibe Standard is prepared in different concentrations. The LOD is then determined from the signal-to-noise ratio, should be at least 3.0

Preparation of LOD establishment stock solution:

Weigh accurately 52 mg of Atorvastatin calcium WS and 50mg of Ezetimibe WS in 50 ml of volumetric flask add 30ml of diluent sonicate to dissolve and make up the volume with diluent. Pipette out 10 ml of above solution in to 200 ml volumetric flask and make up the volume with diluent. Filter the solution through 0.45µ PVDF syringe filter.

Preparation of LOD sample:

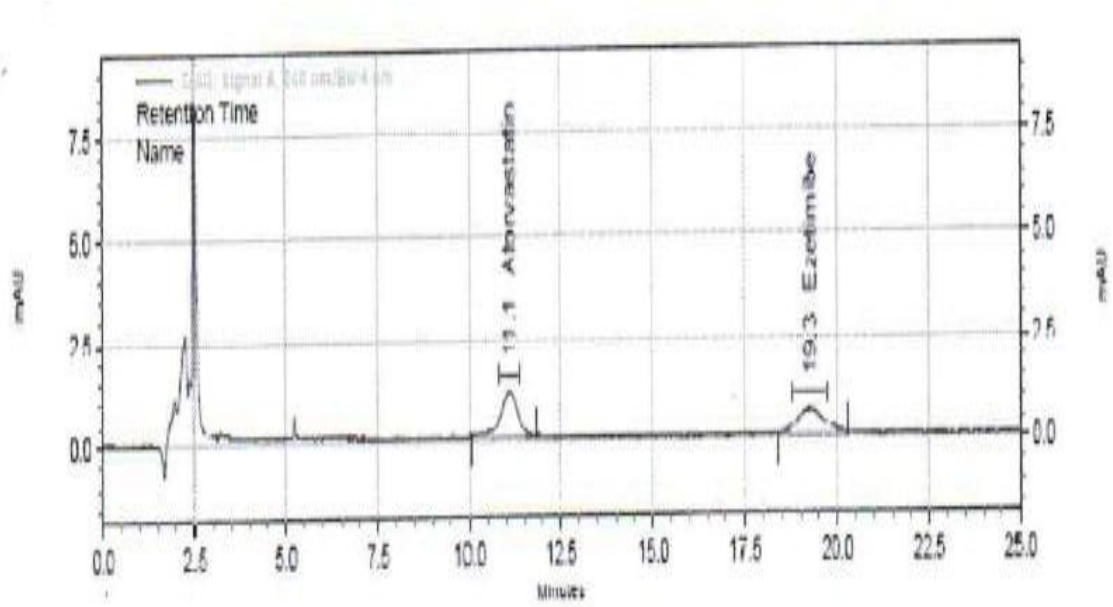
From the stock solution pipetted out 2 ml to 100 ml Volumetric flask and made up the volume with diluent

Acceptance criteria:

The Signal to noise ratio for LOD should be at least 3.0 at lowest concentration.

Result

Chromatogram of Limit of Detection (LOD)



Peak	Conc.(mg/ml)	S/N Ratio
Atorvastatin	0.00104	3.9

INFERENCE

The limit of Detection for Atorvastatin and Ezetimibe is 0.00104 mg/ml of concentration and the signal to noise ratio was found to be 3.9.

8.7. LIMIT OF QUANTIFICATION (LOQ)

The limit of quantitation is defined as the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy. The limit of quantification is determined by calculating the signal to noise ratio and by comparing test results from samples with known concentrations of analyte with those blank samples and establishing the minimum level at which the analyte can be reliably detected. LOQ is determined by injecting solution of known concentration of the analyte and establishing the minimum level at which the analyte can be quantified with a signal to noise ratio should be at least 10.0

ESTABLISHMENT OF LOQ:

Determination of LOQ involves signal-to-noise ratio approach. The Atorvastatin & Ezetimibe Standard is prepared in different concentrations. The LOQ is then determined from the signal-to-noise ratio, should be at least 10.0

Preparation of LOD sample:

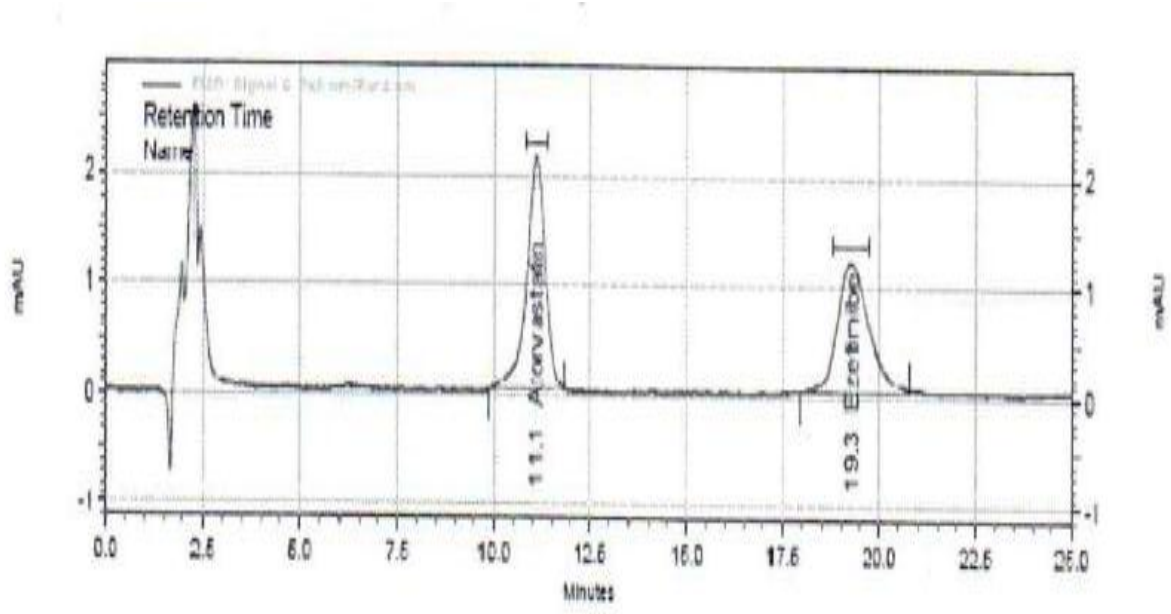
From the stock solution pipetted out 5 ml to 100 ml Volumetric flask and made up the volume with diluent

Acceptance criteria:

The Signal to noise ratio for LOQ should be at least 10.0 at lowest concentration.

Result

Chromatogram of Limit of Quantitation (LOQ)



Peak	Conc.(mg/ml)	S/N Ratio
Atorvastatin	0.0026	10.6

Inference

The limit of Quantitation for Atorvastatin and Ezetimibe is 0.0026 mg/ml of concentration and the signal to noise ratio was found to be 10.6.

Acceptance criteria:

Table: 22

System suitability parameters	Signal to noise ratio	Acceptance criteria
Limit of Detection	3.9	**Should not be less than 3.0**
Limit of Quantification	10.6	**Should not be less than 10.0**

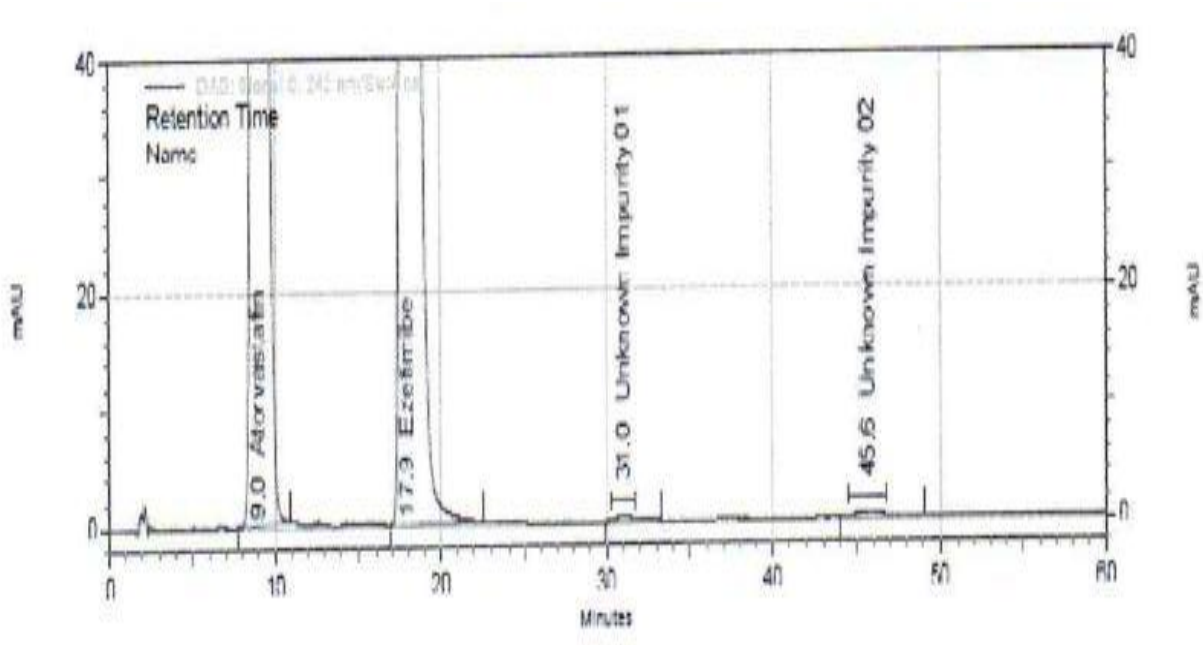
8.8. SOLUTION STABILITY:

To establish the Solution Stability of Atorvastatin and Ezetimibe was measured against 100% of the standard concentration by keeping the solution up to 48 hours at 25°C. The sample was injected at different time intervals (e.g. Initial, 12 hours, 24 hours, and 48 hours). The percentage relative standard deviation of each impurities in Atorvastatin and Ezetimibe at different interval of time was calculated and reported.

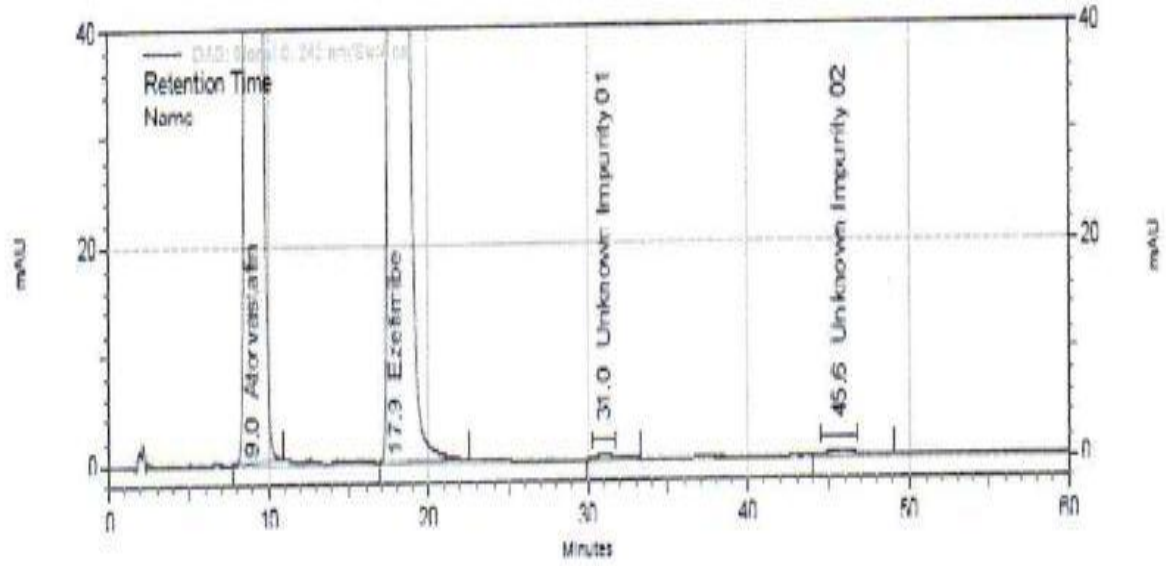
Acceptance Criteria

- The Overall % RSD should not be more than 5.0%
- The Overall % RSD of individual impurity should not be more than 15.0%
- The Overall % RSD of total impurity should not be more than 15.0%

Chromatogram of sample solution – initial



Chromatogram of sample solution-24th hour



Chromatogram of sample solution-48th hour

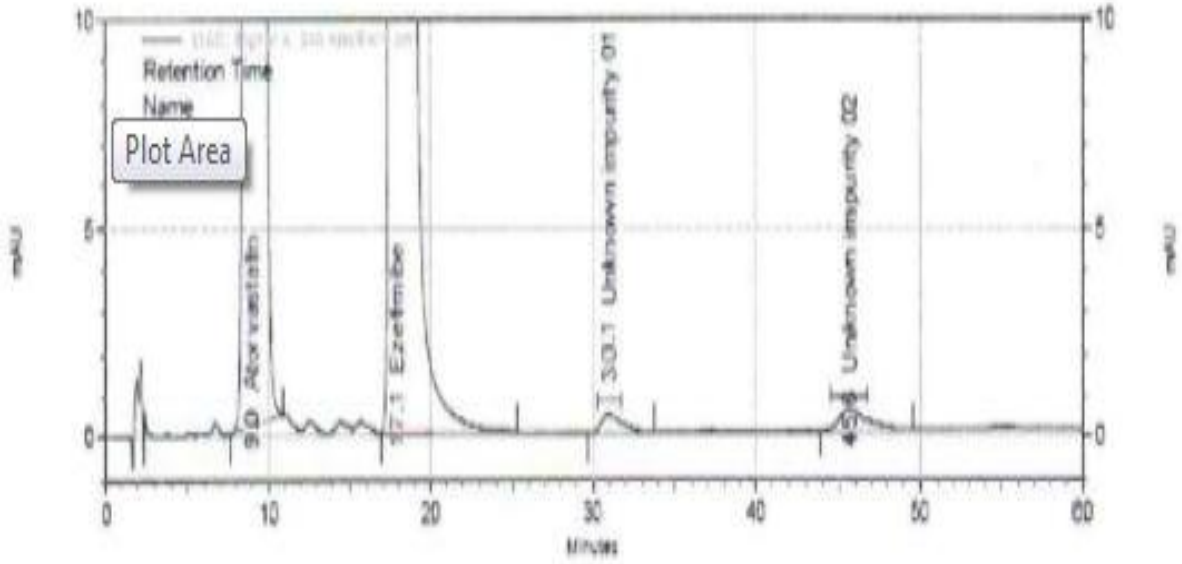


Table: 23 Statistical Data of Solution Stability

S.NO	**Duration**	Single maximum impurity I NMT 1%	Single maximum impurity II NMT 1%	**% Total impurities NMT 4 %**
1	Initial	0.22	0.14	0.36
2	6 Hours	0.22	0.14	0.36
3	12 Hours	0.22	0.14	0.36
4	24 Hours	0.21	0.13	0.34
5	48 Hours	0.21	0.13	0.34
%RSD		2.54	4.03	3.11

Discussion:

The % Individual Impurity I of Atorvastatin and Ezetimibe Tablets 10/10 mg were in the range of 0.21% to 0.22%. Individual Impurity II were in the range of 0.13% to 0.14%. The % RSD between the Total impurities results from initial to 48 hrs in Atorvastatin and Ezetimibe Tablets 10/10 mg were 3.11%, which is within the acceptance limit of 15.0%. Therefore, Atorvastatin and Ezetimibe Tablets 10/10 mg sample solutions are stable up to 48 hours at 25°C.

SUMMARY

AND

CONCLUSION

9. SUMMARY AND CONCLUSION

Table 24: Validation results of optimized method

S.NO	Parameter	RESULTS OBTAINED		Acceptance limit
		Atorvastatin calcium	Ezetimibe	
1	System suitability	%RSD		%RSD NMT 5.0%
		0.17	0.18	
2	Linearity & Range	Correlation coefficient (r2)		Correlation Co-efficient: NLT 0.995
		0.9986	0.9968	
3	Accuracy	% RECOVERY		98%-102%
		100.1%	99.9%	
4	Precision	%RSD		% RSD : NMT 5.0
	System precision	0.17%	0.18%	
		Tailing Factor		Tailing Factor: NMT 2.0
		1.2	1.2	
		Theoretical plates		Theoretical plates : NLT 2000
		2953	4091	
	Method precision			
	Individual Impurity I	0.27% to 0.28%		NMT 1.0 %
		1.89%		%RSD: NMT 10.0
	Individual Impurity II	0.17% to 0.18%		NMT 1.0 %
		2.98%		%RSD: NMT 10.0
	Total impurity	0.44% to 0.45%		NMT 4.0%
		1.16%		%RSD: NMT 10.0
	Intermediate precision (Ruggedness)			
	Individual Impurity I	0.26% to 0.28%		NMT 1.0 %
		2.33%		%RSD: NMT 10.0
	Individual Impurity II	0.16% to 0.18%		NMT 1.0 %
		3.35%		%RSD: NMT 10.

5	**Total Impurity**	**0.43% to 0.45%**	NMT 4.0%
		1.60%	%RSD: NMT 10.0
6	**Limit of Detection**	**3.9 at (0.00104 mg/ml)**	Signal to Noise Ratio should be about 3:1
7	**Limit of Quantitation**	**10.6 at (0.0026mg/ml)**	Signal to Noise Ratio should be about 10:1
8	**Specificity**	The study validates the method is specific	The placebo chromatogram should not show any peak at the retention time of Atorvastatin and Ezetimibe.
9	**Solution stability**	The sample solution was found to be stable Up to 48 hours	
	Individual Impurity I	**0.21% to 0.22%**	NMT 1.0%
		2.54%	%RSD: NMT 10.0
	Individual Impurity II	**0.13%to 0.14%**	NMT 1.0%
		4.03%	%RSD: NMT 10.0
	Total impurity	**0.34% to 0.36%**	NMT 4.0%
		3.11%	%RSD: NMT 10.0

SUMMARY

In this study, RP-HPLC based Related substances method was developed for the simultaneous estimation of Atorvastatin calcium and Ezetimibe in tablet dosage form and validated according to ICH guidelines.

There was no compendial methods available for the determination of Atorvastatin and Ezetimibe in pharmaceutical dosage form. From the literature review, it was evident that very few simultaneous estimation of Related substances methods were reported in articles for determining Atorvastatin and Ezetimibe. The reported simultaneous estimation of Related substances methods make use of C18 and C8 column of different dimension for the separation of analyte and the impurity. Most reversed phase separation are carried out using a polar solvents such as methanol or acetonitrile as mobile phase with peak shape modifiers. Comparatively in our study C18 column with Buffer and organic modifier Acetonitrile alone are utilised for the efficient separation of analyte and the impurity in a single run. In order to obtain the complete separation of Impurity peaks from the significant main peaks the retention time values of both the analytes should be well separated so that there may not be overlapping of peaks. The developed method is superior over other reported methods in terms of resolution between analyte and impurity peak, retention time and sensitivity.

The related substances estimation of this In-house formulation was performed and the percentage purity of Atorvastatin and Ezetimibe was found to be 100.09%. Hence the developed method was reported and was validated as per ICH guidelines.

Conclusion:

A simple, rapid, novel and precise method using RP-HPLC technique was developed for the determination and simultaneous estimation of Related Substances in Atorvastatin calcium and Ezetimibe in tablet dosage form. The proposed method was validated according to ICH guidelines with the validation parameters such as accuracy, precision, robustness, precision, specificity. The percentage Impurities was within the limit for commercial tablets and the Impurity peaks do not interfere with significant main peaks. The proposed method was used for routine analysis and quality control analysis of pharmaceutical preparations. The identification of unknown impurity peaks may be taken up as further research in this study.

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