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STUDIES ON HPLC PROFILE OF SOME PHARMACEUTICAL COMPOUNDS

A THESIS
SUBMITTED TO THE
SAURASHTRA UNIVERSITY
FOR THE DEGREE OF

Doctor of Philosophy

IN

THE FACULTY OF SCIENCE (CHEMISTRY)

BY

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UNDER THE GUIDANCE OF

Dr. Hitendra S. Joshi

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The work included in the thesis is my own work under the supervision of ***Dr. H. S. Joshi*** and leads to some contribution in chemistry subsidized by a number of references.

Date: -09-2008
Place: Rajkot

(*Ashish S. Doshi*)

This is to certify that the present work submitted for the Ph.D. Degree of Saurashtra University by ***Ashish S. Doshi*** his own work and leads to advancement in the knowledge of chemistry. The thesis has been prepared under my supervision.

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DEDICATED TO

MY FATHER

MR. SUMANTBHAI G. DOSHI

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Ashish S. Doshi

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PREFACE

The present thesis work is undertaken to develop reliable, precise and reproducible analytical methods for quantification of certain drugs from its single dosage form as well as combination dosage forms. The data is carefully analyzed and expressed in assorted sections.

The first section explains the importance of drug analysis for health care industry while the second section explains about used separation technique and process of analytical method validation for the same. Third section outlines the name of drugs which are undertaken in the present work.

The fourth section includes the work done on analytical method development, analytical method determination and analytical method validation for an analytical method which is develop for quantification purpose of Ezetimibe through assay and content uniformity determination.

The fifth section considers analytical method development and analytical method determination of the quantification methods of combination dosage forms of Ezetimibe with Statins (Rosuvastatin, Atorvastatin and Simvastatin) & Fibrate (Fenofibrate). The evaluation of the preciseness of these quantification methods is also included in this section.

The sixth section provides conclusion on the performed work. The seventh section represents the references of the current work and the eighth section provides publication details of the performed work.

Hence the work, in a nutshell, is an attempt to evolve analytical methods that are acceptable to quantify the drugs from the pharmaceutical formulations of the recent global market.

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AIM OF WORK:

The aim of the work of the thesis entitled "Studies on HPLC profile of some pharmaceutical compounds" is to develop analytical methods for drug quantification. It has been distinguish in three divisions as under:

1. Selection of analytical technique for drug quantification
2. HPLC method development, method determination and method validation of dosage form of Ezetimibe
3. HPLC method development & method determination of several combination dosage forms of Ezetimibe with Statins and Fibrate and precision evaluation for the same

The research work preformed in above divisions mainly addresses development of stability indicating HPLC methods for drug quantification followed by validating method. Validation activity is carried out according to ICH guidelines with pre-defined acceptance criteria. The strategy for drug quantification is applicable to dosage form and combination dosage forms of drugs.

EXPERIMENTAL WORK:

In the present thesis, HPLC technique is used to develop analytical methods for drug quantification. The experimental work is performed according to following approach:

(A) Analytical Method Development
(B) Analytical Method Determination
(C) Analytical Method Validation

The completion of above approach is described as under:

(A) Analytical Method Development

The development process of an analytical method is followed systemically. It involves selection of various chromatographic parameters which are as under:

- Selection of mobile phase
- Selection of stationary phase
- Selection of flow-mode
- Optimization of flow rate
- Choice of detection method
- Optimization of Injection volume
- Selection of diluent
- Extraction process

(B) Analytical Method Determination

Analytical method determination is harmonized through documentation of Standard Testing Procedure. The parameters which are included in Standard Testing Procedure are as under:

- Aim
- Instrument
- Reagents
- Chromatographic parameters
- Blank preparation
- Standard preparation
- Test preparation
- System suitability
- Procedure
- Calculation

(C) Analytical Method Validation

Analytical method validation is performed according to ICH guidelines and involves following parameters:

- Specificity study
- Limit of detection and Limit of quantification study
- Linearity and range study
- Precision study
- Accuracy study
- Robustness study
- Solution stability study
- System suitability

SELECTION OF DOSAGE FORMS AND DETERMINATION METHOD:

The pharmaceutical formulations undertaken for the present work are dosage form and combination dosage forms which are as under:

- Ezetimibe
- Ezetimibe and Rosuvastatin
- Ezetimibe and Atorvastatin
- Ezetimibe and Simvastatin
- Ezetimibe, Atorvastatin and Fenofibrate

The quantification of Ezetimibe is developed by means of assay and content uniformity determination for single dosage form (tablet). The developed procedures has been evaluated over the specificity, limit of detection & limit of quantification, linearity, precision, accuracy, robustness, solution stability and system suitability in order to ascertain the validity of the analytical method. It has been proved that the developed procedures for assay & content uniformity quantification of Ezetimibe from its tablet

dosage form is specific, linear, precise, accurate, robust and hence stability indicating. The developed method is recommended for quality control analysis to pharmaceutical industry.

The quantification of Ezetimibe with Statins and Fibrates is also developed by means of assay determination of the drugs from combination dosage forms (tablet) for the same. In order to ascertain the preciseness of the developed methods, the same are evaluated for the method precision and system suitability. The developed method is found accurate for the quantification of Ezetimibe with Statins and Fibrates from their pharmaceutical formulations. The developed method is recommended for quality control analysis of Ezetimibe with Statins (Rosuvastatin, Atorvastatin, Simvastatin and Fenofibrate) to pharmaceutical industry.

1.0 Importance of drug analysis

'Health is wealth'. It is vital fact that a healthy body is desire of every human being. Good health is first condition to enjoy the life and all other things which mankind is having. Nowadays peoples are more concentrating towards health. Even governmental bodies of different countries and World health organization (WHO) are also focusing for health of human being. Health care is prevention, treatment and management of illness and preservation of mental and physical well being. Health care embraces all the goods and services designed to promote health including preventive, curative and palliative in interventions. The Health care industry is considered an industry or profession which includes people's exercise of skill or judgment or providing of a service related to the prevention or improvement of the health of the individuals or the treatment or care of individuals who are injured, sick, disabled or infirm. The delivery of modern health care depends on an Interdisciplinary Team.

The medical model of health focuses on the eradication of illness through diagnosis and effective treatment. A traditional view is that improvement in health results from advancements in medical science. Advancements in medical science bring varieties of medicines. Medicines are key part of the health care system. The numerous medicines are introducing into the world-market and also, that is increasing every year. These medicines are being either new entities or partial structural modification of the existing one. So, to evaluate quality and efficacy of these medicines is also important factor. Right from the beginning of discovery of any medicine quality and efficacy of the same are checked by quantification means. Quality and efficacy are checked by either observing effect of drug on various animal models or analytical means. The option of animal models is not practically suitable for every batch of medicine as it's require long time, high cost and more man-power. Later option of analytical way is more suitable, highly precise, safe and selective.

The analytical way deals with quality standards which are assigned for products to have desirable efficacy of the medicines. Sample representing any batch are analyzed for these standards and it is assumed that drug/medicine which is having such standards are having desire effect on use. Quality control is a concept, which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different stage of production. The decision to release or reject a product is based on one or more type of control action.

Due to rapid growth of pharmaceutical industry during last several years, number of pharmaceutical formulations are enter as a part of health care system and thus, there has been rapid progress in the field of pharmaceutical analysis. Developing analytical method for newly introduced pharmaceutical formulation is a matter of most importance because drug or drug combination may not be official in any pharmacopoeias and thus, no analytical method for quantification is available. To check the quality standards of the medicine various analytical methods are used. Modern analytical techniques are playing key role in assessing chemical quality standards of medicine. Thus analytical techniques are required for fixing standards of medicines and its regular checking. Out of all analytical techniques, the technique which is widely used to check the quality of drug is known as 'CHROMATOGRAPHY'.

2.0 Chromatography

In 1906, the Russian botanist M.S. Tswett reported separation of different colored constituents of extract of green leaves into a series of colored bands by allowing a solvent to percolate through column bed of powdered calcium carbonate[1]. He termed this technique as 'chromatography' from the Greek words meaning 'color (chroma)' and 'writing (graphy)'. As a matter of above fact, chromatography owes its origin to the efforts of him. Tswett's this technique was virtually unnoticed in the literature until the early 1940s, when the well-known paper of Martin and Synge was published[2]. They reported the discovery of liquid-liquid partition chromatography, both on columns and on paper. They also provided a theoretical frame work for the basic chromatographic process, and they received the Nobel Prize in chemistry in 1952 for their work. The next major step that led to progress in this field was the development of gas-liquid chromatography by James and Martin[3]. The success of modern chromatography is greatly due to the excellent extensive treatment of chromatographic theory by Giddings in 1965 through his book entitled Dynamics of Chromatography[4]. Afterwards, a number of well-known scientists whose contributions are too numerous to be recounted here, and their work has led to the development of modern liquid chromatography, which is often called high-pressure or high-performance liquid chromatography. This technique is also called as HPLC, or simply LC.

General classification of chromatographic methods is as below[5]:

1) Column chromatography
2) Paper chromatography
3) Thin-layer chromatography
4) Gas chromatography
5) High pressure liquid chromatography
6) Ion-exchange chromatography
7) Gel filtration chromatography
8) Supercritical fluid chromatography

2.1 High-Performance Liquid Chromatography[6,7]

Based on the preceding discussion, chromatography can be simply defined as: 'Chromatography is the technique in which the components of a mixture are separated based upon the rates at which they are distributed through two phases, one of which does not move (stationary phase) and the other that moves (mobile phase)'. When mobile phase is liquid, this technique is known as 'liquid chromatography'. Liquid chromatography (LC) is a method of chromatographic separation based on the difference in the distribution of species between two non-miscible phases, in which the mobile phase is a liquid which percolates through a stationary phase contained in a column. It is mainly based on mechanisms of adsorption, mass distribution, ion exchange, size exclusion or stereochemical interaction. Early liquid chromatography is carried out in long glass columns with wide diameter. Now days with the help of advent of latest technology, the particle diameters were reduced as small as to below 10 μm with replacement of glass columns to steel ones. The flow rate of the mobile phase was improved by applying high pressure to the column using pumps and hence the performance was improved. This development led to be mostly called as 'high-performance liquid chromatography' or 'high-pressure liquid chromatography' (HPLC).

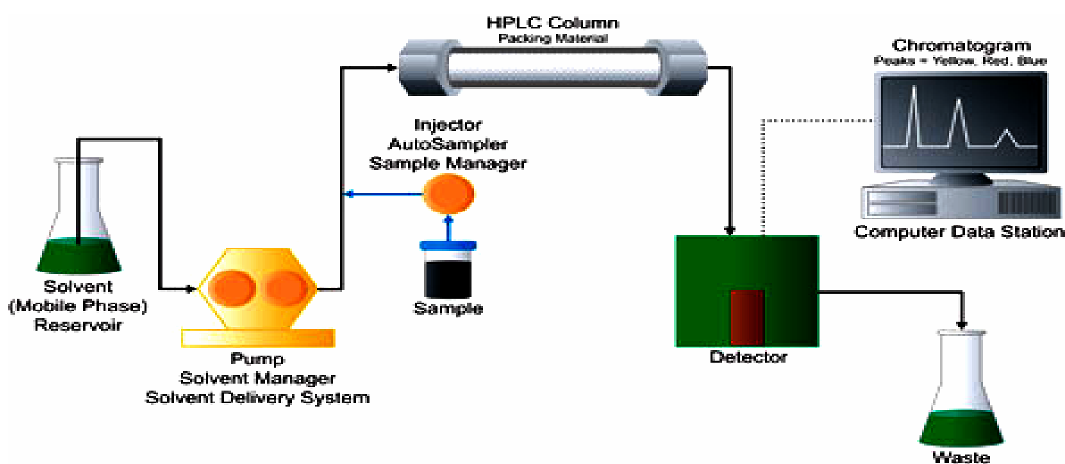
HPLC is most widely used analytical separation technique that offers major improvements over the old chromatography. The technique is more popular because it is non-destructive and may be applied to thermally liable compounds (unlike GC). HPLC is ideally suitable for the separation of macromolecules and ionic species of biomedical interest, liable natural products, and diverse less stable and/or high molecular weight compounds. The majority of difficult separations are often more readily attained by HPLC because both phases used in HPLC participate in the chromatographic process (as opposed to only one in GC) to increase more selective interactions with the sample molecule. Short, small-bore columns containing densely packed particles of stationary phase provide for the rapid exchange of compounds between the mobile and stationary

phases. A large variety of unique column packings (stationary phase) provide a wide range of selectivity to separation through HPLC. HPLC also offers wide choice of detection methods as number of unique detectors are available. HPLC can easily be extended to trace determination of compounds which do not usually provide adequate detector response with the use of post-column derivatization methods that improve selectivity and detection limits. Facility to arrange gradient flow of mobile phase is often use during method development and it is also provide the possibility to achieve difficult separation in reduced run-time. HPLC contains automatic instrumentation and calculation which is carried out by integrator itself that offers saving of manual labor. In addition to receiving and reporting detector output, computers are used to control chromatographic settings and operations, thus providing for long periods of unattended operation. All these advantages make HPLC more efficient over the all remaining chromatographic techniques in case of separation, speed, sensitivity, easy sample recovery, automation, integration, handling and maintenance. The wide applicability of HPLC makes it as a most important separation tool in scientific the field of analysis.

2.1.1 Instrumentation of HPLC

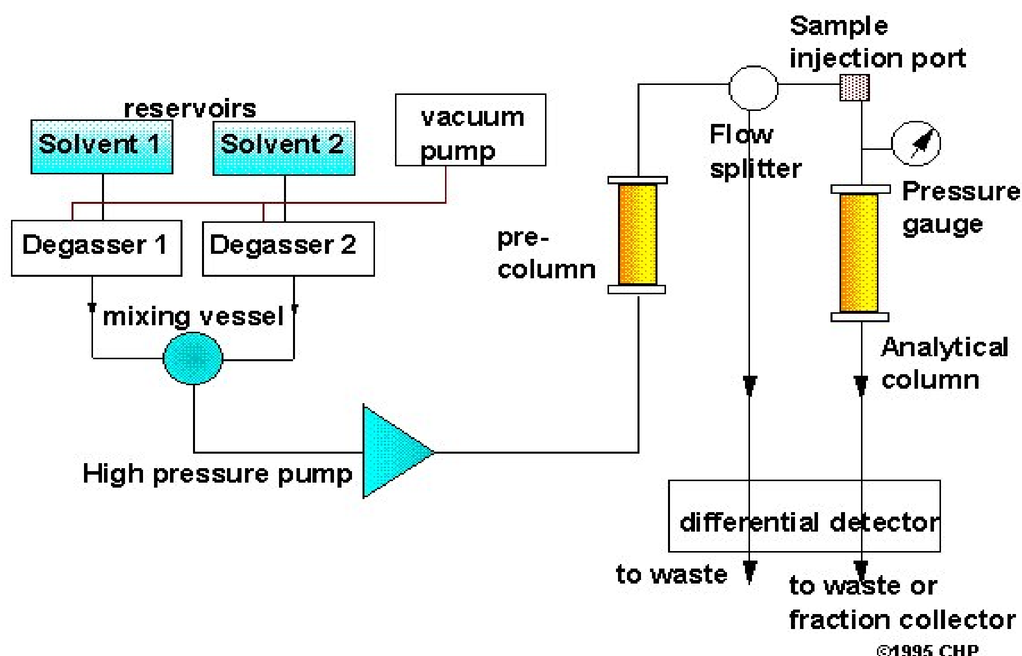
A schematic instrumentation of HPLC is given through figure-1 as under:

Figure-1: A schematic instrumentation of HPLC equipment



A liquid chromatograph consists of a reservoir containing the mobile phase, a pump to force the mobile phase through the system at high pressure, an injector to introduce the sample into the mobile phase, a chromatographic column (a column temperature controller may be also used) to attain retention, a detector to detect analyte response, and a data collection device such as a computer, integrator, or recorder. Further, in some cases, degasser with vacuum pump and pre-column facility can implement in the modern HPLC; represented as figure-2:

Figure-2: Designing of HPLC path



A brief introduction of HPLC components is given as under:

(a) Pumping Systems

HPLC pumping system delivers measured amounts of mobile phase from the solvent reservoirs to the column through high-pressure tubing and fittings. Operating pressures up to 5000 psi or higher, with delivery rates up to about 10 ml/minute is typical. Pumps used for quantitative analysis should be constructed of materials that inert to corrosive mobile phase components and be capable of delivering the mobile phase at a constant rate with minimal fluctuations over

extended periods of time. Modern systems consist of microprocessor controlled metering pumps that can be programmed to deliver either constant (isocratic) flow of mobile phase or vary the ratio of mobile phase components, as is required for gradient run. Advanced pumping system is equipped with a degasser to remove dissolved air and other gases from the solvent through solvent delivery system.

(b) Injectors

After dissolution in mobile phase or suitable diluent, compounds to be chromatographed are injected into the mobile phase, either manually by syringe/loop injectors or automatically by autosampler. Autosampler consist of a carousel or rack to hold sample vials with tops that have a pierceable septum or stopper and an injection device to transfer sample from the vials to a loop from which it is loaded into the chromatograph. Autosampler can be programmed to control sample volume, the number of injections, the interval between injections and rinse cycles.

(c) Columns[8-16]

The column is usually made up of stainless steel to withstand high pressure. Columns used for analytical separations usually have 10-30 cm length and 4-10 mm inside diameter containing stationary phase having particle diameter of 3-10 μm. Particles may range up to 50 μm or more for preparative columns. Stationary phases for modern, reverse-phase liquid chromatography typically consist of an organic phase chemically bound to silica or other materials. Small particles thinly coated with organic phase provide for low mass transfer resistance and, hence, rapid transfer of compounds between the stationary and mobile phases. Columns may be heated to give more efficient separations, but only rarely are they used at temperatures above 60° C. Unmodified silica, porous graphite or polar chemically modified silica, e.g. cyanopropyl or diol, used as the stationary phase for normal-phase liquid chromatography.

Most of separations for reversed-phase liquid chromatography are based upon partition mechanisms that utilize chemically modified silica as the stationary phase and polar solvents as the mobile phase. The surface of the support, e.g. the silanol groups of silica, is reacted with various silane reagents to produce covalently bound silyl derivatives covering a varying number of active sites on the surface of the support. The nature of the bonded phase is an important parameter for determining the separation properties of the chromatographic system.

Commonly used bonded phases are shown below:

Octyl (C_8)	= $Si\text{-}[CH_2]_7\text{-}CH_3$
Octadecyl (C_{18})	= $Si\text{-}[CH_2]_{17}\text{-}CH_3$
Phenyl (C_6H_5)	= $Si\text{-}[CH_2]_n\text{-}C_6H_5$
Cyanopropyl (*CN*)	= $Si\text{-}[CH_2]_3\text{-}CN$
Aminopropyl (NH_2)	= $Si\text{-}[CH_2]_3\text{-}NH_2$
Diol (*OH*)	= $Si\text{-}[CH_2]_3\text{-}O\text{-}CH(OH)\text{-}CH_2\text{-}OH$

In ion-exchange chromatography, the stationary phases are usually synthetic organic resins; cation-exchange resins contain negatively charged active sites and is used to separate basic substances, while anion-exchange resins have positively charged active sites for separation of compounds with negatively charged groups. Water-soluble ionic or ionizable compounds are attracted to the resins, and differences in affinity bring about the chromatographic separation.

In size-exclusion chromatography, columns are packed with a porous stationary phase. Smaller molecules enter the pores and are increasingly retained as molecular size decreases. These columns are typically used to measure aggregation and degradation of large molecules.

For chiral chromatography, special chemically modified stationary phases, e.g. cellulose or amylose derivatives, proteins or peptides, cyclodextrins etc., use for the separation of enantiomers.

(d) Detectors[17-22]

UV/Vis spectrophotometers, including diode array detectors, are the most commonly employed detectors. Fluorescence spectrophotometers, differential refractometers, electrochemical detectors, mass spectrometers, light scattering detectors, radioactivity detectors or other special detectors may also be used.

Detector consists of a flow-through cell mounted at the end of the column. A beam of UV radiation passes through the flow cell and into the detector. As compounds elute from the column, they pass through the cell and absorb the radiation, resulting in measurable energy level changes. Fixed (mercury lamp), variable (deuterium or high-pressure xenon lamp), and multi-wavelength detectors are widely available. Modern variable wavelength detectors can be programmed to change wavelength while an analysis is in progress. Multi-wavelength detectors measure absorbance at two or more wavelengths simultaneously. In diode array multi-wavelength detectors, continuous radiation is passed through the sample cell, and then resolved into its constituent wavelengths, which are individually detected by the photodiode array. These detectors acquire absorbance data over the entire UV-visible range, thus providing the analyst with chromatograms at multiple, selectable wavelengths, spectra of the eluting peaks and also peak purity.

Differential refractometer detectors measure the difference between the refractive index of the mobile phase alone and that of the mobile phase containing chromatographed compounds as it emerges from the column. Refractive index detectors are used to detect non-UV absorbing compounds.

Fluorometric detectors are sensitive to compounds that are inherently fluorescent or that can be converted to fluorescent derivatives either by chemical transformation of the compound or by coupling with fluorescent reagents at specific functional groups.

Potentiometric, voltametric, or polarographic electrochemical detectors are useful for the quantitation of species that can be oxidized or reduced at a working electrode. These detectors are selective, sensitive, and reliable, but require conducting mobile phases free of dissolved oxygen and reducible metal ions. Electrochemical detectors with carbon-paste electrodes may be used advantageously to measure nanogram quantities of easily oxidized compounds, notably phenols and catechols.

(e) Data Collection Devices

Modern data stations receive and store detector output and print out chromatograms complete with peak heights, peak areas, sample identification, and method variables. They are also used to program the liquid chromatograph, controlling most of variables and providing for long periods of unattended operation.

2.1.2 **Mode of HPLC**

Various modes of HPLC utilized to separate compounds are classified as follows:

1) Adsorption chromatography
2) Normal-phase chromatography
3) Reversed-phase chromatography
4) Ion-pair chromatography
5) Ion-exchange chromatography
6) Size exclusion chromatography

1. Adsorption chromatography

Adsorption chromatography uses polar stationary phases with relatively nonpolar mobile phases. Separations in adsorption chromatography result to a great extent from the interaction of sample polar functional groups with discrete adsorption sites on the stationary phase. Adsorption chromatography is usually considered appropriate for the separation of nonionic molecules that are soluble in organic solvents.

2. Normal-phase chromatography

In HPLC, if stationary phase is more polar than the mobile phase, it is termed as normal-phase liquid chromatography. Polar bonded phases that have a diol, cyano, diethylamino, amino, or diamino functional groups are used as stationary phase in normal-phase chromatography. Due to lower affinity of nonpolar compounds to the stationary phases used, nonpolar compounds are elute first while polar compounds are retained for longer time. Normal-phase chromatography is widely applied for chiral separations.

3. Reversed-phase chromatography

In HPLC, if stationary phase is less polar than the mobile phase, it is termed as reversed-phase liquid chromatography. In this technique, C18, C8, Phenyl, and cyano-propyl functional groups that chemically bonded to microporous silica particles are used as stationary phase. Retention in reversed-phase chromatography occurs by nonspecific hydrophobic interactions of the solute with stationary phase. The ubiquitous application of reversed-phase chromatography arise from the fact that practically all organic molecules have hydrophobic regions in their structures and effectively interact with the stationary phase. It is estimated that over 65% (possibly as high as 90%) of all HPLC

separations are executed in the reversed-phase mode. The rationale for this includes the simplicity, versatility, and scope of the reversed-phase method[23].

4. Ion-pair chromatography

Ionic or partially ionic compounds can be chromatographed on reversed-phase columns by using ion-pairing reagents. These reagents are typically long-chain alkyl anions or cations that, when used in dilute concentrations, can increase the retention of analyte ions. C-5 to C-10 alkylsulfonates are commonly used for cationic compounds while C-5 to C-8 alkyl ammonium salts are generally used in the cases of anionic solutes.

5. Ion-exchange chromatography

Ion-exchange chromatography is an adaptable technique used primarily for the separation of ionic or easily ionizable species. The stationary phase is characterized by the presence of charged centers having exchangeable counterions. Both anions and cations can be separated by choosing the suitable ion-exchange medium. Ion-exchange chromatography employs the dynamic interactions between charged solute ions and stationary phases that have oppositely charged groups.

6. Size exclusion chromatography

Size exclusion chromatography separates molecules according to their molecular mass. In Size exclusion chromatography, column is filled with material having precisely controlled pore sizes and the sample is simply screened or filtered according to its solvated molecular size. Largest molecules are eluted first and the smallest molecules last. This method is generally used when a mixture contains compounds with a molecular mass difference of at least 10%. This mode can be further subdivided into gel permeation chromatography (with organic

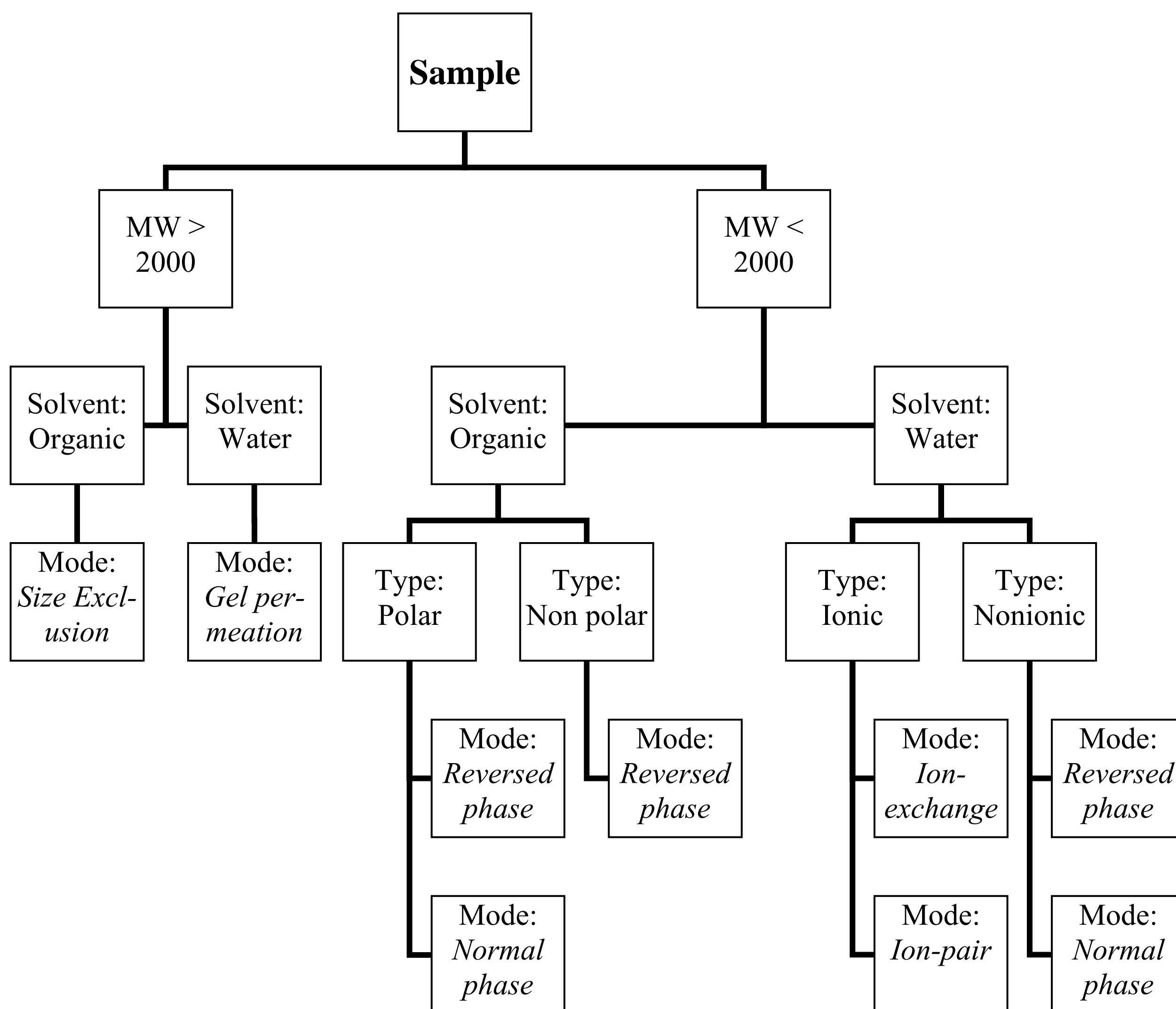
solvents) and gel filtration chromatography (with aqueous solvents).

2.1.3 **HPLC method development:**

A variety of patterns have been used to develop analytical separations. The discussion here is limited to conventional chromatographic approaches. These approaches are advanced and often based on intuitive judgment and the knowledge of the chromatographer. Where as individual approaches may exhibit considerable diversity, method development often follows the series of steps summarized below.

Before starting the method development, it is need to review what is known about the sample. The aim of method development should also define at separation stage. The kinds of sample-related information that can be important that are nature of the sample, number of compounds present, chemical structure and molecular weight of the analytes, pKa values of the compounds, sample solubility and sample type (ionic or non-ioinic).

The choice of the mode of HPLC method should be made principally from the properties of the sample that has been determined about molecular weight (MW). On the basis of the solubility of the sample in polar or nonpolar solvents, mode of HPLC can be select as per below flow-chart:



After selecting mode of HPLC, the choice of the column (stationary phase) should be made after thorough consideration of mode of chromatography, column-to-column variability, and a number of other considerations. The silica particles may be acquired in a variety of sizes, shapes, and degrees of porosity. In addition, various functional groups or polymeric layers can easily be attached to the silica surface, extending the usefulness of these particles for applications to any specific HPLC method. Sufficient time is also allowed for column equilibration before starting the analysis.

The selection of mobile phase is very important parameter in HPLC

method development as the selectivity is altered by changing the mobile phase. When selecting organic solvents for use in mobile phases, various physical and chemical properties of the solvent should be consider. Selected solvent will have low viscosity, be compatible with the detection system, be easily available in pure form, and, if possible, have low flammability and toxicity. UV cutoff values of solvents are also important consideration from the standpoint of detection.

The term polarity concerns the ability of a sample or solvent molecule to interact by combination of dispersion, dipole, hydrogen boding, and dielectric interactions. The combination of these four intermolecular attractive forces constitutes the solvent polarity. Polarity is measure strength of solvent that affected selectivity. The changes in selectivity may be affected by making use of the following solvent properties:

Proton acceptors: amines, ethers, sulfoxides, amides, esters, and alcohols
Proton donors: alcohols, carboxylic acids, phenols, and chloroform
Large dipole solvents: methylene chloride, nitrites, sulfoxides, and ketones

Application of reagents such as buffers, ion-pairing reagents, or other modifiers (such as triethylamine) to the mobile phase is carried out to improve reproducibility, selectivity, or peak shape. Buffers are used primarily to regulate the pH and the acid-base equilibrium of the solute in the mobile phase. They may also be utilized to affect the retention times of ionizable compounds. The buffer capacity should be at maximum and should not vary in the pH range of 2 to 8 commonly used in HPLC. The buffers should be soluble, stable, inert to analytes and compatible to the detector.

By employing gradient elution mode, required % of the organic phase can be estimated rapidly. For aqueous sample mixtures, the best way to start is with gradient reversed phase chromatography. Gradient run can be started with 5 to 10% organic phase (acetonitile or methanol) in the mobile phase and can be

increased up to 90% within 30 to 40 minutes. Separation can then be optimized by changing the initial mobile phase composition and the slope of the gradient according to the chromatogram obtained from the preliminary run. The initial mobile phase composition can be estimated on the basis of where the compounds of interest were eluted at what proportion of organic and aqueous component of the mobile phase.

For each samples, good analytical results will be obtained by careful selection of the wavelength used for detection. This choice requires known UV spectra of the sample. If analyte standards are available, their UV spectra can be measured prior to HPLC method development. Alternatively, PDA detector permits the acquisition of UV spectra for all sample components during method development. The wavelength chosen for detection must provide acceptable response by the various analytes in the sample and there is no interference of baseline noise. In most cases HPLC method development is carried out with UV detector. Alterative detectors are required when sample have low or no UV absorbance.

Diluent for test preparation shall be selected at initial stage of development on the basis of solubility of the drug. However, optimization of the diluent is based on its extraction efficiency, effect on peak symmetry, peak interference in estimation and stability of analyte in diluent. Test concentration and injection volume shall be set according to suitability with extraction process and detector response. Wherever it is necessary to filter the test preparation, filter compatibility shall be check for selection of proper type of filter.

To finalize HPLC method development, optimization can be started only after obtaining preliminary chromatogram that means with more or less symmetrical peaks of all analytes detected in the chromatogram. By slight change of the mobile phase composition, the position of the peaks can be predicted within the range of investigation. A finalized chromatographic condition is that one in

which all the peaks having good theoretical plates are symmetrical and well separated in less run time in the produced chromatogram. With application of more specific column, good symmetry and resolution can be achieved. The parameters that are considered for good chromatographic condition and being optimized are:

1) Resolution (R)
2) Theoretical plates (N)
3) Tailing factor (T)
4) Capacity factor (k')
5) Selectivity (α)

All above terms are explain according to figure-3 and figure-4 as below:

Figure-3: Explanation for resolution, theoretical plates, capacity factor and selectivity

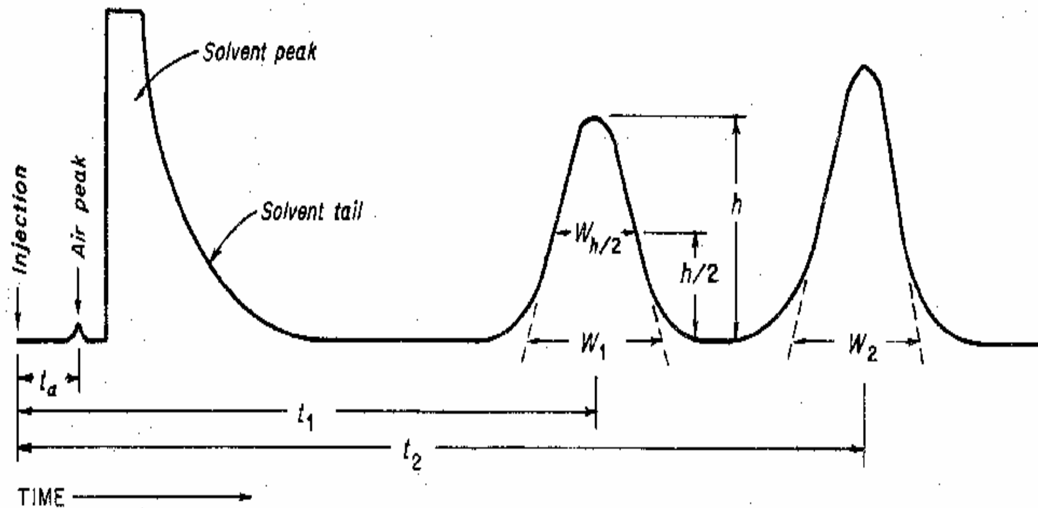
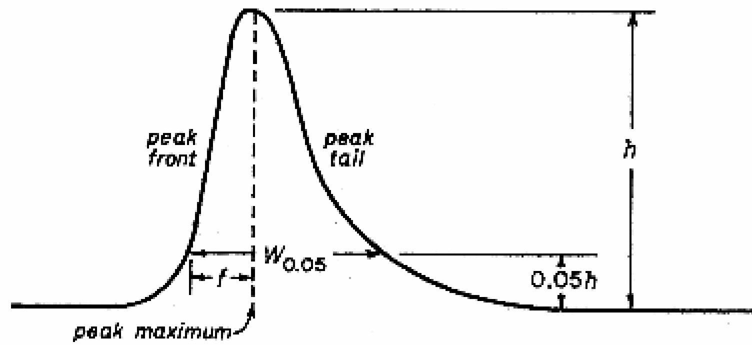


Figure-4: Explanation for tailing factor



1. Resolution (R):

Resolution expresses the separation of two components in a mixture that determined by the following equation:

$$R = \frac{2(t_2 - t_1)}{W_2 + W_1}$$

Where by t_2 and t_1 are the retention times of the two components, and W_2 and W_1 are the corresponding widths at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline.

Where electronic integrators are used, it may be convenient to determine the resolution, R, by the following equation:

$$R = \frac{2\,(t_2 - t_1)}{1.70\,(W_{2h/2} + W_{1h/2})}$$

Where by $W_{1h/2}$ and $W_{2h/2}$ are the width at half-height of corresponding peaks. For better separation, the ideal value of R is 1.5

2. Theoretical plates (N)

The number of theoretical plates is a measure of column efficiency. It is expressed by following equation:

$$N = 16 \left(\frac{t}{W}\right)^2 = 5.54 \left(\frac{t}{W_{h/2}}\right)^2$$

Where t is retention time of the peak and W is the with for the peak. $W_{h/2}$ is width at half-height of the peak. Value of theoretical plates, higher than 4000 is indicating good column performance.

3. Tailing factor (T)

The tailing factor (T) is a measure of peak symmetry. It is unity for perfectly symmetrical peak to value of 1.0 and its value increases as tailing becomes more pronounced. It is determined by following formula:

$$T = \frac{W_{0.05}}{2f}$$

Where by $W_{0.05}$ is width of the peak at 5% height and f is distance from the peak maximum to the leading edge of the peak which being measured at a point of 5% of the peak height from the baseline. In general, value of tailing factor should be less than 2.0

4. Capacity Factor (k')

Capacity factor is the ratio of the reduced retention volume to the dead volume. Capacity factor is a measure of how well the sample molecule is retained by a column during an isocratic separation. It is determined by using following formula:

$$k' = \frac{t_1 - t_0}{t_0}$$

Where by t_0 is the void volume of the column and t_1 is the retention time of the corresponding peak. The ideal value of k' ranges from 2-10.

5. Selectivity (α)

The selectivity is a measure of relative retention of two components in a mixture. Selectivity is the ratio of the capacity factors of both corresponding peaks. It can be calculated by following formula:

$$\alpha = \frac{t_2 - t_1}{t_1 - t_0}$$

Where by t_0 is the void volume of the column and t_1 and t_2 are the retention times of the corresponding peak. The ideal value of α should not be less than 1.

2.2 Analytical method validation

The developed analytical procedure used to measure the quality of pharmaceutical products. It is necessary to assure that the performance characteristics of the developed analytical procedure meet the requirements for the intended analytical application. The procedure which provides assurance for the same by the means of laboratory studies is defined as method validation. Method validation is the process of demonstrating that analytical procedures are suitable for their intended use and that they support the identity, strength and quality, for the quantification of the drug substances and drug products.

Method validation has received considerable attention in the literature and from industrial committees and regulatory agencies. The U.S. FDA CGMP[24]

states for validation for the test methods employed by the firm. The U.S. FDA has also proposed industry guidance for Analytical Procedures and Methods Validation[25]. ISO/IEC 17025 includes a chapter on the validation of methods[26] with list validation parameters. The ICH[27] has developed a consensus text on the validation of analytical procedures. ICH also developed guidance with detailed methodology[28]. The U.S. EPA prepared guidance for method's development and validation for the Resource Conservation and Recovery Act (RCRA)[29]. The AOAC, the EPA and other scientific organizations provide methods that are validated through multi-laboratory studies. The USP has published specific guidelines for method validation for compound evaluation[30]. The WHO published validation guidelines under the title, 'Validation of analytical procedures used in the examination of pharmaceutical materials' in the 32nd report of the WHO expert committee on 'specifications for pharmaceutical preparations'.

Representatives of the pharmaceutical and chemical industry have published papers on the validation of analytical methods. Hokanson[31,32] applied the life cycle approach, developed for computerized systems, to the validation and revalidation of methods. Green[33] gave a practical guide for analytical method validation, with a description of a set of minimum requirements for a method. Wegscheider[34] has published procedures for method validation with a special focus on calibration, recovery experiments, method comparison and investigation of ruggedness. Seno et al.[35] have described how analytical methods are validated in a Japanese QC laboratory. The AOAC[36] has developed a Peer-Verified Methods validation program with detailed guidelines on exactly which parameters should be validated. Winslow and Meyer[37] recommend the definition and application of a master plan for validating analytical methods. J. Breaux and colleagues have published a study on analytical methods development and validation[38].

2.2.1 **Strategy for the Validation of Methods**

Method development and validation are an iterative process. The influence of operating parameters on the performance of the method can be assessed at the validation stage which was not done during development/optimization stage of the method. The most significant point raised for validation is that the validity of a method can be demonstrated only through laboratory studies. It is not sufficient to simply review historical results; instead, laboratory studies must be conducted which are intended to validate the specific method, and those studies should be pre-planned and described in a suitable documentation. This documentation should clearly indicate the method's intended use and principles of operation, as well as the validation parameters to be studied, and a rationale for why this method and these parameters were chosen. It also must include pre-defined acceptance criteria and a description of the analytical procedure.

2.2.2 **Parameters for Method Validation**

The parameters for method validation have been defined in different working groups of national and international committees and are described in the literature. An attempt at harmonization was made for pharmaceutical applications through the ICH[27-28]. The defined validation parameters by the ICH and other regulatory bodies are summarized as under:

a) Specificity study
b) Linearity and range study
c) Limit of detection and Limit of quantitation study
d) Precision study
e) Accuracy study
f) Robustness study
g) Solution stability study
h) System suitability

A brief introduction of above parameters is as below:

a) Specificity study

Specificity of an analytical method is its ability to measure accurately an analyte in the presence of interference, such as synthetic precursors, excipients, enantiomers and known (or likely) degradation products that may be expected to be present in the sample matrix. The term specificity is also referring to selectivity when a number of chemical entities that may or may not be distinguished from each other.

Specificity study should also assess interferences that may be caused by the matrix, e.g., urine, blood, soil, water or food. Optimized sample preparation can eliminate most of the matrix components, e.g. placebo. The absence of matrix interferences for a quantitative method should be demonstrated by the analysis of control matrix in specificity. An investigation of specificity should be conducted during the validation of identification tests, the determination of impurities and the assay. In order to check the interference of degradation products, analyte is forcely subject to chemical (acid, alkali and oxidative) and physical (thermal and photolytic) degradation, known as stress application. In each stress application, peak purity of the analyte peak is also evaluated[39].

b) Linearity and range study

Linearity

The linearity of an analytical method is its ability to elicit test results that are directly proportional to the concentration (amount) of analyte in samples within a given range. Linearity may be demonstrated directly on the test substance (by dilution of a standard stock solution) and/or by using separate weighings of synthetic mixtures of the test product components, using the proposed procedure.

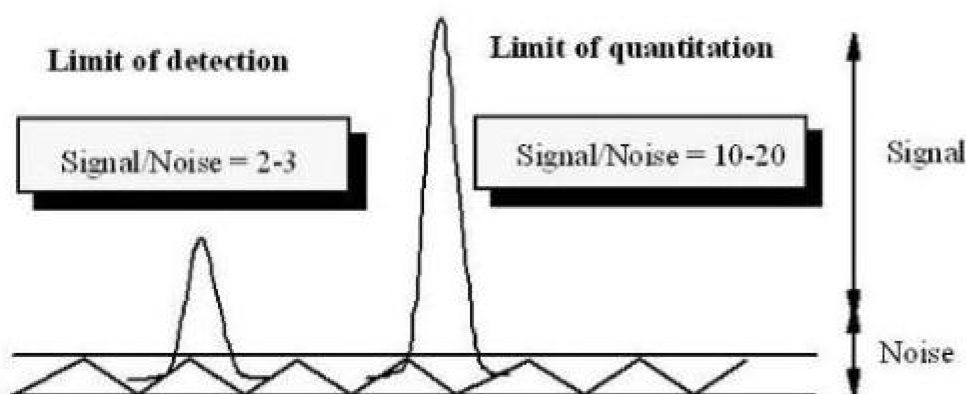
Linearity is determined by replicate injections of 5 or more concentrations level within the range of 40–160 %. The response should be directly proportional to the concentrations of the analytes or proportional by means of a well-defined mathematical calculation. Linearity is evaluated graphically by plotting a graph of the relative responses on the y-axis and the corresponding concentrations on the x-axis. A linear regression equation is applied to the results to evaluate correlation coefficient. In addition, y-intercept, slope of the regression line and residual sum of squares should also calculate.

Range

The range of an analytical method is the interval between the upper and lower concentrations (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. The range is normally expressed in the same units as the test results (e.g., percentage, parts per million) obtained by the analytical method.

c) Limit of detection (LOD) and Limit of quantitation (LOQ) study

Figure-5: Explanation for the LOD and LOQ



Limit of detection:

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

In chromatography, the detection limit is the injected amount that results in a peak with a height at least two or three times as high as the baseline noise level. Besides this signal/noise method, LOD can be measured by another three different methods; (i) visual inspection (ii) standard deviation of the blank response (iii) standard deviation of the response based on the slope of the calibration curve.

Limit of quantitation:

The quantitation limit of an analytical method is the lowest amount of analyte in a sample which can be quantitated with suitable precision and accuracy.

In chromatography, the quantitation limit is the minimum injected amount that produces quantitative measurements in the target matrix with acceptable precision, typically requiring peak heights 10 to 20 times higher than the baseline noise. Beside this signal/noise method, LOQ can be measured by another three different methods; (i) visual inspection (ii) standard deviation of the response (iii) standard deviation of the response based on the slope of the calibration curve.

d) Precision study

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

The measurement of precision of an analytical method is performed on replicate standard preparations and replicate sample preparations. The results for the same are usually expressed as the variance, standard deviation or confidence level of a series of measurements. Precision is performed by means of repeatability, reproducibility and intermediate precision (ruggedness).

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

Reproducibility: Reproducibility expresses the precision between laboratories. The reproducibility of an analytical method is determined by analyzing aliquots from same homogeneous lots.

Intermediate Precision: Intermediate precision expresses within-laboratories variations; different days, different analysts, different equipment, etc. The objective of intermediate precision validation is to verify that in the same laboratory the method will provide the same results once the development phase is over. The objective is also extent to verify that the method will provide the same results in different laboratories (ruggedness).

e) Accuracy study

The accuracy of the analytical method is 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. The accuracy of an analytical method is the extent to which test results generated by the method and the true value agree.

The true value for accuracy assessment can be assessed by analyzing a sample with known concentrations (e.g., a control sample or certified reference

material) and comparing the measured value with the true value as supplied with the material. If certified reference materials or control samples are not available, a blank sample matrix of interest can be spiked with a known concentration by weight or volume. After extraction of the analyte from the matrix and injection into the analytical instrument, its recovery can be determined by comparing the response of the extract with the response of the reference material dissolved in a pure solvent (without matrix).

The ICH document on validation methodology recommends accuracy to be assessed using a minimum of nine determinations over a minimum of three concentration levels covering the specified range (e.g., three concentrations/three replicates each). Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value.

f) Robustness study

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.

Robustness tests examine the effect that operational parameters have on the analysis results. For the determination of a method’s robustness, method parameters like pH, flow rate, column temperature, column lot or mobile phase composition, are varied within a realistic range, and the quantitative influence of the variables is determined. If the influence of the parameter is within a previously specified tolerance, the parameter is said to be within the method’s robustness range.

g) Solution stability study

Many solutes readily decompose prior to chromatographic investigations, for example, during the preparation of the sample solutions, extraction, cleanup, phase transfer or storage of prepared vials (in refrigerators or in an automatic sampler). Under these circumstances, method validation should investigate the stability of the analytes and standards in solution form (in analytical preparations). The standard and test preparations are stored up to specified period at specified temperature and its stability is evaluated by comparing solution preparations at different time intervals to that of initial.

h) System suitability study

In addition, prior to the start of laboratory studies to demonstrate method validity, some type of system suitability must be done to demonstrate that the analytical system is performing properly. System suitability should be determined by replicate analysis of the standard or reference solution. System suitability is considered appropriate when the RSD, theoretical plates, tailing factor and resolution parameters calculated on the results obtained at different time intervals, does not exceed more than of specified limit of the corresponding value of the system precision.

2.2.3 Prior steps of Validation

Prior to start method validation, validation aim should be a well-planned according to scientific soundness and completeness with pre-defined acceptance criteria. Because the type of analysis and the other information of a sample have so much influence on the validation, the objective and scope of the method should always be defined as the first step of any method validation. For an efficient

validation process, it is of utmost importance to specify the right validation parameters.

Subsequent to the execution of the validation, results, conclusions and deviations should present in report. Provided the pre-defined acceptance criteria are met, and the deviations (if any) do not affect the scientific interpretation of the data, then the developed analytical method can be considered as valid.

3.0 Objective of the work

The prime and specific objective of the work to develop and validate analytical methods for the quantification of drug from its pharmaceutical dosage form. This objective is further extending to combined pharmaceutical dosage form of the drug. The details are as under:

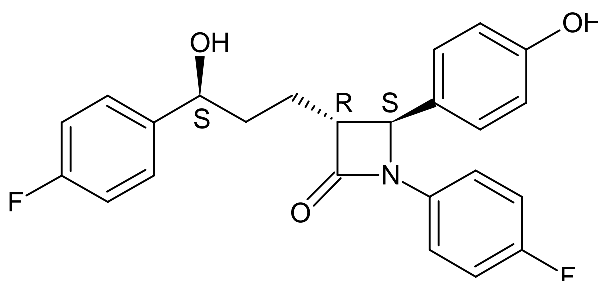
- To develop analytical method for assay and content uniformity determination of Ezetimibe from pharmaceutical dosage form
- To develop analytical method for assay determination of Ezetimibe and Rosuvastatin from their combined pharmaceutical dosage form
- To develop analytical method for assay determination of Ezetimibe and Atorvastatin from their combined pharmaceutical dosage form
- To develop analytical method for assay determination of Ezetimibe and Simvastatin from their combined pharmaceutical dosage form
- To develop analytical method for assay determination of Ezetimibe, Atorvastatin and Fenofibrate from their combined pharmaceutical dosage form

4.0 Method development and validation for assay and content uniformity determination of Ezetimibe from its pharmaceutical dosage form

4.1 Introduction to Ezetimibe

4.1.1 Description

Ezetimibe is chemically *1-(4-flurophenyl)-3(R)-[3-(4-flourophenyl)-3(S)-hydroxypropyl]-4(S)-(4-hydroxyphenyl)-2-azetidinone*. Its molecular formula is $C_{24}H_{21}F_2NO_3$ and it has a molecular weight of 409.43 g mol^{-1}. Its structural formula is as under:



Ezetimibe is a white crystalline powder that is practically insoluble in water and freely to very soluble in ethanol, methanol, and acetone[40].

4.1.2 Clinical pharmacology and mechanism of action

Ezetimibe is the first in a new class of lipid-lowering drug, known as selective cholesterol absorption inhibitors (CAI), that inhibit intestinal absorption of cholesterol and related phytosterols[41] by inhibiting absorption of dietary and biliary cholesterol from the intestinal lumen. The drug has been found useful for treatment of hyperlipoproteinemia. Ezetimibe, either as monotherapy or in combination with Statins (3-hydroxyl-3-methylglutaryl-coenzyme A (HMGCoA) reductase inhibitors)[42], is used for reduction of low-density lipoproteincholesterol (LDL-C), total cholesterol (TC), and apolipoprotein B (Apo B) when combined with dietary measures for treatment of primary hypercholesterolemia. As

monotherapy, Ezetimibe seems to reduce overall LDL-C by 17%; in combination therapy with Statins it reduces LDL-C by an additional 12–20%[43-48] and is useful for the treatment of homozygous familial hypercholesterolemia (HoFH). It also reduces elevated levels of sitosterol and campesterol in the treatment of homozygous familial sitosterolemia[49]. Ezetimibe, administered in combination with Fenofibrate, is used as adjunctive therapy to diet for the reduction of elevated TC, LDL-C, Apo B, and non-HDL-C (high-density lipoprotein–cholesterol) in patients with mixed hyperlipidemia[41].

Ezetimibe has a mechanism of action that differs from those of other classes of cholesterol-reducing compounds. A new mechanism of cholesterol-lowering is the inhibition of intestinal cholesterol absorption by inhibiting absorption of dietary and biliary cholesterol from intestinal lumen. After oral administration, Ezetimibe is rapidly metabolized to Ezetimibe-Glucuronide. Ezetimibe and Ezetimibe-Glucuronide are the major drug delivered compounds in plasma, constituting approximately 10 to 20% and 80 to 90% of the total drug in plasma, respectively. Both Ezetimibe and Ezetimibe-Glucuronide are slowly eliminated from plasma with a half-life of approximately 22 hours. Plasma concentration-time profiles exhibit multiple peaks, suggesting enterohepatic recycling.

4.1.3 **Literature review**

The literature reviews regarding Ezetimibe suggest that analytical methods were reported for its determination as API and pharmaceutical formulation. Brief details for the same are as under:

(1) Oliveira PR, Brum L Jr, Fronza M, Bernardi LS, Masiero SMK, and Dalmora SL have developed an analytical method based on liquid chromatography-tandem mass spectrometry (LC-MS-MS) for the determination of Ezetimibe in human plasma. Ezetimibe and Etoricoxib (internal standard) were extracted

from the plasma by liquid-liquid extraction and separated on a C_{18} analytical column (50 × 3.0 mm I.D.) with Acetonitrile:Water (85:15, *v/v*) as mobile phase. Detection was carried out by positive electrospray ionization (ESI+) in multiple reaction monitoring (MRM) mode[50].

(2) Sistla R, Tata VSSK, Kashyap YV, Chandrasekar D, Diwan PV have developed reversed-phase HPLC method for assaying Ezetimibe in pharmaceutical dosage forms. The assay involved an isocratic elution of Ezetimibe in a Kromasil 100 C_{18} column using a mobile phase composition of water (pH 6.8, 0.05%, w/v 1-heptane sulfonic acid) and Acetonitrile (30:70, v/v). The flow rate was 0.5 ml/min and the analyte monitored at 232 nm[51].

(3) Singh S, Singh B, Bahuguna R, Wadhwa L, Saxena R have developed an analytical method with C_8 column and a mobile phase composed of ammonium acetate buffer (0.02 M, pH adjusted to 7.0 with ammonium hydroxide) and Acetonitrile, which was pushed through the column in a gradient mode. The detection was carried out at 250 nm[52].

4.1.4 **Aim of work**

Methods reported in the literature for analysis of Ezetimibe include determination of the drug in human plasma by LC-tandem MS[50] and a reversed-phase HPLC method for determination of the pharmaceutical form of the drug[51]. The latter report includes a limited investigation of the effect of stress; another briefly described study of the effect of stress used active pharmaceutical ingredient only[52]. There are no reports of methods for study of the effect of stress on pharmaceutical dosage forms and there is no validated LC method, which enables both assay and determination of content uniformity of Ezetimibe in pharmaceutical dosage forms.

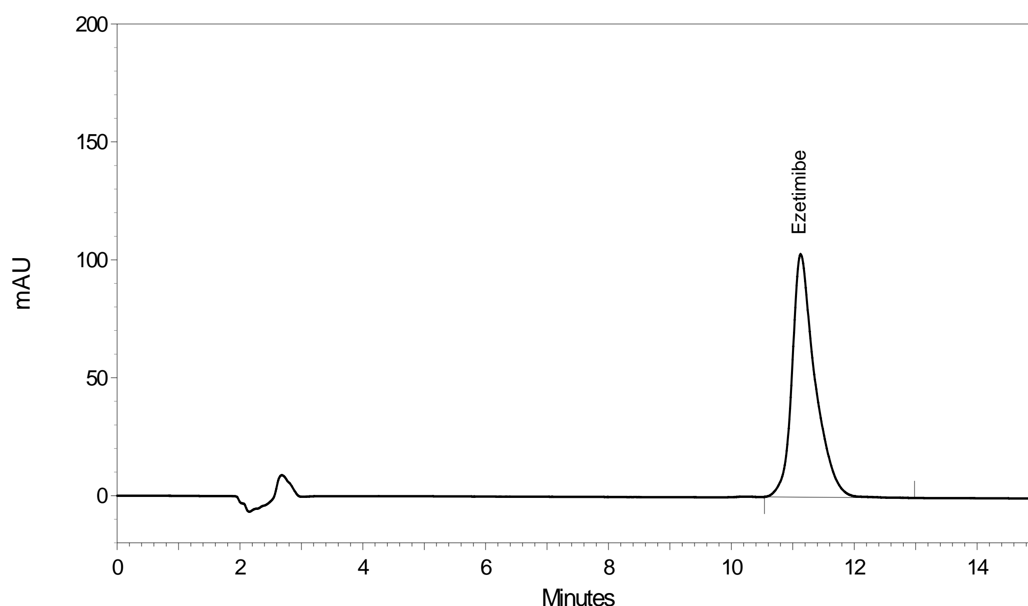
The objective of this work was to develop a stability-indicating liquid chromatographic analytical method for assay of Ezetimibe and for determination of the content uniformity of a tablet formulation, to validate the method in accordance with ICH guidelines[53], and to investigate the effect of applying degradative stress to the product. Reported work in the literature[51] includes application of limited stress in which a single product seemed to be formed under the action of alkali. In the current work a more intensive stress study was performed on the pharmaceutical dosage form and showed that the drug decomposed into numerous products under different stress conditions. Accordingly, a stability- indicating method was established in which the analyte peak was well resolved from those of all the degradation products formed under all stress conditions. The stress study also furnished information about the percentage degradation of the drug under different stress conditions, information which is not reported in the literature[52].

The validation procedure followed the guidelines of USP 30[54]. The method was successfully used for assay of Ezetimibe and determination of the content uniformity of the tablet formulation. Determination of content uniformity is, nowadays, an important test included in USP 30[54]. Because there is no literature report of a validated analytical method for determination of the content uniformity of Ezetimibe in pharmaceutical dosage forms this was performed in the current work to enhance the imperative for the same.

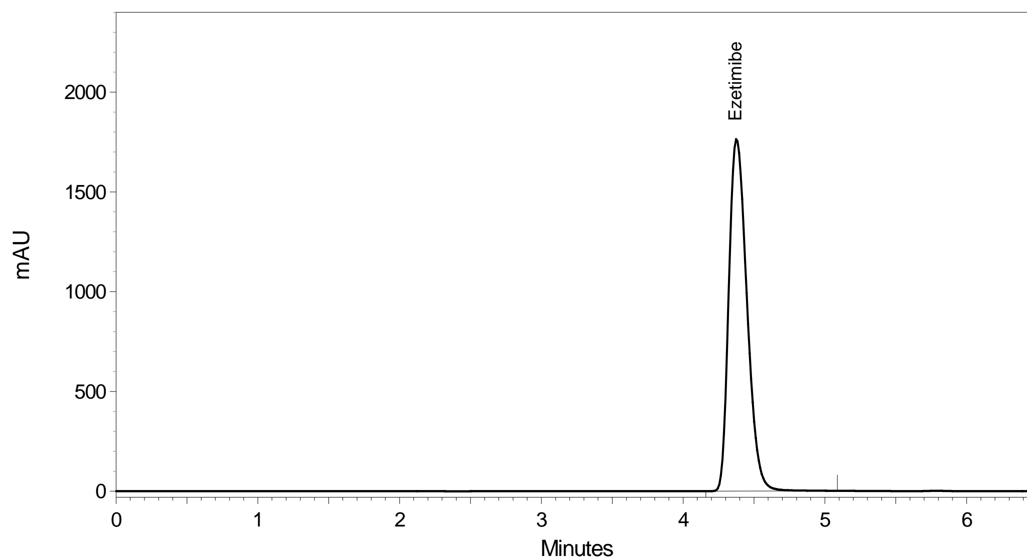
4.2 Analytical method development for the method of assay and content uniformity determination of Ezetimibe

In the present work, an analytical method based on LC was developed and validated for assay and content uniformity determination of Ezetimibe in tablet formulation. The basic chromatographic conditions used for this method were designed to be simple and easy to use and reproduce. The analytical conditions were selected after testing the different parameters that influence LC analysis,

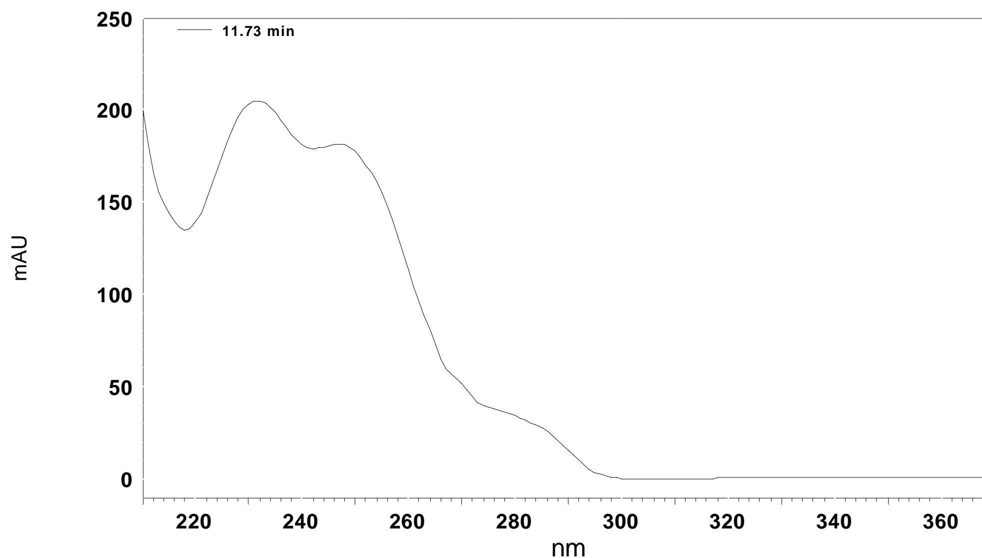
such as column, aqueous and organic phase for mobile phase, mobile phase proportion, wavelength, diluent, concentration of analyte and other chromatographic parameters. A C18 Phenomenex column (250 x 4.6mm) having 5 µm particle size was used because of its advantages of high degree of retention, high resolution capacity, better reproducibility, ability to produce lower back pressure, and low degree of tailing. For mobile phase selection, the preliminary trials using different compositions of mobile phases consisting of phosphate buffer and acetonitile gave poor peak shape. The representative chromatogram for the same is shown as under:



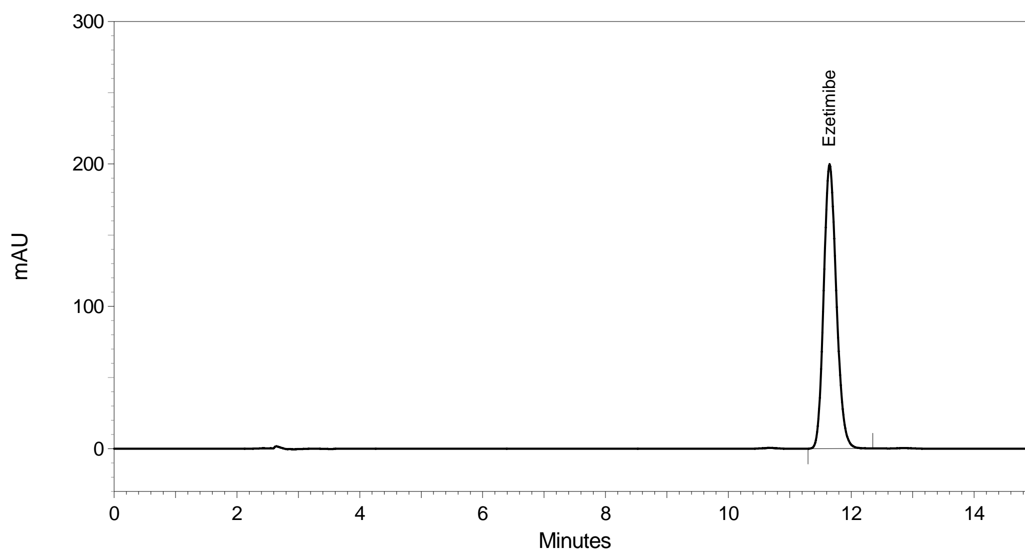
Above chromatogram clearly indicate that the peak is not symmetrical and value of theoretical plates is lower side. In focus to develop good symmetrical peak, phosphate buffer was replaced by water which is adjusted to acidic pH by orthophosphoric acid and thus, better peak shape was obtained. The representative chromatogram for the same is shown as under:



Further, the mobile phase proportion was optimized to retain analyte properly that provide good resolution between Ezetimibe and its degradation impurities obtained in alkali degradation. Proportion of acetonitrile is finalized to 50 % of the mobile phase. A detection wavelength of 232 nm was selected by scanning the standard solution over the range of 190 nm to 370 nm by PDA. Detection at 232 nm wavelength offered high response, good linearity, and the best option for detection conditions. The representative spectrum for the same is shown as under:



As a diluent, the mixture of water-acetonitrile-methanol (40:50:10, *v/v*) was made. Injection volume was fixed to 20 µl and the flow rate of the mobile phase is set to 1.0 ml/minute. On this finalized chromatographic condition, obtained chromatogram was having of good peak symmetry and higher theoretical plates. The representative chromatogram for the same is shown as under:



The drug substance was easily extracted from pharmaceutical dosage using diluent as water-acetonitrile-methanol (40:50:10, *v/v*). Tablet was easily dispersed using water and the drug substance is freely to very soluble in methanol. Extraction trials are finalized to keep sonication time for 30 minutes. Solutions of standard preparation and test preparation were found stable in diluent. By keeping same concentration of analyte for assay and content uniformity determination, validation study was clubbed in all validation parameters except precision study.

4.3 Analytical method validation for the method of assay determination of Ezetimibe

4.3.1 Objective

To perform analytical method validation of the developed chromatographic method for assay determination of Ezetimibe from Ezetimibe tablets.

4.3.2 Scope

This protocol is applicable to the standard testing procedure for assay quantification of Ezetimibe from Ezetimibe tablets developed by Department of Chemistry, Saurashtra University.

4.3.3 Standard testing procedure

Aim:

To determine assay of Ezetimibe from Ezetimibe tablets through High performance liquid chromatography

Instrument:

High performance liquid chromatograph (Shimadzu; LC-10AT*vp*) equipped with PDA detector (SPD-M10A*vp*) and connected to multi-instrument data-acquisition and data-processing system (Class-*VP* 6.13 SP2)

Reagents:

Acetonitrile (HPLC grade)

Methanol (HPLC grade)

Orthophosphoric acid (GR grade)

Water (HPLC grade)

Chromatographic condition:

Mobile phase	:-	0.1% Orthophosphoric acid *(v/v)*:Acetonitrile (50:50, *v/v*)
Column	:-	Phenomenex Luna (2) C_{18}, 4.6 x 250 mm, 5 µm
Flow rate	:-	1.0 ml/min
Detection	:-	232 nm
Injection volume	:-	20 µl
Diluent	:-	Water:Acetonitrile:Methanol (40:50:10, *v/v*)

Blank preparation:

Use diluent as blank.

Standard preparation:

Stock solution: Weigh accurately about 50 mg Ezetimibe reference standard and transfer into 100 ml volumetric flask. Add 70 ml of diluent to dissolve the substance by sonication for one minute and then dilute to volume with diluent. The concentration obtained is 500 µg/ml of Ezetimibe.

Pipette out 5 ml of above standard stock solution and transfer into 50 ml volumetric flask and dilute to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe.

Test Preparation:

Weigh accurately 20 tablets and find out the average weight. Weigh accurately 10 tablets and transfer into 200 ml volumetric flask. Add 140 ml of diluent into the flask and sonicate of 30 minutes with normal hand-shaking. Cool the flask to room temperature and dilute to volume with diluent. Filter 10 ml of this solution through 0.45 µm nylon syringe filter. The concentration obtained is 500 µg/ml of Ezetimibe.

Pipette out 5 ml of above test stock solution and transfer into 50 ml volumetric flask and dilute to volume with diluent. The concentration obtained is 50 µg/ml of

Ezetimibe.

System suitability:

- % RSD for replicate standard preparation should be less than 2.0 % for analyte peak.
- Asymmetry of the analyte peak should be less than 2.0 in standard preparation.
- Theoretical plates of the analyte peak should be more than 5000 in standard preparation.

Procedure:

- Inject the blank preparation and record the chromatogram.
- Inject five replicate of standard preparation and check for the system suitability and record the chromatograms.
- Inject test preparation in duplicate and record the chromatograms.
- Calculate the % assay of the sample.

Calculation:

Calculate the % assay of the sample using following formula:

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

Where by,

A_T = Average area of Ezetimibe obtained in test preparation

A_S = Average area of Ezetimibe obtained in standard preparation

W_1 = Weight taken of Ezetimibe reference standard (mg)

W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

LC = Label claim (mg)

P = Potency of Ezetimibe reference standard (%)

4.3.4 Product Information

(1) Name:- Ezetimibe tablets (2) Label claim:- 10 mg

4.3.5 Reason for validation

To establish intended applicability of the developed analytical method

4.3.6 Validation Approach

Validation of analytical method will be done by using Ezetimibe tablets 10 mg formulation to establish by laboratory studies, that the performance characteristic of the method meets the requirement for the intended analytical application. The whole experiment of validation is applicable to above mentioned Standard Testing Procedure for determination of assay.

4.3.7 Chemicals and Reagents used in validation process

Acetonitrile (HPLC grade)

Methanol (HPLC grade)

Orthophosphoric acid (GR grade)

Water (HPLC grade)

Hydrochloric acid (GR grade)

Sodium hydroxide (GR grade)

Hydrogen peroxide (AR grade)

4.3.8 Validation Parameters

a) Specificity study
b) Linearity and range study
c) Limit of detection and Limit of quantitation study
d) Precision study
e) Accuracy study
f) Robustness study
g) Solution stability study
h) System suitability study

4.4 Validation procedure for assay determination of Ezetimibe

4.4.1 Specificity study

The evaluation of the specificity of the method was determined against placebo and stress (forced degradation) application. The interference of the excipients of the claimed placebo present in the pharmaceutical dosage form was derived from placebo solution. Further the specificity of the method toward the drug was established by means of the interference of the degradation products against drug during the forced degradation study.

Blank, standard and sample preparation was prepared as per analytical method.

Blank preparation:
Diluent was used as blank.

Standard preparation:
Stock solution: 50.9 mg of Ezetimibe reference standard was accurately weighed and transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask and the standard substance was dissolved by sonication for one minute. Then, volume of the flask was make up with diluent. The concentration obtained is 509 µg/ml of Ezetimibe.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.9 µg/ml of Ezetimibe.

Test Preparation:
Test stock solution: 10 Tablets were accurately weighed (1010.2 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceed for sonication of 30 minutes

with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is about 500 µg/ml of Ezetimibe.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is about 50 µg/ml of Ezetimibe.

Average weight of the tablets:
Randomly selected 20 tablets were weighed accurately (2030.0 mg) and the mean weight was calculated for the same. This mean weight (101.5 mg) was used as average weight through out all experiments.

Placebo preparation:
Stock solution: Placebo equivalent to 10 times of average placebo weight was weighed (915.4 mg) and transferred into 200 ml volumetric flask.140 ml of diluent was added into the volumetric flask and sonicated of 30 minutes with normal hand-shaking. The volumetric flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter.

5 ml of above placebo stock solution was pipette out and transferred into 50 ml volumetric flask followed by dilute to volume with diluent.

Application of the Stress (forced degradation) Study:

Stress study was carried out by application of chemical and physical forced degradation. To perform forced degradation study, the drug content equivalent to 50 mg was employed for acidic, alkaline and oxidant media and also for thermal and photolytic conditions. After the degradation treatments were

completed, the stress content were allowed to equilibrate to room temperature and diluted with diluent to attain 50 µg/ml concentration. Pattern of stress (degradation) conditions and preparation for same was described as under:

4.4.1.1 **Chemical forced degradation**

(i) Acidic Condition

Application: Acidic degradation study was performed by refluxing the drug content in 1 N HCl at 80˚ C for 1 hour and then the mixture was neutralized.

Sample preparation for acidic degradation:
Stock solution: 10 Tablets were accurately weighed (1016.2 mg) and transferred into 250 ml round bottom flask. About 20 ml of diluent was added into the round bottom flask and sonicated of 10 minutes with normal hand-shaking for dispersion the tablets. About 30 ml of 1 N HCl was added to the round bottom flask and the mixture was refluxed at 80˚ C for 1 hour. After, the mixture was cooled to room temperature and neutralized with NaOH solution. The mixture was transferred into 200 ml volumetric flask and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is about 500 µg/ml of Ezetimibe.

5 ml of above stock solution was pipette out and transferred into 50 ml volumetric flask followed by dilute to volume with diluent. The concentration obtained is about 50 µg/ml of Ezetimibe.

Placebo preparation and blank preparation was also performed for acidic degradation to identify the peaks which arise due to placebo and blank. Placebo and blank was subjected under same acid-stress condition as that of sample. Preparations are as under:

Placebo preparation for acidic degradation:

Stock solution: Placebo equivalent to 10 times of average placebo weight was weighed (914.6 mg) and transferred into 250 ml round bottom flask. About 20 ml of diluent was added into the round bottom flask and sonicated of 10 minutes with normal hand-shaking. About 30 ml of 1 N HCl was added to the round bottom flask and the mixture was refluxed at 80˚ C for 1 hour. After, the mixture was cooled to room temperature and neutralized with NaOH solution. The mixture was transferred into 200 ml volumetric flask and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter.

5 ml of above stock solution was pipette out and transferred into 50 ml volumetric flask followed by dilute to volume with diluent.

Blank preparation for acidic degradation:

Stock solution: About 20 ml of diluent and about 30 ml of 1 N HCl was added into the round bottom flask and the mixture was refluxed at 80˚ C for 1 hour. After, the mixture was cooled to room temperature and neutralized with NaOH solution. The mixture was transferred into 200 ml volumetric flask and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter.

5 ml of above stock solution was pipette out and transferred into 50 ml volumetric flask followed by dilute to volume with diluent.

(ii) Alkaline Condition

Application: Alkaline degradation study was performed by keeping the drug content in 0.1 N NaOH at room temperature for 90 minutes and then the mixture was neutralized.

Sample preparation for alkaline degradation:

Stock solution: 10 Tablets were accurately weighed (1017.7 mg) and transferred into 200 ml volumetric flask. About 20 ml of diluent was added into the volumetric flask and sonicated of 10 minutes with normal hand-shaking for dispersion the tablets. About 80 ml of 0.1 N NaOH was added to the volumetric flask and the mixture was kept at room temperature up to 90 minutes. After, the mixture was neutralized with HCl solution and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 μm nylon syringe filter. The concentration obtained is about 500 μg/ml of Ezetimibe.

5 ml of above stock solution was pipette out and transferred into 50 ml volumetric flask followed by dilute to volume with diluent. The concentration obtained is about 50 μg/ml of Ezetimibe.

Placebo preparation and blank preparation was also performed for alkaline degradation to identify the peaks which arise due to placebo and blank. Placebo and blank was subjected under same alkali-stress condition as that of sample. Preparations are as under:

Placebo preparation for alkaline degradation:

Stock solution: Placebo equivalent to 10 times of average placebo weight was weighed (917.6 mg) and transferred into 200 ml volumetric flask. About 20 ml of diluent was added into the volumetric flask and sonicated of 10 minutes with normal hand-shaking. About 80 ml of 0.1 N NaOH was added to the volumetric flask and the mixture was kept at room temperature up to 90 minutes. After, the mixture was neutralized with HCl solution and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 μm nylon syringe filter.

5 ml of above stock solution was pipette out and transferred into 50 ml volumetric flask followed by dilute to volume with diluent.

Blank preparation for alkali degradation:

Stock solution: About 20 ml of diluent and about 80 ml of 0.1 N NaOH was added into the 200 ml volumetric flask and the mixture was kept at room temperature up to 90 minutes. After, the mixture was neutralized with HCl solution and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter.

5 ml of above stock solution was pipette out and transferred into 50 ml volumetric flask followed by dilute to volume with diluent.

(iii) Oxidative Condition

Application: Oxidative degradation study was performed by refluxing the drug content in 3% *v/v* H_2O_2 at 80˚ C for 1 hour.

Sample preparation for oxidative degradation:

Stock solution: 10 Tablets were accurately weighed (1016.2 mg) and transferred into 250 ml round bottom flask. About 20 ml of diluent was added into the round bottom flask and sonicated of 10 minutes with normal hand-shaking for dispersion the tablets. About 30 ml of 3% *v/v* H_2O_2 was added to the round bottom flask and the mixture was refluxed at 80˚C for 1 hour. After, the mixture was cooled to room temperature. The mixture was transferred into 200 ml volumetric flask and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is about 500 µg/ml of Ezetimibe.

5 ml of above stock solution was pipette out and transferred into 50 ml volumetric flask followed by dilute to volume with diluent. The concentration obtained is about 50 µg/ml of Ezetimibe.

Placebo preparation and blank preparation was also performed for oxidative degradation to identify the peaks which arise due to placebo and blank. Placebo and blank was subjected under same oxidative-stress condition as that of sample. Preparations are as under:

Placebo preparation for oxidative degradation:

Stock solution: Placebo equivalent to 10 times of average placebo weight was weighed (914.9 mg) and transferred into 250 ml round bottom flask. About 20 ml of diluent was added into the round bottom flask and sonicated of 10 minutes with normal hand-shaking. About 30 ml of 3% *v/v* H_2O_2 was added to the round bottom flask and the mixture was refluxed at 80˚ C for 1 hour. After, the mixture was cooled to room temperature. The mixture was transferred into 200 ml volumetric flask and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter.

5 ml of above stock solution was pipette out and transferred into 50 ml volumetric flask followed by dilute to volume with diluent.

Blank preparation for oxidative degradation:

Stock solution: About 20 ml of diluent and about 30 ml of 3% *v/v* H_2O_2 was added into the round bottom flask and the mixture was refluxed at 80˚ C for 1 hour. After, the mixture was cooled to room temperature. The mixture was transferred into 200 ml volumetric flask and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter.

5 ml of above stock solution was pipette out and transferred into 50 ml volumetric flask followed by dilute to volume with diluent.

4.4.1.2 **Physical forced degradation**

(i) Thermal Condition

Application: Thermal degradation study was performed by keeping the powdered drug content at 70˚ C for 72 hours.

Sample preparation for thermal degradation:
Stock solution: 20 Tablets were taken and then powdered. The powdered drug content was kept at 70• C for 72 hours. Thermally degraded drug content equivalent to 10 times of average weight was weighed (1015.7 mg) and transferred into 200 ml volumetric flask. About 140 ml of diluent was added into the volumetric flask and sonicated of 30 minutes with normal hand-shaking. After, the mixture was cooled to room temperature and diluted to volume. 10 ml of this solution was filtered through 0.45 μm nylon syringe filter. The concentration obtained is about 500 μg/ml of Ezetimibe.

5 ml of above stock solution was pipette out and transferred into 50 ml volumetric flask followed by dilute to volume with diluent. The concentration obtained is about 50 μg/ml of Ezetimibe.

Placebo preparation was also performed for thermal degradation to identify the peaks which arise due to placebo. Placebo was subjected under same thermal-stress condition as that of sample. Preparation is as under:

Placebo preparation for thermal degradation:
Stock solution: Placebo equivalent to 10 times of average weight (916.4 mg) which was previously kept at 70• C for 72 hours was weighed and transferred into 200 ml volumetric flask. About 140 ml of diluent was added into the volumetric flask and sonicated of 30 minutes with normal hand-shaking. After, the mixture

was cooled to room temperature and diluted to volume. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter.

5 ml of above stock solution was pipette out and transferred into 50 ml volumetric flask followed by dilute to volume with diluent.

Blank preparation for thermal degradation:
Diluent was used as blank.

(v) Photolytic Condition

Application: Photolytic degradation study was performed by exposing the powdered drug content in sun-light for 72 hour.

Sample preparation for photolytic degradation:
Stock solution: 20 Tablets were taken and then powdered. The powdered drug content was exposed to sun-light for 72 hours. Photolytically degraded drug content equivalent to 10 times of average weight was weighed (1016.1 mg) and transferred into 200 ml volumetric flask. About 140 ml of diluent was added into the volumetric flask and sonicated of 30 minutes with normal hand-shaking. After, the mixture was cooled to room temperature and diluted to volume. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is about 500 µg/ml of Ezetimibe.

5 ml of above stock solution was pipette out and transferred into 50 ml volumetric flask followed by dilute to volume with diluent. The concentration obtained is about 50 µg/ml of Ezetimibe.

Placebo preparation was also performed for photolytic degradation to identify the peaks which arise due to placebo. Placebo was subjected under same photolytic-stress condition as that of sample. Preparation is as under:

Placebo preparation for photolytic degradation:

Stock solution: Placebo equivalent to 10 times of average placebo weight (915.8 mg) which was previously exposed at sun-light for 72 hours was weighed and transferred into 200 ml volumetric flask. About 140 ml of diluent was added into the volumetric flask and sonicated of 30 minutes with normal hand-shaking. After, the mixture was cooled to room temperature and diluted to volume. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter.

5 ml of above stock solution was pipette out and transferred into 50 ml volumetric flask followed by dilute to volume with diluent.

Blank preparation for photolytic degradation:

Diluent was used as blank.

The peak purity index of drug in all above stress samples of test preparations (*i.e.*, acidic, alkali, oxidative, thermal and photolytic) was measured using PDA detector.

Chromatographic sequence for Specificity study is represented through Table 1:

Table 1: Sequence of Specificity study

No.	Description	Injection replicate	Remark
1	Blank	1	As Such
2	Standard preparation	5	
3	Test preparation	2	
4	Bracketing standard	1	
5	Blank preparation of Acidic stress	1	Acidic forced degradation
6	Placebo preparation of Acidic stress	1	
7	Test preparation of Acidic stress	2	
8	Bracketing standard	1	
9	Blank preparation of Alkali stress	1	Alkali forced degradation
10	Placebo preparation of Alkali stress	1	
11	Test preparation of Alkali stress	2	
12	Bracketing standard	1	
13	Blank preparation of Oxidative stress	1	Oxidative forced degradation
14	Placebo preparation of Oxidative stress	1	
15	Test preparation of Oxidative stress	2	
16	Bracketing standard	1	
17	Blank preparation of thermal stress	1	Thermal forced degradation
18	Placebo preparation of thermal stress	1	
19	Test preparation of thermal stress	2	
20	Bracketing standard	1	
21	Blank preparation of photolytic stress	1	Photolytic forced degradation
22	Placebo preparation of photolytic stress	1	
23	Test preparation of photolytic stress	2	
24	Bracketing standard	1	

Observation, calculation and chromatograms:

Table 2: Summary of standard and test preparation

Observation				
Data for Standard preparation			*Data for Test preparation*	
Replicate	Area		Replicate	Area
1	2677187		1	2630628
2	2673551		2	2628587
3	2674330		Average	2629608
4	2673621			
5	2672927			
Average	2674323			
Stdev	1676.32			
% RSD	0.06			
Standard weight	50.9 mg		Test weight	1010.2 mg
Standard potency	99.8 %		Label claim	10 mg

% Assay calculation for test sample is as under:

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2629608}{2674323} x \frac{50.9}{100} x \frac{5}{50} x \frac{200}{1010.2} x \frac{50}{5} x \frac{101.5}{10} x\, 99.8$$

$$= 100.4$$

Where by,

A_T = Average area of Ezetimibe obtained in test preparation

A_S = Average area of Ezetimibe obtained in standard preparation

W_1 = Weight taken of Ezetimibe reference standard (mg)

W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

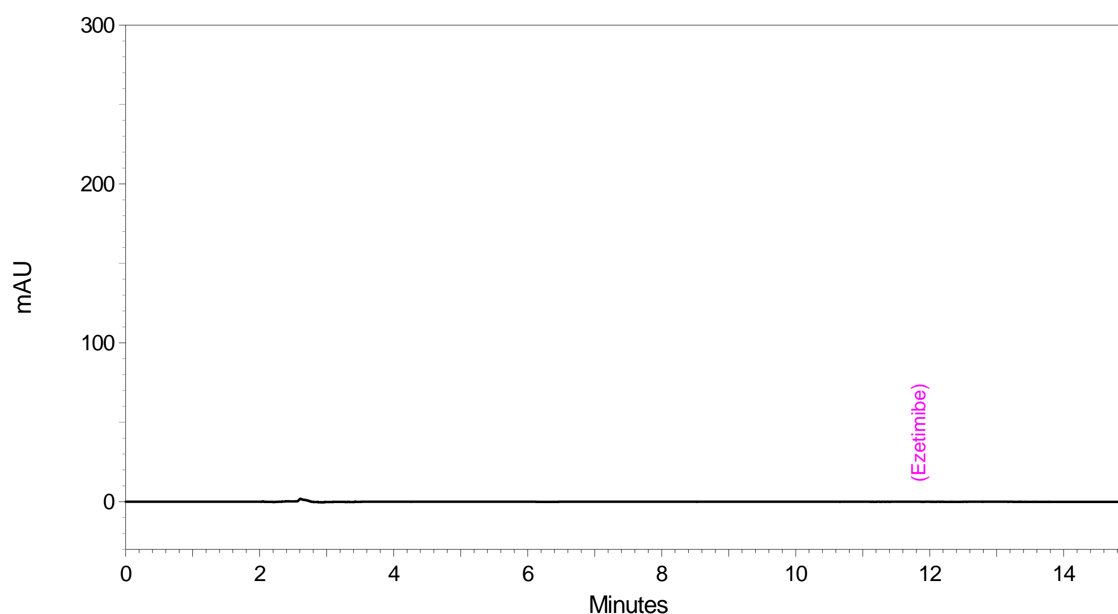
LC = Label claim (mg)

P = Potency of Ezetimibe reference standard (%)

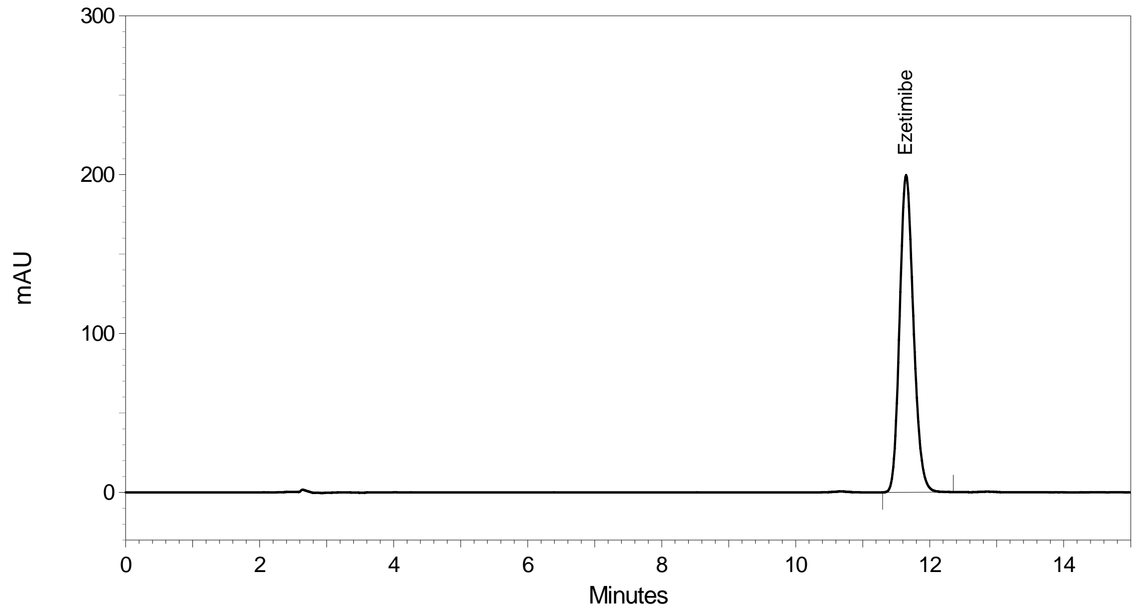
Table 3. Summary of forced degradation profile in specificity study

Degradation condition	Peak purity	Total Degradation, %	Major impurity, %	RRT of major impurity
Acidic	1.0000	73.89	36.58	1.6
Alkali	1.0000	23.87	20.74	1.6
Oxidative	1.0000	8.75	2.05	1.6
Thermal	1.0000	0.94	0.32	0.3
Photolytic	1.0000	3.90	2.31	0.3

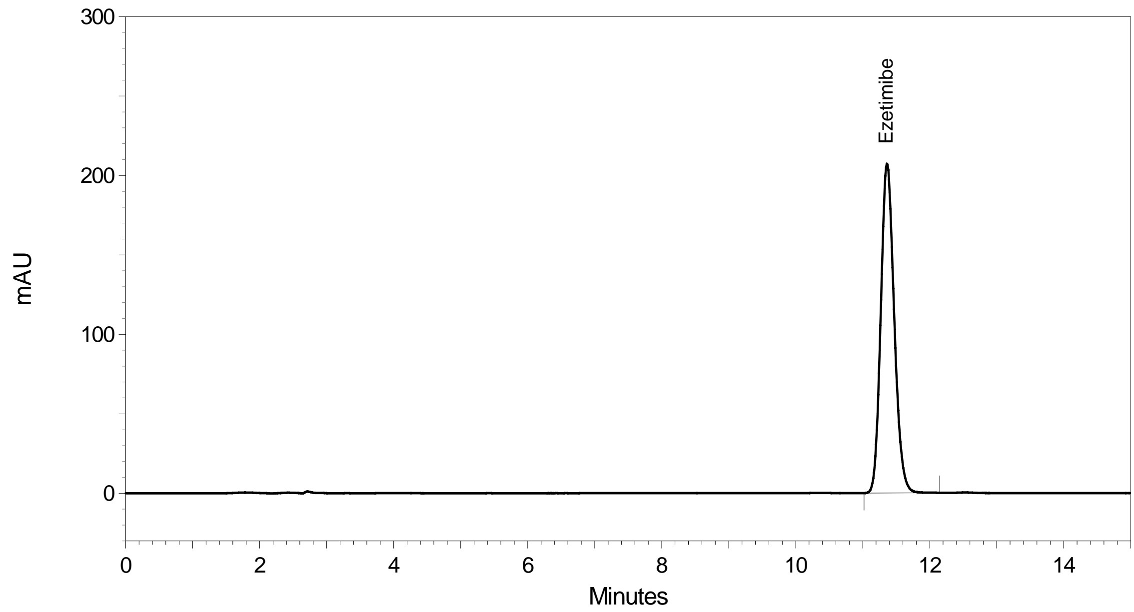
Chromatogram of blank preparation of specificity study:



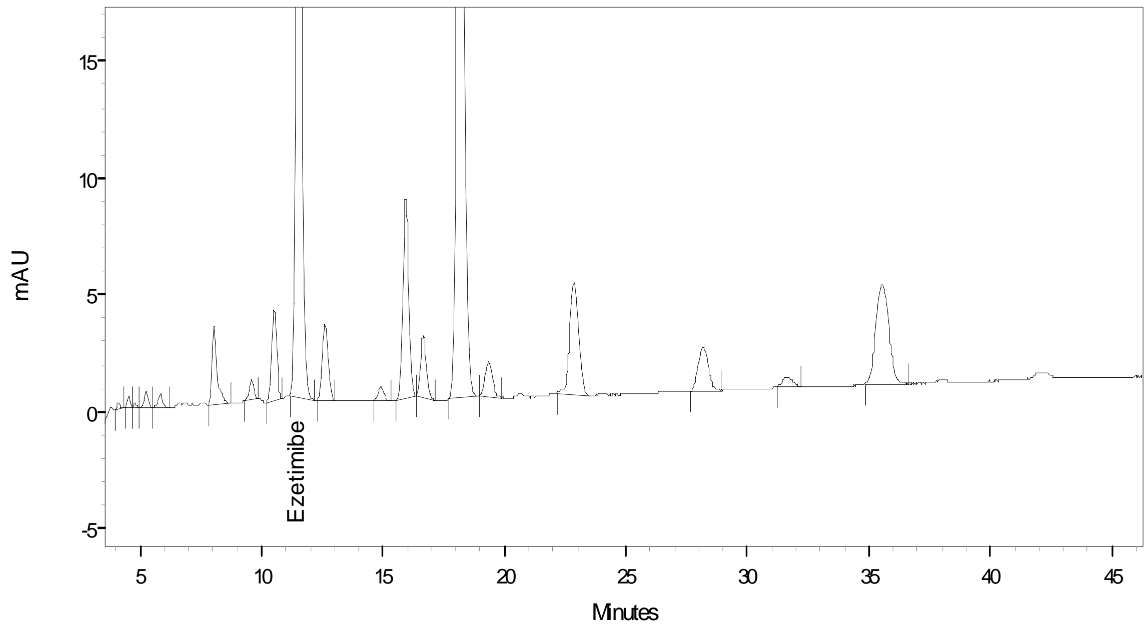
Chromatogram of standard preparation of specificity study:



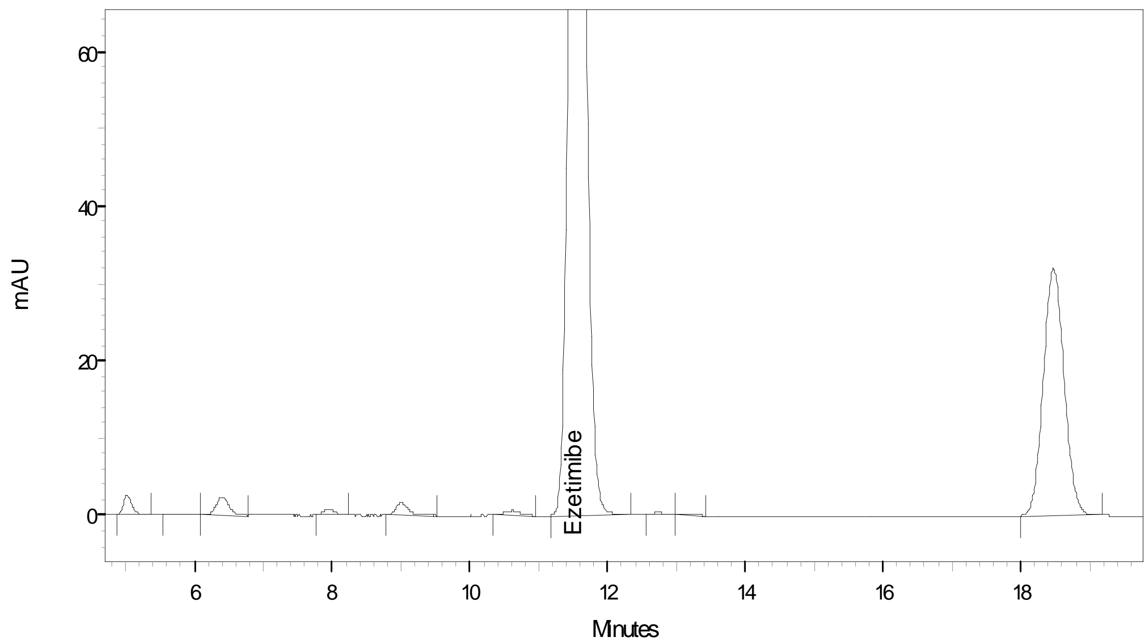
Chromatogram of test preparation of specificity study:



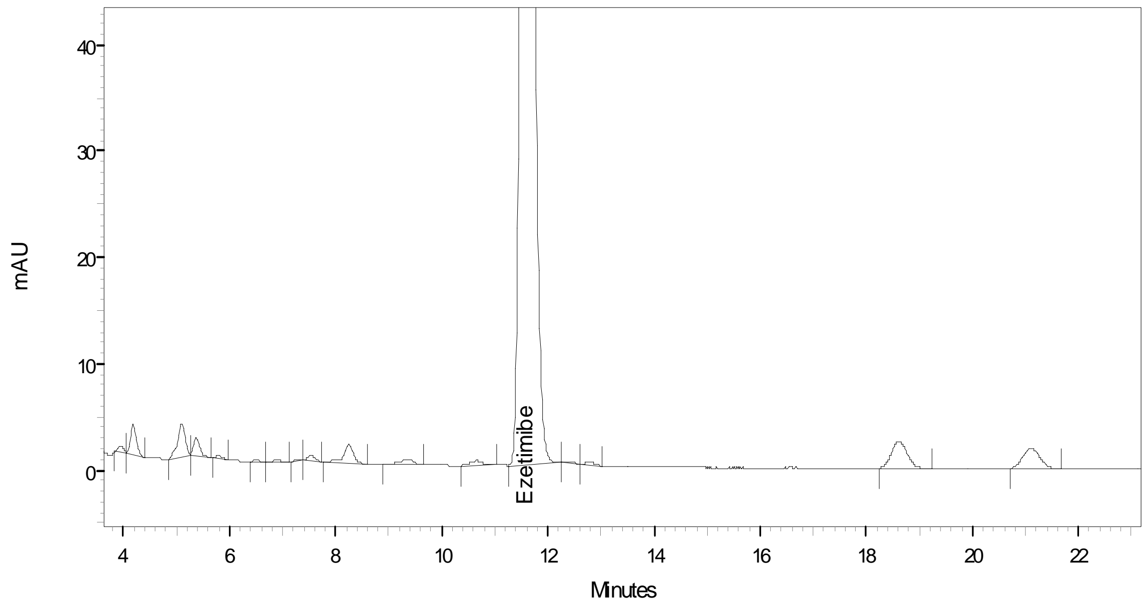
Chromatogram of acidic stress test preparation of specificity study (Zoom view):



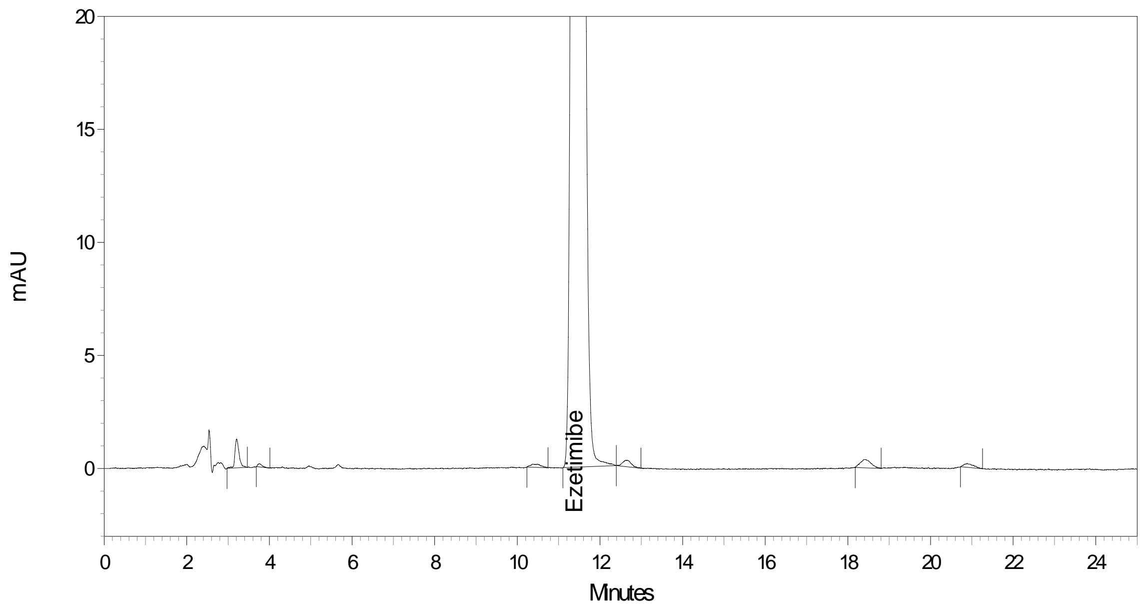
Chromatogram of alkali stress test preparation of specificity study (Zoom view):



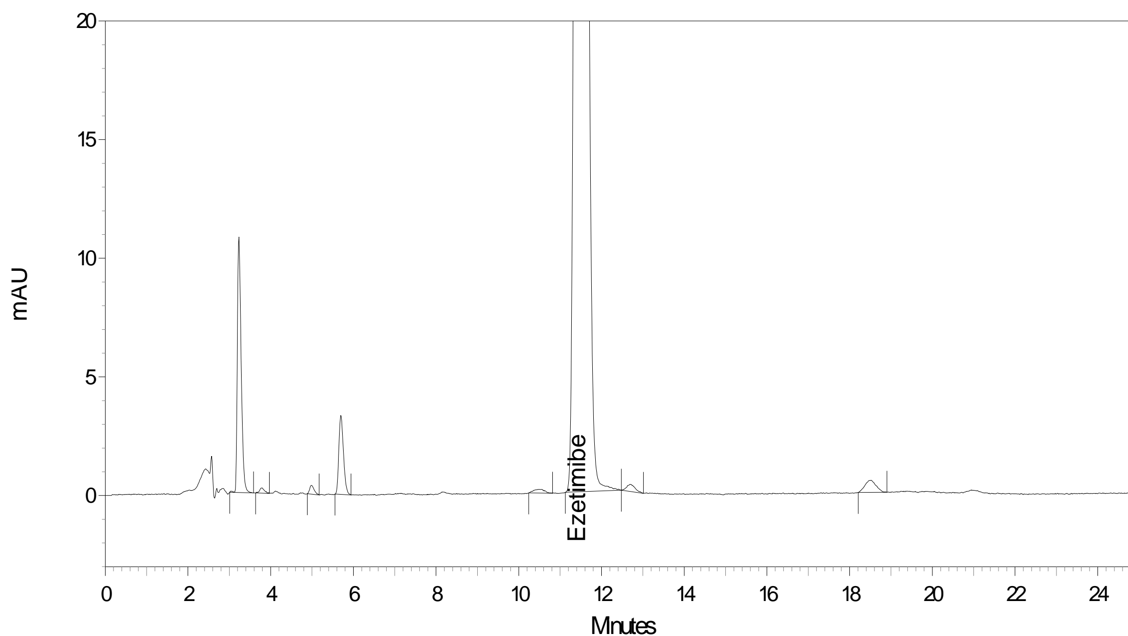
Chromatogram of oxidative stress test preparation of specificity study (Zoom view):



Chromatogram of thermal stress test preparation of specificity study (Zoom view):



Chromatogram of photolytic stress test preparation of specificity study (Zoom view):



Acceptance criteria:

1) There should not be any interference from blank peaks, placebo peaks and impurity peaks with the analyte peak in test preparation and stress test preparations.
2) The peak purity of the analyte peak in standard preparation, test preparation and stress test preparations should be more than 0.995

Results:

1) There is no any interference of blank peaks, placebo peaks and impurity peaks with the analyte peak in test preparation and stress test preparations.
2) The peak purity of the analyte peak in standard preparation, test preparation and stress test preparations is well with-in the acceptance criteria.

Conclusion:

All the results are well with-in the acceptance criteria. Hence, the analytical method is found specific.

4.4.2 **Linearity and range study**

The linearity plot was prepared with 7 concentration levels (20, 30, 40, 50, 60, 70, and 80 µg/ml of Ezetimibe). These concentration levels were respectively corresponding to 40, 60, 80, 100, 120, 140, and 160% of test solution concentration.

Stock solution for Linearity study was prepared and further diluted to attain concentration of about 40, 60, 80, 100, 120, 140, and 160% of test solution concentration.

Blank and standard preparation is prepared as per method as under:

Blank preparation:
Diluent was used as blank.

Standard preparation:
Stock solution: 50.1 mg of Ezetimibe reference standard was accurately weighed and transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask and the standard substance was dissolved by sonication for one minute. Then, volume of the flask was making up with diluent. The concentration obtained is 501 µg/ml of Ezetimibe.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.1 µg/ml of Ezetimibe.

Sample preparations for linearity levels are as under:

Stock Solution for Linearity:

50.3 mg of Ezetimibe standard was weighed and transferred into 100 ml volumetric flask. About 70 ml of diluent was added into the volumetric flask and substance was dissolved by sonication of one minute. After, the mixture was diluted to volume with diluent. The concentration obtained is 503 µg/ml of Ezetimibe.

Linearity Level 1 (40%):-

2 ml of stock solution was pipette out and transferred into 50 ml volumetric flak. The solution was diluted to volume with diluent. The concentration obtained is 20.12 µg/ml of Ezetimibe.

Linearity Level 2 (60%):-

3 ml of stock solution was pipette out and transferred into 50 ml volumetric flak. The solution was diluted to volume with diluent. The concentration obtained is 30.18 µg/ml of Ezetimibe.

Linearity Level 3 (80%):-

4 ml of stock solution was pipette out and transferred into 50 ml volumetric flak. The solution was diluted to volume with diluent. The concentration obtained is 40.24 µg/ml of Ezetimibe.

Linearity Level 4 (100%):-

5 ml of stock solution was pipette out and transferred into 50 ml volumetric flak. The solution was diluted to volume with diluent. The concentration obtained is 50.30 µg/ml of Ezetimibe.

Linearity Level 5 (120%):-

6 ml of stock solution was pipette out and transferred into 50 ml volumetric flak. The solution was diluted to volume with diluent. The concentration obtained is 60.36 µg/ml of Ezetimibe.

Linearity Level 6 (140%):-

7 ml of stock solution was pipette out and transferred into 50 ml volumetric flak. The solution was diluted to volume with diluent. The concentration obtained is 70.42 µg/ml of Ezetimibe.

Linearity Level 7 (160%):-

8 ml of stock solution was pipette out and transferred into 50 ml volumetric flak. The solution was diluted to volume with diluent. The concentration obtained is 80.48 µg/ml of Ezetimibe.

For each linearity level, the solution was injected in duplicate. Linearity was evaluated by linear regression analysis.

Chromatographic sequence for Linearity study is represented through Table 4 as under:

Table 4: Sequence of Linearity and range study

Sr. No.	Description	Injection replicate
1	Blank	1
2	Standard preparation	5
3	Linearity level 1 (40 %)	2
4	Linearity level 2 (60 %)	2
5	Linearity level 3 (80 %)	2
6	Linearity level 4 (100 %)	2
7	Linearity level 5 (120 %)	2
8	Linearity level 6 (140 %)	2
9	Linearity level 7 (160 %)	2
10	Bracketing standard	1

Observation, calculation and chromatograms:

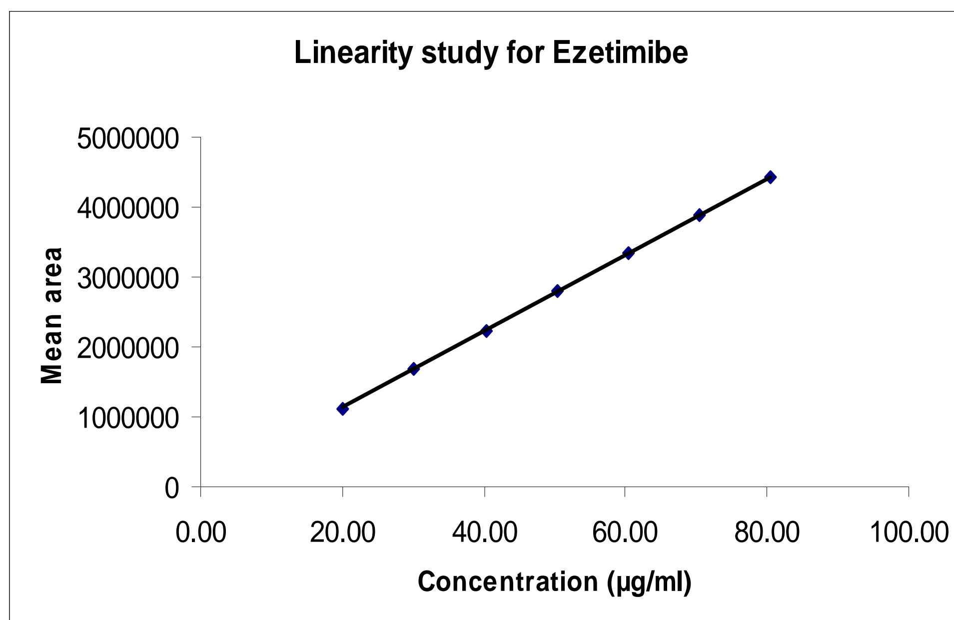
Table 5: Summary of Linearity and range study

Observation				
Data for Standard preparation				
Replicate	Area		Standard weight	50.1 mg
1	2784996		Standard potency	99.8 %
2	2783794			
3	2778503			
4	2783355			
5	2782413			
Average	2782612			
Stdev	2477.71			
% RSD	0.09			
Data for Linearity Level preparations				
Linearity Level	Replicate	Area	Mean area	
Level 1 (40 %)	1	1114515	1113461	
	2	1112406		
Level 2 (60 %)	1	1689608	1690737	
	2	1691866		
Level 3 (80 %)	1	2234876	2236387	
	2	2237897		
Level 4 (100 %)	1	2804842	2803771	
	2	2802699		
Level 5 (120 %)	1	3336594	3334340	
	2	3332086		
Level 6 (140 %)	1	3894257	3892370	
	2	3890482		
Level 7 (160 %)	1	4412854	4418228	
	2	4423602		

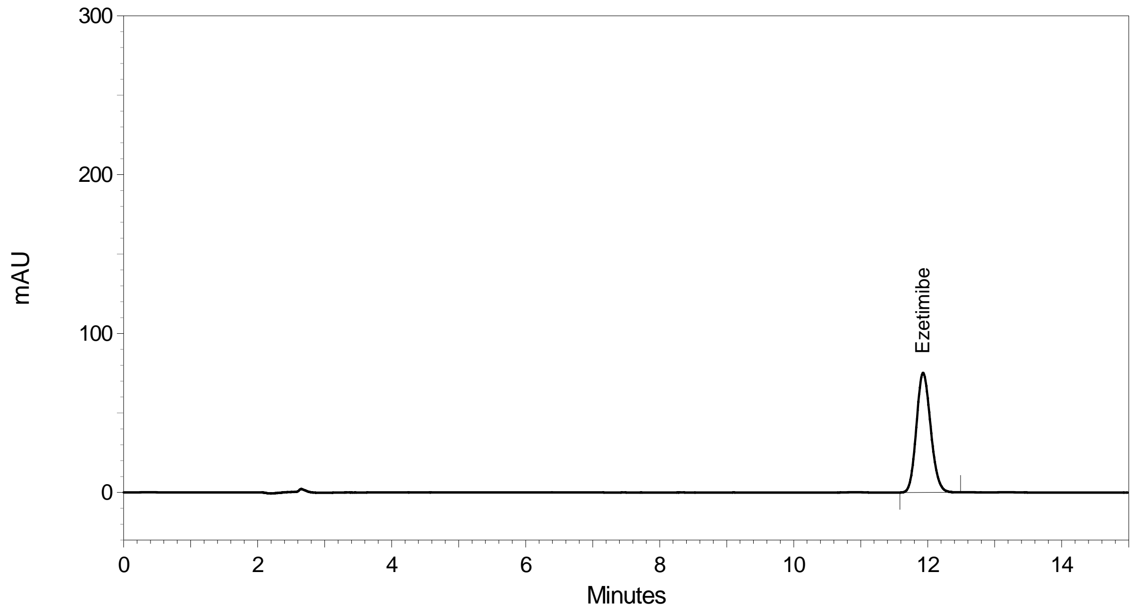
Table 6: Summary of concentration and linearity evaluation

Linearity Level	% of Level	Concentration (µg/ml)	Mean area
1	40	20.12	1113461
2	60	30.18	1690737
3	80	40.24	2236387
4	100	50.30	2803771
5	120	60.36	3334340
6	140	70.42	3892370
7	160	80.48	4418228
		Correlation co-efficient	0.9999
		Slope	54727.06
		Intercept	31412.96

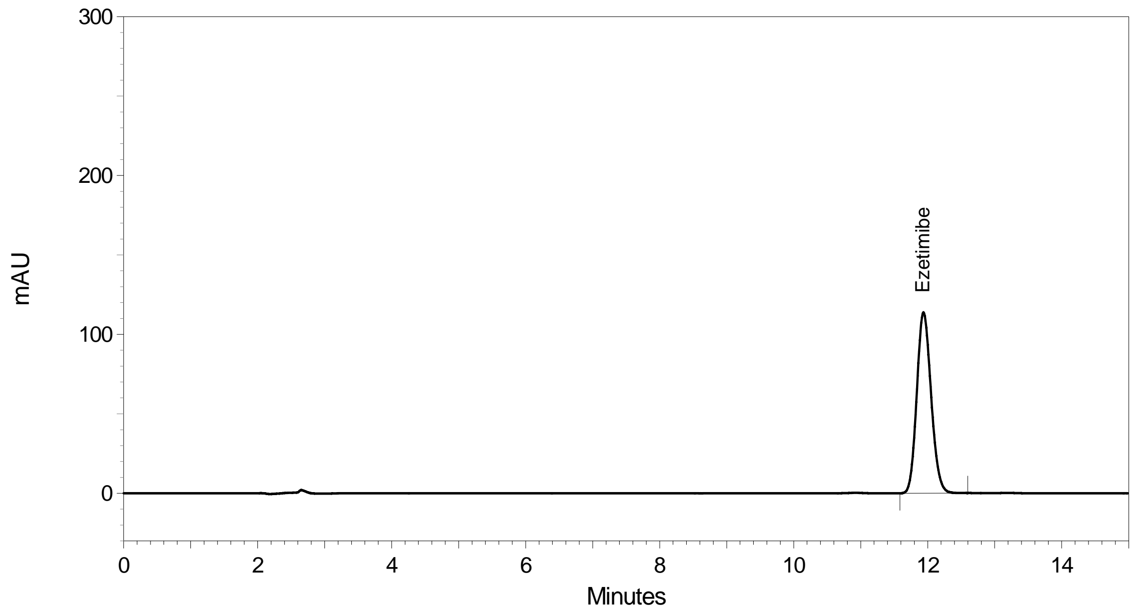
Chart 1: Evaluation of linearity



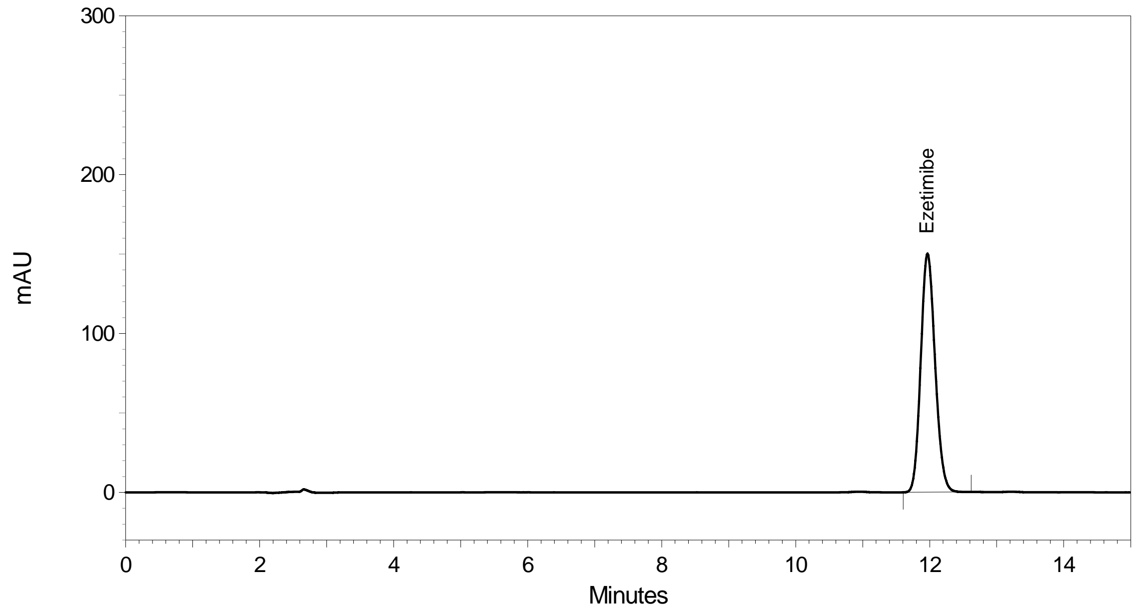
Chromatogram of 40% Linearity level:



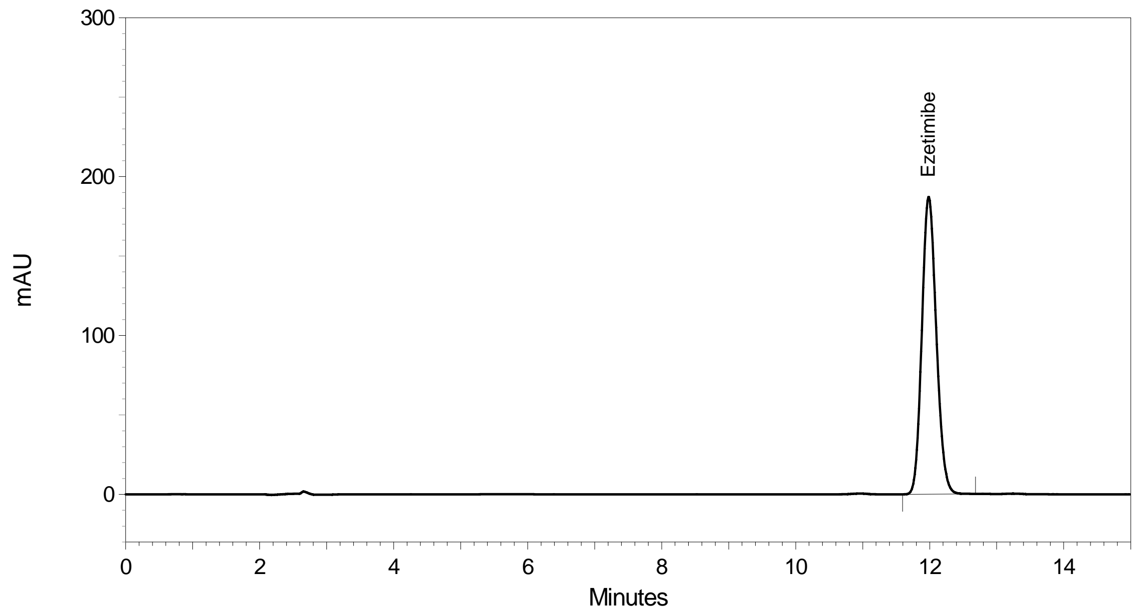
Chromatogram of 60% Linearity level:



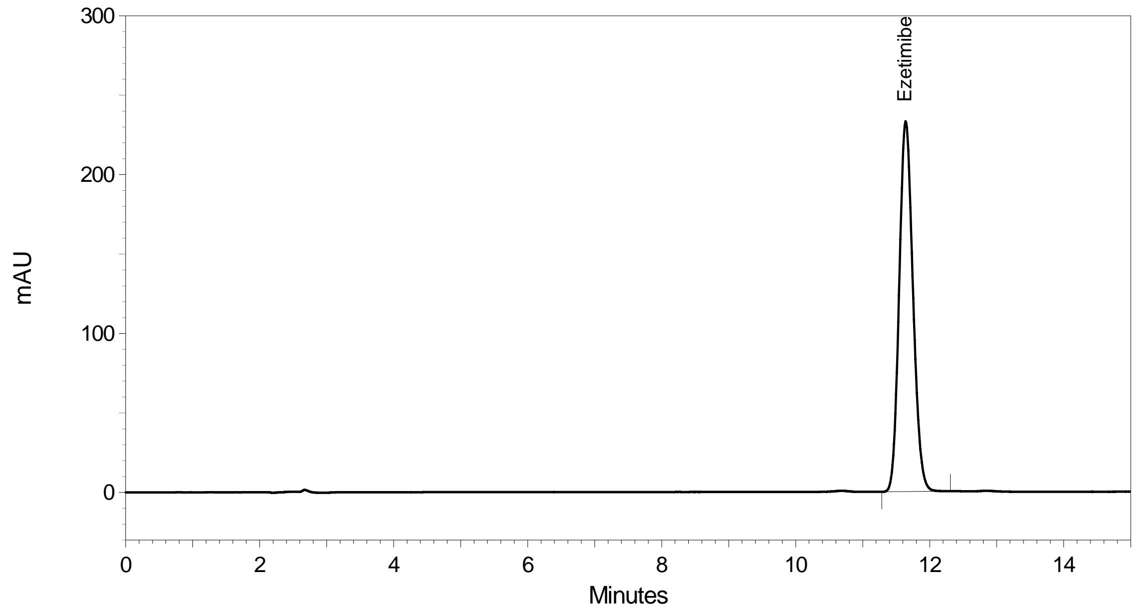
Chromatogram of 80% Linearity level:



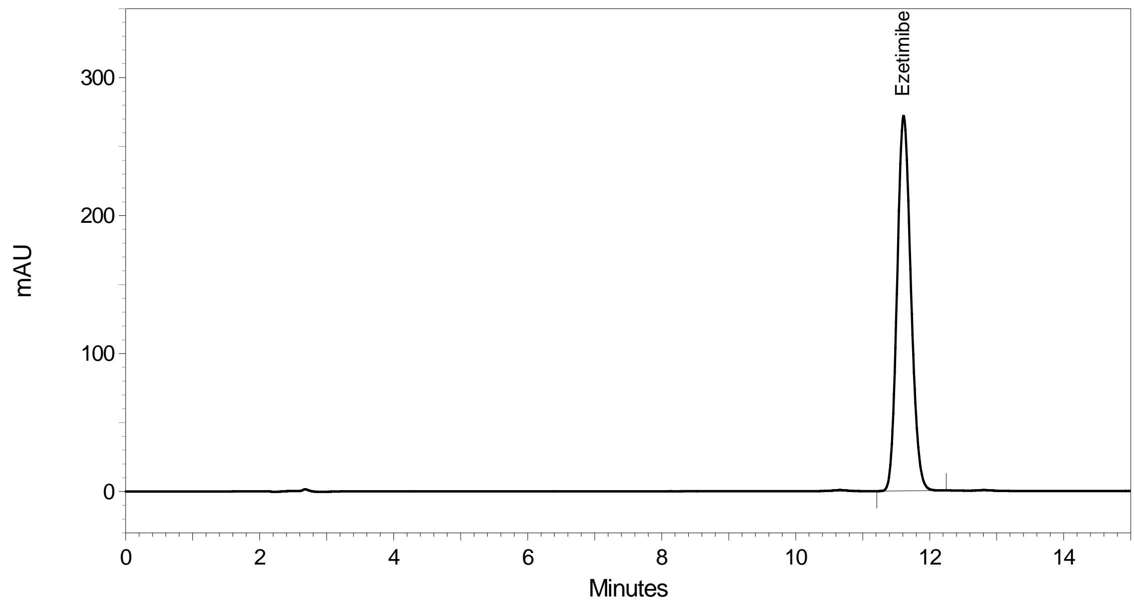
Chromatogram of 100% Linearity level:



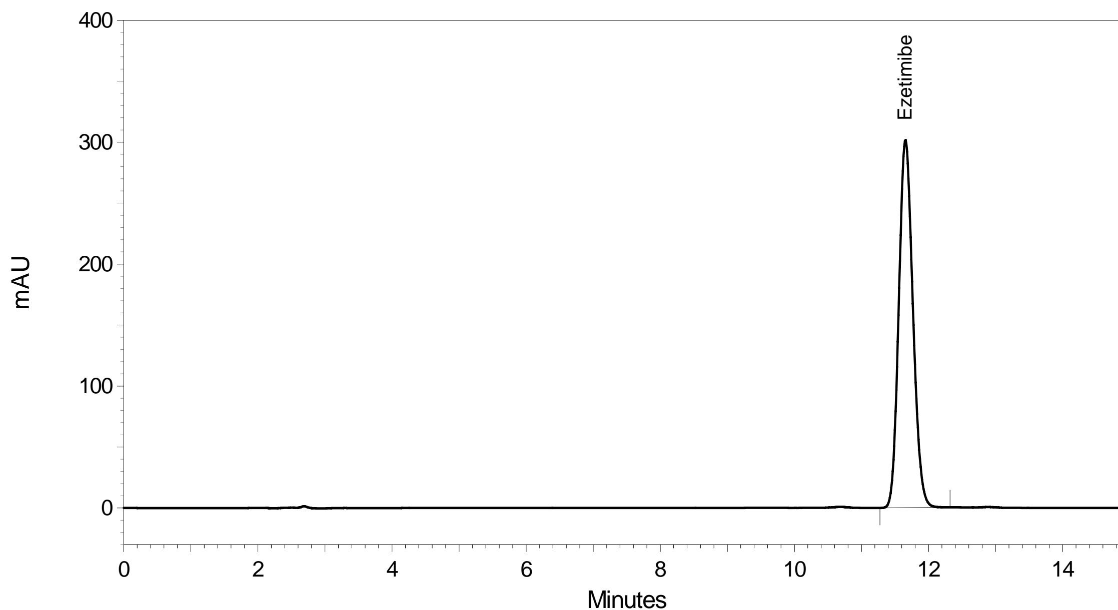
Chromatogram of 120% Linearity level:



Chromatogram of 140% Linearity level:



Chromatogram of 160% Linearity level:



Acceptance criteria:

The correlation coefficient value should not be less than 0.995 over the working range.

Results:

The correlation coefficient value of the analytical method is 0.9999 over the working range of 20 µg/ml to 80 µg/ml.

Conclusion:

All the results are well with-in the acceptance criteria. Hence, the analytical method is found linear.

4.4.3 **Limit of detection and Limit of quantitation study**

The limit of detection (LOD) and limit of quantitation (LOQ) were determined by calculating the signal to noise (S/N) ratio of the LOD preparation and LOQ preparation.

LOQ value is precised by six replicate injections and checked for linear response with respect to other linearity levels by extended linearity curve.

For LOD and LOQ study, blank, standard preparation, LOD preparation and LOQ preparation was prepared as under:

Blank preparation:
Diluent was used as blank.

Standard preparation:
Stock solution: 49.8 mg of Ezetimibe reference standard was accurately weighed and transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask and the standard substance was dissolved by sonication for one minute. Then, volume of the flask was make up with diluent. The concentration obtained is 498 µg/ml of Ezetimibe.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 49.8 µg/ml of Ezetimibe.

LOD and LOQ preparation:
Solution-A: 50.0 mg of Ezetimibe reference standard was accurately weighed and transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask and the standard substance was dissolved by sonication for one minute. Then, volume of the flask was make up with diluent. The concentration obtained is 500 µg/ml of Ezetimibe. 5 ml of this solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.0 µg/ml of Ezetimibe. 5 ml of this solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 5.0 µg/ml of Ezetimibe. This solution is designated as Solution-A.

LOD preparation:

1 ml of above Solution-A was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 0.1 µg/ml of Ezetimibe.

LOQ preparation:

2 ml of above Solution-A was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 0.2 µg/ml of Ezetimibe.

Chromatographic sequence for LOD and LOQ study is represented through Table 7 as under:

Table 7: Sequence of LOD and LOQ study

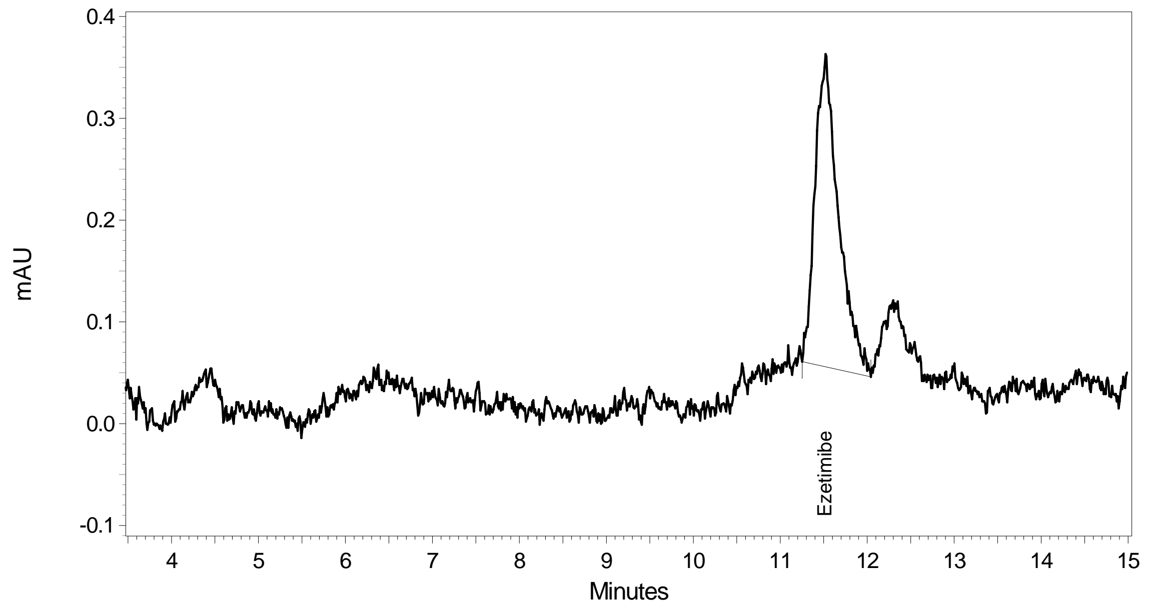
Sr. No.	Description	Injection replicate
1	Blank	1
2	Standard preparation	5
3	Blank	1
4	LOD preparation	2
5	LOQ preparation	6
6	Bracketing standard	1

<u>Observation, calculation and chromatograms:</u>

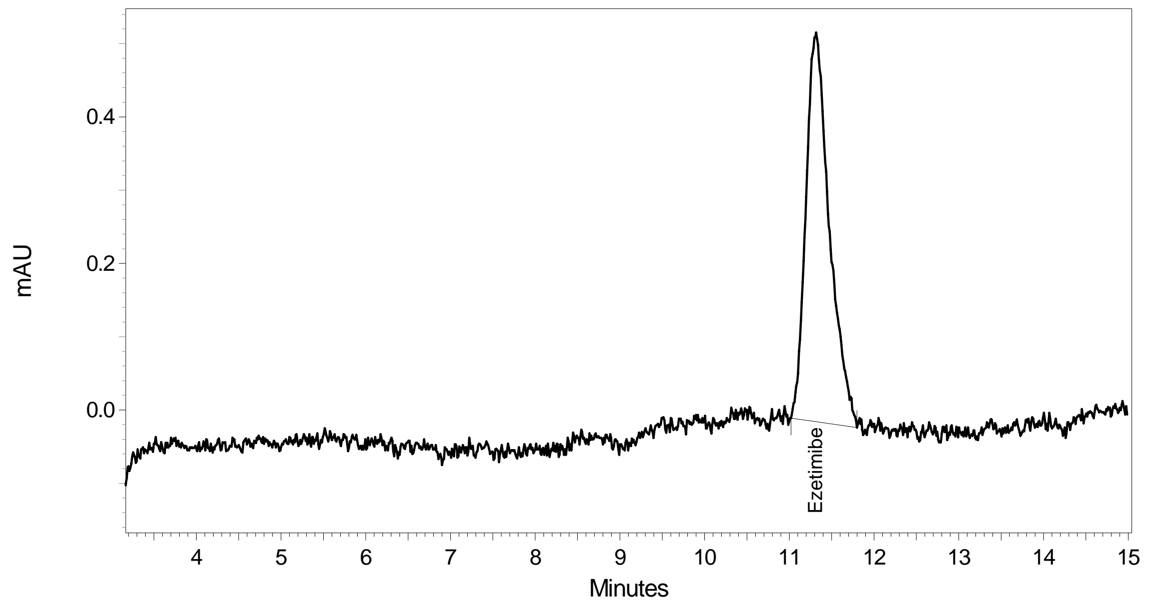
Table 8: Summary of LOQ study

Observation				
Data for Standard preparation			*Data for LOQ preparation*	
Replicate	Area		Replicate	Area
1	2724800		1	10312
2	2733707		2	10257
3	2731324		3	10275
4	2720542		4	10123
5	2723842		5	11050
Average	2726843		6	10277
Stdev	5478.97		Average	10382
% RSD	0.20		Stdev	333.56
			% RSD	3.21

Chromatogram of LOD preparation:



Chromatogram of LOQ preparation:



Signal-to-noise ratio calculation:

For LOD preparation:

$$\text{S/N ratio} = \frac{\textit{Signal Height}}{\textit{Noise Height}}$$

$$= \frac{3.8\text{ cm}}{1.0\text{ cm}}$$

$$= 3.8$$

For LOQ preparation:

$$\text{S/N ratio} = \frac{\textit{Signal Height}}{\textit{Noise Height}}$$

$$= \frac{4.8\text{ cm}}{0.4\text{ cm}}$$

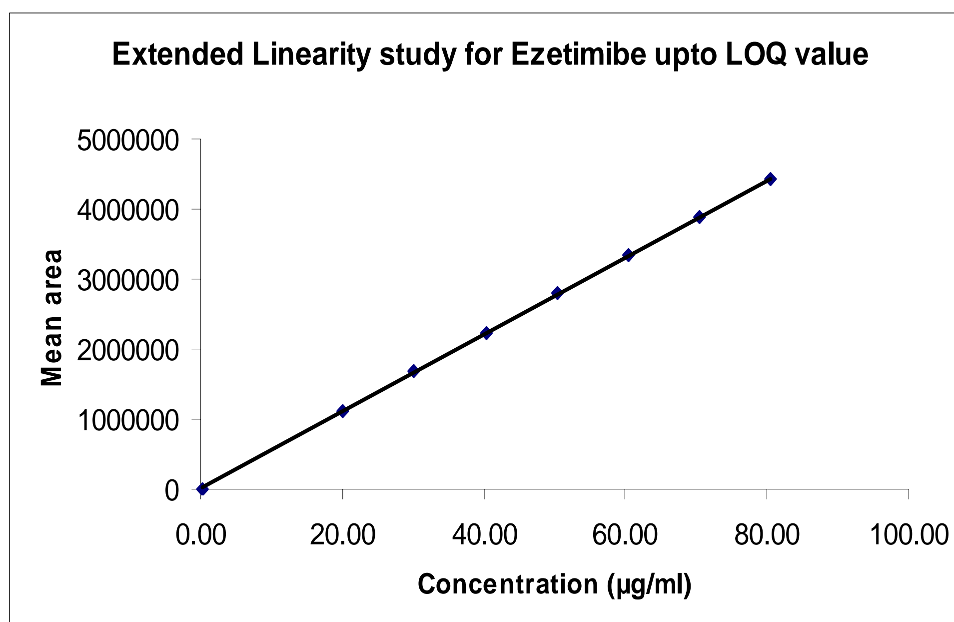
$$= 12.0$$

LOQ value of the analytical method is evaluated by establish linearity upto LOQ value. Hence linearity study is extended to LOQ value as under:

Table 9: Summary of LOQ study by evaluating linearity upto LOQ concentration

Linearity Level	% of Level	Concentration (µg/ml)	Mean area
1	LOQ	0.20	10382
2	40	20.12	1113461
3	60	30.18	1690737
4	80	40.24	2236387
5	100	50.30	2803771
6	120	60.36	3334340
7	140	70.42	3892370
8	160	80.48	4418228
		Correlation co-efficient	0.9999
		Slope	55005.75
		Intercept	15143.64

Chart 2: Confirmation of LOQ value by extended linearity study upto LOQ level



Acceptance criteria:

1) Signal-to-noise ratio for LOD level should not be less than 3.
2) Signal-to-noise ratio for LOQ level should not be less than 10.
3) % RSD of six replicate injections of LOQ level should be less than 15.

Results:

1) Signal-to-noise ratio for LOD level is 3.8
2) Signal-to-noise ratio for LOQ level is 12.0
3) % RSD of six replicate injections of LOQ level is 3.21

Conclusion:

All the results are well with-in the acceptance criteria. Hence, the LOD and LOQ values of the analytical method are 0.1 µg/ml and 0.2 µg/ml respectively which correspond to 0.2 % and 0.4 % of working concentration.

4.4.4 **Precision study**

Precision study was established by evaluating method precision and intermediate precision study.

Method precision of the analytical method was determined by analyzing six sets of sample preparation. Assay of all six replicate sample preparations was determined and mean % assay value, standard deviation, % relative standard deviation and 95% confidence interval for the same was calculated.

Intermediate precision of the analytical method was determined by performing method precision on another day by another analyst using different make of raw materials under same experimental condition. Assay of all six replicate sample preparations was determined and mean % assay value, standard deviation, % relative standard deviation and 95% confidence interval for the same was calculated. Overall assay value of method precision and intermediate precision was compared and % difference and overall % relative standard deviation was calculated.

For method precision, blank, standard preparation and six sets of test preparations was prepared as per method as under:

Blank preparation:
Diluent was used as blank.

Standard preparation:
Stock solution: 49.0 mg of Ezetimibe reference standard was accurately weighed and transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask and the standard substance was dissolved by sonication for one minute. Then, volume of the flask was make up with diluent. The concentration obtained is 490 µg/ml of Ezetimibe.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 49.0 µg/ml of Ezetimibe.

Test Preparation (Set 1):

Test stock solution: 10 Tablets were accurately weighed (1020.2 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is about 500 µg/ml of Ezetimibe.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is about 50 µg/ml of Ezetimibe.

Test Preparation (Set 2):

Test stock solution: 10 Tablets were accurately weighed (1024.8 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceeding for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is about 500 µg/ml of Ezetimibe.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is about 50 µg/ml of Ezetimibe.

Test Preparation (Set 3):

Test stock solution: 10 Tablets were accurately weighed (1014.0 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceeding for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is about 500 µg/ml of Ezetimibe.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is about 50 µg/ml of Ezetimibe.

Test Preparation (Set 4):

Test stock solution: 10 Tablets were accurately weighed (1012.8 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceeding for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is about 500 µg/ml of Ezetimibe.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is about 50 µg/ml of Ezetimibe.

Test Preparation (Set 5):

Test stock solution: 10 Tablets were accurately weighed (1020.0 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceeding for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room

temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is about 500 µg/ml of Ezetimibe.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is about 50 µg/ml of Ezetimibe.

Test Preparation (Set 6):

Test stock solution: 10 Tablets were accurately weighed (1019.8 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceeding for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is about 500 µg/ml of Ezetimibe.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is about 50 µg/ml of Ezetimibe.

Chromatographic sequence for Method precision study is represented through Table 10 as under:

Table 10: Sequence of Method precision study

Sr. No.	Description	Injection replicate
1	Blank	1
2	Standard preparation	5
3	Test Preparation (Set 1)	2
4	Test Preparation (Set 2)	2
5	Test Preparation (Set 3)	2
6	Test Preparation (Set 4)	2
7	Test Preparation (Set 5)	2
8	Test Preparation (Set 6)	2
9	Bracketing standard	1

For intermediate precision, blank, standard preparation and six sets of test preparations was prepared as per method as under:

Blank preparation:
Diluent was used as blank.

Standard preparation:
Stock solution: 49.1 mg of Ezetimibe reference standard was accurately weighed and transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask and the standard substance was dissolved by sonication for one minute. Then, volume of the flask was make up with diluent. The concentration obtained is 491 µg/ml of Ezetimibe.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 49.1 µg/ml of Ezetimibe.

Test Preparation (Set 1):
Test stock solution: 10 Tablets were accurately weighed (1017.8 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceeding for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is about 500 µg/ml of Ezetimibe.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is about 50 µg/ml of Ezetimibe.

Test Preparation (Set 2):

Test stock solution: 10 Tablets were accurately weighed (1008.2 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceeding for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is about 500 µg/ml of Ezetimibe.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is about 50 µg/ml of Ezetimibe.

Test Preparation (Set 3):

Test stock solution: 10 Tablets were accurately weighed (1025.0 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceeding for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is about 500 µg/ml of Ezetimibe.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is about 50 µg/ml of Ezetimibe.

Test Preparation (Set 4):

Test stock solution: 10 Tablets were accurately weighed (1015.4 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceeding for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room

temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is about 500 µg/ml of Ezetimibe.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is about 50 µg/ml of Ezetimibe.

Test Preparation (Set 5):

Test stock solution: 10 Tablets were accurately weighed (1012.2 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceeding for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is about 500 µg/ml of Ezetimibe.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is about 50 µg/ml of Ezetimibe.

Test Preparation (Set 6):

Test stock solution: 10 Tablets were accurately weighed (1017.4 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceeding for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is about 500 µg/ml of Ezetimibe.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is about 50 µg/ml of Ezetimibe.

Chromatographic sequence for Intermediate precision study is represented through Table 11 as under:

Table 11: Sequence of Intermediate precision study

Sr. No.	Description	Injection replicate
1	Blank	1
2	Standard preparation	5
3	Test Preparation (Set 1)	2
4	Test Preparation (Set 2)	2
5	Test Preparation (Set 3)	2
6	Test Preparation (Set 4)	2
7	Test Preparation (Set 5)	2
8	Test Preparation (Set 6)	2
9	Bracketing standard	1

Observation, calculation and chromatograms:

Table 12: Summary of method precision study

Observation				
Data for Standard preparation				
Replicate	Area		Standard weight	49.0 mg
1	2801242		Standard potency	99.8 %
2	2802113			
3	2799597			
4	2805735			
5	2805914			
Average	2802920			
Stdev	2801.67			
% RSD	0.10			

Data for Test preparations				
Set No.	Replicate	Area	Mean area	Weight of the sample
1	1	2900000	2900685	1020.2 mg
	2	2901370		
2	1	2947850	2948078	1024.8 mg
	2	2948305		
3	1	2904173	2906175	1014.0 mg
	2	2908177		
4	1	2911627	2918995	1012.8 mg
	2	2926363		
5	1	2902090	2899559	1020.0 mg
	2	2897027		
6	1	2917721	2919589	1019.8 mg
	2	2921456		

% Assay calculation for each set is as under:

For Set 1:-

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2900685}{2802920} x \frac{49.0}{100} x \frac{5}{50} x \frac{200}{1020.2} x \frac{50}{5} x \frac{101.5}{10} x\, 99.8$$

= 100.7

For Set 2:-

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2948078}{2802920} x \frac{49.0}{100} x \frac{5}{50} x \frac{200}{1024.8} x \frac{50}{5} x \frac{101.5}{10} x\, 99.8$$

= 101.9

For Set 3:-

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2906175}{2802920} x \frac{49.0}{100} x \frac{5}{50} x \frac{200}{1014.0} x \frac{50}{5} x \frac{101.5}{10} x\, 99.8$$

= 101.5

For Set 4:-

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2918995}{2802920} x \frac{49.0}{100} x \frac{5}{50} x \frac{200}{1012.8} x \frac{50}{5} x \frac{101.5}{10} x\, 99.8$$

= 102.1

For Set 5:-

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2899559}{2802920} x \frac{49.0}{100} x \frac{5}{50} x \frac{200}{1020.0} x \frac{50}{5} x \frac{101.5}{10} x\, 99.8$$

$$= 100.7$$

For Set 6:-

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2919589}{2802920} x \frac{49.0}{100} x \frac{5}{50} x \frac{200}{1019.8} x \frac{50}{5} x \frac{101.5}{10} x\, 99.8$$

$$= 101.4$$

Where by,

A_T = Average area of Ezetimibe obtained in test preparation

A_S = Average area of Ezetimibe obtained in standard preparation

W_1 = Weight taken of Ezetimibe reference standard (mg)

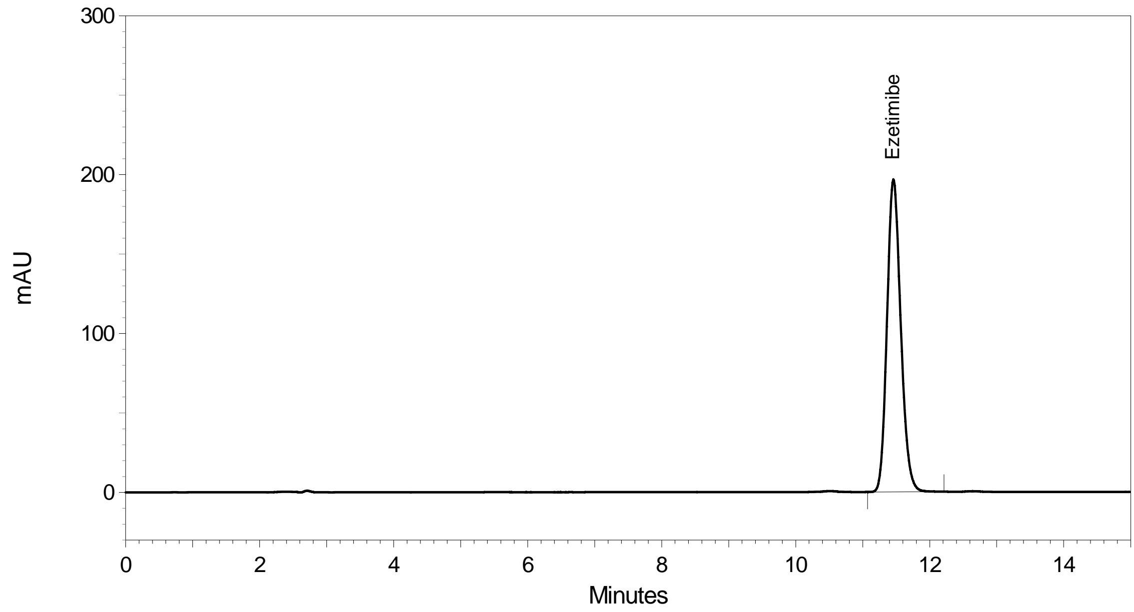
W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

LC = Label claim (mg)

P = Potency of Ezetimibe reference standard (%)

Chromatogram of standard preparation of method precision study:



Prototype chromatogram of test preparation (set 1) of method precision study:

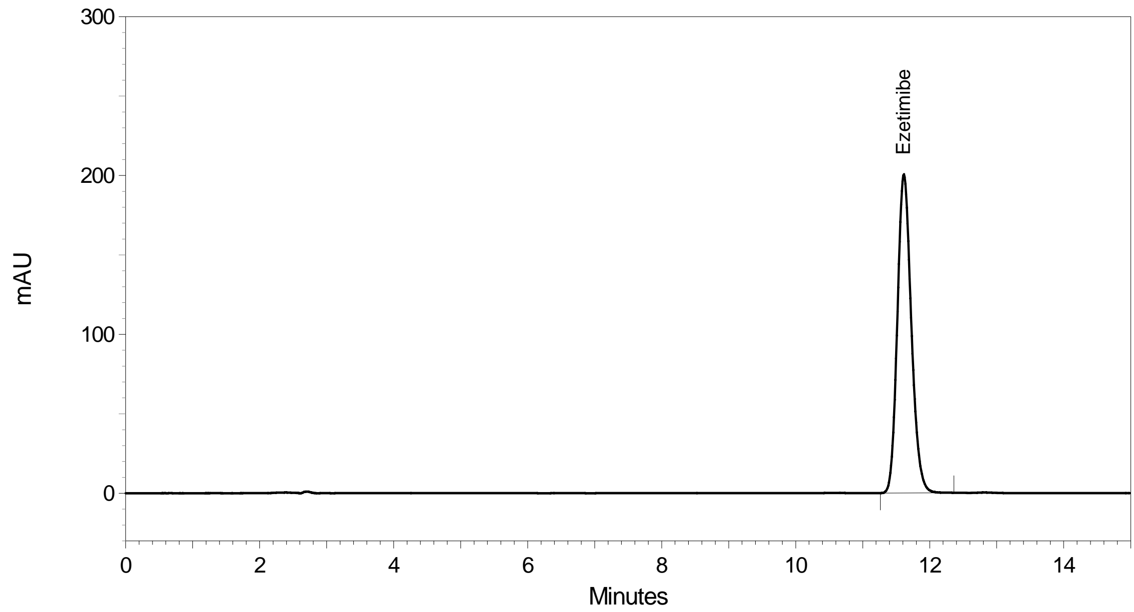


Table 13: Summary of Intermediate precision study

Observation				
Data for Standard preparation				
Replicate	Area		Standard weight	49.1 mg
1	2787178		Standard potency	99.8 %
2	2790033			
3	2783101			
4	2785738			
5	2785229			
Average	2786256			
Stdev	2568.70			
% RSD	0.09			

Data for Test preparations				
Set No.	Replicate	Area	Mean area	Weight of the sample
1	1	2886135	2880677	1017.8 mg
	2	2875218		
2	1	2889707	2890815	1008.2 mg
	2	2891922		
3	1	2874049	2872628	1025.0 mg
	2	2871206		
4	1	2887173	2887020	1015.4 mg
	2	2886867		
5	1	2872424	2883852	1012.2 mg
	2	2895279		
6	1	2907535	2905633	1017.4 mg
	2	2903730		

% Assay calculation for each set is as under:

For Set 1:-

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2880677}{2786256} x \frac{49.1}{100} x \frac{5}{50} x \frac{200}{1017.8} x \frac{50}{5} x \frac{101.5}{10} x\, 99.8$$

$= 101.0$

For Set 2:-

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2890815}{2786256} x \frac{49.1}{100} x \frac{5}{50} x \frac{200}{1008.2} x \frac{50}{5} x \frac{101.5}{10} x\, 99.8$$

$= 102.4$

For Set 3:-

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2872628}{2786256} x \frac{49.1}{100} x \frac{5}{50} x \frac{200}{1025.0} x \frac{50}{5} x \frac{101.5}{10} x\, 99.8$$

$= 100.1$

For Set 4:-

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2887020}{2786256} x \frac{49.1}{100} x \frac{5}{50} x \frac{200}{1015.4} x \frac{50}{5} x \frac{101.5}{10} x\, 99.8$$

$= 101.5$

For Set 5:-

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2883852}{2786256} x \frac{49.1}{100} x \frac{5}{50} x \frac{200}{1012.2} x \frac{50}{5} x \frac{101.5}{10} x\, 99.8$$

$= 101.7$

For Set 6:-

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2905633}{2786256} x \frac{49.1}{100} x \frac{5}{50} x \frac{200}{1017.4} x \frac{50}{5} x \frac{101.5}{10} x\, 99.8$$

$$= 102.0$$

Where by,

A_T = Average area of Ezetimibe obtained in test preparation

A_S = Average area of Ezetimibe obtained in standard preparation

W_1 = Weight taken of Ezetimibe reference standard (mg)

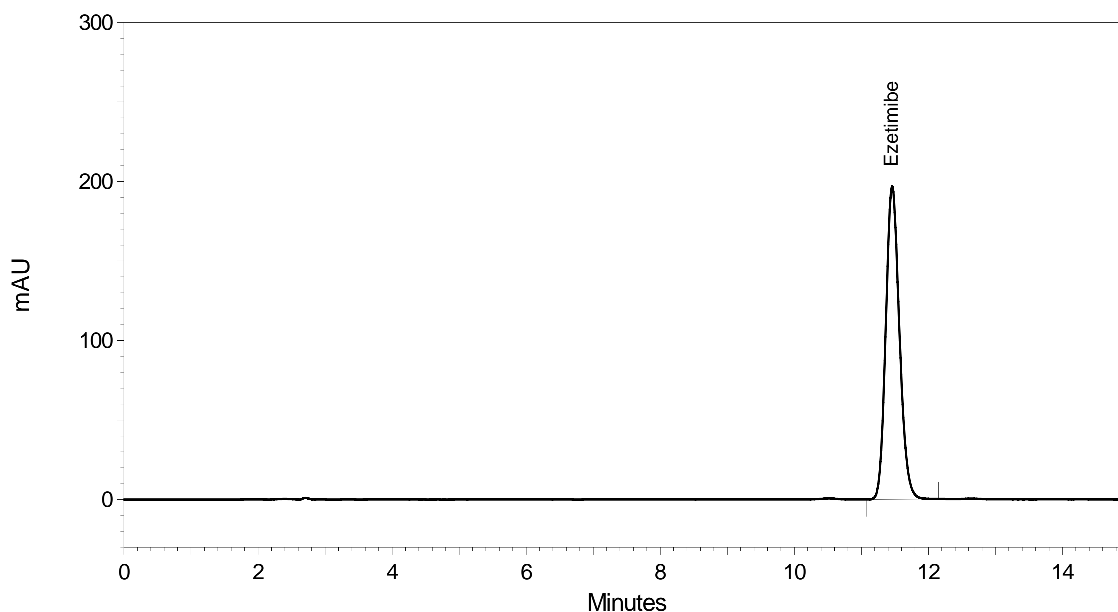
W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

LC = Label claim (mg)

P = Potency of Ezetimibe reference standard (%)

Chromatogram of standard preparation of intermediate precision study:



Prototype chromatogram of test preparation (set 1) of intermediate precision study:

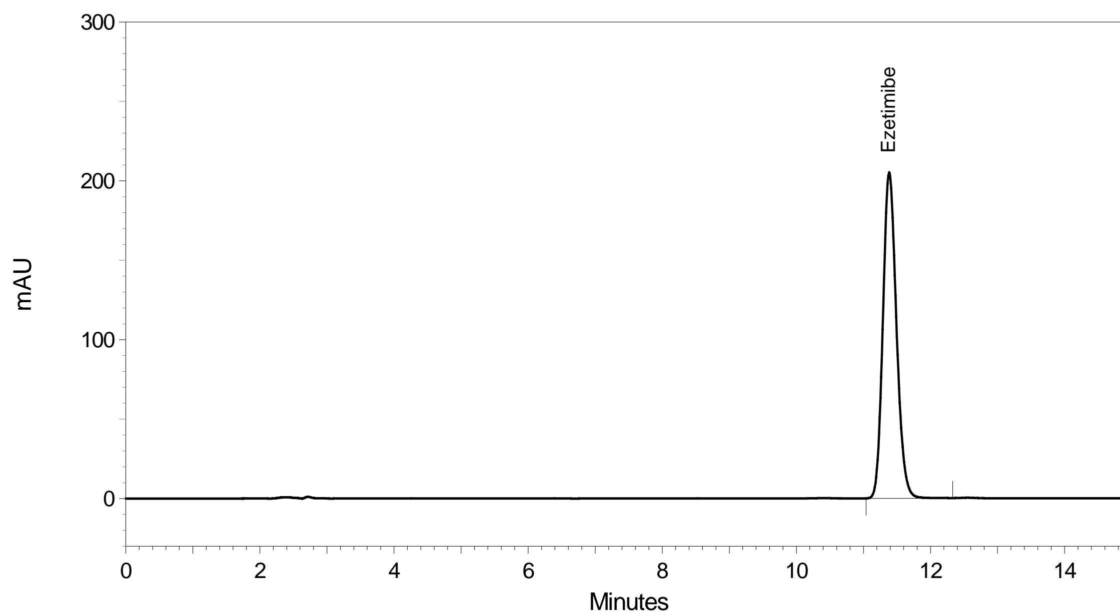


Table 14: Summary of Precision study

Study	Set No.	Assay (%)	Mean Assay (%)	Stdev	RSD (%)	95% Confidence Interval
Method Precision	1	100.7	101.4	0.59	0.58	0.62
	2	101.9				
	3	101.5				
	4	102.1				
	5	100.7				
	6	101.4				
Intermediate Precision	1	101.0	101.0	0.81	0.80	0.85
	2	102.4				
	3	100.1				
	4	101.5				
	5	101.7				
	6	102.0				
Overall	Mean	101.0		Absolute difference between mean % assay values of method precision and Intermediate precision = 0.4		
	Stdev	0.68				
	RSD (%)	0.67				

Acceptance criteria:

1) %RSD of six replicate sets of method precision study should be less than 2.0
2) %RSD of six replicate sets of intermediate precision study should be less than 2.0
3) Overall %RSD of replicate sets of method and intermediate precision study should not be more than 2.0
4) Absolute difference between mean % assay values of method precision and Intermediate precision should not be more than 2.0

Results:

1) %RSD of six replicate sets of method precision study is 0.58
2) %RSD of six replicate sets of intermediate precision study is 0.80
3) Overall %RSD of replicate sets of method and intermediate precision study is 0.67
4) Absolute difference between mean % assay values of method precision and Intermediate precision is 0.4

Conclusion:

All the results are well with-in the acceptance criteria. Hence, the analytical method is found precise.

4.4.5 **Accuracy study**

This parameter was determined by the recovery test. Recovery of the method is evaluated at 3 different concentration levels (corresponding to 50, 100, and 150% of test solution concentration) by addition of known amounts of standard to placebo preparation. For each concentration level, 3 sets were prepared and injected in duplicate.

Blank and standard preparation is prepared as per method as under:

Blank preparation:

Diluent was used as blank.

Standard preparation:

Stock solution: 49.5 mg of Ezetimibe reference standard was accurately weighed and transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask and the standard substance was dissolved by sonication for one minute. Then, volume of the flask was making up with diluent. The concentration obtained is 495 µg/ml of Ezetimibe.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 49.5 µg/ml of Ezetimibe.

Sample preparations for accuracy levels are as under:

Accuracy level 1 (50%) – Set 1:

Test stock solution: 50.8 mg of Ezetimibe reference standard was accurately weighed and transferred into 200 ml volumetric flask. 915.9 mg placebo (equivalent of 10 average placebo weight) was weighed and transferred into this 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceeding for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 254 µg/ml of Ezetimibe.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 25.4 µg/ml of Ezetimibe.

Accuracy level 1 (50%) – Set 2:

Test stock solution: 50.4 mg of Ezetimibe reference standard was accurately weighed and transferred into 200 ml volumetric flask. 914.5 mg placebo (equivalent of 10 average placebo weight) was weighed and transferred into this 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceeding for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 252 µg/ml of Ezetimibe.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 25.2 µg/ml of Ezetimibe.

Accuracy level 1 (50%) – Set 3:

Test stock solution: 50.7 mg of Ezetimibe reference standard was accurately weighed and transferred into 200 ml volumetric flask. 914.6 mg placebo (equivalent of 10 average placebo weight) was weighed and transferred into this 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceeding for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 253.5 µg/ml of Ezetimibe.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 25.35 µg/ml of Ezetimibe.

Accuracy level 2 (100%) – Set 1:

Test stock solution: 100.6 mg of Ezetimibe reference standard was accurately weighed and transferred into 200 ml volumetric flask. 915.1 mg placebo

(equivalent of 10 average weight) was weighed and transferred into this 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceeding for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 503 µg/ml of Ezetimibe.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.3 µg/ml of Ezetimibe.

Accuracy level 2 (100%) – Set 2:
Test stock solution: 100.8 mg of Ezetimibe reference standard was accurately weighed and transferred into 200 ml volumetric flask. 914.6 mg placebo (equivalent of 10 average placebo weight) was weighed and transferred into this 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceeding for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 504 µg/ml of Ezetimibe.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.4 µg/ml of Ezetimibe.

Accuracy level 2 (100%) – Set 3:
Test stock solution: 100.9 mg of Ezetimibe reference standard was accurately weighed and transferred into 200 ml volumetric flask. 915.7 mg placebo (equivalent of 10 average placebo weight) was weighed and transferred into this 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceeding for sonication of 30 minutes with normal hand-

shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 504.5 µg/ml of Ezetimibe.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.45 µg/ml of Ezetimibe.

Accuracy level 3 (150%) – Set 1:

Test stock solution: 150.4 mg of Ezetimibe reference standard was accurately weighed and transferred into 200 ml volumetric flask. 915.2 mg placebo (equivalent of 10 average placebo weight) was weighed and transferred into this 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceeding for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 752 µg/ml of Ezetimibe.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 75.2 µg/ml of Ezetimibe.

Accuracy level 3 (150%) – Set 2:

Test stock solution: 150.2 mg of Ezetimibe reference standard was accurately weighed and transferred into 200 ml volumetric flask. 915.0 mg placebo (equivalent of 10 average placebo weight) was weighed and transferred into this 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceeding for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 751 µg/ml of Ezetimibe.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 75.1 µg/ml of Ezetimibe.

Accuracy level 3 (150%) – Set 3:

Test stock solution: 150.9 mg of Ezetimibe reference standard was accurately weighed and transferred into 200 ml volumetric flask. 915.4 mg placebo (equivalent of 10 average placebo weight) was weighed and transferred into this 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceeding for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 754.5 µg/ml of Ezetimibe.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 75.45 µg/ml of Ezetimibe.

Chromatographic sequence for Accuracy study is represented through Table 15 as under:

Table 15: Sequence of Accuracy study

Sr. No.	Description	Injection replicate
1	Blank	1
2	Standard preparation	5
3	Accuracy Level 1 Preparation ; (Set 1)	2
4	Accuracy Level 1 Preparation ; (Set 2)	2
5	Accuracy Level 1 Preparation ; (Set 3)	2
6	Accuracy Level 2 Preparation ; (Set 1)	2
7	Accuracy Level 2 Preparation ; (Set 2)	2
8	Accuracy Level 2 Preparation ; (Set 3)	2
9	Accuracy Level 3 Preparation ; (Set 1)	2
10	Accuracy Level 3 Preparation ; (Set 2)	2
11	Accuracy Level 3 Preparation ; (Set 3)	2
12	Bracketing standard	1

<u>Observation, calculation and chromatograms:</u>

Table 16: Summary of accuracy study

Observation				
Data for Standard preparation				
Replicate	Area		Standard weight	49.5 mg
1	2870232		Standard potency	99.8 %
2	2871017		Standard conc.	49.5 µg/ml
3	2872907			
4	2870484			
5	2872130			
Average	2871354			
Stdev	1133.95			
% RSD	0.04			
Data for Test preparations				
Accuracy Level	Set No.	Replicate	Area	Mean area
I (50 %)	1	1	1481614	1480344
		2	1479073	
	2	1	1479204	1479064
		2	1478923	
	3	1	1479737	1481501
		2	1483265	
II (100 %)	1	1	2999951	3000708
		2	3001464	
	2	1	3000890	3002721
		2	3004552	
	3	1	2999563	2999863
		2	3000162	
III (150 %)	1	1	4409716	4404905
		2	4400094	
	2	1	4404162	4403775
		2	4403388	
	3	1	4409067	4413254
		2	4417441	

Table 17: Summary for added amount

Accuracy Level	Set No.	Wt. taken (mg)	Volume 1 (ml)	Volume 2 (ml)	Volume 3 (ml)	Amount added (µg/ml)
I (50 %)	1	50.8	200	5	50	25.40
	2	50.4	200	5	50	25.20
	3	50.7	200	5	50	25.35
II (100 %)	1	100.6	200	5	50	50.30
	2	100.8	200	5	50	50.40
	3	100.9	200	5	50	50.45
III (150 %)	1	150.4	200	5	50	75.20
	2	150.2	200	5	50	75.10
	3	150.9	200	5	50	75.45

Where by, Amount added (µg/ml) = $\frac{Wt.taken}{Volume\,1} x \frac{Volume\,2}{Volume\,3} x\,1000$

Prototype calculation for Set-1 of Accuracy Level – I is as under:

Amount added (µg/ml) = $\frac{Wt.taken}{Volume\,1} x \frac{Volume\,2}{Volume\,3} x\,1000$

$= \frac{50.8}{200} x \frac{5}{50} x\,1000$

= 25.4

Amount added for remaining all sets is calculated as per above formula and recorded in Table 17.

Table 18: Summary for % recovery

Accuracy Level	Set No.	Amount added (µg/ml)	Amount found (µg/ml)	Recovery (%)	Mean Recovery (%)	Stdev	RSD (%)
I (50 %)	1	25.40	25.52	100.47	100.80	0.36	0.36
	2	25.20	25.50	101.19			
	3	25.35	25.54	100.75			
II (100 %)	1	50.30	51.73	102.84	102.69	0.16	0.16
	2	50.40	51.76	102.70			
	3	50.45	51.72	102.52			
III (150 %)	1	75.20	75.94	100.98	100.97	0.13	0.13
	2	75.10	75.92	101.09			
	3	75.45	76.08	100.83			

Prototype calculation for Set-1 of Accuracy Level – I is as under:

$$\text{Amount found } (\mu g/ml) = \frac{Mean\ area\ of\ test\ preparation}{Average\ area\ of\ standard\ preparation} x\ standard \text{ conc.}$$

$$= \frac{1480344}{2871354} x\ 49.5$$

$$= 25.52$$

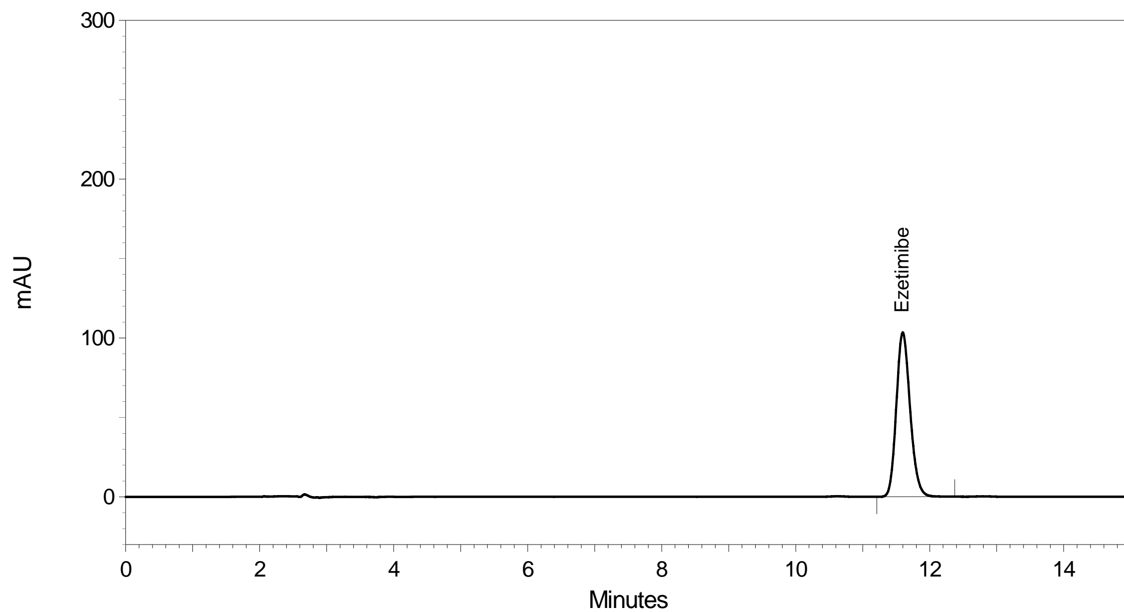
$$\%\ \text{Recovery} = \frac{Amount\ found}{Amount\ added} x\ 100$$

$$= \frac{25.52}{25.40} x\ 100$$

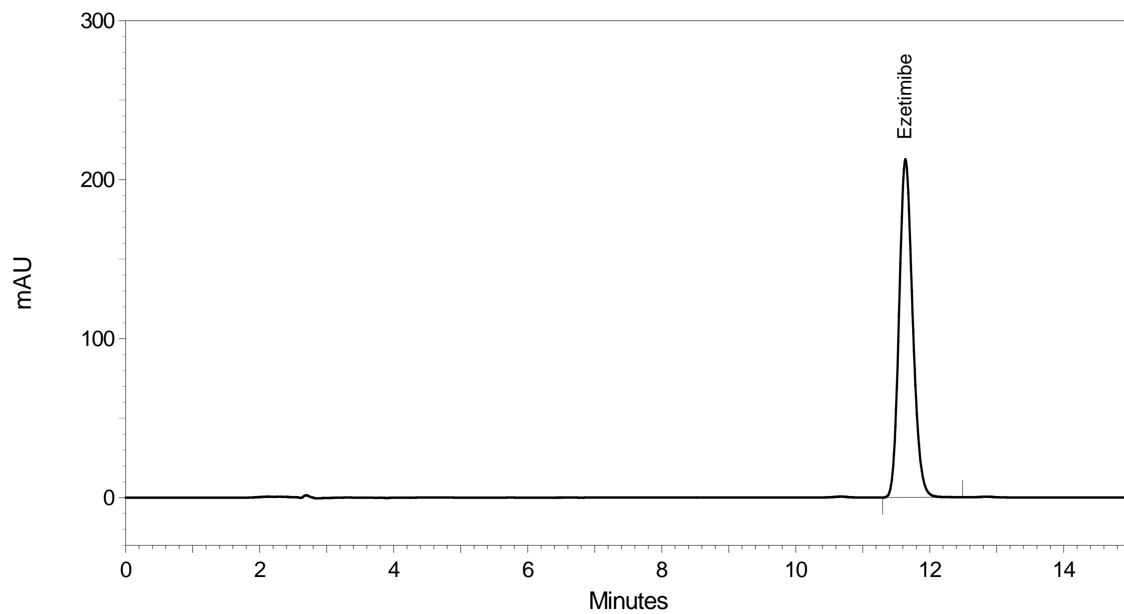
$$= 100.47$$

Amount added for remaining all sets is calculated as per above formula and recorded in Table 18.

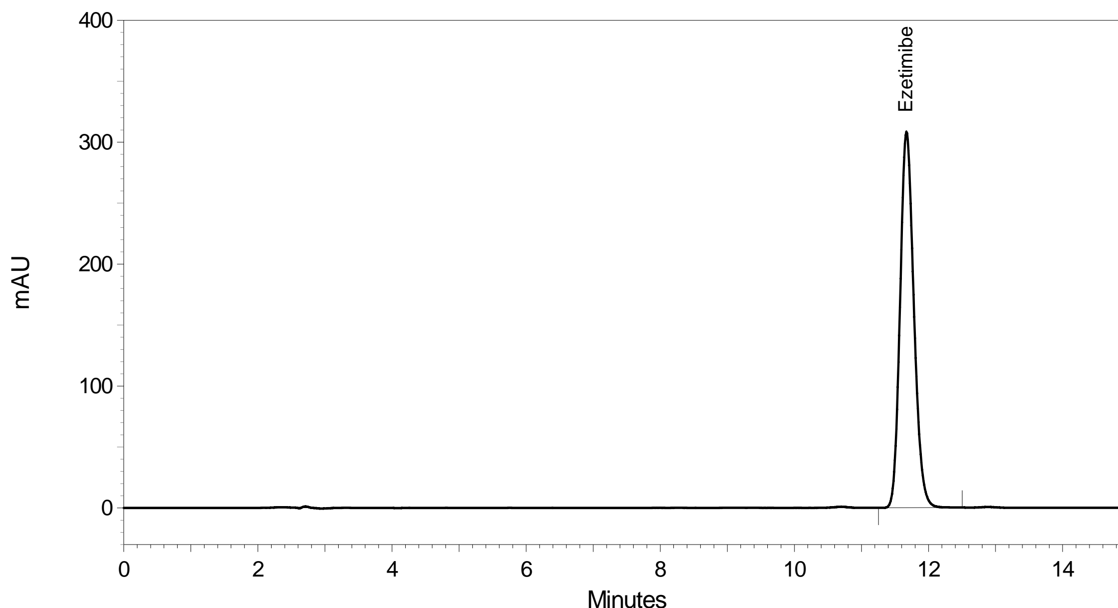
Prototype chromatogram of accuracy level-I (50 %):



Prototype chromatogram of accuracy level-II (100 %):



Prototype chromatogram of accuracy level-III (150 %):



Acceptance criteria:

1) % Recovery and mean % Recovery should between 97.0 to 103.0
2) % RSD of % Recovery of replicate sets of each level should not be more than 2.0

Results:

1) % Recovery and mean % Recovery values found between 97.0 to 103.0
2) % RSD of % Recovery of replicate sets of each level found less than 2.0

Conclusion:

All results are well with-in the limit. Hence, analytical method is found accurate.

4.4.6 **Robustness study**

Robustness of the method was evaluated by assaying test solutions under slight but deliberate changes in analytical conditions, such as change in flow rate

(± 0.1 ml min^{-1}), change in proportions of Buffer-Acetonitrile (52:48 and 48:52, *v/v*), and change in column-lot.

4.4.6.1 **Robust parameter: Change in Flow rate** – ‘At 0.9 ml min^{-1} flow’ and ‘At 1.1 ml min^{-1} flow’

In this parameter, analytical method was deliberately changed to flow rate. Sample was assayed by changing flow rate to 0.9 ml min^{-1} and 1.1 ml min^{-1} flow respectively.

Blank preparation:
Diluent was used as blank.

Standard preparation:
Stock solution: 50 mg of Ezetimibe reference standard was accurately weighed and transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask and the standard substance was dissolved by sonication for one minute. Then, volume of the flask was making up with diluent. The concentration obtained is 500 µg/ml of Ezetimibe.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe.

Test Preparation:
Test stock solution: 10 Tablets were accurately weighed (1020.8 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceeding for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered

through 0.45 μm nylon syringe filter. The concentration obtained is about 500 μg/ml of Ezetimibe.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is about 50 μg/ml of Ezetimibe.

Chromatographic sequence for Flow change parameter of Robustness study is represented through Table 19 as under:

Table 19: Sequence of Flow change parameter of Robustness study

Sr. No.	Description	Replicate	Chromatographic parameter
1	Blank	1	At 0.9 ml/min flow rate
2	Standard preparation	5	
3	Test Preparation	2	
4	Bracketing standard	1	
5	Blank	1	At 1.1 ml/min flow rate
6	Standard preparation	5	
7	Test Preparation	2	
8	Bracketing standard	1	

Observation, calculation and chromatograms:

Table 20: Summary for Flow change parameter of Robustness study

At 0.9 ml/min flow rate			At 1.1ml/min flow rate	
Data for Standard preparation			*Data for Standard preparation*	
Replicate	Area		Replicate	Area
1	3093202		1	2503168
2	3090125		2	2496820
3	3091170		3	2502338
4	3125283		4	2497781
5	3100228		5	2501978
Mean	3100002		Mean	2500417
Stdev	14671.23		Stdev	2897.49
% RSD	0.47		% RSD	0.12
Data for Test preparation			*Data for Test preparation*	
Replicate	Area		Replicate	Area
1	3182421		1	2563620
2	3183593		2	2564373
Mean	3183007		Mean	2563997
Standard wt.(mg)	50.0		Standard wt.(mg)	50.0
Test wt.(mg)	1020.8		Test wt.(mg)	1020.8
Label claim	10		Label claim	10
Average wt.(mg)	101.5		Average wt.(mg)	101.5
% Assay	101.9		% Assay	101.8

% Assay calculation for flow rate change is as under:

At 0.9 ml/min flow rate:

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{3183007}{3100002} x \frac{50.0}{100} x \frac{5}{50} x \frac{200}{1020.8} x \frac{50}{5} x \frac{101.5}{10} x\, 99.8$$

$$= 101.9$$

At 1.1 ml/min flow rate:

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2563997}{2500417} x \frac{50.0}{100} x \frac{5}{50} x \frac{200}{1020.8} x \frac{50}{5} x \frac{101.5}{10} x 99.8$$

$$= 101.8$$

Where by,

A_T = Average area of Ezetimibe obtained in test preparation

A_S = Average area of Ezetimibe obtained in standard preparation

W_1 = Weight taken of Ezetimibe reference standard (mg)

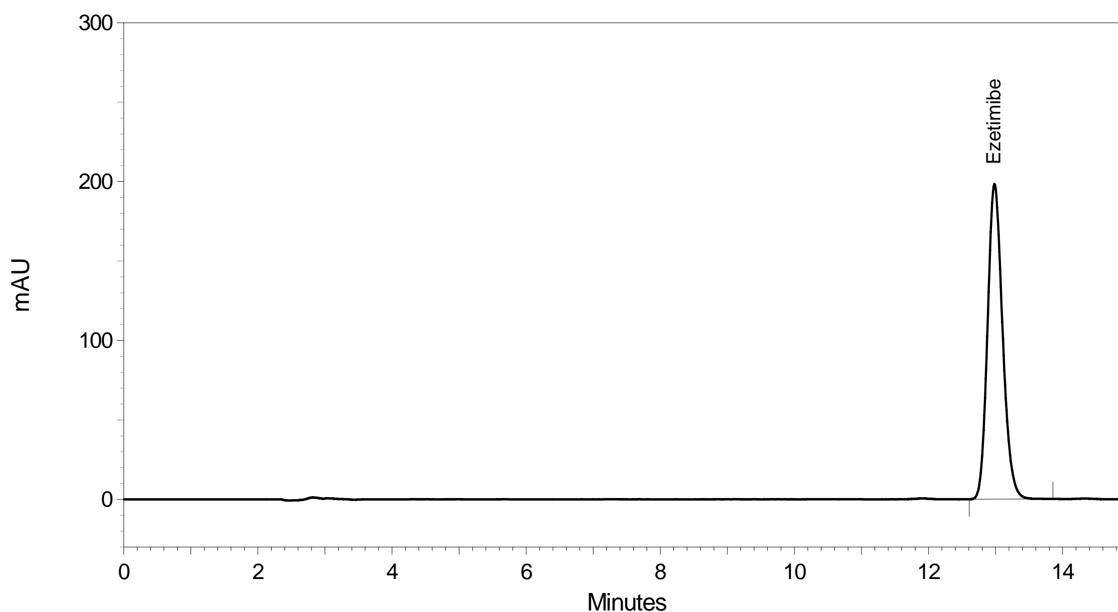
W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

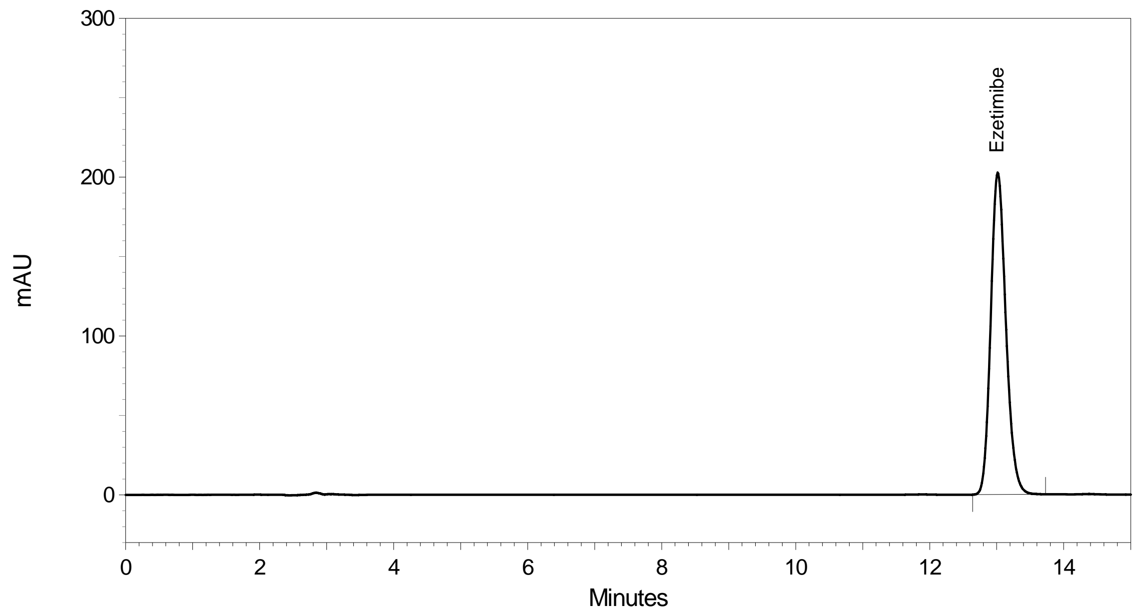
LC = Label claim (mg)

P = Potency of Ezetimibe reference standard (%)

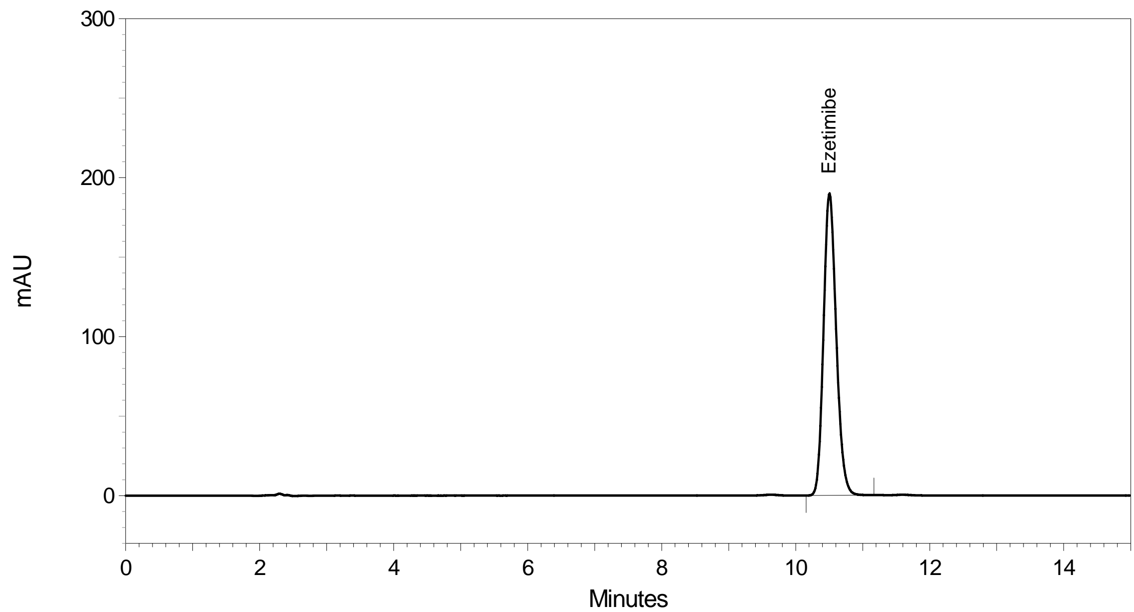
Chromatogram of standard preparation at 0.9 ml/min flow rate:



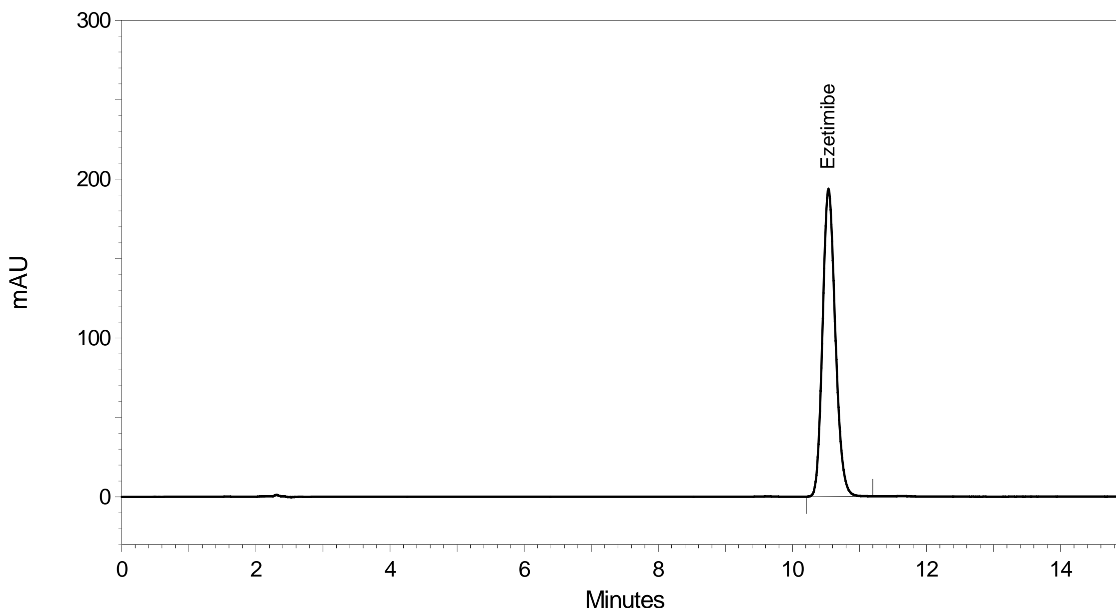
Chromatogram of test preparation at 0.9 ml/min flow rate:



Chromatogram of standard preparation at 1.1 ml/min flow rate:



Chromatogram of test preparation at 1.1 ml/min flow rate:



4.4.6.2 **Robust parameter: Change in mobile phase composition** – 'Buffer-Acetonitrile (52:48, *v/v*)' and 'Buffer-Acetonitrile (48:52, *v/v*)'

In this parameter, analytical method was deliberately changed for composition of mobile phase. Sample was assayed by changing mobile phase composition to Buffer-Acetonitrile (52:48, *v/v*) as aqueous component of mobile phase was increased and organic component was decreased in same proportion. Then after sample was assayed by changing mobile phase composition to Buffer-Acetonitrile (48:52, *v/v*) as aqueous component of mobile phase was decreased and organic component was increased in same proportion.

Blank preparation:
Diluent was used as blank.

Standard preparation:
Stock solution: 50.4 mg of Ezetimibe reference standard was accurately weighed and transferred into 100 ml volumetric flask. 70 ml of diluent was added into the

volumetric flask and the standard substance was dissolved by sonication for one minute. Then, volume of the flask was making up with diluent. The concentration obtained is 504 µg/ml of Ezetimibe.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.4 µg/ml of Ezetimibe.

Test Preparation:

Test stock solution: 10 Tablets were accurately weighed (1015.2 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceeding for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 500 µg/ml of Ezetimibe.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe.

Chromatographic sequence for mobile phase composition change parameter of Robustness study is represented through Table 21.

Table 21: Sequence of mobile phase composition change parameter of Robustness study

Sr. No.	Description	Replicate	Chromatographic parameter
1	Blank	1	Buffer-Acetonitrile (48:52, *v/v*)
2	Standard preparation	5	
3	Test Preparation	2	
4	Bracketing standard	1	
5	Blank	1	Buffer-Acetonitrile (52:48, *v/v*)
6	Standard preparation	5	
7	Test Preparation	2	
8	Bracketing standard	1	

Observation, calculation and chromatograms:

Table 22: Summary for mobile phase composition change parameter

Buffer-Acetonitrile (48:52, *v/v*)			Buffer-Acetonitrile (52:48, *v/v*)	
Data for Standard preparation			*Data for Standard preparation*	
Replicate	Area		Replicate	Area
1	2817022		1	2825466
2	2816139		2	2805835
3	2818778		3	2806197
4	2814871		4	2807975
5	2816953		5	2839509
Mean	2816753		Mean	2816996
Stdev	1425.82		Stdev	15009.50
% RSD	0.05		% RSD	0.53
Data for Test preparation			*Data for Test preparation*	
Replicate	Area		Replicate	Area
1	2850600		1	2841871
2	2848424		2	2867886
Mean	2849512		Mean	2854879
Standard wt.(mg)	50.4		Standard wt.(mg)	50.4
Test wt.(mg)	1015.2		Test wt.(mg)	1015.2
Label claim	10		Label claim	10
Average wt.(mg)	101.5		Average wt.(mg)	101.5
% Assay	101.7		% Assay	101.9

% Assay calculation for mobile phase composition change is as under:

For mobile phase composition of Buffer-Acetonitrile (48:52, *v/v*):

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} x P$$

$$= \frac{2849512}{2816753} x \frac{50.4}{100} x \frac{5}{50} x \frac{200}{1015.2} x \frac{50}{5} x \frac{101.5}{10} x\, 99.8$$

$$= 101.7$$

For mobile phase composition of Buffer-Acetonitrile (52:48, *v/v*):

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2854879}{2816996} x \frac{50.4}{100} x \frac{5}{50} x \frac{200}{1015.2} x \frac{50}{5} x \frac{101.5}{10} x\, 99.8$$

$$= 101.9$$

Where by,

A_T = Average area of Ezetimibe obtained in test preparation

A_S = Average area of Ezetimibe obtained in standard preparation

W_1 = Weight taken of Ezetimibe reference standard (mg)

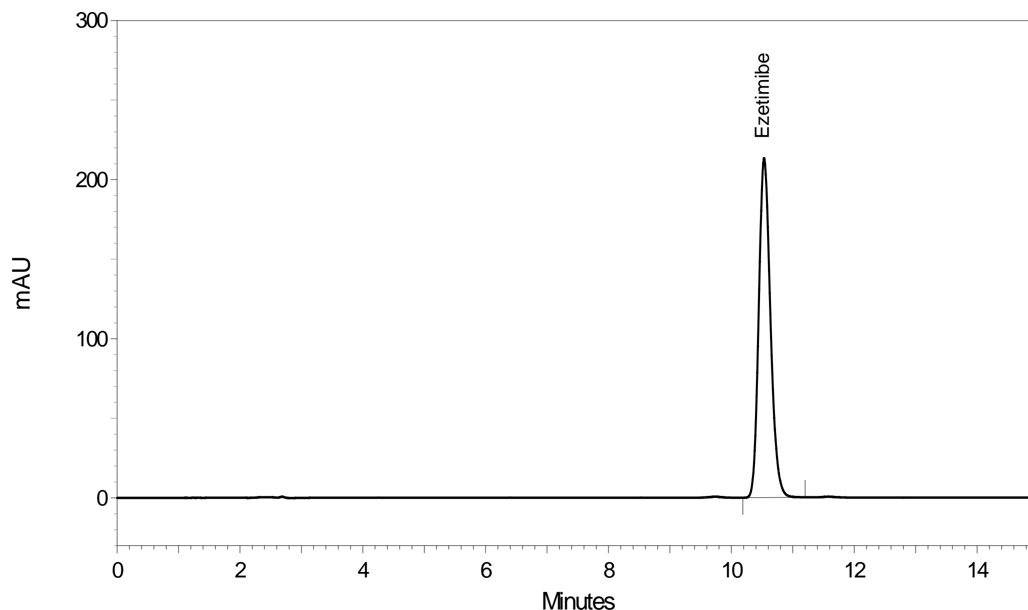
W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

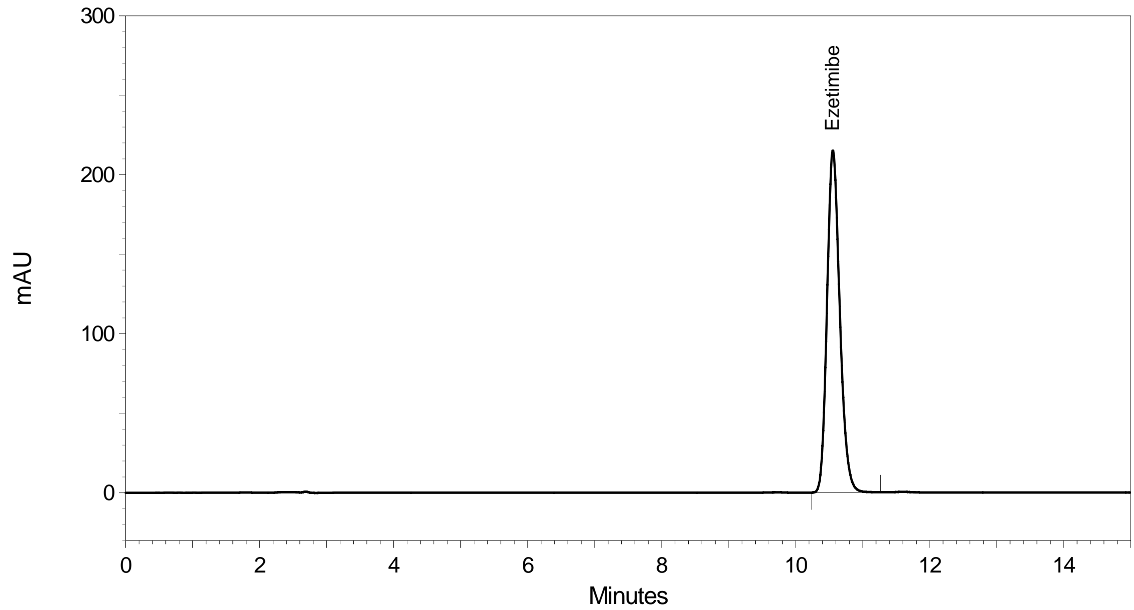
LC = Label claim (mg)

P = Potency of Ezetimibe reference standard (%)

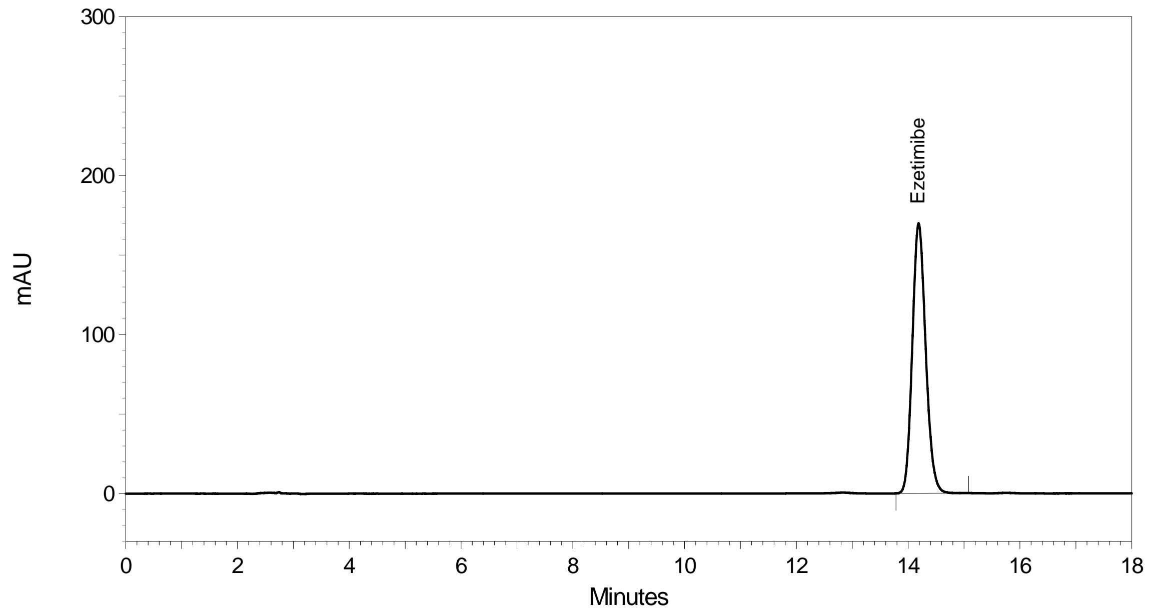
Chromatogram of standard preparation of Buffer-Acetonitrile (48:52, *v/v*):



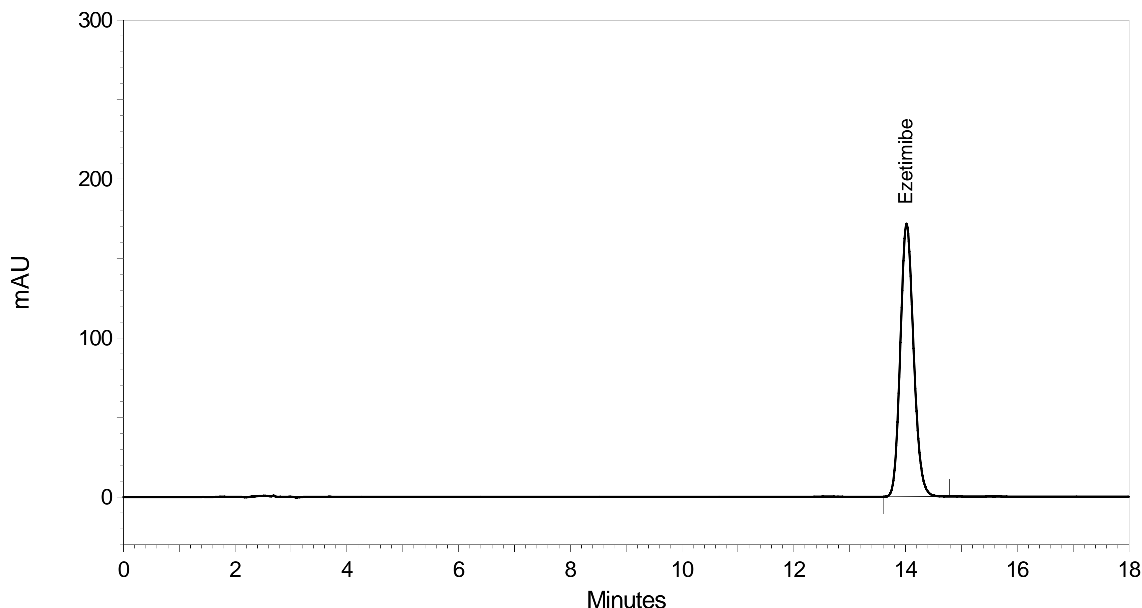
Chromatogram of test preparation of Buffer-Acetonitrile (48:52, *v/v*)



Chromatogram of standard preparation of Buffer-Acetonitrile (52:48, *v/v*):



Chromatogram of test preparation of Buffer-Acetonitrile (52:48, *v/v*):



4.4.6.3 **Robust parameter: Change in column lot**

In this parameter, column used in analytical method was changed to different lot. Sample was assayed by changing the lot of column.

Blank preparation:
Diluent was used as blank.

Standard preparation:
Stock solution: 50.1 mg of Ezetimibe reference standard was accurately weighed and transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask and the standard substance was dissolved by sonication for one minute. Then, volume of the flask was making up with diluent. The concentration obtained is 501 µg/ml of Ezetimibe.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.1 µg/ml of Ezetimibe.

Test Preparation:

Test stock solution: 10 Tablets were accurately weighed (1011.8 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceeding for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is about 500 µg/ml of Ezetimibe.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is about 50 µg/ml of Ezetimibe.

Chromatographic sequence for column change parameter of Robustness study is represented through Table 23 as under:

Table 23: Sequence of column change parameter of Robustness study

Sr. No.	Description	Replicate	Chromatographic parameter
1	Blank	1	Column change
2	Standard preparation	5	
3	Test Preparation	2	
4	Bracketing standard	1	

Observation, calculation and chromatograms:

Table 24: Summary for column change parameter of Robustness study

Column Lot change	
Data for Standard preparation	
Replicate	Area
1	2800700
2	2804678
3	2803786
4	2802343
5	2806376
Mean	2803577
Stdev	2173.14
% RSD	0.08
Data for Test preparation	
Replicate	Area
1	2853872
2	2856813
Mean	2855343
Standard wt.(mg)	50.1
Test wt.(mg)	1011.8
Label claim	10
Average wt.(mg)	101.5
% Assay	102.2

% Assay calculation for column change is as under:

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2855343}{2803577} x \frac{50.1}{100} x \frac{5}{50} x \frac{200}{1011.8} x \frac{50}{5} x \frac{101.5}{10} x\, 99.8$$

$$= 102.2$$

Where by,

A_T = Average area of Ezetimibe obtained in test preparation

A_S = Average area of Ezetimibe obtained in standard preparation

W_1 = Weight taken of Ezetimibe reference standard (mg)

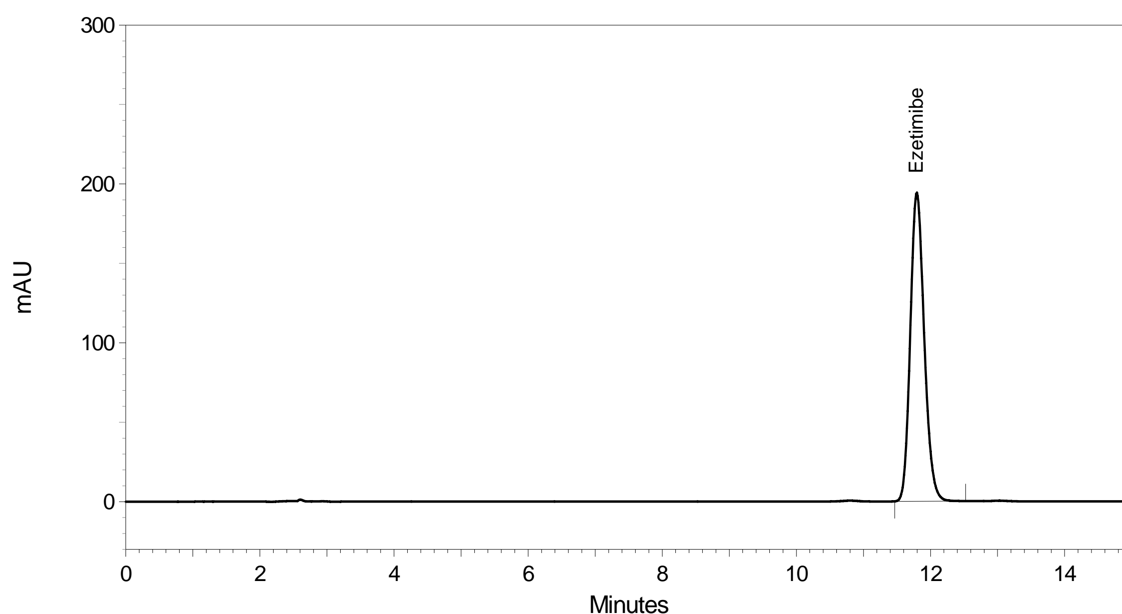
W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

LC = Label claim (mg)

P = Potency of Ezetimibe reference standard (%)

Chromatogram of standard preparation of column change parameter



Chromatogram of test preparation of column change parameter

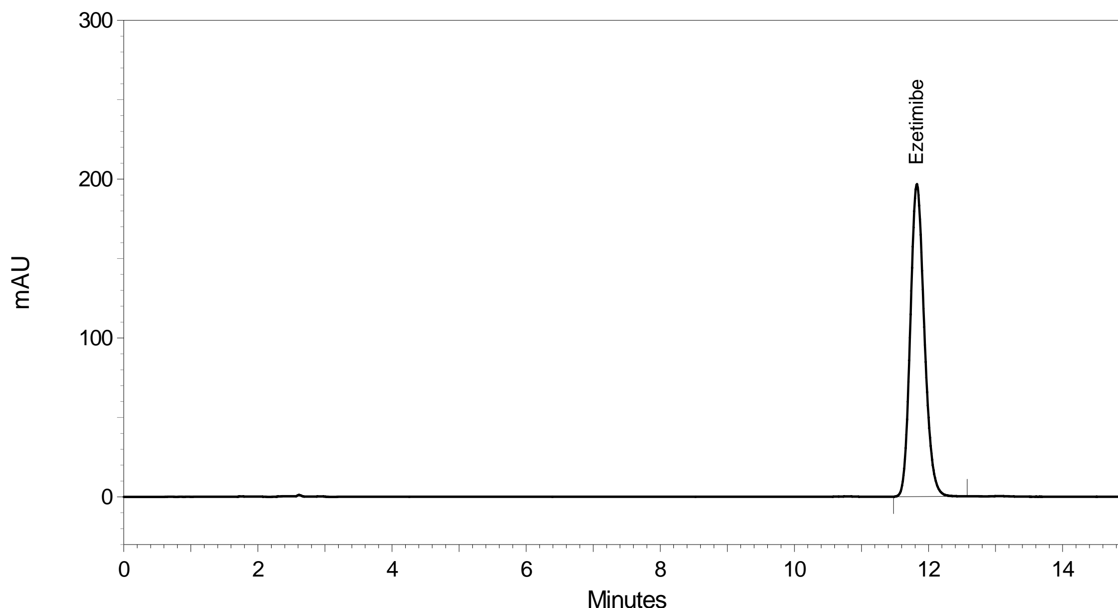


Table 25: Summary of robustness study

Robust conditions	Assay (%)	% Assay difference	RT, minute	System suitability parameters	
				Theoretical plates	Asymmetry
0.9 ml/min flow	101.9	0.5	12.98	15750	1.19
1.1 ml/min flow	101.8	0.4	10.51	14274	1.15
Buffer-acetonitrile (48:52,*v/v*)	101.7	0.3	10.53	14359	1.22
Buffer-acetonitrile (52:48,*v/v*)	101.9	0.5	14.19	16284	1.15
Column (lot change)	102.2	0.8	11.80	15011	1.16
% Assay difference is calculated with respect to mean value of method precision					

Acceptance criteria:

1) % Assay value of the sample should not differ than ± 2.0 % from the actual value during each robust parameter.
2) System suitability should comply during each robust parameter.

Result:

1) % Assay value of the sample is well with-in the acceptance criteria.
2) System suitability is complies during each robust parameter.

4.4.7 **Solution stability study**

Solution stability period for the solutions of standard preparation and test preparation was evaluated. The solutions were stored at 5˚ C and ambient temperature without protection against light and tested at interval of 6, 12, 24, 36, and 48 h. The responses for the aged solution were evaluated using a freshly prepared standard solution[55].

Stage of solution stability study: Initial

Blank preparation:
Diluent was used as blank.

Standard preparation:
Stock solution: 51.3 mg of Ezetimibe reference standard was accurately weighed and transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask and the standard substance was dissolved by sonication for one minute. Then, volume of the flask was make up with diluent. The concentration obtained is 513 µg/ml of Ezetimibe.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 51.3 µg/ml of Ezetimibe.

Test Preparation:
Test stock solution: 10 Tablets were accurately weighed (1008.1 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the

volumetric flask. The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is about 500 µg/ml of Ezetimibe.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is about 50 µg/ml of Ezetimibe.

Chromatographic sequence for Initial stage of solution stability study is represented through Table 26 as under:

Table 26: Sequence for Initial stage of solution stability study

Sr. No.	Description	Replicate
1	Blank	1
2	Standard preparation	5
3	Test Preparation	2
4	Bracketing standard	1

Observation, calculation and chromatograms:

Table 27: Summary of Initial stage of solution stability study

Observation				
Standard Details			*Test Details*	
Data for Standard preparation			*Data for Test preparation*	
Replicate	Area		Replicate	Area
1	2902135		1	2814998
2	2901226		2	2813349
3	2900660		Average	2814174
4	2900068			
5	2897362			
Average	2900290			
Stdev	1805.51			
% RSD	0.06			
Standard weight	51.3 mg		Test weight	1008.1 mg
Standard potency	99.8 %		Label claim	10 mg

%Assay of test solution at 'Initial' stage:

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2814174}{2900290} x \frac{51.3}{100} x \frac{5}{50} x \frac{200}{1008.1} x \frac{50}{5} x \frac{101.5}{10} x 99.8$$

$= 100.03$

Where by,

A_T = Average area of Ezetimibe obtained in test preparation

A_S = Average area of Ezetimibe obtained in standard preparation

W_1 = Weight taken of Ezetimibe reference standard (mg)

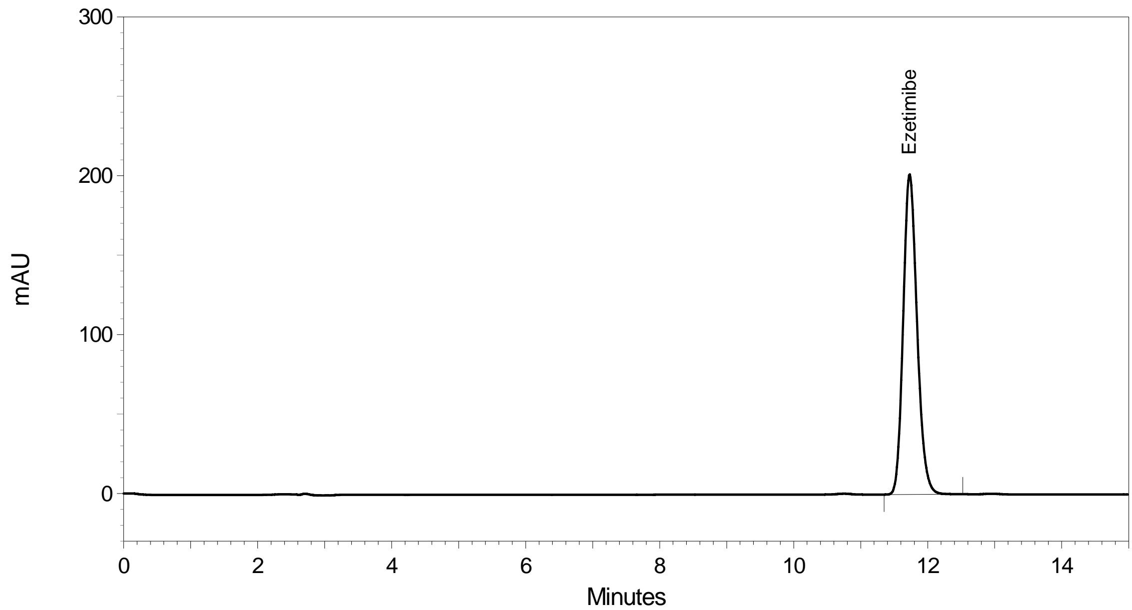
W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

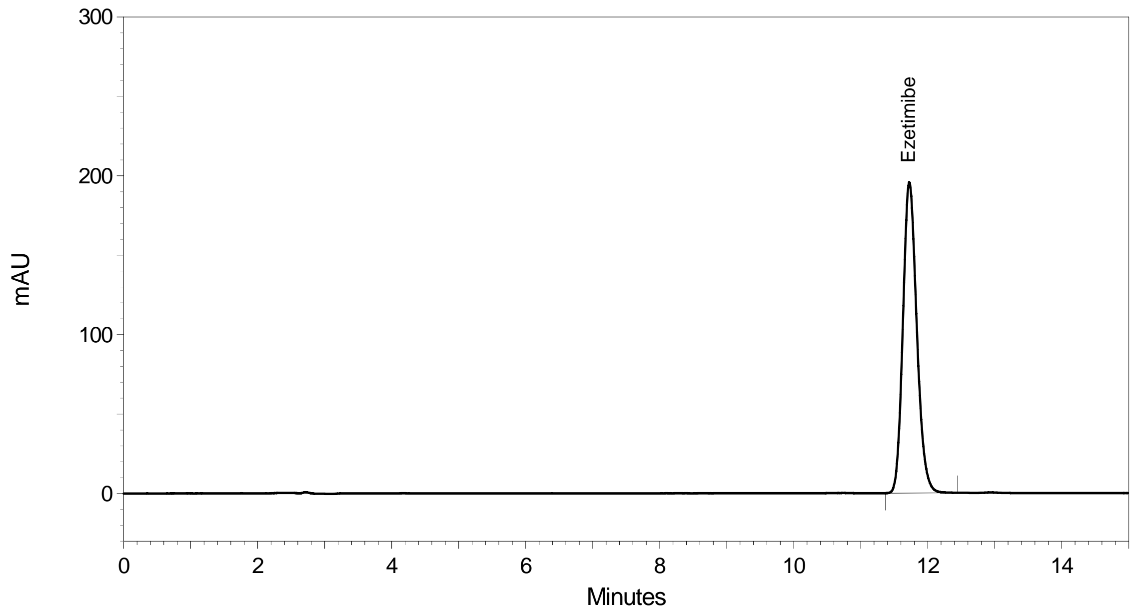
LC = Label claim (mg)

P = Potency of Ezetimibe reference standard (%)

Chromatogram of standard preparation of Initial stage:



Chromatogram of test preparation of Initial stage:



<u>Stage of solution stability study: After 6 hours</u>

Blank preparation:

Diluent was used as blank.

Standard preparation (freshly prepared):

Stock solution: 50.7 mg of Ezetimibe reference standard was accurately weighed and transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask and the standard substance was dissolved by sonication for one minute. Then, volume of the flask was make up with diluent. The concentration obtained is 507 µg/ml of Ezetimibe.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.7 µg/ml of Ezetimibe.

Standard preparation (of 5˚ C) for stability:

Standard preparation solution which is stored at 5˚ C for 6 hours was injected (and % RSD for the same solution was measured against initially injected standard).

Standard preparation (of room temperature) for stability:

Standard preparation solution which is stored at room temperature for 6 hours was injected (and % RSD for the same solution was measured against initially injected standard).

Test Preparation (of 5˚ C) for stability:

Test preparation solution which is stored at 5˚ C for 6 hours was injected (and assay for the same solution was measured against freshly prepared standard followed by comparison of that of initial).

Test Preparation (of room temperature) for stability:

Test preparation solution which is stored at room temperature for 6 hours was injected (and assay for the same solution was measured against freshly prepared standard followed by comparison of that of initial).

Chromatographic sequence for 'After 6 hours' stage of solution stability study is represented through Table 28 as under:

Table 28: Sequence for 'After 6 hours' stage of solution stability study

Sr. No.	Description	Replicate
1	Blank	1
2	Standard preparation	5
3	Standard preparation (At 5˚ C)	2
4	Standard preparation (At room temperature)	2
5	Test preparation (At 5˚ C)	2
6	Test preparation (At room temperature)	2
7	Bracketing standard	1

<u>Observation, calculation and chromatograms:</u>

Table 29: Summary of 'After 6 hours' stage of solution stability study

Observation				
Standard Details			*Test Details*	
Data for Standard preparation			*Data for Test preparation (at 5˚ C temperature)*	
Replicate	Area		Replicate	Area
1	2857361		1	2810384
2	2859906		2	2811034
3	2861165		Average	2810709
4	2867835			
5	2864644		*Data for Test preparation (at room temperature)*	
Average	2862182		Replicate	Area
Stdev	4107.14		1	2808649
% RSD	0.14		2	2809183
			Average	2808916
Standard weight	50.7 mg		Test weight	1008.1 mg
Standard potency	99.8 %		Label claim	10 mg

% Assay of Test preparation solution stored at 5˚ C temperature:

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2810709}{2862182} x \frac{50.7}{100} x \frac{5}{50} x \frac{200}{1008.1} x \frac{50}{5} x \frac{101.5}{10} x\, 99.8$$

$$= 100.06$$

% Assay of Test preparation solution stored at room temperature:

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2808916}{2862182} \text{x} \frac{50.7}{100} \text{x} \frac{5}{50} \text{x} \frac{200}{1008.1} \text{x} \frac{50}{5} \text{x} \frac{101.5}{10} \text{x } 99.8$$

$$= 99.99$$

Where by,

A_T = Average area of Ezetimibe obtained in test preparation

A_S = Average area of Ezetimibe obtained in standard preparation

W_1 = Weight taken of Ezetimibe reference standard (mg)

W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

LC = Label claim (mg)

P = Potency of Ezetimibe reference standard (%)

Table 30: Summary for standard solution stability at 'After 6 hours' stage:

Observation						
Data for Standard preparation (at 5˚ C temperature)				*Data for Standard preparation (at room temperature)*		
Stage	Replicate	Area		Stage	Replicate	Area
Initial	1	2902135		Initial	1	2902135
	2	2901226			2	2901226
	3	2900660			3	2900660
	4	2900068			4	2900068
	5	2897362			5	2897362
After 6 hours	1	2899098		After 6 hours	1	2894301
	2	2906204			2	2898904
	Average	2900965			Average	2899237
	Stdev	2776.36			Stdev	2678.95
	% RSD	0.10			% RSD	0.09

Stage	Mean area		Stage	Mean area
Initial	2900290		Initial	2900290
After 6 hours	2902651		After 6 hours	2896603
Absolute Difference (%)	0.08		Absolute Difference (%)	0.13

For standard preparation (at 5˚ C temperature):

$$\text{Absolute Difference (\%)} = \left| 100 - \left[\frac{A_R}{A_I} x\, 100 \right] \right|$$

$$= \left| 100 - \left[\frac{2902651}{2900290} x\, 100 \right] \right|$$

$$= 0.08$$

For standard preparation (at room temperature):

$$\text{Absolute Difference (\%)} = \left| 100 - \left[\frac{A_R}{A_I} x\, 100 \right] \right|$$

$$= \left| 100 - \left[\frac{2896603}{2900290} x\, 100 \right] \right|$$

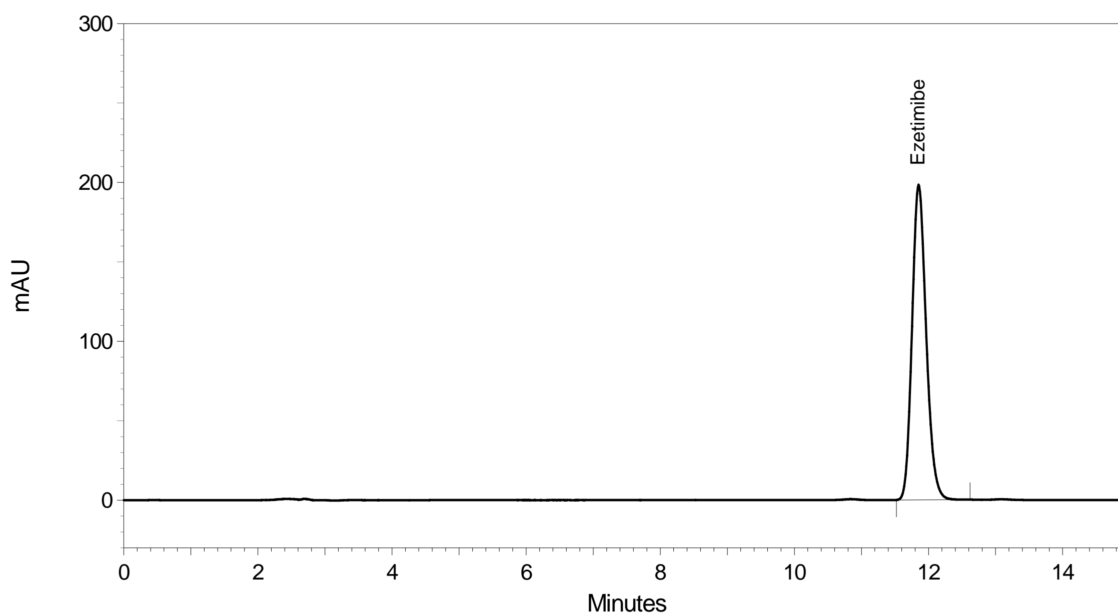
$$= 0.13$$

Where by,

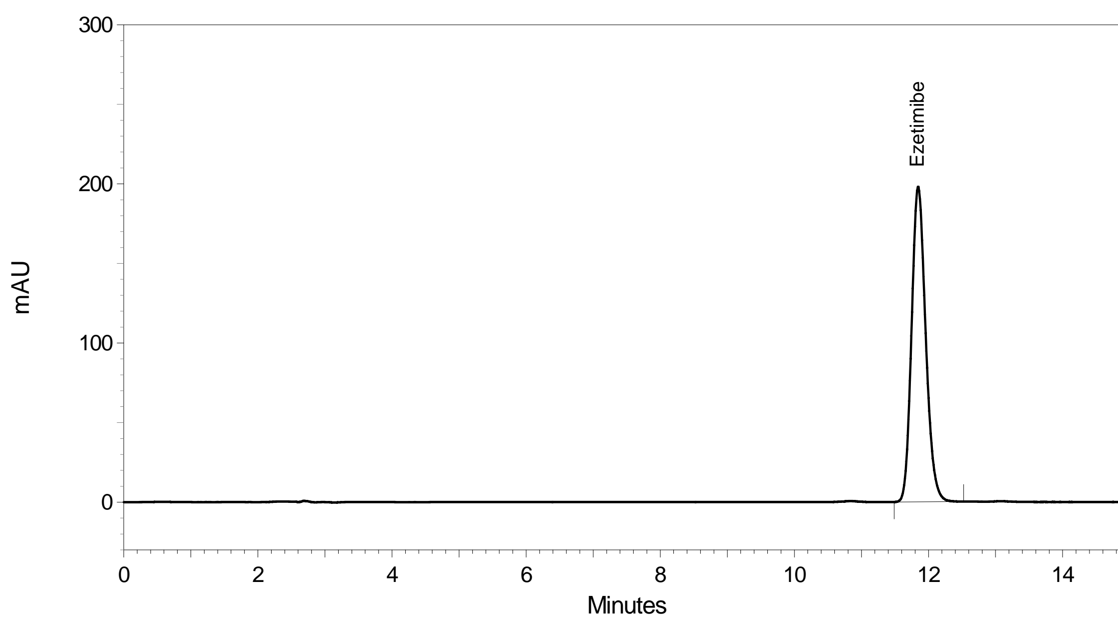
A_R = Standard mean area of respective time interval stage

A_I = Standard mean area of initial stage

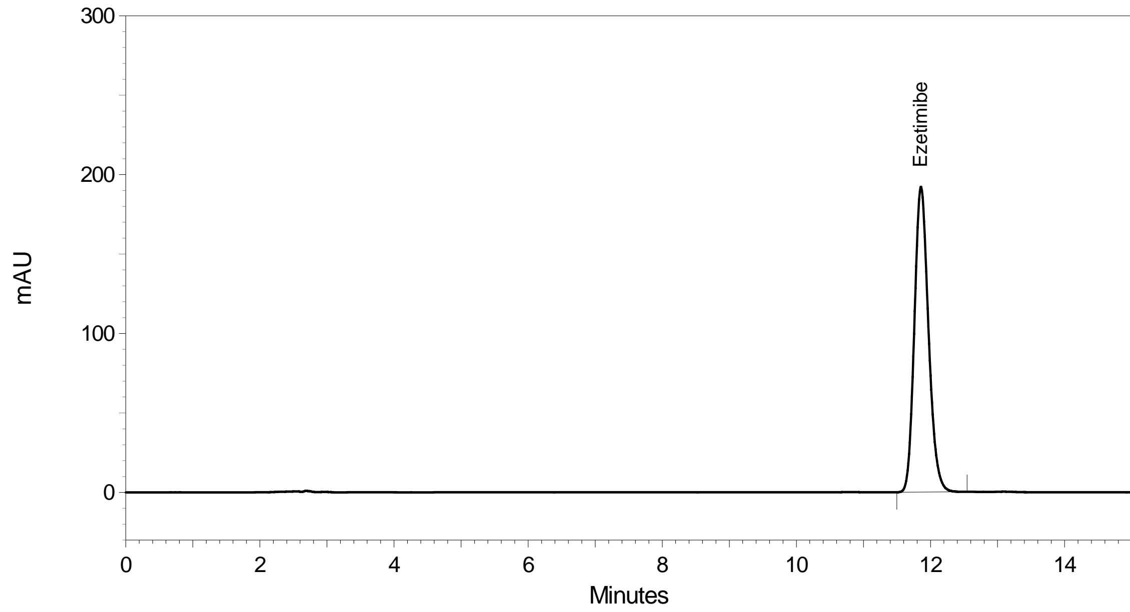
Chromatogram of standard preparation of 'After 6 hours stage' at 5˚ C:



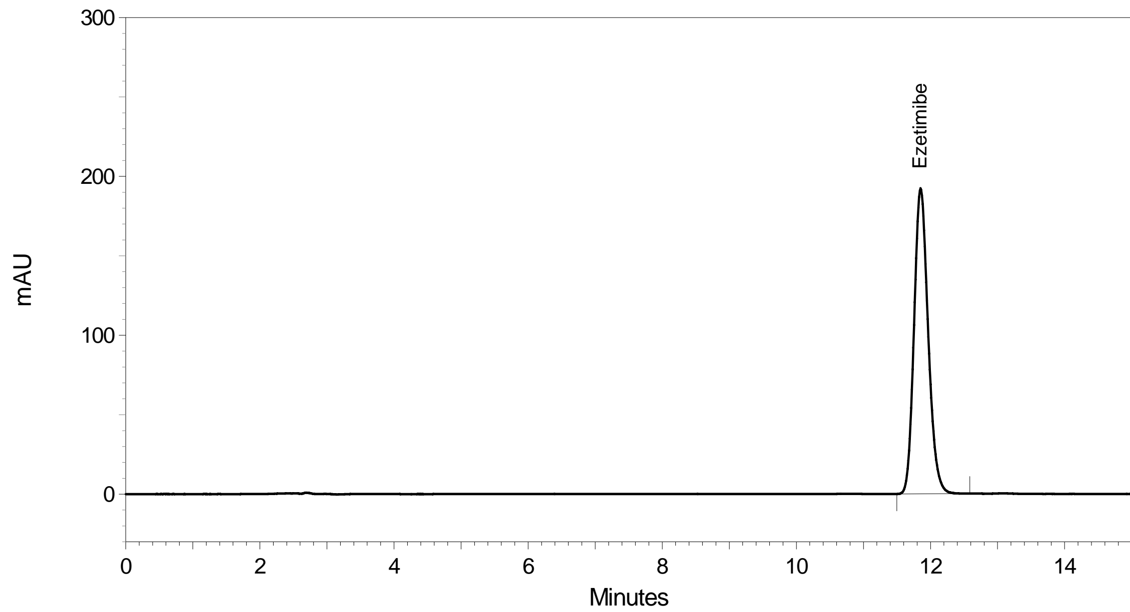
Chromatogram of standard preparation of 'After 6 hours stage' at room temp.:



Chromatogram of test preparation of ‘After 6 hours stage’ at 5˚ C:



Chromatogram of test preparation of ‘After 6 hours stage’ at room temperature:



Stage of solution stability study: After 12 hours

Blank preparation:
Diluent was used as blank.

Standard preparation (freshly prepared):
Stock solution: 51 mg of Ezetimibe reference standard was accurately weighed and transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask and the standard substance was dissolved by sonication for one minute. Then, volume of the flask was make up with diluent. The concentration obtained is 510 μg/ml of Ezetimibe.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 51 μg/ml of Ezetimibe.

Standard preparation (of 5˚ C) for stability:
Standard preparation solution which is stored at 5˚ C for 12 hours was injected (and % RSD for the same solution was measured against initially injected standard).

Standard preparation (of room temperature) for stability:
Standard preparation solution which is stored at room temperature for 12 hours was injected (and % RSD for the same solution was measured against initially injected standard).

Test Preparation (of 5˚ C) for stability:
Test preparation solution which is stored at 5˚ C for 12 hours was injected (and assay for the same solution was measured against freshly prepared standard followed by comparison of that of initial).

Test Preparation (of room temperature) for stability:

Test preparation solution which is stored at room temperature for 12 hours was injected (and assay for the same solution was measured against freshly prepared standard followed by comparison of that of initial).

Chromatographic sequence for ‘After 12 hours’ stage of solution stability study is represented through Table 31 as under:

Table 31: Sequence for ‘After 12 hours’ stage of solution stability study

Sr. No.	Description	Replicate
1	Blank	1
2	Standard preparation	5
3	Standard preparation (At 5˚ C)	2
4	Standard preparation (At room temperature)	2
5	Test preparation (At 5˚ C)	2
6	Test preparation (At room temperature)	2
7	Bracketing standard	1

Observation, calculation and chromatograms:

Table 32: Summary of 'After 12 hours' stage of solution stability study

Observation				
Standard Details			*Test Details*	
Data for Standard preparation			*Data for Test preparation (at 5˚ C temperature)*	
Replicate	Area		Replicate	Area
1	2880077		1	2810711
2	2886772		2	2814680
3	2892030		Average	2812696
4	2885316			
5	2887232		*Data for Test preparation (at room temperature)*	
Average	2886285			
Stdev	4290.02		Replicate	Area
% RSD	0.15		1	2805281
			2	2810676
			Average	2807979
Standard weight	51.0 mg		Test weight	1008.1 mg
Standard potency	99.8 %		Label claim	10 mg

% Assay of Test preparation solution stored at 5˚ C temperature:

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2812696}{2886285} x \frac{51.0}{100} x \frac{5}{50} x \frac{200}{1008.1} x \frac{50}{5} x \frac{101.5}{10} x\, 99.8$$

$$= 99.88$$

% Assay of Test preparation solution stored at room temperature:

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2807979}{2886285} x \frac{51.0}{100} x \frac{5}{50} x \frac{200}{1008.1} x \frac{50}{5} x \frac{101.5}{10} x\, 99.8$$

$$= 99.71$$

Where by,

A_T = Average area of Ezetimibe obtained in test preparation

A_S = Average area of Ezetimibe obtained in standard preparation

W_1 = Weight taken of Ezetimibe reference standard (mg)

W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

LC = Label claim (mg)

P = Potency of Ezetimibe reference standard (%)

Table 33: Summary for standard solution stability at 'After 12 hours' stage

Observation						
Data for Standard preparation (at 5° C temperature)				*Data for Standard preparation (at room temperature)*		
Stage	Replicate	Area		Stage	Replicate	Area
Initial	1	2902135		Initial	1	2902135
	2	2901226			2	2901226
	3	2900660			3	2900660
	4	2900068			4	2900068
	5	2897362			5	2897362
After 12 hours	1	2912657		After 12 hours	1	2884149
	2	2904476			2	2887159
	Average	2902655			Average	2896108
	Stdev	4905.05			Stdev	7343.88
	% RSD	0.17			% RSD	0.25
Stage	Mean area			Stage	Mean area	
Initial	2900290			Initial	2900290	
After 12 hours	2908567			After 12 hours	2885654	
Absolute Difference (%)	0.29			Absolute Difference (%)	0.50	

For standard preparation (at 5° C temperature):

$$\text{Absolute Difference (\%)} = \left| 100 - \left[\frac{A_R}{A_I} x\, 100 \right] \right|$$

$$= \left| 100 - \left[\frac{2908567}{2900290} x\, 100 \right] \right|$$

$$= 0.29$$

For standard preparation (at room temperature):

$$\text{Absolute Difference (\%)} = \left| 100 - \left[\frac{A_R}{A_I} \, x\, 100 \right] \right|$$

$$= \left| 100 - \left[\frac{2885654}{2900290} \, x\, 100 \right] \right|$$

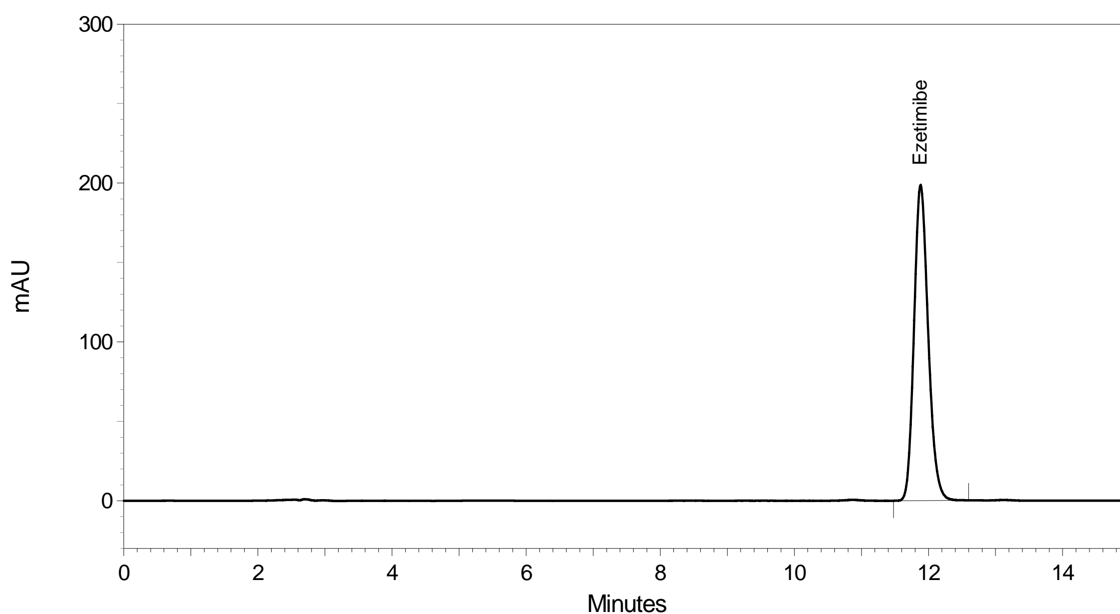
$$= 0.50$$

Where by,

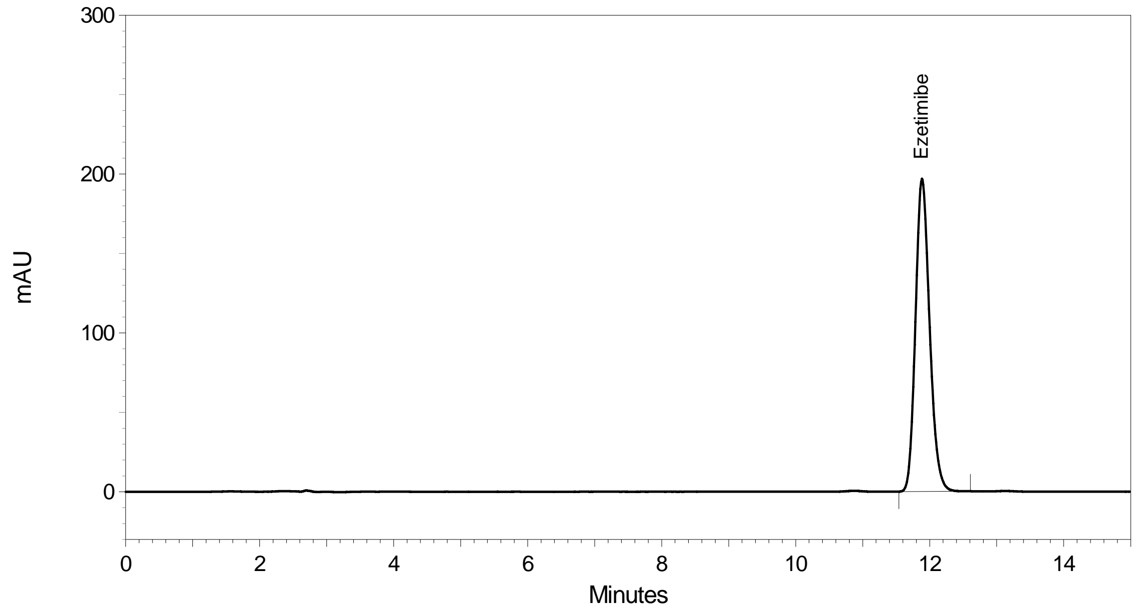
A_R = Standard mean area of respective time interval stage

A_I = Standard mean area of initial stage

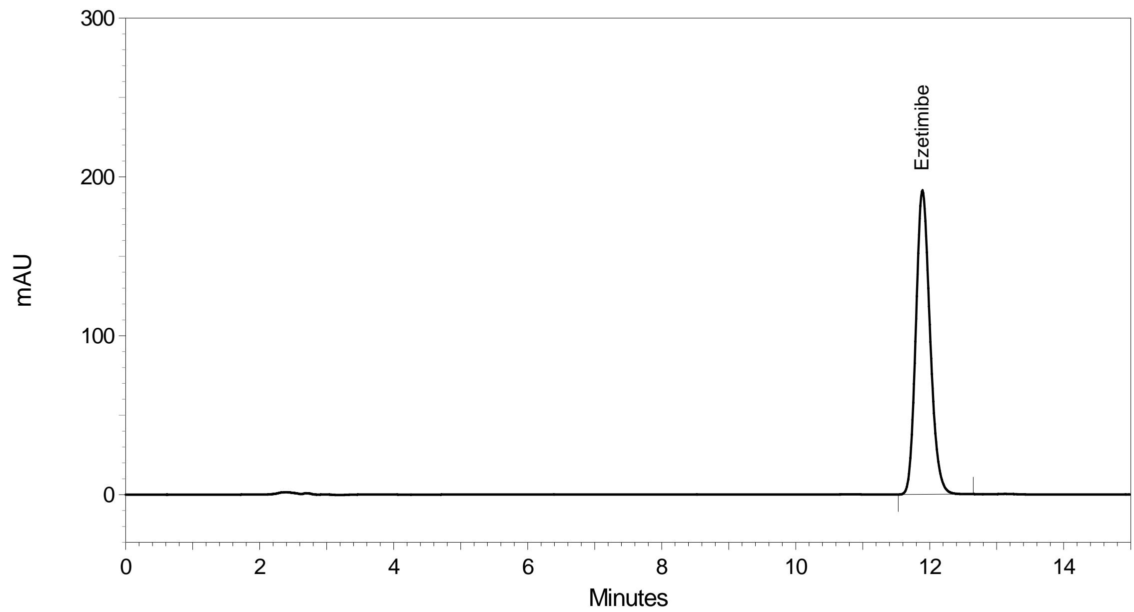
Chromatogram of standard preparation of 'After 12 hours stage' at 5° C:



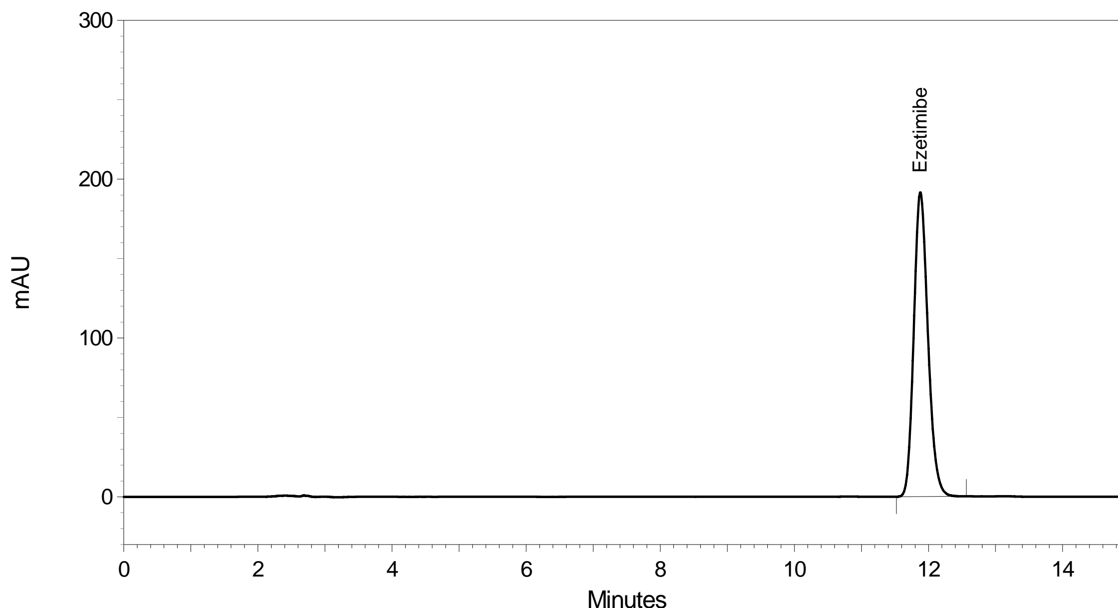
Chromatogram of standard preparation of 'After 12 hours stage' at room temperature:



Chromatogram of test preparation of 'After 12 hours stage' at 5˚ C:



Chromatogram of test preparation of 'After 12 hours stage' at room temperature:



Stage of solution stability study: After 24 hours

Blank preparation:
Diluent was used as blank.

Standard preparation (freshly prepared):
Stock solution: 49.3 mg of Ezetimibe reference standard was accurately weighed and transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask and the standard substance was dissolved by sonication for one minute. Then, volume of the flask was make up with diluent. The concentration obtained is 493 µg/ml of Ezetimibe.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 49.3 µg/ml of Ezetimibe.

Standard preparation (of 5˚ C) for stability:

Standard preparation solution which is stored at 5˚ C for 24 hours was injected (and % RSD for the same solution was measured against initially injected standard).

Standard preparation (of room temperature) for stability:

Standard preparation solution which is stored at room temperature for 24 hours was injected (and % RSD for the same solution was measured against initially injected standard).

Test Preparation (of 5˚ C) for stability:

Test preparation solution which is stored at 5˚ C for 24 hours was injected (and assay for the same solution was measured against freshly prepared standard followed by comparison of that of initial).

Test Preparation (of room temperature) for stability:

Test preparation solution which is stored at room temperature for 24 hours was injected (and assay for the same solution was measured against freshly prepared standard followed by comparison of that of initial).

Chromatographic sequence for 'After 24 hours' stage of solution stability study is represented through Table 34 as under:

Table 34: Sequence for 'After 24 hours' stage of solution stability study

Sr. No.	Description	Replicate
1	Blank	1
2	Standard preparation	5
3	Standard preparation (At 5˚ C)	2
4	Standard preparation (At room temperature)	2
5	Test preparation (At 5˚ C)	2
6	Test preparation (At room temperature)	2
7	Bracketing standard	1

Observation, calculation and chromatograms:

Table 35: Summary for 'After 24 hours' stage of solution stability study

Observation				
Standard Details			*Test Details*	
Data for Standard preparation			*Data for Test preparation (at 5° C temperature)*	
Replicate	Area		Replicate	Area
1	2790226		1	2816760
2	2795948		2	2816535
3	2791627		Average	2816648
4	2791489			
5	2791716		*Data for Test preparation (at room temperature)*	
Average	2792201			
Stdev	2180.15		Replicate	Area
% RSD	0.08		1	2791101
			2	2797839
			Average	2794470
Standard weight	49.3 mg		Test weight	1008.1 mg
Standard potency	99.8 %		Label claim	10 mg

% Assay of Test preparation solution stored at 5° C temperature:

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2816648}{2792201} x \frac{49.3}{100} x \frac{5}{50} x \frac{200}{1008.1} x \frac{50}{5} x \frac{101.5}{10} x\, 99.8$$

$$= 99.94$$

% Assay of Test preparation solution stored at room temperature:

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2794470}{2792201} x \frac{49.3}{100} x \frac{5}{50} x \frac{200}{1008.1} x \frac{50}{5} x \frac{101.5}{10} x\, 99.8$$

$$= 99.16$$

Where by,

A_T = Average area of Ezetimibe obtained in test preparation

A_S = Average area of Ezetimibe obtained in standard preparation

W_1 = Weight taken of Ezetimibe reference standard (mg)

W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

LC = Label claim (mg)

P = Potency of Ezetimibe reference standard (%)

Table 36: Summary for standard solution stability at 'After 24 hours' stage

Observation					
Data for Standard preparation (at 5˚ C temperature)			*Data for Standard preparation (at room temperature)*		
Stage	Replicate	Area	Stage	Replicate	Area
Initial	1	2902135	Initial	1	2902135
	2	2901226		2	2901226
	3	2900660		3	2900660
	4	2900068		4	2900068
	5	2897362		5	2897362
After 24 hours	1	2896367	After 24 hours	1	2873645
	2	2901055		2	2884018
	Average	2899839		Average	2894159
	Stdev	2144.40		Stdev	10989.86
	% RSD	0.07		% RSD	0.38
Stage	Mean area		Stage	Mean area	
Initial	2900290		Initial	2900290	
After 24 hours	2898711		After 24 hours	2878832	
Absolute Difference (%)	0.05		Absolute Difference (%)	0.74	

For standard preparation (at 5˚ C temperature):

$$\text{Absolute Difference (\%)} = \left| 100 - \left[\frac{A_R}{A_I} x\, 100 \right] \right|$$

$$= \left| 100 - \left[\frac{2898711}{2900290} \, x \, 100 \right] \right|$$

$$= 0.05$$

For standard preparation (at room temperature):

$$\text{Absolute Difference (\%)} = \left| 100 - \left[\frac{A_R}{A_I} \, x \, 100 \right] \right|$$

$$= \left| 100 - \left[\frac{2878832}{2900290} \, x \, 100 \right] \right|$$

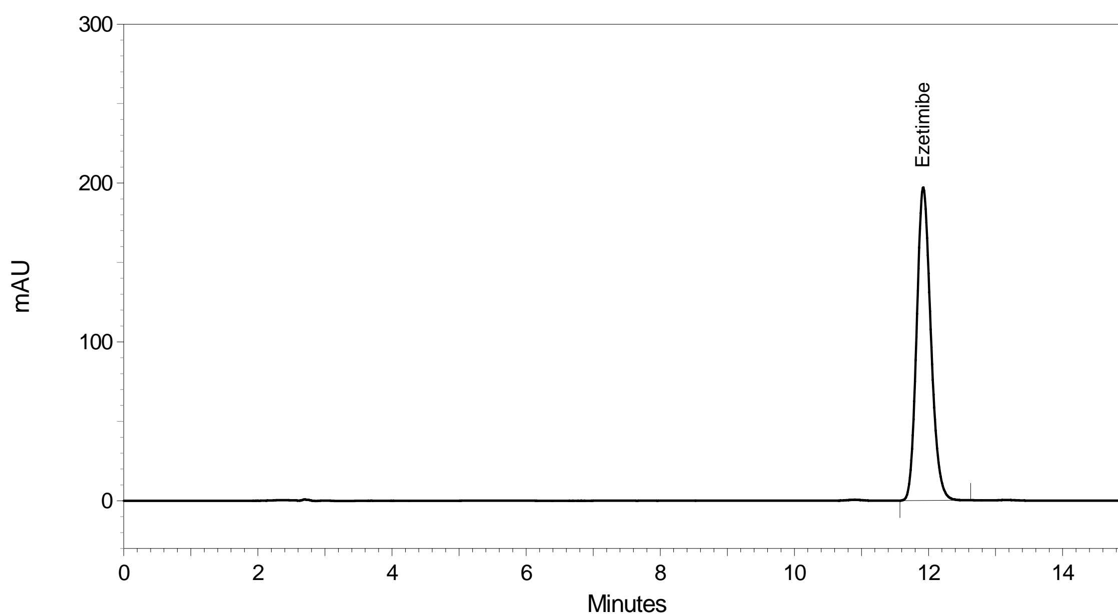
$$= 0.74$$

Where by,

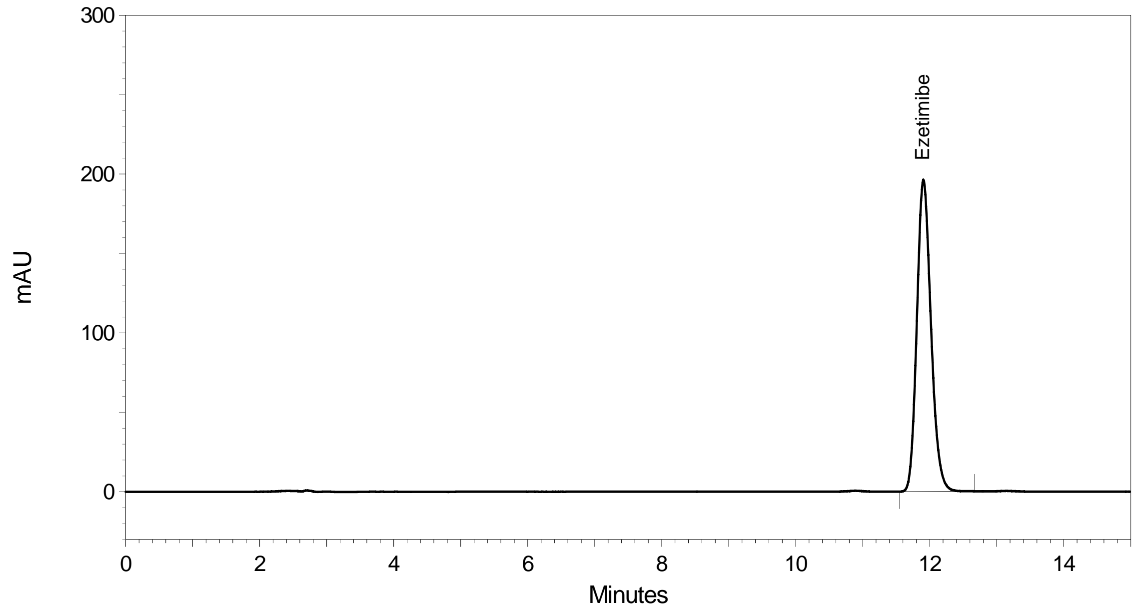
A_R = Standard mean area of respective time interval stage

A_I = Standard mean area of initial stage

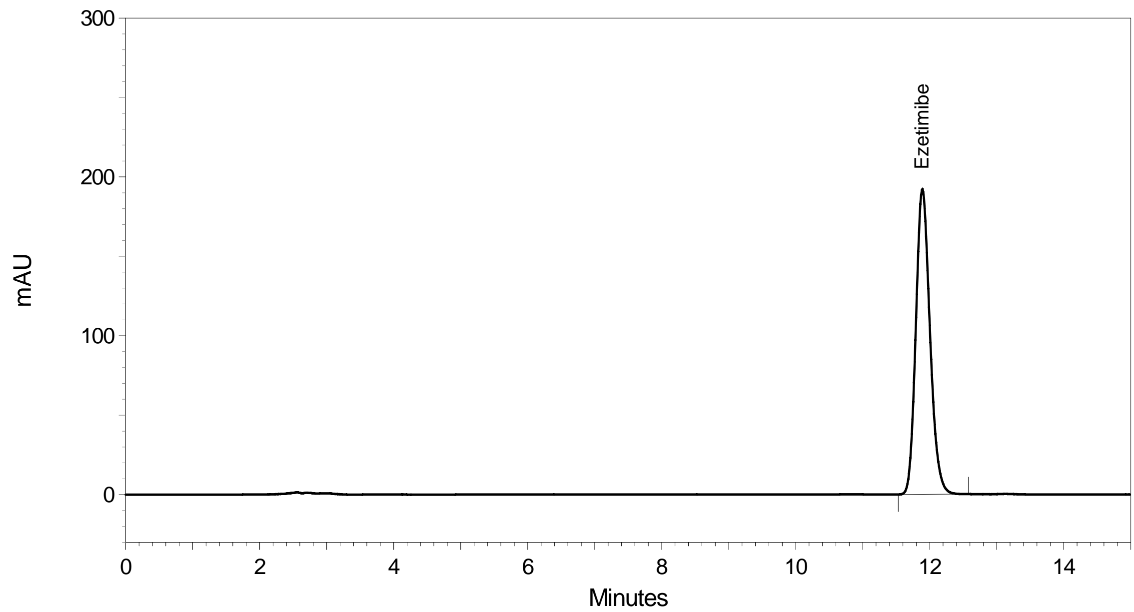
Chromatogram of standard preparation of 'After 24 hours stage' at 5° C:



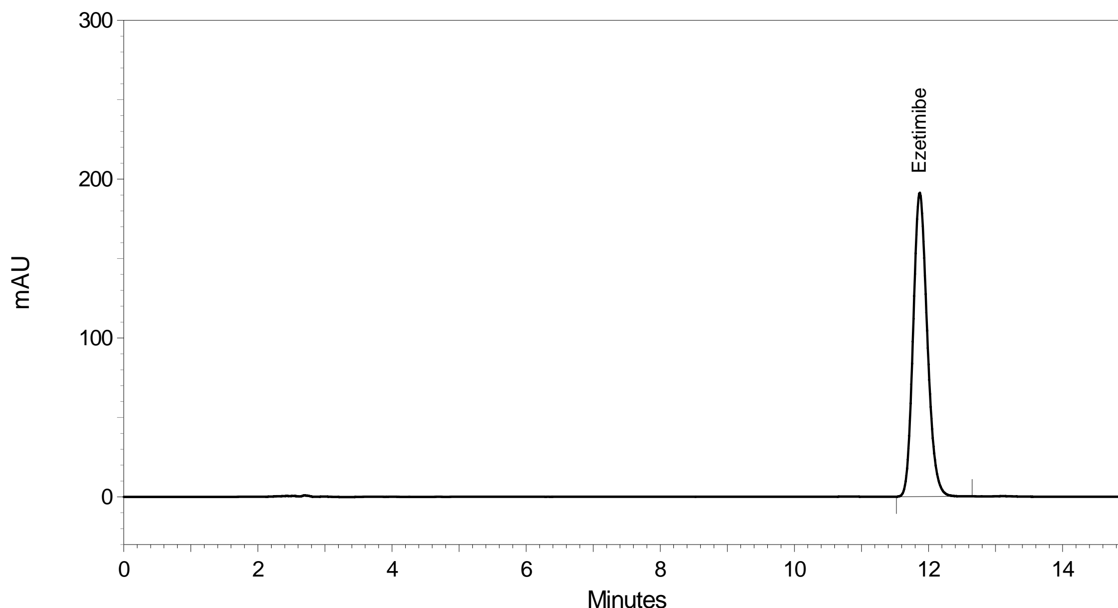
Chromatogram of standard preparation of 'After 24 hours stage' at room temperature:



Chromatogram of test preparation of 'After 24 hours stage' at 5˚ C:



Chromatogram of test preparation of 'After 24 hours stage' at room temperature:



<u>Stage of solution stability study: After 36 hours</u>

Blank preparation:
Diluent was used as blank.

Standard preparation (freshly prepared):
Stock solution: 50.5 mg of Ezetimibe reference standard was accurately weighed and transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask and the standard substance was dissolved by sonication for one minute. Then, volume of the flask was make up with diluent. The concentration obtained is 505 µg/ml of Ezetimibe.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.5 µg/ml of Ezetimibe.

Standard preparation (of 5˚ C) for stability:

Standard preparation solution which is stored at 5˚ C for 36 hours was injected (and % RSD for the same solution was measured against initially injected standard).

Standard preparation (of room temperature) for stability:

Standard preparation solution which is stored at room temperature for 36 hours was injected (and % RSD for the same solution was measured against initially injected standard).

Test Preparation (of 5˚ C) for stability:

Test preparation solution which is stored at 5˚ C for 36 hours was injected (and assay for the same solution was measured against freshly prepared standard followed by comparison of that of initial).

Test Preparation (of room temperature) for stability:

Test preparation solution which is stored at room temperature for 36 hours was injected (and assay for the same solution was measured against freshly prepared standard followed by comparison of that of initial).

Chromatographic sequence for 'After 36 hours' stage of solution stability study is represented through Table 37 as under:

Table 37: Sequence for 'After 36 hours' stage of solution stability study

Sr. No.	Description	Replicate
1	Blank	1
2	Standard preparation	5
3	Standard preparation (At 5˚ C)	2
4	Standard preparation (At room temperature)	2
5	Test preparation (At 5˚ C)	2
6	Test preparation (At room temperature)	2
7	Bracketing standard	1

Observations, calculation and chromatograms:

Table 38: Summary for 'After 36 hours' stage of solution stability study

Observation				
Standard Details			*Test Details*	
Data for Standard preparation			*Data for Test preparation (at 5° C temperature)*	
Replicate	Area		Replicate	Area
1	2857063		1	2818290
2	2854169		2	2814714
3	2854215		Average	2816502
4	2861988			
5	2854899		*Data for Test preparation (at room temperature)*	
Average	2856467			
Stdev	3303.32		Replicate	Area
% RSD	0.12		1	2788190
			2	2786973
			Average	2787582
Standard weight	50.5 mg		Test weight	1008.1 mg
Standard potency	99.8 %		Label claim	10 mg

% Assay of Test preparation solution stored at 5° C temperature:

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2816502}{2856467} x \frac{50.5}{100} x \frac{5}{50} x \frac{200}{1008.1} x \frac{50}{5} x \frac{101.5}{10} x\, 99.8$$

$$= 100.07$$

% Assay of Test preparation solution stored at room temperature:

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2787582}{2856467} x \frac{50.5}{100} x \frac{5}{50} x \frac{200}{1008.1} x \frac{50}{5} x \frac{101.5}{10} x\, 99.8$$

$$= 99.04$$

Where by,

A_T = Average area of Ezetimibe obtained in test preparation

A_S = Average area of Ezetimibe obtained in standard preparation

W_1 = Weight taken of Ezetimibe reference standard (mg)

W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

LC = Label claim (mg)

P = Potency of Ezetimibe reference standard (%)

Table 39: Summary for standard solution stability at 'After 36 hours' stage

Observation						
Data for Standard preparation (at 5˚ C temperature)				*Data for Standard preparation (at room temperature)*		
Stage	Replicate	Area		Stage	Replicate	Area
Initial	1	2902135		Initial	1	2902135
	2	2901226			2	2901226
	3	2900660			3	2900660
	4	2900068			4	2900068
	5	2897362			5	2897362
After 36 hours	1	2898857		After 36 hours	1	2869288
	2	2903724			2	2877686
	Average	2900576			Average	2892632
	Stdev	2094.15			Stdev	13382.86
	% RSD	0.07			% RSD	0.46
Stage	Mean area			Stage	Mean area	
Initial	2900290			Initial	2900290	
After 36 hours	2901291			After 36 hours	2873487	
Absolute Difference (%)	0.03			Absolute Difference (%)	0.92	

For standard preparation (at 5˚ C temperature):

$$\text{Absolute Difference (\%)} = \left| 100 - \left[\frac{A_R}{A_I} x\, 100 \right] \right|$$

$$= \left| 100 - \left[\frac{2901291}{2900290} \, x \, 100 \right] \right|$$

$= 0.03$

For standard preparation (at room temperature):

$$\text{Absolute Difference (\%)} = \left| 100 - \left[\frac{A_R}{A_I} \, x \, 100 \right] \right|$$

$$= \left| 100 - \left[\frac{2873487}{2900290} \, x \, 100 \right] \right|$$

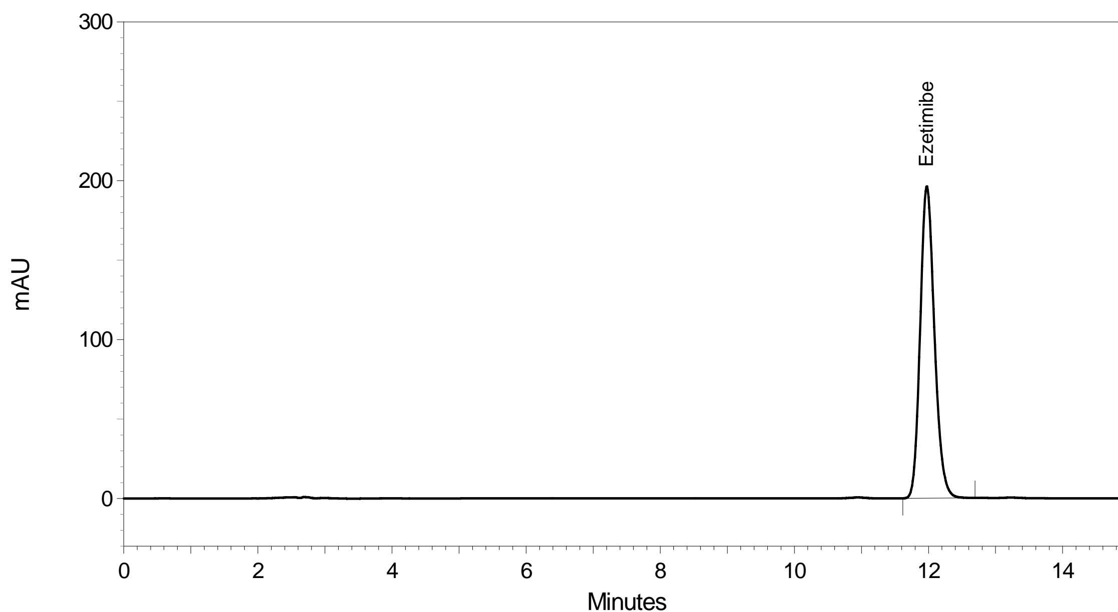
$= 0.92$

Where by,

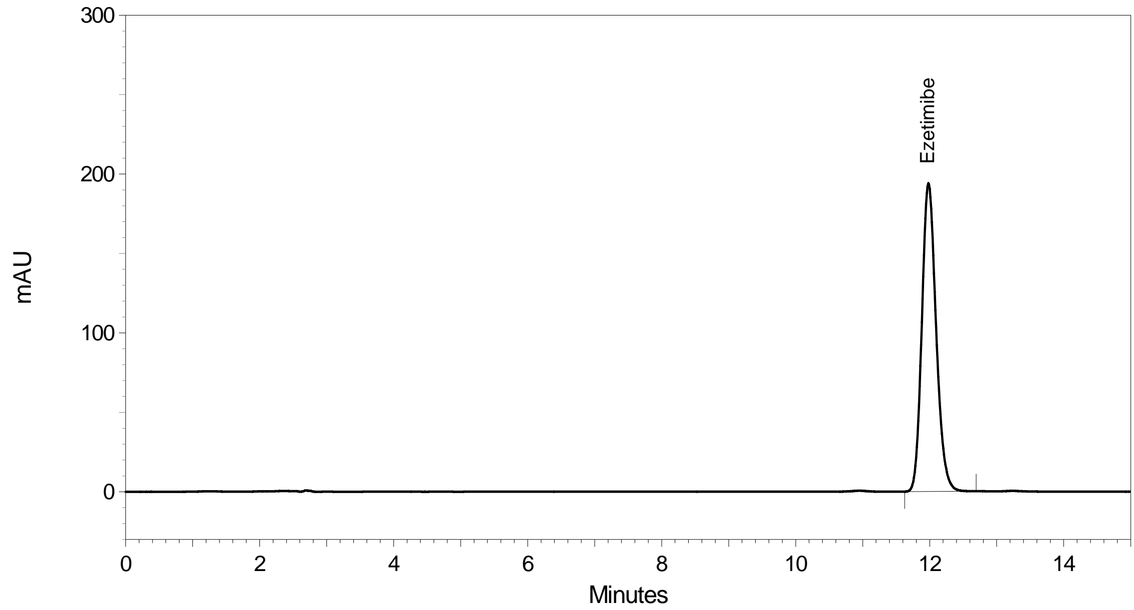
A_R = Standard mean area of respective time interval stage

A_I = Standard mean area of initial stage

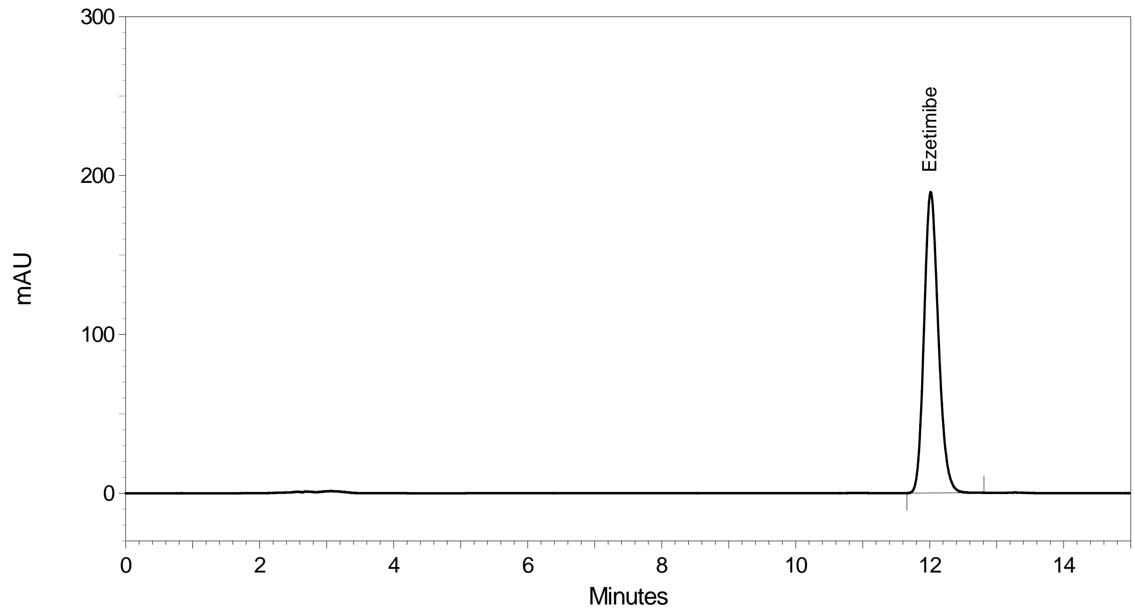
Chromatogram of standard preparation of 'After 36 hours stage' at 5˚ C:



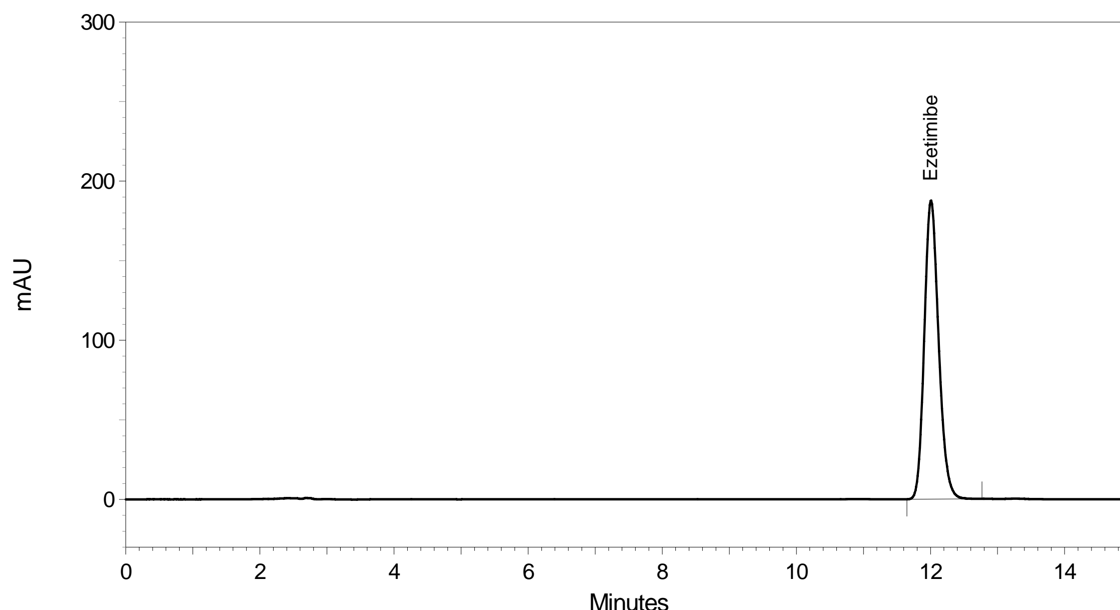
Chromatogram of standard preparation of 'After 36 hours stage' at room temperature:



Chromatogram of test preparation of 'After 36 hours stage' at 5° C:



Chromatogram of test preparation of 'After 36 hours stage' at room temperature:



Stage of solution stability study: After 48 hours

Blank preparation:
Diluent was used as blank.

Standard preparation (freshly prepared):
Stock solution: 50.9 mg of Ezetimibe reference standard was accurately weighed and transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask and the standard substance was dissolved by sonication for one minute. Then, volume of the flask was make up with diluent. The concentration obtained is 509 µg/ml of Ezetimibe.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.9 µg/ml of Ezetimibe.

Standard preparation (of 5˚ C) for stability:

Standard preparation solution which is stored at 5˚ C for 48 hours was injected (and % RSD for the same solution was measured against initially injected standard).

Standard preparation (of room temperature) for stability:

Standard preparation solution which is stored at room temperature for 48 hours was injected (and % RSD for the same solution was measured against initially injected standard).

Test Preparation (of 5˚ C) for stability:

Test preparation solution which is stored at 5˚ C for 48 hours was injected (and assay for the same solution was measured against freshly prepared standard followed by comparison of that of initial).

Test Preparation (of room temperature) for stability:

Test preparation solution which is stored at room temperature for 48 hours was injected (and assay for the same solution was measured against freshly prepared standard followed by comparison of that of initial).

Chromatographic sequence for 'After 48 hours' stage of solution stability study is represented through Table 40 as under:

Table 40: Sequence for 'After 48 hours' stage of solution stability study

Sr. No.	Description	Replicate
1	Blank	1
2	Standard preparation	5
3	Standard preparation (At 5˚ C)	2
4	Standard preparation (At room temperature)	2
5	Test preparation (At 5˚ C)	2
6	Test preparation (At room temperature)	2
7	Bracketing standard	1

Observations, calculation and chromatograms:

Table 41: Summary for 'After 48 hours' stage of solution stability study

Observation				
Standard Details			*Test Details*	
Data for Standard preparation			*Data for Test preparation (at 5° C temperature)*	
Replicate	Area		Replicate	Area
1	2846944		1	2757505
2	2845652		2	2758820
3	2848059		Average	2758163
4	2848430			
5	2858337		*Data for Test preparation (at room temperature)*	
Average	2849484			
Stdev	5065.89		Replicate	Area
% RSD	0.18		1	2720017
			2	2723838
			Average	2721928
Standard weight	50.9 mg		Test weight	1008.1 mg
Standard potency	99.8 %		Label claim	10 mg

% Assay of Test preparation solution stored at 5° C temperature:

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2758163}{2849484} x \frac{50.9}{100} x \frac{5}{50} x \frac{200}{1008.1} x \frac{50}{5} x \frac{101.5}{10} x\, 99.8$$

$$= 99.01$$

% Assay of Test preparation solution stored at room temperature:

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC} xP$$

$$= \frac{2721928}{2849484} x \frac{50.9}{100} x \frac{5}{50} x \frac{200}{1008.1} x \frac{50}{5} x \frac{101.5}{10} x\, 99.8$$

$$= 97.71$$

Where by,

A_T = Average area of Ezetimibe obtained in test preparation

A_S = Average area of Ezetimibe obtained in standard preparation

W_1 = Weight taken of Ezetimibe reference standard (mg)

W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

LC = Label claim (mg)

P = Potency of Ezetimibe reference standard (%)

Table 42: Summary for standard solution stability at 'After 48 hours' stage

Observation						
Data for Standard preparation (at 5˚ C temperature)				*Data for Standard preparation (at room temperature)*		
Stage	Replicate	Area		Stage	Replicate	Area
Initial	1	2902135		Initial	1	2902135
	2	2901226			2	2901226
	3	2900660			3	2900660
	4	2900068			4	2900068
	5	2897362			5	2897362
After 48 hours	1	2841627		After 48 hours	1	2786973
	2	2841658			2	2856708
	Average	2883534			Average	2877876
	Stdev	28655.09			Stdev	43275.21
	% RSD	0.99			% RSD	1.50
Stage	Mean area			Stage	Mean area	
Initial	2900290			Initial	2900290	
After 48 hours	2841643			After 48 hours	2821841	
Absolute Difference (%)	2.02			Absolute Difference (%)	2.70	

For standard preparation (at 5˚ C temperature):

$$\text{Absolute Difference (\%)} = \left| 100 - \left[\frac{A_R}{A_I} x\, 100 \right] \right|$$

$$= \left| 100 - \left[\frac{2841643}{2900290} \, x \, 100 \right] \right|$$

$$= 2.02$$

For standard preparation (at room temperature):

$$\text{Absolute Difference (\%)} = \left| 100 - \left[\frac{A_R}{A_I} \, x \, 100 \right] \right|$$

$$= \left| 100 - \left[\frac{2821841}{2900290} \, x \, 100 \right] \right|$$

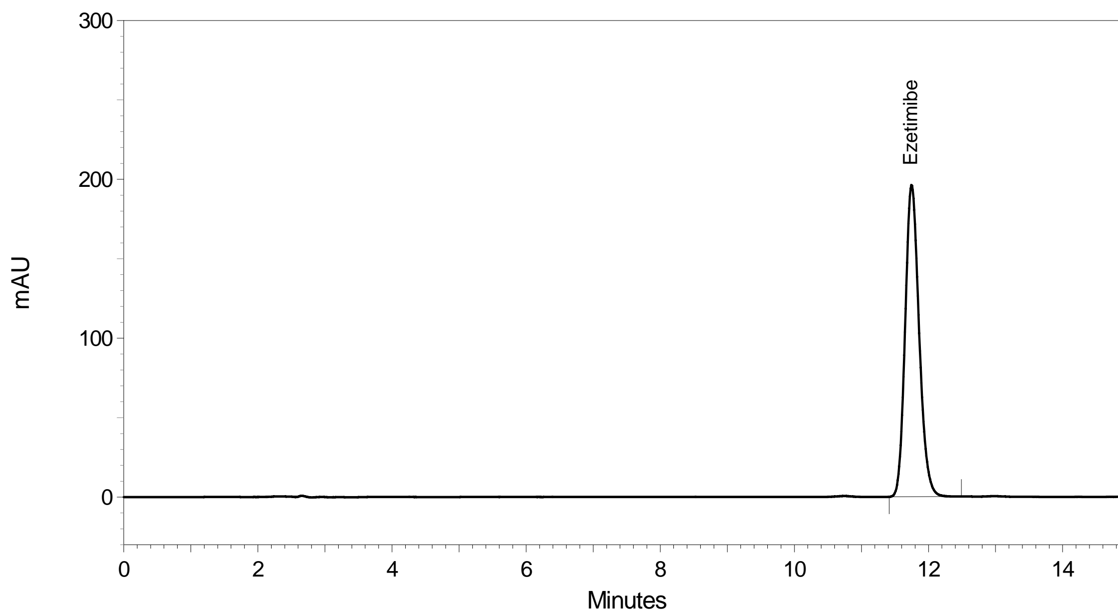
$$= 2.70$$

Where by,

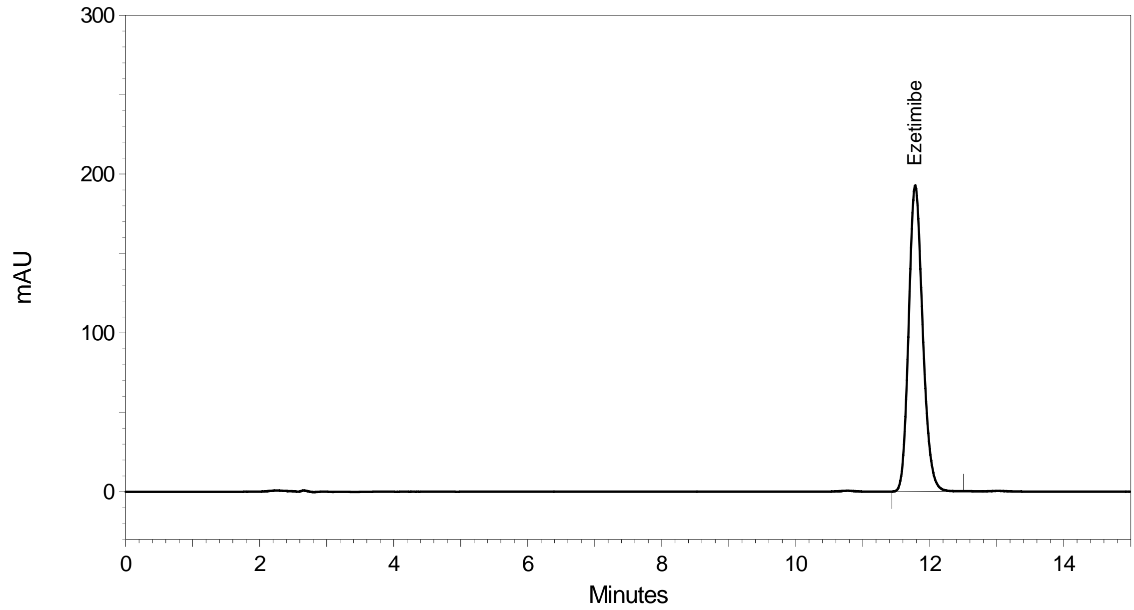
A_R = Standard mean area of respective time interval stage

A_I = Standard mean area of initial stage

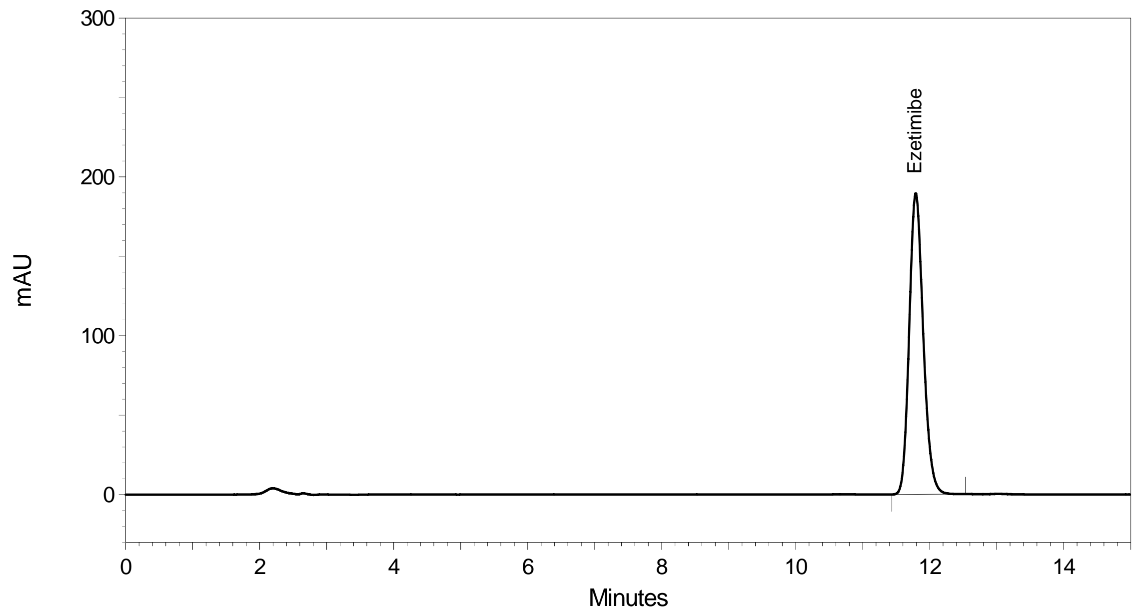
Chromatogram of standard preparation of 'After 48 hours stage' at 5˚ C:



Chromatogram of standard preparation of 'After 48 hours stage' at room temperature:



Chromatogram of test preparation of 'After 48 hours stage' at 5° C:



Chromatogram of test preparation of 'After 48 hours stage' at room temperature:

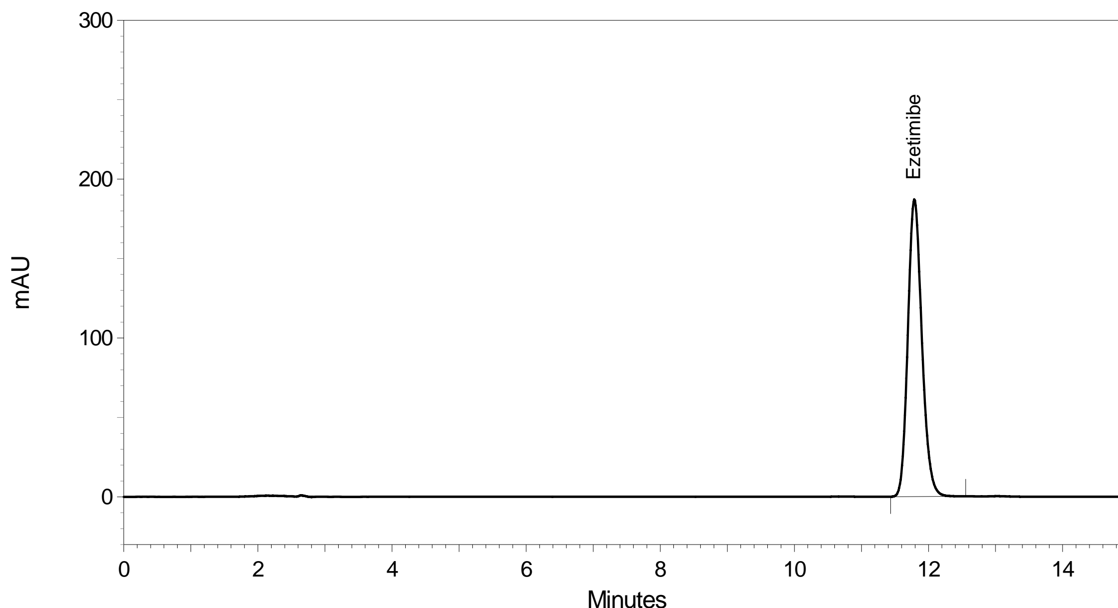


Table 43: Summary for solution stability study

Time intervals	Absolute difference in assay for standard solution, %		Absolute difference in assay for test solution, %	
	At 5°C	At room temperature	At 5°C	At room temperature
After 6 hours	0.08	0.13	0.03	0.04
After 12 hours	0.29	0.50	0.15	0.32
After 24 hours	0.05	0.74	0.09	0.87
After 36 hours	0.03	0.92	0.04	0.99
After 48 hours	2.02	2.70	1.02	2.32

Acceptance criteria:

1) For standard solution, absolute difference between Initial stage and value obtained at different time interval should be not more than ± 2.0 %. Overall % RSD for area between Initial stage and different time interval should be not more than ± 2.0.
2) For test solution, absolute difference between Initial assay value and assay value obtained at different time interval should be not more than ± 2.0 %.

Result: Up to certain time period,

1) For standard solution, results are well with-in the acceptance criteria.
2) For test solution, results are well with-in the acceptance criteria.

Conclusion:

Solution stability time period for Standard solution is 36 hours at 5 °C and room temperature. Solution stability time period for test solution is 48 hours at 5 °C and 36 hours at room temperature.

4.4.8 System suitability study

A system suitability test for the chromatographic system was performed before each validation parameter. Five replicate injections of standard preparation were injected and asymmetry, theoretical plate and % RSD of peak area were determined for same.

Table 44: Summary for system suitability study

System suitability parameters (In-house limit)	% RSD[a] (NMT[b] 2.0)	Theoretical plates (NLT[c] 5000)	Asymmetry (NMT[b] 2.0)
Validation parameters			
Specificity	0.06	15488	1.19
Linearity and range	0.09	14411	1.13
LOD and LOQ	0.20	14109	1.15
Precision	0.10	14667	1.17
Intermediate precision	0.09	14843	1.18
Accuracy	0.04	14787	1.16
Solution stability	0.06	14902	1.15
Robustness	0.12	14556	1.16

[a]Relative standard deviation
[b]Not more than
[c]Not less than

Acceptance criteria:

1) Theoretical plates of the analyte peak should be more than 5000 for standard preparation during each validation parameter.
2) Asymmetry of the analyte peak should be less than 2.0 for standard preparation during each validation parameter.
3) RSD of peak area of five replicate standard preparations should be less than 2.0 % during each validation parameter.

Result:

1) Theoretical plates of the analyte peak are found more than 5000 for standard preparation during each validation parameter.
2) Asymmetry of the analyte peak is found less than 2.0 for standard preparation during each validation parameter.
3) RSD of peak area of five replicate standard preparations should be less than 2.0 % during each validation parameter.

Conclusion:

System suitability was found satisfactory during each validation parameter. Hence, system is suitable for the same.

4.5 Reporting of deviation

The entire validation activity was performed in accordance with the acceptance criteria and no deviation was observed.

4.6 Discussion and conclusion

The observation and result obtained for each validation parameter including specificity, linearity, LOD and LOQ, precision, accuracy, robustness, solution stability and system suitability lies well with-in the acceptance criteria. Since, all the results are with-in the limit, the developed analytical method is considered as validated and suitable for intended use.

4.7 Analytical method validation for the method of content uniformity determination of Ezetimibe

4.7.1 **Objective:**

To perform analytical method validation of the developed chromatographic method for content uniformity determination of Ezetimibe from Ezetimibe tablets.

4.7.2 **Scope:**

This protocol is applicable to the standard testing procedure for content uniformity quantification of Ezetimibe from Ezetimibe tablets developed by Department of Chemistry, Saurashtra University.

4.7.3 **Standard testing procedure:**

Aim:

To determine content uniformity of Ezetimibe from Ezetimibe tablets through High performance liquid chromatography.

Instrument:

High performance liquid chromatograph (Shimadzu; LC-10AT*vp*) equipped with PDA detector (SPD-M10A*vp*) and connected to multi-instrument data-acquisition and data-processing system (Class-*VP* 6.13 SP2)

Reagents:

Acetonitrile (HPLC grade)

Methanol (HPLC grade)

Orthophosphoric acid (GR grade)

Water (HPLC grade)

Chromatographic condition:

Mobile phase	:-	0.1% Orthophosphoric acid *(v/v)*:Acetonitrile (50:50, *v/v*)
Column	:-	Phenomenex Luna (2) C_{18}, 4.6 x 250 mm, 5 µm
Flow rate	:-	1.0 ml/min
Detection	:-	232 nm
Injection volume	:-	20 µl
Diluent	:-	Water:Acetonitrile:Methanol (40:50:10, *v/v*)

Blank preparation:

Use diluent as blank.

Standard preparation:

Stock solution: Weigh accurately about 50 mg Ezetimibe reference standard and transfer into 100 ml volumetric flask. Add 70 ml of diluent to dissolve the substance by sonication for one minute and then dilute to volume with diluent. The concentration obtained is 500 µg/ml of Ezetimibe.

Pipette out 5 ml of above standard stock solution and transfer into 50 ml volumetric flask and dilute to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe.

Test Preparation:

Weigh accurately 1 tablet and transfer into 200 ml volumetric flask. Add 140 ml of diluent into the flask and sonicate of 30 minutes with normal hand-shaking. Cool the flask to room temperature and dilute to volume with diluent. Filter 10 ml of this solution through 0.45 µm nylon syringe filter. The concentration obtained is 50 µg/ml of Ezetimibe.

Repeat the same above procedure for other nine tablets.

System suitability:

- % RSD for replicate standard preparation should be less than 2.0 % for analyte peak.
- Asymmetry of the analyte peak should be less than 2.0 in standard preparation.
- Theoretical plates of the analyte peak should be more than 5000 in standard preparation.

Procedure:

- Inject the blank preparation and record the chromatogram.
- Inject five replicate of standard preparation and check for the system suitability and record the chromatograms.
- Inject test preparations in single and record the chromatograms.
- Calculate the % content uniformity of the sample and also calculate % RSD for the same.

Calculation:

Calculate the % content of the tablet using following formula.

$$\% \text{ Content} = \frac{A_T}{A_S} x \frac{W}{100} x \frac{5}{50} x \frac{200}{LC} xP$$

Where by,

A_T = Area of Ezetimibe obtained in test preparation

A_S = Average area of Ezetimibe obtained in standard preparation

W = Weight taken of Ezetimibe reference standard (mg)

LC = Label claim (mg)

P = Potency of Ezetimibe reference standard (%)

4.7.4 **Product Information:**

(1) Name:- Ezetimibe tablets (2) Label claim:- 10 mg

4.7.5 Reason for validation:

To establish intended applicability of the developed analytical method

4.7.6 Validation Approach:

Validation of analytical method will be done by using Ezetimibe tablets 10 mg formulation to establish by laboratory studies, that the performance characteristic of the method meets the requirement for the intended analytical application.

This whole experiment of validation is applicable to above mentioned Standard Testing Procedure for determination of content uniformity. As chromatographic parameters and concentration of content uniformity test are same as that of assay method, only precision study is performed for analytical method validation of content uniformity determination.

4.7.7 Chemicals and Reagents used in validation process

Acetonitrile (HPLC grade)

Methanol (HPLC grade)

Orthophosphoric acid (GR grade)s

Water (HPLC grade)

4.7.8 Validation Parameters

a) Precision study

b) System suitability study

4.8 Validation procedure for content uniformity determination of Ezetimibe

4.8.1 Precision study

Precision study was established by evaluating method precision and intermediate precision study. Method precision of the analytical method was determined by analyzing test preparations. Content of all test preparations was

determined and mean % content uniformity value, standard deviation, % relative standard deviation and 95% confidence interval for the same was calculated.

Intermediate precision of the analytical method was determined by performing method precision on another day by another analyst using different make of raw materials under same experimental condition. Content of all test preparations was determined and mean % content uniformity value, standard deviation, % relative standard deviation and 95% confidence interval for the same was calculated. Overall content value of method precision and intermediate precision was compared and % difference and overall % relative standard deviation was calculated.

For method precision, blank, standard preparation and test preparations was prepared as per method as under:

Blank preparation:
Diluent was used as blank.

Standard preparation:
Stock solution: 51.6 mg of Ezetimibe reference standard was accurately weighed and transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask and the standard substance was dissolved by sonication for one minute. Then, volume of the flask was make up with diluent. The concentration obtained is 516 µg/ml of Ezetimibe.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 51.6 µg/ml of Ezetimibe.

Test Preparation (Unit 1):
1 Tablet was accurately weighed (99.9 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The

volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is about 50 µg/ml of Ezetimibe.
Above preparation was repeated for other nine tablets.

Chromatographic sequence for Method precision study is represented through Table 1 as under:

Table 1: Sequence of Method precision study:

Sr. No.	Description	Injection replicate
1	Blank	1
2	Standard preparation	5
3	Test Preparation (Unit 1)	1
4	Test Preparation (Unit 2)	1
5	Test Preparation (Unit 3)	1
6	Test Preparation (Unit 4)	1
7	Test Preparation (Unit 5)	1
8	Test Preparation (Unit 6)	1
9	Test Preparation (Unit 7)	1
10	Test Preparation (Unit 8)	1
11	Test Preparation (Unit 9)	1
12	Test Preparation (Unit 10)	1
13	Bracketing standard	1

For intermediate precision, blank, standard preparation and test preparations was prepared as per method as under:

Blank preparation:
Diluent was used as blank.

Standard preparation:
Stock solution: 51.5 mg of Ezetimibe reference standard was accurately weighed and transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask and the standard substance was dissolved by sonication for one

minute. Then, volume of the flask was make up with diluent. The concentration obtained is 515 µg/ml of Ezetimibe.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 51.5 µg/ml of Ezetimibe.

Test Preparation (Unit 1):

1 Tablet was accurately weighed (100.4 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is about 50 µg/ml of Ezetimibe.

Above preparation was repeated for other nine tablets.

Chromatographic sequence for Intermediate precision study is represented through Table 2.

Table 2: Sequence of Intermediate precision study

Sr. No.	Description	Injection replicate
1	Blank	1
2	Standard preparation	5
3	Test Preparation (Unit 1)	1
4	Test Preparation (Unit 2)	1
5	Test Preparation (Unit 3)	1
6	Test Preparation (Unit 4)	1
7	Test Preparation (Unit 5)	1
8	Test Preparation (Unit 6)	1
9	Test Preparation (Unit 7)	1
10	Test Preparation (Unit 8)	1
11	Test Preparation (Unit 9)	1
12	Test Preparation (Unit 10)	1
13	Bracketing standard	1

<u>Observation, calculation and chromatograms:</u>

Table 3: Summary of method precision study

Observation				
Data for Standard preparation				
Replicate	Area		Standard weight	51.6 mg
1	2768197		Standard potency	99.8 %
2	2779313			
3	2767094			
4	2775563			
5	2768436			
Average	2771721			
Stdev	5408.75			
% RSD	0.20			

Unit No.	Area	% Assay	Weight of the tablet (mg)
Data for Test preparations			
Unit No.	Area	% Assay	Weight of the tablet (mg)
1	2736272	101.7	99.9
2	2724166	101.2	99.3
3	2730031	101.4	101.6
4	2747213	102.1	102.4
5	2757922	102.5	103.3
6	2728942	101.4	101.4
7	2747151	102.1	101.8
8	2734399	101.6	100.2
9	2733805	101.6	100.3
10	2744383	102.0	101.3
	Mean	101.8	
	Stdev	0.40	
	% RSD	0.39	

Calculation for % Content for each unit is as under:

For Unit 1:-

$$\% \text{ Content} = \frac{A_T}{A_S} x \frac{W}{100} x \frac{5}{50} x \frac{200}{LC} xP$$

$$= \frac{2736272}{2771721} x \frac{51.6}{100} x \frac{5}{50} x \frac{200}{10} x\, 99.8$$

= 101.7

For Unit 2:-

$$\% \text{ Content} = \frac{A_T}{A_S} x \frac{W}{100} x \frac{5}{50} x \frac{200}{LC} xP$$

$$= \frac{2724166}{2771721} x \frac{51.6}{100} x \frac{5}{50} x \frac{200}{10} x\, 99.8$$

= 101.2

For Unit 3:-

$$\% \text{ Content} = \frac{A_T}{A_S} x \frac{W}{100} x \frac{5}{50} x \frac{200}{LC} xP$$

$$= \frac{2730031}{2771721} x \frac{51.6}{100} x \frac{5}{50} x \frac{200}{10} x\, 99.8$$

= 101.4

For Unit 4:-

$$\% \text{ Content} = \frac{A_T}{A_S} x \frac{W}{100} x \frac{5}{50} x \frac{200}{LC} xP$$

$$= \frac{2747213}{2771721} x \frac{51.6}{100} x \frac{5}{50} x \frac{200}{10} x\, 99.8$$

= 102.1

For Unit 5:-

$$\% \text{ Content} = \frac{A_T}{A_S} x \frac{W}{100} x \frac{5}{50} x \frac{200}{LC} xP$$

$$= \frac{2757922}{2771721} x \frac{51.6}{100} x \frac{5}{50} x \frac{200}{10} x\, 99.8$$

= 102.5

For Unit 6:-

$$\% \text{ Content} = \frac{A_T}{A_S} x \frac{W}{100} x \frac{5}{50} x \frac{200}{LC} xP$$

$$= \frac{2728942}{2771721} x \frac{51.6}{100} x \frac{5}{50} x \frac{200}{10} x\, 99.8$$

$$= 101.4$$

For Unit 7:-

$$\% \text{ Content} = \frac{A_T}{A_S} x \frac{W}{100} x \frac{5}{50} x \frac{200}{LC} xP$$

$$= \frac{2747151}{2771721} x \frac{51.6}{100} x \frac{5}{50} x \frac{200}{10} x\, 99.8$$

$$= 102.1$$

For Unit 8:-

$$\% \text{ Content} = \frac{A_T}{A_S} x \frac{W}{100} x \frac{5}{50} x \frac{200}{LC} xP$$

$$= \frac{2734399}{2771721} x \frac{51.6}{100} x \frac{5}{50} x \frac{200}{10} x\, 99.8$$

$$= 101.6$$

For Unit 9:-

$$\% \text{ Content} = \frac{A_T}{A_S} x \frac{W}{100} x \frac{5}{50} x \frac{200}{LC} xP$$

$$= \frac{2733805}{2771721} x \frac{51.6}{100} x \frac{5}{50} x \frac{200}{10} x\, 99.8$$

$$= 101.6$$

For Unit 10:-

$$\% \text{ Content} = \frac{A_T}{A_S} x \frac{W}{100} x \frac{5}{50} x \frac{200}{LC} xP$$

$$= \frac{2744383}{2771721} x \frac{51.6}{100} x \frac{5}{50} x \frac{200}{10} x\, 99.8$$

= 102.0

Where by,

A_T = Area of Ezetimibe obtained in test preparation

A_S = Average area of Ezetimibe obtained in standard preparation

W = Weight taken of Ezetimibe reference standard (mg)

LC = Label claim (mg)

P = Potency of Ezetimibe reference standard (%)

Prototype chromatogram of test preparation for method precision study:

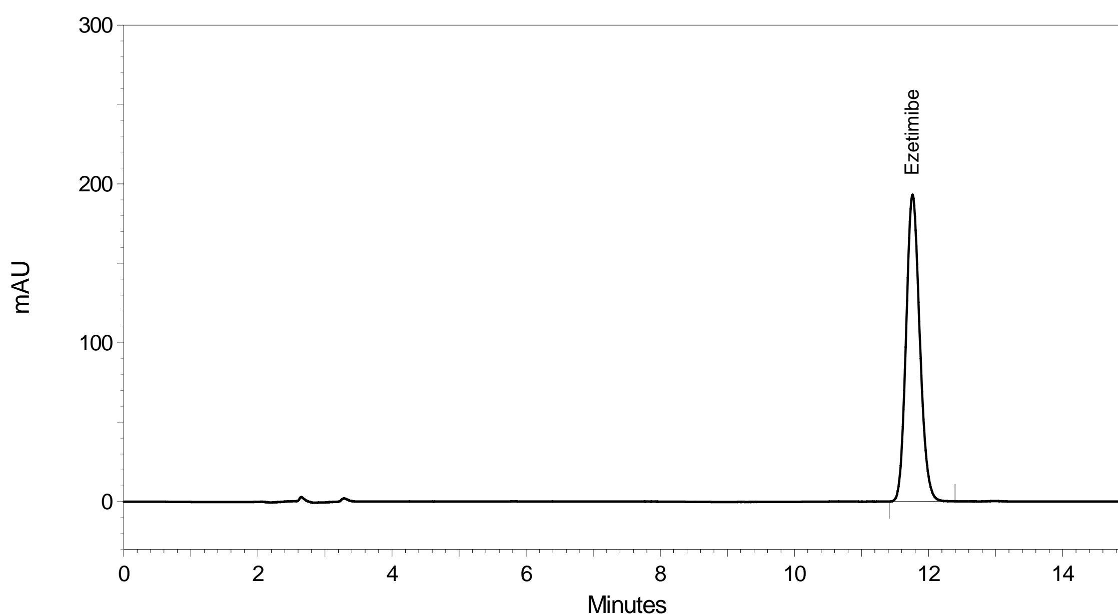


Table 4: Summary of Intermediate precision study

Observation				
Data for Standard preparation				
Replicate	Area		Standard weight	51.5 mg
1	2759154		Standard potency	99.8 %
2	2760810			
3	2758683			
4	2762901			
5	2765190			
Average	2761348			
Stdev	2710.08			
% RSD	0.10			

Data for Test preparations			
Unit No.	Area	% Assay	Weight of the tablet (mg)
1	2722333	101.3	100.4
2	2750077	102.4	102.3
3	2720555	101.3	100.8
4	2735428	101.8	100.7
5	2735867	101.8	101.7
6	2715231	101.1	101.8
7	2724759	101.4	101.1
8	2741880	102.1	102.4
9	2726593	101.5	100.9
10	2746359	102.2	101.9
	Mean	101.7	
	Stdev	0.44	
	% RSD	0.43	

Calculation for % Content for each unit is as under:

For Unit 1:-

$$\% \text{ Content} = \frac{A_T}{A_S} x \frac{W}{100} x \frac{5}{50} x \frac{200}{LC} xP$$

$$= \frac{2722333}{2761348} x \frac{51.5}{100} x \frac{5}{50} x \frac{200}{10} x\, 99.8$$

$$= 101.3$$

For Unit 2:-

$$\% \text{ Content} = \frac{A_T}{A_S} x \frac{W}{100} x \frac{5}{50} x \frac{200}{LC} xP$$

$$= \frac{2750077}{2761348} x \frac{51.5}{100} x \frac{5}{50} x \frac{200}{10} x\, 99.8$$

$$= 102.4$$

For Unit 3:-

$$\% \text{ Content} = \frac{A_T}{A_S} x \frac{W}{100} x \frac{5}{50} x \frac{200}{LC} xP$$

$$= \frac{2720555}{2761348} x \frac{51.5}{100} x \frac{5}{50} x \frac{200}{10} x\, 99.8$$

$$= 101.3$$

For Unit 4:-

$$\% \text{ Content} = \frac{A_T}{A_S} x \frac{W}{100} x \frac{5}{50} x \frac{200}{LC} xP$$

$$= \frac{2735428}{2761348} x \frac{51.5}{100} x \frac{5}{50} x \frac{200}{10} x\, 99.8$$

$$= 101.8$$

For Unit 5:-

$$\% \text{ Content} = \frac{A_T}{A_S} x \frac{W}{100} x \frac{5}{50} x \frac{200}{LC} xP$$

$$= \frac{2735867}{2761348} x \frac{51.5}{100} x \frac{5}{50} x \frac{200}{10} x\, 99.8$$

$$= 101.8$$

For Unit 6:-

$$\% \text{ Content} = \frac{A_T}{A_S} x \frac{W}{100} x \frac{5}{50} x \frac{200}{LC} xP$$

$$= \frac{2715231}{2761348} x \frac{51.5}{100} x \frac{5}{50} x \frac{200}{10} x\, 99.8$$

$= 101.1$

For Unit 7:-

$$\% \text{ Content} = \frac{A_T}{A_S} x \frac{W}{100} x \frac{5}{50} x \frac{200}{LC} xP$$

$$= \frac{2724759}{2761348} x \frac{51.5}{100} x \frac{5}{50} x \frac{200}{10} x\, 99.8$$

$= 101.4$

For Unit 8:-

$$\% \text{ Content} = \frac{A_T}{A_S} x \frac{W}{100} x \frac{5}{50} x \frac{200}{LC} xP$$

$$= \frac{2741880}{2761348} x \frac{51.5}{100} x \frac{5}{50} x \frac{200}{10} x\, 99.8$$

$= 102.1$

For Unit 9:-

$$\% \text{ Content} = \frac{A_T}{A_S} x \frac{W}{100} x \frac{5}{50} x \frac{200}{LC} xP$$

$$= \frac{2726593}{2761348} x \frac{51.5}{100} x \frac{5}{50} x \frac{200}{10} x\, 99.8$$

$= 101.5$

For Unit 10:-

$$\% \text{ Content} = \frac{A_T}{A_S} x \frac{W}{100} x \frac{5}{50} x \frac{200}{LC} xP$$

$$= \frac{2746359}{2761348} x \frac{51.5}{100} x \frac{5}{50} x \frac{200}{10} x\, 99.8$$

$= 102.2$

Where by,

A_T = Area of Ezetimibe obtained in test preparation

A_S = Average area of Ezetimibe obtained in standard preparation

W = Weight taken of Ezetimibe reference standard (mg)

LC = Label claim (mg)

P = Potency of Ezetimibe reference standard (%)

Prototype chromatogram of test preparation of intermediate precision study:

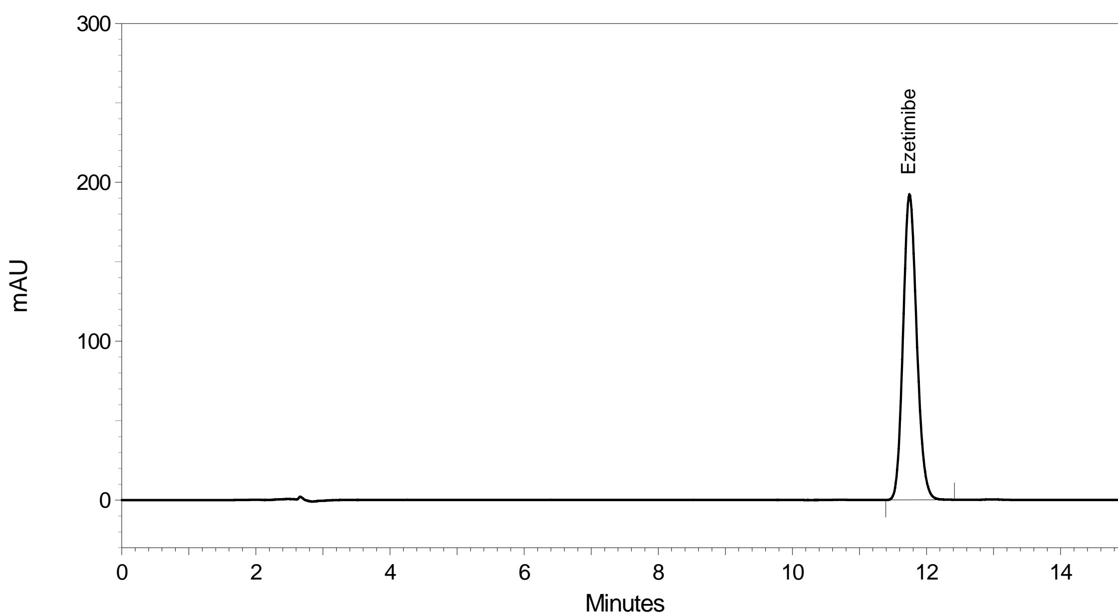


Table 5: Summary of Precision study

Study	Set No.	Assay (%)	Mean Assay (%)	Stdev	RSD (%)	95% Confidence Interval
Method Precision	1	101.7	101.8	0.40	0.39	0.28
	2	101.2				
	3	101.4				
	4	102.1				
	5	102.5				
	6	101.4				
	7	102.1				
	8	101.6				
	9	101.6				
	10	102.0				
Intermediate Precision	1	101.3	101.7	0.44	0.43	0.31
	2	102.4				
	3	101.3				
	4	101.8				
	5	101.8				
	6	101.1				
	7	101.4				
	8	102.1				
	9	101.5				
	10	102.2				
Overall	Mean	101.7		Absolute Difference between mean % content values of method precision and Intermediate precision = 0.1		
	Stdev	0.40				
	RSD(%)	0.39				

Acceptance criteria:

1) %RSD of six replicate sets of method precision study should be less than 6.0
2) %RSD of six replicate sets of intermediate precision study should be less than 6.0
3) Overall %RSD of replicate sets of method and intermediate precision study should not be more than 6.0
4) Absolute difference between mean % content values of method precision and Intermediate precision should not be more than 5.0

Results:

1) %RSD of six replicate sets of method precision study is 0.39
2) %RSD of six replicate sets of intermediate precision study is 0.43
3) Overall %RSD of replicate sets of method and intermediate precision study is 0.39
4) Absolute difference between mean % content values of method precision and Intermediate precision is 0.1

Conclusion:

All the results are well with-in the acceptance criteria. Hence, the analytical method is found precise.

4.8.2 **System suitability study:**

A system suitability test for the chromatographic system was performed before each validation parameter. Five replicate injections of standard preparation were injected and asymmetry, theoretical plate and % RSD of peak area were determined for same.

Table 6: Summary for system suitability study

System suitability parameters (In-house limit)	% RSD[a] (NMT[b] 2.0)	Theoretical plates (NLT[c] 5000)	Asymmetry (NMT[b] 2.0)
Validation parameters			
Method precision	0.20	15846	1.17
Intermediate precision	0.10	15548	1.12
[a]Relative standard deviation [b]Not more than [c]Not less than			

Acceptance criteria:

1) Theoretical plates of the analyte peak should be more than 5000 for standard preparation during each validation parameter.
2) Asymmetry of the analyte peak should be less than 2.0 for standard preparation during each validation parameter.
3) RSD of peak area of five replicate standard preparations should be less than 2.0 % during each validation parameter.

Result:

1) Theoretical plates of the analyte peak are found more than 5000 for standard preparation during each validation parameter.
2) Asymmetry of the analyte peak is found less than 2.0 for standard preparation during each validation parameter.
3) RSD of peak area of five replicate standard preparations should be less than 2.0 % during each validation parameter.

Conclusion:

System suitability was found satisfactory during each validation parameter. Hence, system is suitable for the same.

4.9 Reporting of deviation

The entire validation activity was performed in accordance with the acceptance criteria and no deviation was observed.

4.10 Discussion and conclusion

The observation and result obtained for each validation parameter including specificity, linearity, LOD and LOQ, precision, accuracy, robustness, solution stability and system suitability lies well with-in the acceptance criteria. Since, all the results are with-in the limit, the developed analytical method is considered as validated and suitable for intended use.

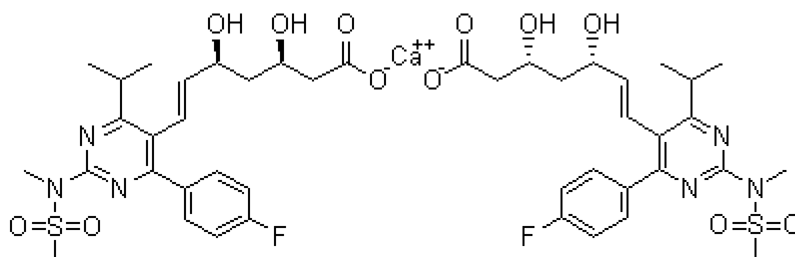
5.0 Method development and precision study for assay determination of Ezetimibe, Statins and Fibrate from their combined pharmaceutical dosage forms

5.1 Introduction to combination dosage forms of Ezetimibe with Statins and Fibrate

5.1.1 Description of Stains and Fibrate:

Hypolipidemic drugs belonging to the class of pharmaceuticals is called as 'Statins'. Statins are used to control hypercholesterolemia (elevated cholesterol levels) and to prevent cardiovascular disease. Rosuvastatin, Atorvastatin and Simvastatin are the compounds which fall in the category of Statins.

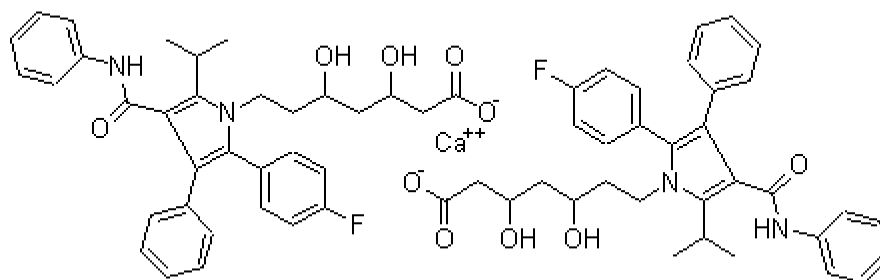
Rosuvastatin is chemically *7-[4-(4-fluorophenyl)-6-(1-methylethyl)-2-(methyl-methylsulfonyl-amino)-pyrimidin-5-yl]-3,5-dihydroxy-hept-6-enoic acid*. It is avail in its calcium salt and termed as Rosuvastatin calcium. Molecular formula of Rosuvastatin calcium is $C_{44}H_{54}CaF_2N_6O_{12}S_2$ and it has a molecular weight of 1001.14 g mol^{-1}. Its structural formula is:



Rosuvastatin calcium is off-white to creamish white crystalline powder that is soluble in acetonitrile and slightly soluble in acetone[56].

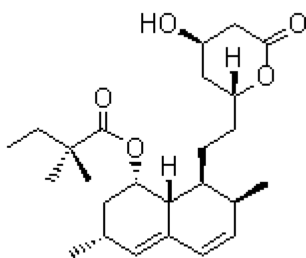
Atorvastatin is chemically *[R-(R*, R*)]-2-(4-fluorophenyl)-β, δ-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino) carbonyl]-1H- pyrrole-1-heptanoic acid*. It is avail in its calcium salt and termed as Atorvastatin calcium.

Molecular formula of Atorvastatin calcium is $C_{66}H_{68}CaF_2N_4O_{10}$ and it has a molecular weight of 1155.36 g mol^{-1}. Its structural formula is:



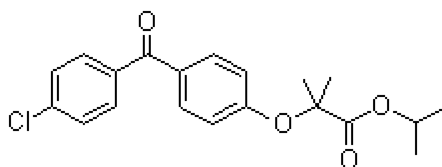
Atorvastatin calcium is a white to off-white crystalline powder that is very slightly soluble in water and acetonitrile, slightly soluble in ethanol (95%) and freely soluble in methanol[57].

Simvastatin is chemically *(1S,3R,7S,8S,8aR)-8-[2-[(2R,4R)-4-hydroxy-6-oxotetrahydro-2H-pyran-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl 2,2-dimethylbutanoate*. Its molecular formula is $C_{25}H_{38}O_5$ and it has a molecular weight of 418.57 g mol^{-1}. Its structural formula is:



Simvastatin is a white or almost white crystalline powder that is practically insoluble in water, very soluble in methylene chloride and freely soluble in alcohol[58].

Fenofibrate is chemically *1-methylethyl 2-[4-(4-chlorobenzoyl) phenoxy]-2-methylpropanoate*. Its molecular formula is $C_{20}H_{21}ClO_4$ and it has a molecular weight of 360.84 g mol^{-1}. Its structural formula is:



Fenofibrate is a white or almost white crystalline powder that is practically insoluble in water, very soluble in methylene chloride and slightly soluble in alcohol[59].

5.1.2 Clinical pharmacology and mechanism of action of Statins and Fibrates

Clinical studies have demonstrated that elevated levels of total cholesterol (total-C), low-density lipoprotein cholesterol (LDL-C) and apolipoprotein B (Apo B), the major protein constituent of LDL, promote human atherosclerosis. In addition, decreased levels of high-density lipoprotein cholesterol (HDL-C) are associated with the development of atherosclerosis. Epidemiologic studies have established that cardiovascular morbidity and mortality vary directly with the level of total-C and LDL-C and inversely with the level of HDL-C. Like LDL, cholesterol-enriched triglyceride-rich lipoproteins, including very-low- density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), and remnants, can also promote atherosclerosis. The independent effect of raising HDL-C or lowering triglycerides (TG) on the risk of coronary and cardiovascular morbidity and mortality has not been determined[60].

Statins are the inhibitors of 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA). HMG-CoA reductase is the rate-limiting step in cholesterol biosynthesis that catalyzes the conversion of HMG-CoA to mevalonate, a precursor of cholesterol. By inhibiting cholesterol biosynthesis, Statins lead to increased hepatic LDL receptor activity and accelerated clearance of circulating LDL. Statins inhibits hepatic synthesis of VLDL, which reduces the total number of VLDL and LDL particles. Statins reduces total cholesterol (total-C), LDL-C, Apo B, intermediate density lipoprotein cholesterol (IDL-C), non-HDL-C in

patients with homozygous and heterozygous familial hypercholesterolemia (FH), non familial forms of hypercholesterolemia, mixed dyslipidemia and dysbetalipoproteinemia. Statins also reduces plasma triglycerides in a dose-dependent fashion, which is proportional to their LDL-C lowering effects [if the triglycerides are less than 3.9 mmol/L (<350 mg/dL). Statins have a modest HDL-raising effect (5 to 10%)[61-62].

Statins currently available differ in their LDL-C reducing effect. Numbers of patients have been treated with Statins for over last 8 years as a part of large randomized controlled clinical trials and no increase in any major noncardiac diseases have been seen in these individuals. Statins are the drug class of choice for LDL-C reduction and are by far the most widely used class of lipid-lowering drugs[62].

Fibric acid derivatieves, or fibrates, are agonists of PPARα (Peroxisome proliferator activated receptor α), a nuclear receptor involved in the regulation of carbohydrate and lipid metabolism. Fibrates stimulates LPL activity (enhancing triglyceride hydrolysis), reduce apoC-III synthesis (enhancing lipoprotein remnant clearance), and may reduce VLDL production. Fibrates are the most effective drugs available for reducing triglyceride levels and they also raise HDL-C levels. Fibrates are the drug class of choice in patients with severe hypertriglyceridemia [11.3 mmol/L (>1000 mg/dL)] and are a reasonable considerations in patients with moderate hypertriglyceridemia [4.5 to 11.3 mmol/L (400 to 1000 mg/dL)] [62].

Fenofibric acid, the active metabolite of Fenofibrate, produces reductions in total cholesterol, LDL cholesterol, apoprotein B, total glycerides and triglyceride rich lipoprotein (VLDL) in treated patients. In addition, treatment with Fenofibrate results in increases in high density lipoprotein (HDL), and apolipoproteins - apo AI and apo AII. The effect of fenofibric acid as seen in clinical practice has been explained *in vitro* in human hepaStocyte cultures by the activation of PPARα. Through this mechanism, Fenofibrate increases lipolysis

and elimination of triglyceriderich particles from plasma by activating lipoprotein lipase and reducing production of apoprotein C-III. The resulting produces larger particles which have a greater affinity for cholesterol receptors and are catabolized rapidly. Activation of PPARα also induces an increase in the synthesis of apoproteins A-I, A-II, and HDL-Cholesterol. Fenofibrate also reduces serum uric acid levels in hyperuricemic and normal individuals by increasing the urinary excretions of uric acid[63].

The relative successful indications of Ezetimibe with Statins and Fibrate in the role of combined therapy are being determined by clinical trials also[64-84].

5.1.3 Literature review

The literature reviews regarding combined dosage forms of Ezetimibe suggest that analytical methods were reported for its simultaneous determination as combined pharmaceutical formulation with Statins. Brief details for the same are as under:

(1) Rajkondawar V. V. developed an analytical method based on reversed-phase liquid chromatography for the simultaneous determination of Ezetimibe and Rosuvastatin. Separation was achieved on a C_{18} analytical column (150 × 4.6 mm i.d.) with mobile phase consisting of 50 mM KH_2PO_4 buffer-acetonitrile (55:45, *v/v*). The flow rate was 1.0 ml/min and the analyte monitored at 235 nm[85].

(2) Syed S. Q., Syed N. R., Islam U. K., Muhammad A. and Zeba A. S. have developed reversed-phase HPLC method for the simultaneous quantitation of Ezetimibe and Atorvastatin in pharmaceutical formulations. The chromatographic separation involved an elution on a Hypersil phenyl-2 column (250 × 4.6 mm i.d.) using a mobile phase composition of 0.1 M

ammonium acetate (pH 6.5) and acetonitrile (28:72, v/v). Eluent was monitored by absorbance at 242 nm[86].

(3) Chaudhari B. G., Patel N. M., Shah P. B., Patel L. J. and Patel V. P. have developed an analytical method on LiChrospher 100 C_{18} column and a mobile phase composed of acetonitrile-water-methanol (45:40:15, *v/v/v*) with apparent pH to 4.0; flow rate was 1.0 ml/min and UV detection was carried out at 250 nm. The proposed method was useful for simultaneous determination for Ezetimibe and Atorvastatin in combine pharmaceutical dosage forms [87].

(4) Unnam S. and Chandrasekhar B. K. have developed an analytical method by revered-phase HPLC for simultaneous determination of Ezetimibe and Atorvastatin 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[88].

(5) N. Ozaltin and E. Ucakturk have developed an analytical method through liquid chromatography for simultaneous determination of Ezetimibe and Simvastatin. The chromatographic separation was achieved on C_8 column (200 × 4.6 mm i.d.) by application of dual-mode solvent through gradient pattern. The developed method was successfully applied to pharmaceutical formulations[89].

(6) Chaudhari B. G., Patel N. M., Shah P. B. have developed an analytical method through the reversed-phase liquid chromatography for simultaneous determination of Ezetimibe and Simvastatin in pharmaceutical dosage forms. The proposed method utilizes a LiChrospher 100 C_{18} column (250 × 4.6 mm i.d.) and mobile phase consisting of acetonitrile-water-methanol (60:25:15, *v/v/v*) of apparent pH of 4.0 ; flow rate was 1.5 ml/min and detection was monitored at 238 nm[90].

(7) Oliveira P. R., Barth T., Todeschini V., and Dalmora S. L. have developed an analytical method for simultaneous determination of Ezetimibe and Simvastatin in pharmaceutical dosage forms. A reversed-phase liquid chromatographic separation was achieved on a Synergi fusion C_{18} column using mobile phase consisting of 0.03 M phosphate buffer (pH 4.5)-acetonitrile (35:65, *v/v*). The flow rate was 0.6 ml/min and detection was 234 nm by PDA[91].

5.1.4 **Aim of work**

Methods reported in the literature for analysis of Ezetimibe with Statins have reversed-phase HPLC technique for the determination of the drug in combined pharmaceutical form. All analytical methods are differing in stationary phases, components of mobile phase, flow rate and detection pattern. There is no similarity in these methods as those are differ according to its combined dosage form with different type of Statin (i.e. Rosuvastatin, Atorvastatin and Simvastatin). The aim of present work is to develop such type of analytical methods that are similar in stationary phase, components of mobile phase, flow rate and detection pattern and able to determine the combined pharmaceutical dosage forms of Ezetimibe with mentioned type of Statins. The advantage of these newly developed analytical methods is that different combination dosage forms of Statins with Ezetimibe will be analyzed simultaneously on a single HPLC instrument by keeping just different composition of mobile phase's components according to each combination dosage forms. Selection of different composition of mobile phase's components can be performed through the dual mode of pump-ports of modern HPLC system. The proposed arrangement reduces labor work of preparing different mobile phase for each combined dosage forms and also infers the requirement of saturation of different type of stationary phase accordingly. Hence, it saves considerable time duration of the analysis and results can be achieved in short period of time.

Further, study of literature reveals that there are no reports of analytical methods for determination of Ezetimibe, Statins and Fibrates simultaneously. In the present work, an analytical method is developed that estimate Ezetimibe, Atorvastatin and Fenofibrate simultaneously from their combined triple dosage form of pharmaceutical formulation.

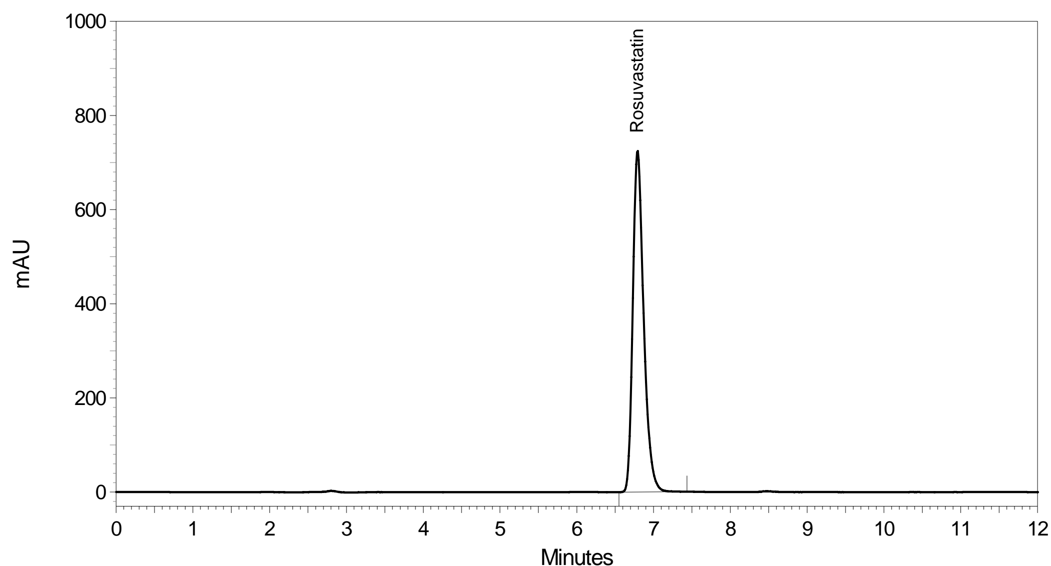
5.2 Analytical method development for assay determination of combination dosage forms of Ezetimibe with Statins and Fibrate

Analytical methods based on LC were developed for assay determination of combination dosage forms of Ezetimibe named by as under:

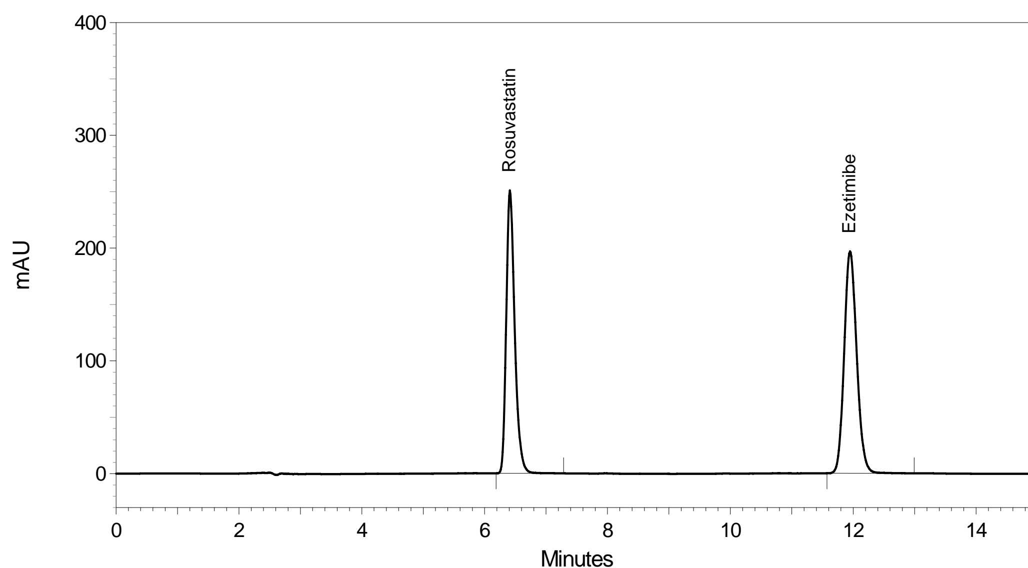
(1) Ezetimibe + Rosuvastatin
(2) Ezetimibe + Atorvastatin
(3) Ezetimibe + Simvastatin
(4) Ezetimibe + Atorvastatin + Fenofibrate

The basic chromatographic conditions used for analytical separations of above combination dosage forms were designed to be simple and easy to use and reproduce. The analytical conditions were selected after testing the different parameters that influence LC analysis, such as column, aqueous and organic phase for mobile phase, mobile phase proportion, wavelength, diluent, concentration of analyte and other chromatographic parameters.

In focus to develop analytical separation for 'Ezetimibe and Rosuvastatin', solution of Rosuvastatin is injected under the same chromatographic condition which is developed for determination of Ezetimibe as earlier mentioned. Under this chromatographic condition, the retention of Rosuvastatin peak is achieved. The representative chromatogram for the same is shown as under:



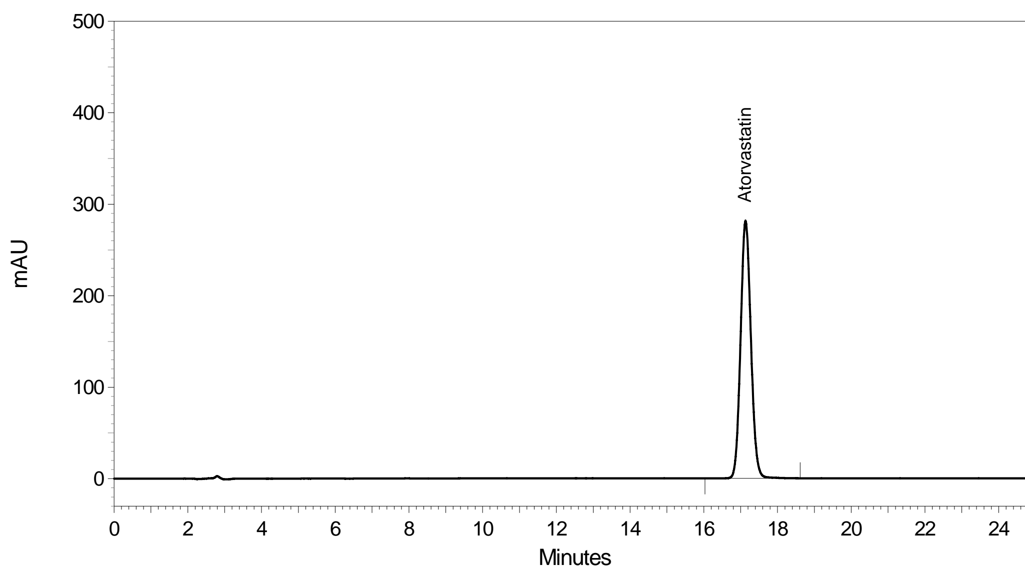
All other parameters of chromatographic condition are being kept same as that of method of Ezetimibe. A schematic chromatogram which states for obtained analytical separation of Ezetimibe and Rosuvastatin is shown as under:



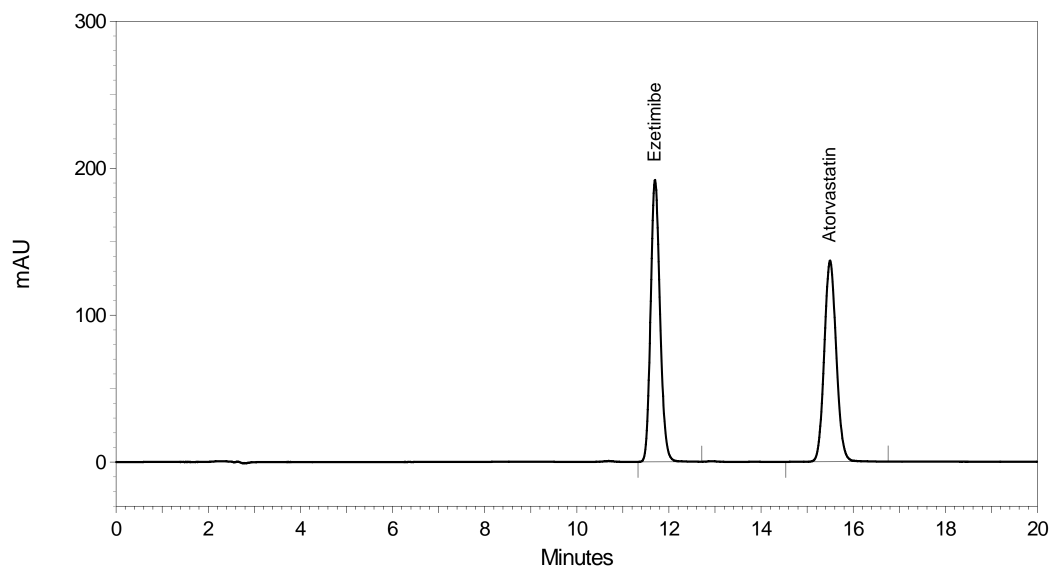
Above chromatogram is clearly indicated that both peaks are symmetrical and resolution between the both peaks is very good. Rosuvastatin elutes first and

Ezetimibe elutes later in developed analytical method. The preciseness of the analytical method is evaluated and determined through Section 5.3

In focus to develop analytical separation for 'Ezetimibe and Atorvastatin', solution of Atorvastatin is injected under the same chromatographic condition which is developed for determination of Ezetimibe as earlier mentioned. Under this chromatographic condition, the retention of Atorvastatin peak is achieved. The representative chromatogram for the same is shown as under:

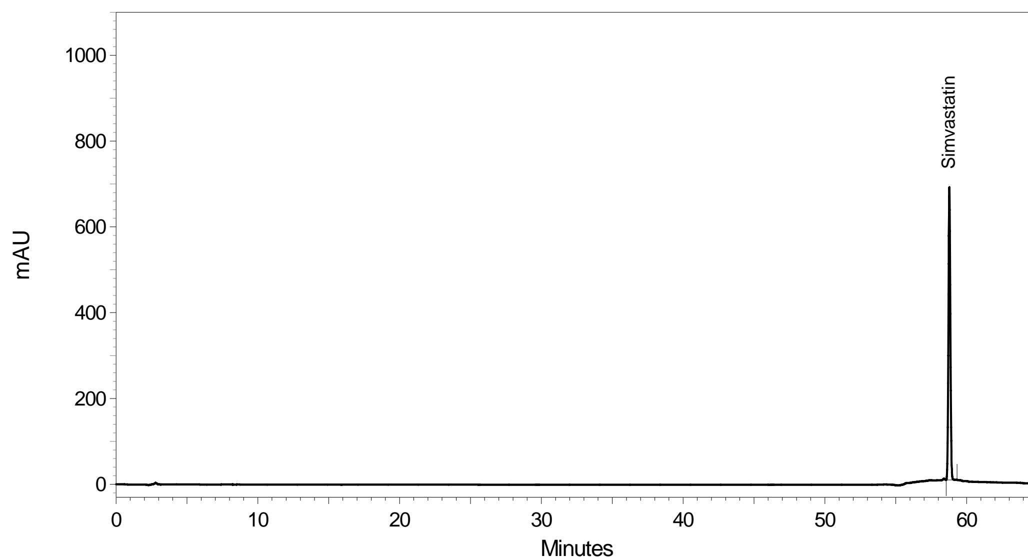


All other parameters of chromatographic condition are being kept same as that of method of Ezetimibe. A schematic chromatogram which states for obtained analytical separation of Ezetimibe and Atorvastatin is shown as under:

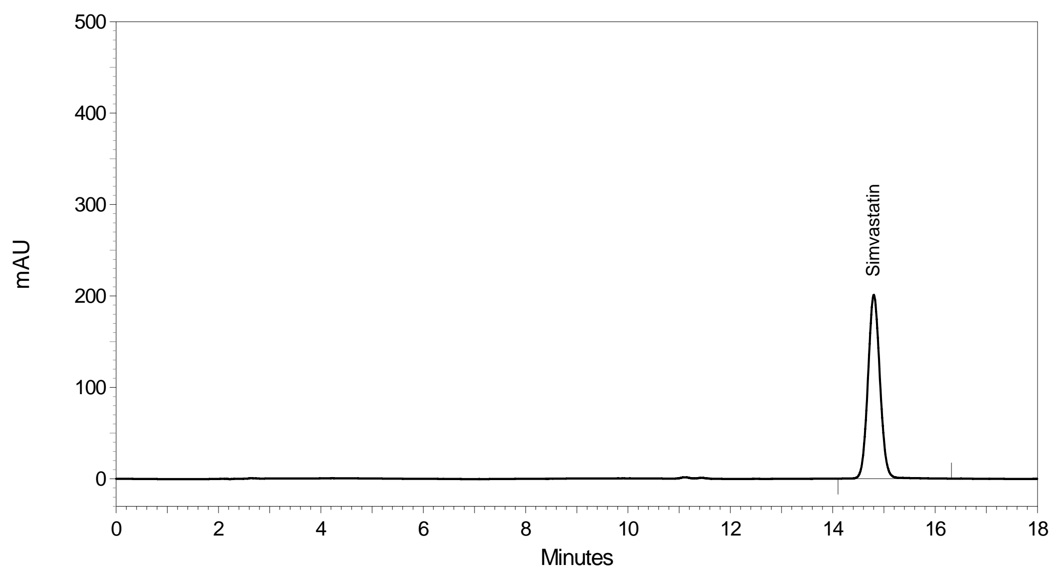


Above chromatogram is clearly indicated that both peaks are symmetrical and resolution between the both peaks is very good. Ezetimibe elutes first and Atorvastatin elutes later in developed analytical method. The preciseness of the analytical method is evaluated and determined through Section 5.4

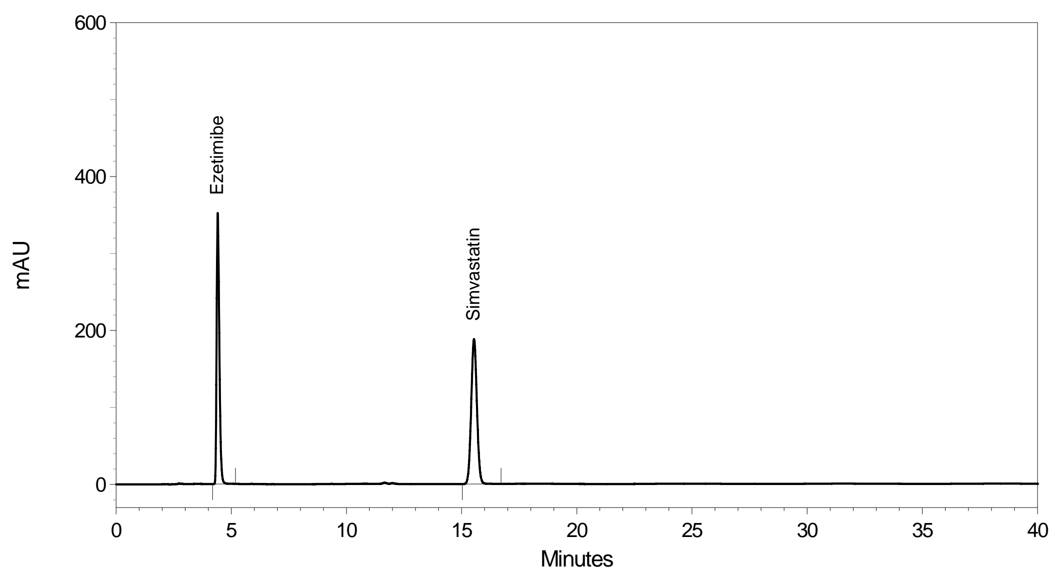
In focus to develop analytical separation for 'Ezetimibe and Simvastatin', solution of Simvastatin is injected under the same chromatographic condition which is developed for determination of Ezetimibe as earlier mentioned. Under this chromatographic condition, the retention of Simvastatin peak is achieved but being very late eluted. The representative chromatogram for the same is shown as under:



Above chromatogram states that retention time of Simvastatin is very longer. An attempt was made to reduce late elution of Simvastatin by increasing proportion of organic phase (Acetonitrile) of the mobile phase. After different trials, it was achieved at proportion of 70% of organic phase. The representative chromatogram for the same is shown as under:

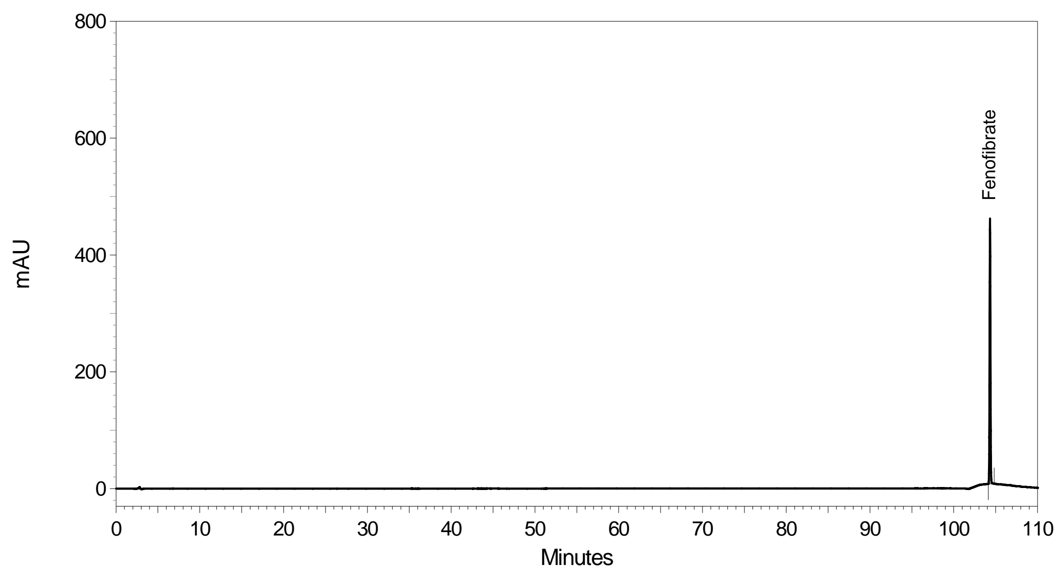


Except mobile phase proportion, all other parameters of chromatographic condition are being kept same as that of method of Ezetimibe. At present mobile phase proportion, elution of Ezetimibe is changed to early. A schematic chromatogram which states for obtained analytical separation of Ezetimibe and Simvastatin is shown as under:



Above chromatogram is clearly indicated that both peaks are symmetrical and resolution between the both peaks is very good. Ezetimibe elutes first and Simvastatin elutes later in developed analytical method. The preciseness of the analytical method is evaluated and determined through Section 5.5

In focus to develop analytical separation for 'Ezetimibe, Atorvastatin and Fenofibrate', solution of Fenofibrate is injected under the same chromatographic condition which is developed for determination of Ezetimibe as earlier mentioned. Under this chromatographic condition, the retention of Fenofibrate peak is achieved but being very late eluted. The representative chromatogram for the same is shown as under:

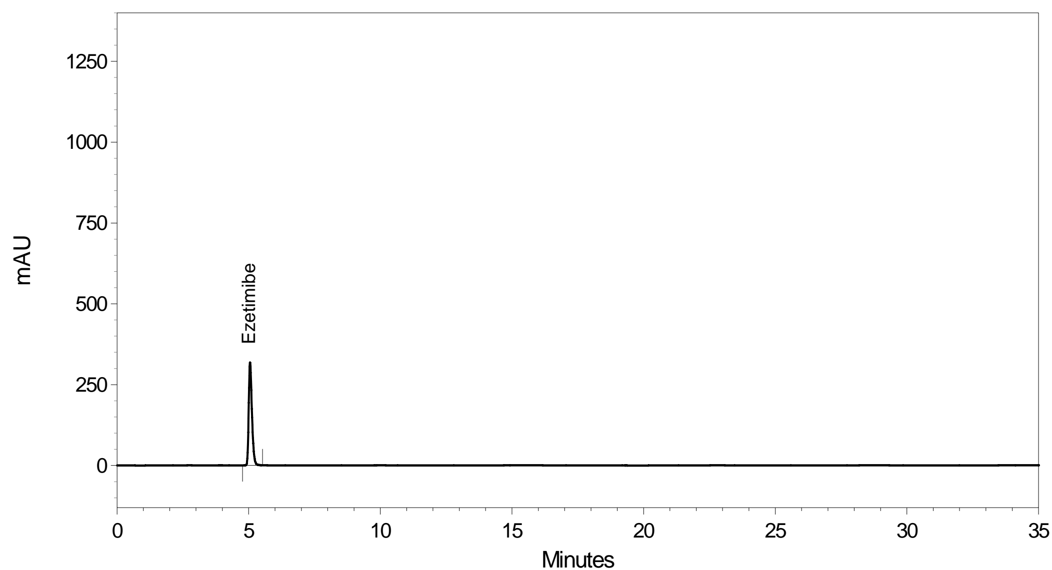


Above chromatogram states that elution of Fenofibrate is too much late. This chromatographic condition is not suitable even though peak of Fenofibrate is found symmetrical. An attempt was made to reduce late elution of Fenofibrate by increasing proportion of organic phase (Acetonitrile) of the mobile phase. After different trials, it was achieved at proportion of 70% of organic phase but at this condition, peak of Ezetimibe and Atorvastatin are merged. Further, an attempt was made to separate the peaks of Ezetimibe and Atorvastatin by decreasing proportion of organic phase vice versa increasing proportion of aqueous phase (Buffer). At 35% proportion of aqueous phase (Buffer), peaks of Ezetimibe and Atorvastatin are found to be completely resolved. In this similar chromatographic condition, solution of Fenofibrate is injected. All analyte peaks are found symmetrical and completely resolved to each other.

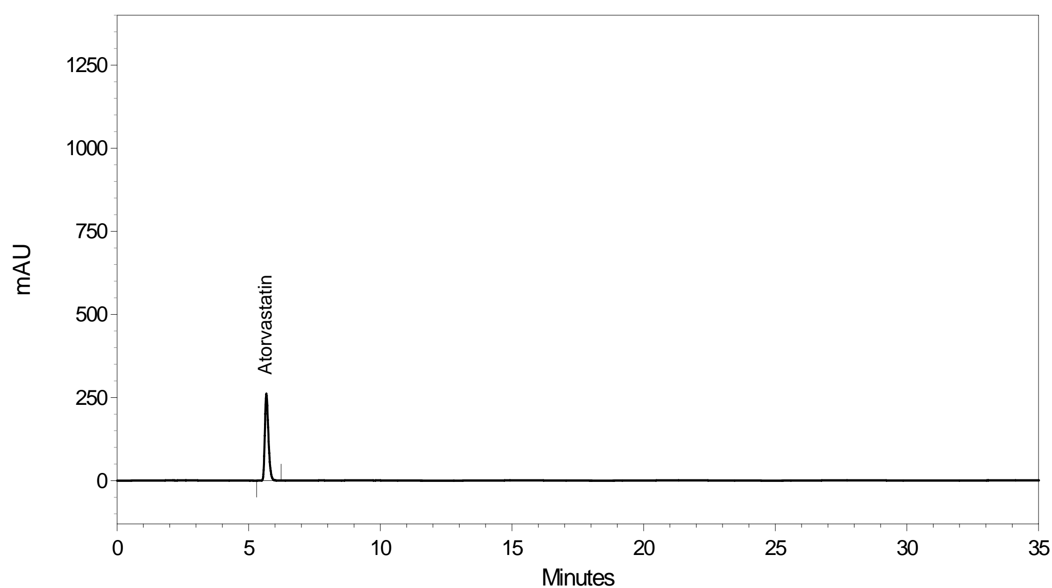
During the extraction trials, the preliminary diluent (Water:Acetonitrile:Methanol :: 40:50:10) which is used for extraction of all earlier dosage form is found unsuitable for this triple dosage form. The present dosage form does not produce complete dispersion of tablet in selected diluent. Afterwards on the basis of other experiments, it is concluded that presence of water in diluent is being interfere in the extraction process. Hence, the diluent is modified to (Acetonitrile:Methanol :: 50:50) by removing water and complete

extraction is achieved. At the optimized chromatographic condition, the representative chromatograms of all analytes are shown as under:

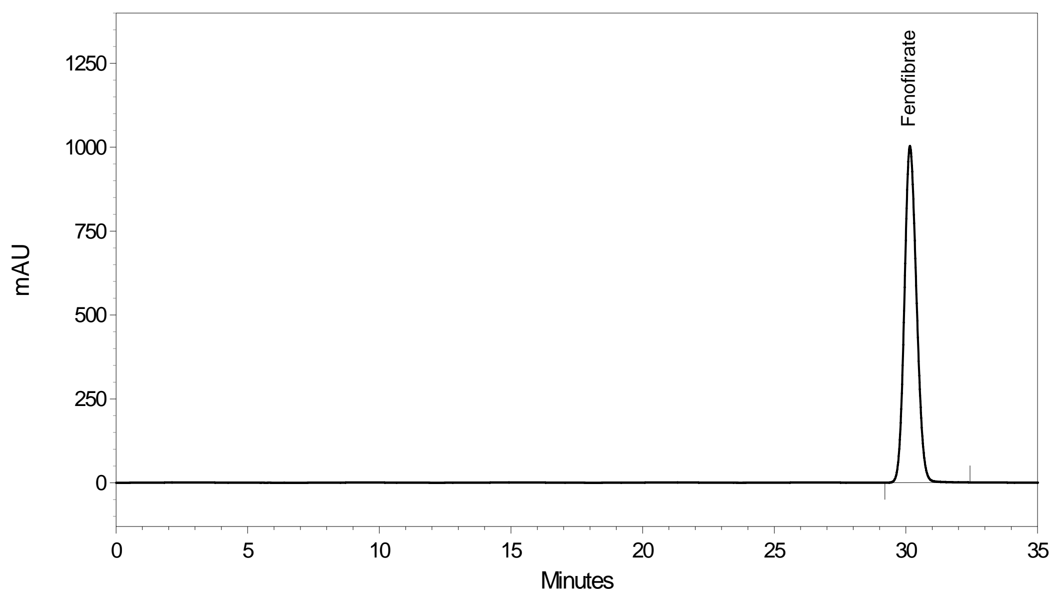
For Ezetimibe:-



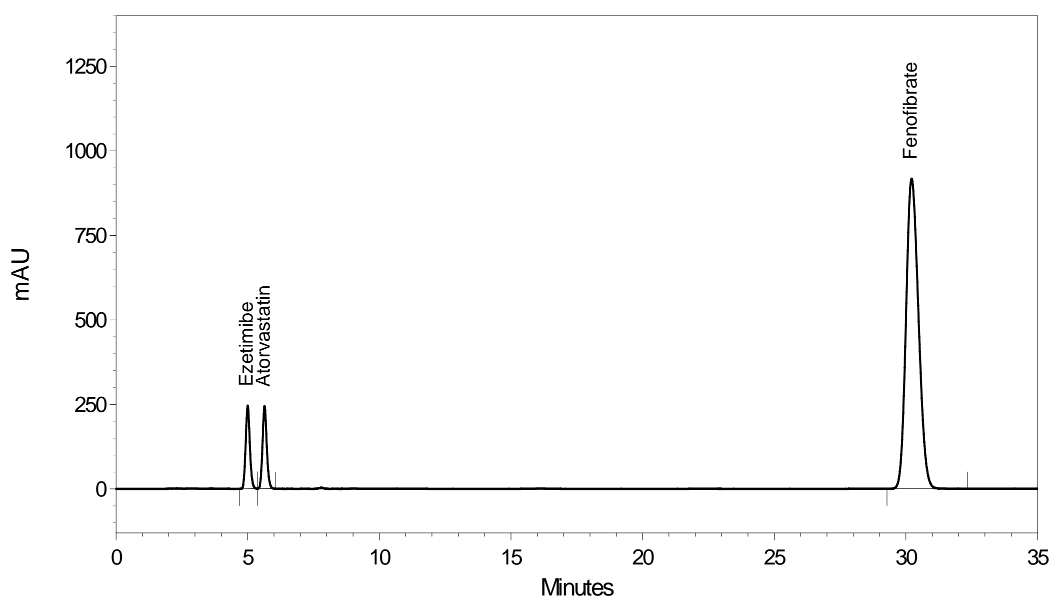
For Atorvastatin:-



For Fenofibrate:-



Hence, the chromatographic method to evaluate all three analyte simultaneously is developed. The chromatogram that represent simultaneous evaluation for the same is shown as under:



Above chromatogram is clearly indicated that all peaks are symmetrical and completely resolved. Except mobile phase proportion and components of diluent, all other parameters of chromatographic condition are being kept same as

that of the method of Ezetimibe. At present mobile phase proportion, the elution order is in the sequence of Ezetimibe, Atorvastatin and Fenofibrate. The preciseness of the analytical method is evaluated and determined through Section 5.6

5.3 Determination method of Ezetimibe and Rosuvastatin

5.3.1 Objective:

To develop analytical method for the assay determination of Ezetimibe and Rosuvastatin from combination dosage form

5.3.2 Standard testing procedure:

Aim:

To determine assay of Ezetimibe and Rosuvastatin from its combination dosage form (tablet) through High performance liquid chromatography

Instrument:

High performance liquid chromatograph (Shimadzu; LC-10AT*vp*) equipped with PDA detector (SPD-M10A*vp*) and connected to multi-instrument data-acquisition and data-processing system (Class-*VP* 6.13 SP2)

Reagents:

Acetonitrile (HPLC grade)

Methanol (HPLC grade)

Orthophosphoric acid (GR grade)

Water (HPLC grade)

Chromatographic condition:

Mobile phase :- 0.1% Orthophosphoric acid *(v/v)*:Acetonitrile (50:50, *v/v*)

Column :- Phenomenex Luna (2) C_{18}, 4.6 x 250 mm, 5 µm

Flow rate :- 1.0 ml/min

Detection :- 232 nm

Injection volume :- 20 µl

Diluent :- Water:Acetonitrile:Methanol (40:50:10, *v/v*)

Blank preparation:

Use diluent as blank.

Standard preparation:

Stock solution: Weigh accurately about 50 mg Ezetimibe reference standard and 52.09 Rosuvastatin calcium reference standard (equivalent to 50 mg Rosuvastatin) and transfer into 100 ml volumetric flask. Add 70 ml of diluent to dissolve the substance by sonication for one minute and then dilute to volume with diluent. The concentration obtained is 500 µg/ml of Ezetimibe and 500 µg/ml of Rosuvastatin.

Pipette out 5 ml of above standard stock solution and transfer into 50 ml volumetric flask and dilute to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe and 50 µg/ml of Rosuvastatin.

Test Preparation:

Weigh accurately 20 tablets and find out the average weight. Weigh accurately 10 tablets and transfer into 200 ml volumetric flask. Add 140 ml of diluent into the flask and sonicate of 30 minutes with normal hand-shaking. Cool the flask to room temperature and dilute to volume with diluent. Filter 10 ml of this solution through 0.45 µm nylon syringe filter. The concentration obtained is 500 µg/ml of Ezetimibe and 500 µg/ml of Rosuvastatin.

Pipette out 5 ml of above test stock solution and transfer into 50 ml volumetric flask and dilute to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe and 50 µg/ml of Rosuvastatin.

System suitability:

- % RSD for replicate standard preparation should be less than 2.0 % for both analyte peaks.
- Asymmetry of the both analyte peaks should be less than 2.0 in standard preparation.
- Theoretical plates of the both analyte peaks should be more than 5000 in standard preparation.
- Resolution of the both analyte peak should be more than 10.0 in standard preparation.

Procedure:

- Inject the blank preparation and record the chromatogram.
- Inject five replicate of standard preparation and check for the system suitability and record the chromatograms.
- Inject test preparation in duplicate and record the chromatograms.
- Calculate the % assay of the sample.

Elution order:

1. Rosuvastatin
2. Ezetimibe

Calculation:

Calculate the % assay of Ezetimibe and Rosuvastatin in the sample using following formula.

For Ezetimibe:

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_1} x P_1$$

Where by,

A_{T1} = Average area of Ezetimibe obtained in test preparation

A_{S1} = Average area of Ezetimibe obtained in standard preparation

W_1 = Weight taken of Ezetimibe reference standard (mg)

W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

LC_1 = Label claim of Ezetimibe (mg)

P_1 = Potency of Ezetimibe reference standard (%)

For Rosuvastatin:

$$\% \text{ Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{961.06}{1001.14} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_2} x P_2$$

(Equivalency: 1001.14 mg of Rosuvastatin calcium ≈ 961.06 mg of Rosuvastatin)

Where by,

A_{T2} = Average area of Rosuvastatin obtained in test preparation

A_{S2} = Average area of Rosuvastatin obtained in standard preparation

W_3 = Weight taken of Rosuvastatin calcium reference standard (mg)

W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

LC_2 = Label claim of Rosuvastatin (mg)

P_2 = Potency of Rosuvastatin calcium reference standard (%)

5.3.3 **Product Information:**

Name :- Ezetimibe and Rosuvastatin tablet

Label claim :- 10 mg for Ezetimibe ; 10 mg for Rosuvastatin

5.3.4 **Method precision study:**

Method precision of the analytical method was determined by analyzing six sets of sample preparation. Assay of all six replicate sample preparations was determined and mean % assay value, standard deviation, % relative standard deviation and 95% confidence interval for the same was calculated.

For method precision, blank, standard preparation and six sets of test preparations was prepared as per method as under:

Blank preparation:

Diluent was used as blank.

Standard preparation:

Stock solution: Accurately weighed 49.0 mg Ezetimibe reference standard and 54.4 Rosuvastatin calcium reference standard (equivalent to 52.2 mg Rosuvastatin) were transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask to dissolve the substance by sonication for one minute and then diluted to volume with diluent. The concentration obtained is 490 μg/ml of Ezetimibe and 522 μg/ml of Rosuvastatin.

5 ml of above standard stock solution was pipetted out and transferred into 50 ml volumetric flask and diluted to volume with diluent. The concentration obtained is 49.0 μg/ml of Ezetimibe and 52.2 μg/ml of Rosuvastatin.

Test Preparation (Set 1):

Test stock solution: 10 Tablets were accurately weighed (2335.8 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 μm nylon syringe filter. The concentration obtained is 500 μg/ml of Ezetimibe and 500 μg/ml of Rosuvastatin.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50 μg/ml of Ezetimibe and 50 μg/ml of Rosuvastatin.

Test Preparation (Set 2):

Test stock solution: 10 Tablets were accurately weighed (2328.9 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 500 µg/ml of Ezetimibe and 500 µg/ml of Rosuvastatin.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe and 50 µg/ml of Rosuvastatin.

Test Preparation (Set 3):

Test stock solution: 10 Tablets were accurately weighed (2339.1 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 500 µg/ml of Ezetimibe and 500 µg/ml of Rosuvastatin.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe and 50 µg/ml of Rosuvastatin.

Test Preparation (Set 4):

Test stock solution: 10 Tablets were accurately weighed (2331.6 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and

diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 500 µg/ml of Ezetimibe and 500 µg/ml of Rosuvastatin.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe and 50 µg/ml of Rosuvastatin.

Test Preparation (Set 5):

Test stock solution: 10 Tablets were accurately weighed (2325.5 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 500 µg/ml of Ezetimibe and 500 µg/ml of Rosuvastatin.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe and 50 µg/ml of Rosuvastatin.

Test Preparation (Set 6):

Test stock solution: 10 Tablets were accurately weighed (2340.7 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 500 µg/ml of Ezetimibe and 500 µg/ml of Rosuvastatin.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe and 50 µg/ml of Rosuvastatin.

Average weight of tablets:

Average weight of tablets was performed on 20 tablets. 20 tablets are randomly selected and weighed (4670.0 mg) for the same. The average weight (233.5 mg) is calculated.

Chromatographic sequence for Method precision study is represented through Table 1 as under:

Table 1: Sequence of Method precision study:

Sr. No.	Description	Injection replicate
1	Blank	1
2	Standard preparation	5
3	Test Preparation (Set 1)	2
4	Test Preparation (Set 2)	2
5	Test Preparation (Set 3)	2
6	Test Preparation (Set 4)	2
7	Test Preparation (Set 5)	2
8	Test Preparation (Set 6)	2
9	Bracketing standard	1

Observation, calculation and chromatograms:

Table 2: Summary of method precision study for Ezetimibe

Observation				
Data for Standard preparation				
Replicate	Area		Standard weight	49.0 mg
1	2685398		Standard potency	99.8 %
2	2700761			
3	2675829			
4	2688642			
5	2696707			
Average	2689467			
Stdev	9787.80			
% RSD	0.36			

Data for Test preparations				
Set No.	Replicate	Area	Mean area	Weight of the sample
1	1	2790429	2793858	2335.8 mg
	2	2797286		
2	1	2783867	2778629	2328.9 mg
	2	2773390		
3	1	2778223	2778025	2339.1 mg
	2	2777827		
4	1	2783867	2778721	2331.6 mg
	2	2773574		
5	1	2778059	2774153	2325.5 mg
	2	2770246		
6	1	2769313	2774097	2340.7 mg
	2	2778881		

% Assay calculation for each set is as under:

For Set 1:-

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_1} x P_1$$

$$= \frac{2793858}{2689467} x \frac{49.0}{100} x \frac{5}{50} x \frac{200}{2335.8} x \frac{50}{5} x \frac{233.5}{10} x\, 99.8$$

$= 101.6$

For Set 2:-

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_1} x P_1$$

$$= \frac{2778629}{2689467} x \frac{49.0}{100} x \frac{5}{50} x \frac{200}{2328.9} x \frac{50}{5} x \frac{233.5}{10} x\, 99.8$$

$= 101.3$

For Set 3:-

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_1} x P_1$$

$$= \frac{2778025}{2689467} x \frac{49.0}{100} x \frac{5}{50} x \frac{200}{2339.1} x \frac{50}{5} x \frac{233.5}{10} x\, 99.8$$

$= 100.8$

For Set 4:-

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_1} x P_1$$

$$= \frac{2778721}{2689467} x \frac{49.0}{100} x \frac{5}{50} x \frac{200}{2331.6} x \frac{50}{5} x \frac{233.5}{10} x\, 99.8$$

$= 101.2$

For Set 5:-

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_1} x P_1$$

$$= \frac{2774153}{2689467} x \frac{49.0}{100} x \frac{5}{50} x \frac{200}{2325.5} x \frac{50}{5} x \frac{233.5}{10} x\, 99.8$$

$= 101.3$

For Set 6:-

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_1} x P_1$$

$$= \frac{2774097}{2689467} x \frac{49.0}{100} x \frac{5}{50} x \frac{200}{2340.7} x \frac{50}{5} x \frac{233.5}{10} x\, 99.8$$

$= 100.6$

Where by,

A_{T1} = Average area of Ezetimibe obtained in test preparation

A_{S1} = Average area of Ezetimibe obtained in standard preparation

W_1 = Weight taken of Ezetimibe reference standard (mg)

W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

LC_1 = Label claim of Ezetimibe (mg)

P_1 = Potency of Ezetimibe reference standard (%)

Table 3: Summary of method precision study for Rosuvastatin

Observation				
Data for Standard preparation				
Replicate	Area		Standard weight	54.4 mg
1	2460597		Standard potency	98.4 %
2	2462498			
3	2444174			
4	2453446			
5	2453667			
Average	2454876			
Stdev	7226.07			
% RSD	0.29			
Data for Test preparations				
Set No.	Replicate	Area	Mean area	Weight of the sample
1	1	2392226	2388655	2335.8 mg
	2	2385083		
2	1	2397758	2397969	2328.9 mg
	2	2398180		
3	1	2392156	2387103	2339.1 mg
	2	2382049		
4	1	2395432	2393226	2331.6 mg
	2	2391019		
5	1	2384145	2383149	2325.5 mg
	2	2382153		
6	1	2391627	2392730	2340.7 mg
	2	2393832		

% Assay calculation for each set is as under:

For Set 1:-

$$\% \text{ Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{961.06}{1001.14} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_2} x P_2$$

$$= \frac{2388655}{2454876} x \frac{54.4}{100} x \frac{5}{50} x \frac{961.06}{1001.14} x \frac{200}{2335.8} x \frac{50}{5} x \frac{233.5}{10} x\, 98.4$$

$= 100.0$

For Set 2:-

$$\% \text{ Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{961.06}{1001.14} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_2} x P_2$$

$$= \frac{2397969}{2454876} x \frac{54.4}{100} x \frac{5}{50} x \frac{961.06}{1001.14} x \frac{200}{2328.9} x \frac{50}{5} x \frac{233.5}{10} x\, 98.4$$

$= 100.7$

For Set 3:-

$$\% \text{ Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{961.06}{1001.14} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_2} x P_2$$

$$= \frac{2387103}{2454876} x \frac{54.4}{100} x \frac{5}{50} x \frac{961.06}{1001.14} x \frac{200}{2339.1} x \frac{50}{5} x \frac{233.5}{10} x\, 98.4$$

$= 99.8$

For Set 4:-

$$\% \text{ Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{961.06}{1001.14} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_2} x P_2$$

$$= \frac{2393226}{2454876} x \frac{54.4}{100} x \frac{5}{50} x \frac{961.06}{1001.14} x \frac{200}{2331.6} x \frac{50}{5} x \frac{233.5}{10} x\, 98.4$$

$= 100.3$

For Set 5:-

$$\% \text{ Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{961.06}{1001.14} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_2} x P_2$$

$$= \frac{2383149}{2454876} x \frac{54.4}{100} x \frac{5}{50} x \frac{961.06}{1001.14} x \frac{200}{2325.5} x \frac{50}{5} x \frac{233.5}{10} x\, 98.4$$

$= 100.2$

For Set 6:-

$$\% \text{ Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{961.06}{1001.14} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_2} x P_2$$

$$= \frac{2392730}{2454876} x \frac{54.4}{100} x \frac{5}{50} x \frac{961.06}{1001.14} x \frac{200}{2340.7} x \frac{50}{5} x \frac{233.5}{10} x \; 98.4$$

= 99.9

Where by,

A_{T2} = Average area of Rosuvastatin obtained in test preparation

A_{S2} = Average area of Rosuvastatin obtained in standard preparation

W_3 = Weight taken of Rosuvastatin calcium reference standard (mg)

W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

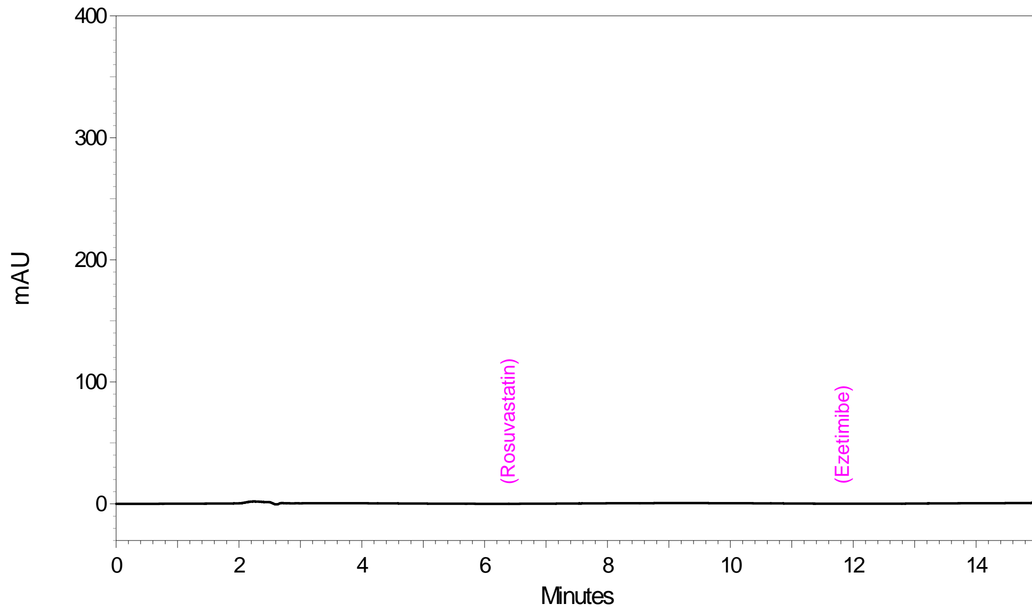
LC_2 = Label claim of Rosuvastatin (mg)

P_2 = Potency of Rosuvastatin calcium reference standard (%)

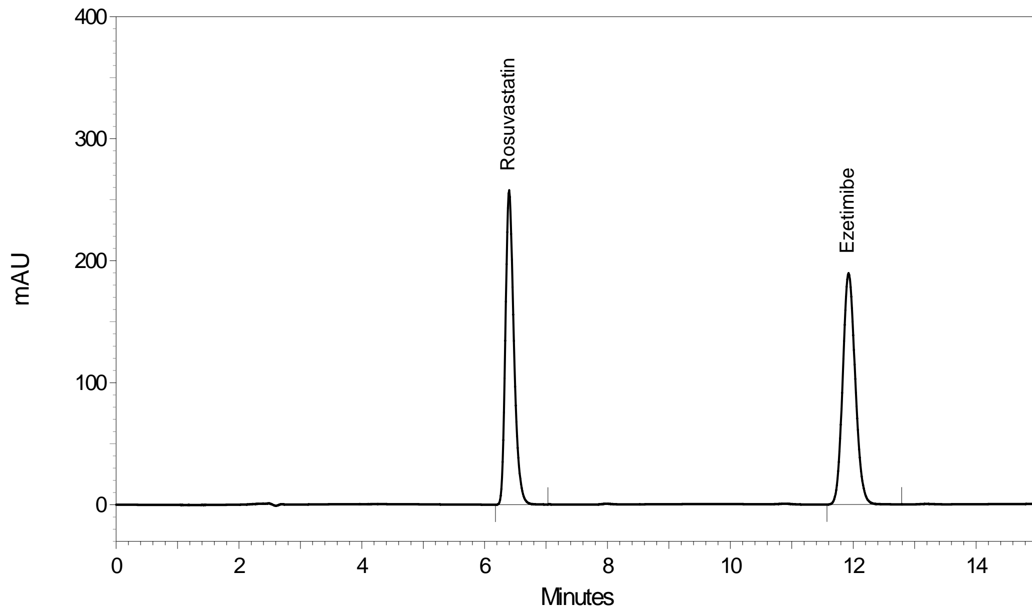
Table 4: Summary of Method Precision study

Analyte	Set No.	Assay (%)	Mean Assay (%)	Stdev	RSD (%)	95% Confidence Interval
Ezetimibe	1	101.6	101.1	0.37	0.37	0.39
	2	101.3				
	3	100.8				
	4	101.2				
	5	101.3				
	6	100.6				
Rosuvastatin	1	100.0	100.2	0.33	0.33	0.34
	2	100.7				
	3	99.8				
	4	100.3				
	5	100.2				
	6	99.9				

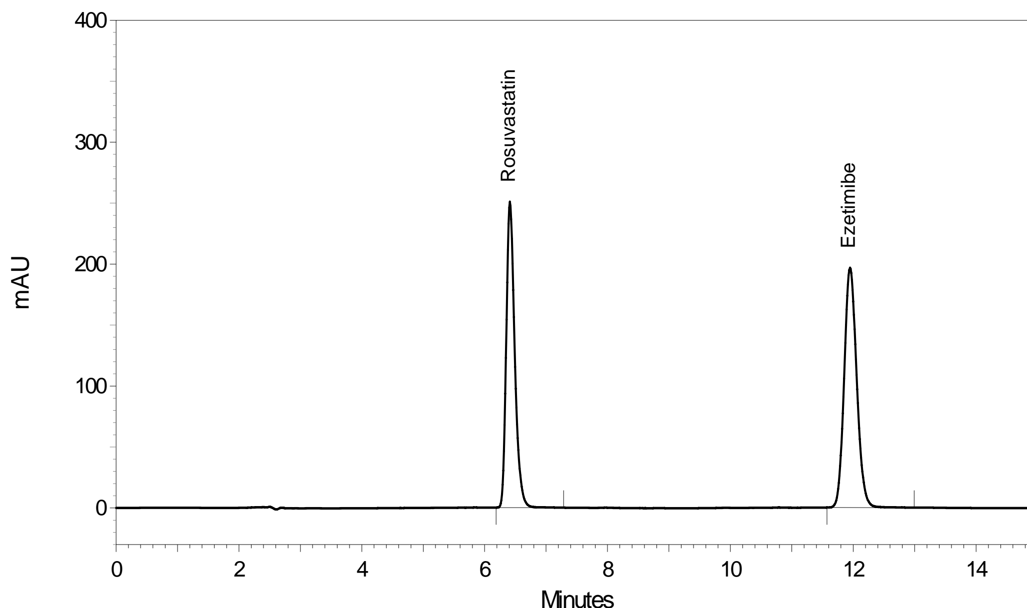
Chromatogram of blank preparation of method precision study:



Chromatogram of standard preparation of method precision study:



Prototype chromatogram of test preparation of method precision study:



Acceptance criteria:

%RSD of six replicate sets of method precision study for both analyte should be less than 2.0

Results:

%RSD of six replicate sets of method precision study is 0.37 for Ezetimibe and 0.33 for Rosuvastatin.

Conclusion:

All the results are well with-in the acceptance criteria. Hence, the analytical method is found precise.

5.3.5 **System suitability study:**

A system suitability test for the chromatographic system was performed before each validation parameter. Five replicate injections of standard preparation were injected and asymmetry, theoretical plate and % RSD of peak area were determined for same.

Table 5: Summary for System suitability of method precision

System suitability parameters (In-house limit)	% RSD[a] (NMT[b] 2.0)	Theoretical plates (NLT[c] 5000)	Asymmetry (NMT[b] 2.0)	Resolution (NLT[c] 10)
Method Precision				
Rosuvastatin	0.29	10147	1.30	--
Ezetimibe	0.36	15865	1.12	17.4

[a]Relative standard deviation
[b]Not more than
[c]Not less than

Acceptance criteria:

1) RSD of peak area of five replicate standard preparations should be less than 2.0 %
2) Theoretical plates of the analyte peak should be more than 5000 for standard preparation.
3) Asymmetry of the analyte peak should be less than 2.0 for standard preparation.
4) Resolution of the both analyte peak should be more than 10.0 in standard preparation.

Result:

1) RSD of peak area of five replicate standard preparations is found less than 2.0 % for both analyte peak.
2) Theoretical plates of the both analyte peak is found more than 5000 in standard preparation.
3) Asymmetry of the both analyte peak is found less than 2.0 for standard preparation.
4) Resolution of the both analyte peak is found more than 10.0 for standard preparation.

Conclusion:

System suitability was found satisfactory during method precision. Hence, system is suitable for the same.

5.3.6 **Reporting of deviation**

The entire precision activity was performed in accordance with the acceptance criteria and no deviation was observed.

5.3.7 **Discussion and conclusion**

The observation and result obtained for precision and system suitability lies well with-in the acceptance criteria. Since, all the results are with-in the limit, the developed analytical method is considered as precise for assay quantification.

5.4 Determination method of Ezetimibe and Atorvastatin

5.4.1 **Objective:**

To develop analytical method for the assay determination of Ezetimibe and Atorvastatin from combination dosage form

5.4.2 **Standard testing procedure:**

Aim:

To determine assay of Ezetimibe and Atorvastatin from its combination dosage form (tablet) through High performance liquid chromatography

Instrument:

High performance liquid chromatograph (Shimadzu; LC-10AT*vp*) equipped with PDA detector (SPD-M10A*vp*) and connected to multi-instrument data-acquisition and data-processing system (Class-*VP* 6.13 SP2)

Reagents:

Acetonitrile (HPLC grade)

Methanol (HPLC grade)

Orthophosphoric acid (GR grade)

Water (HPLC grade)

Chromatographic condition:

Mobile phase	:-	0.1% Orthophosphoric acid *(v/v)*:Acetonitrile (50:50, *v/v*)
Column	:-	Phenomenex Luna (2) C_{18}, 4.6 x 250 mm, 5 µm
Flow rate	:-	1.0 ml/min
Detection	:-	232 nm
Injection volume	:-	20 µl
Diluent	:-	Water:Acetonitrile:Methanol (40:50:10, *v/v*)

Blank preparation:

Use diluent as blank.

Standard preparation:

Stock solution: Weigh accurately about 50 mg Ezetimibe reference standard and 51.8 Atorvastatin calcium reference standard (equivalent to 50 mg Atorvastatin) and transfer into 100 ml volumetric flask. Add 70 ml of diluent to dissolve the substance by sonication for one minute and then dilute to volume with diluent. The concentration obtained is 500 µg/ml of Ezetimibe and 500 µg/ml of Atorvastatin.

Pipette out 5 ml of above standard stock solution and transfer into 50 ml volumetric flask and dilute to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe and 50 µg/ml of Atorvastatin.

Test Preparation:

Weigh accurately 20 tablets and find out the average weight. Weigh accurately 10

tablets and transfer into 200 ml volumetric flask. Add 140 ml of diluent into the flask and sonicate of 30 minutes with normal hand-shaking. Cool the flask to room temperature and dilute to volume with diluent. Filter 10 ml of this solution through 0.45 µm nylon syringe filter. The concentration obtained is 500 µg/ml of Ezetimibe and 500 µg/ml of Atorvastatin.

Pipette out 5 ml of above test stock solution and transfer into 50 ml volumetric flask and dilute to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe and 50 µg/ml of Atorvastatin.

System suitability:

- % RSD for replicate standard preparation should be less than 2.0 % for both analyte peaks.
- Asymmetry of the both analyte peaks should be less than 2.0 in standard preparation.
- Theoretical plates of the both analyte peaks should be more than 5000 in standard preparation.
- Resolution of the both analyte peak should be more than 5.0 in standard preparation.

Procedure:

- Inject the blank preparation and record the chromatogram.
- Inject five replicate of standard preparation and check for the system suitability and record the chromatograms.
- Inject test preparation in duplicate and record the chromatograms.
- Calculate the % assay of the sample.

Elution order:

1. Ezetimibe
2. Atorvastatin

Calculation:

Calculate the % assay of Ezetimibe and Atorvastatin in the sample using following formula.

For Ezetimibe:

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_1} x P_1$$

Where by,

A_{T1} = Average area of Ezetimibe obtained in test preparation

A_{S1} = Average area of Ezetimibe obtained in standard preparation

W_1 = Weight taken of Ezetimibe reference standard (mg)

W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

LC_1 = Label claim of Ezetimibe (mg)

P_1 = Potency of Ezetimibe reference standard (%)

For Atorvastatin:

$$\% \text{ Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{1115.28}{1155.36} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_2} x P_2$$

(Equivalency: 1155.36 mg of Atorvastatin calcium ≈ 1115.28 mg of Atorvastatin)

Where by,

A_{T2} = Average area of Atorvastatin obtained in test preparation

A_{S2} = Average area of Atorvastatin obtained in standard preparation

W_3 = Weight taken of Atorvastatin calcium reference standard (mg)

W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

LC_2 = Label claim of Atorvastatin (mg)

P_2 = Potency of Atorvastatin calcium reference standard (%)

5.4.3 **Product Information:**

Name :- Ezetimibe and Atorvastatin tablet

Label claim :- 10 mg for Ezetimibe ; 10 mg for Atorvastatin

5.4.4 **Method precision study:**

Method precision of the analytical method was determined by analyzing six sets of sample preparation. Assay of all six replicate sample preparations was determined and mean % assay value, standard deviation, % relative standard deviation and 95% confidence interval for the same was calculated.

For method precision, blank, standard preparation and six sets of test preparations was prepared as per method as under:

Blank preparation:
Diluent was used as blank.

Standard preparation:
Stock solution: Weigh accurately about 49.6 mg Ezetimibe reference standard and 51.5 Atorvastatin calcium reference standard (equivalent to 49.7 mg Atorvastatin) and transfer into 100 ml volumetric flask. Add 70 ml of diluent to dissolve the substance by sonication for one minute and then dilute to volume with diluent. The concentration obtained is 496 µg/ml of Ezetimibe and 497 µg/ml of Atorvastatin.

Pipette out 5 ml of above standard stock solution and transfer into 50 ml volumetric flask and dilute to volume with diluent. The concentration obtained is 49.6 µg/ml of Ezetimibe and 49.7 µg/ml of Atorvastatin.

Test Preparation (Set 1):
Test stock solution: 10 Tablets were accurately weighed (1535.9 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the

volumetric flask. The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 500 µg/ml of Ezetimibe and 500 µg/ml of Atorvastatin.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe and 50 µg/ml of Atorvastatin.

Test Preparation (Set 2):

Test stock solution: 10 Tablets were accurately weighed (1526.8 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 500 µg/ml of Ezetimibe and 500 µg/ml of Atorvastatin.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe and 50 µg/ml of Atorvastatin.

Test Preparation (Set 3):

Test stock solution: 10 Tablets were accurately weighed (1517.7 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 500 µg/ml of Ezetimibe and 500 µg/ml of Atorvastatin.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe and 50 µg/ml of Atorvastatin.

Test Preparation (Set 4):

Test stock solution: 10 Tablets were accurately weighed (1529.7 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 500 µg/ml of Ezetimibe and 500 µg/ml of Atorvastatin.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe and 50 µg/ml of Atorvastatin.

Test Preparation (Set 5):

Test stock solution: 10 Tablets were accurately weighed (1525.5 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 500 µg/ml of Ezetimibe and 500 µg/ml of Atorvastatin.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe and 50 µg/ml of Atorvastatin.

Test Preparation (Set 6):

Test stock solution: 10 Tablets were accurately weighed (1514.6 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 500 µg/ml of Ezetimibe and 500 µg/ml of Atorvastatin.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe and 50 µg/ml of Atorvastatin.

Average weight of tablets:

Average weight of tablets was performed on 20 tablets. 20 tablets are randomly selected and weighed (3050.0 mg) for the same. The average weight (152.5 mg) is calculated.

Chromatographic sequence for Method precision study is represented through Table 1 as under:

Table 1: Sequence of Method precision study:

Sr. No.	Description	Injection replicate
1	Blank	1
2	Standard preparation	5
3	Test Preparation (Set 1)	2
4	Test Preparation (Set 2)	2
5	Test Preparation (Set 3)	2
6	Test Preparation (Set 4)	2
7	Test Preparation (Set 5)	2
8	Test Preparation (Set 6)	2
9	Bracketing standard	1

Observation, calculation and chromatograms:

Table 2: Summary of method precision study for Ezetimibe

Observation				
Data for Standard preparation				
Replicate	Area		Standard weight	49.6 mg
1	2717854		Standard potency	99.8 %
2	2718728			
3	2720391			
4	2722413			
5	2721015			
Average	2720080			
Stdev	1816.37			
% RSD	0.07			

Data for Test preparations				
Set No.	Replicate	Area	Mean area	Weight of the sample
1	1	2808145	2810825	1535.9 mg
	2	2813505		
2	1	2772342	2772732	1526.8 mg
	2	2773121		
3	1	2781229	2771816	1517.7 mg
	2	2762402		
4	1	2806070	2804315	1529.7 mg
	2	2802560		
5	1	2778989	2773911	1525.5 mg
	2	2768833		
6	1	2761638	2772356	1514.6 mg
	2	2783073		

% Assay calculation for each set is as under:

For Set 1:-

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_1} x P_1$$

$$= \frac{2810825}{2720080} x \frac{49.6}{100} x \frac{5}{50} x \frac{200}{1535.9} x \frac{50}{5} x \frac{152.5}{10} x\, 99.8$$

$$= 101.6$$

For Set 2:-

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_1} x P_1$$

$$= \frac{2772732}{2720080} x \frac{49.6}{100} x \frac{5}{50} x \frac{200}{1526.8} x \frac{50}{5} x \frac{152.5}{10} x\, 99.8$$

$$= 100.8$$

For Set 3:-

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_1} x P_1$$

$$= \frac{2771816}{2720080} x \frac{49.6}{100} x \frac{5}{50} x \frac{200}{1517.7} x \frac{50}{5} x \frac{152.5}{10} x\, 99.8$$

$$= 101.4$$

For Set 4:-

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_1} x P_1$$

$$= \frac{2804315}{2720080} x \frac{49.6}{100} x \frac{5}{50} x \frac{200}{1529.7} x \frac{50}{5} x \frac{152.5}{10} x\, 99.8$$

$$= 101.8$$

For Set 5:-

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_1} x P_1$$

$$= \frac{2773911}{2720080} x \frac{49.6}{100} x \frac{5}{50} x \frac{200}{1525.5} x \frac{50}{5} x \frac{152.5}{10} x\, 99.8$$

$= 100.9$

For Set 6:-

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_1} x P_1$$

$$= \frac{2772356}{2720080} x \frac{49.6}{100} x \frac{5}{50} x \frac{200}{1514.6} x \frac{50}{5} x \frac{152.5}{10} x\, 99.8$$

$= 101.6$

Where by,

A_{T1} = Average area of Ezetimibe obtained in test preparation

A_{S1} = Average area of Ezetimibe obtained in standard preparation

W_1 = Weight taken of Ezetimibe reference standard (mg)

W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

LC_1 = Label claim of Ezetimibe (mg)

P_1 = Potency of Ezetimibe reference standard (%)

Table 3: Summary of method precision study for Atorvastatin

Observation				
Data for Standard preparation				
Replicate	Area		Standard weight	51.5 mg
1	2488821		Standard potency	99.2 %
2	2496533			
3	2503887			
4	2491552			
5	2494948			
Average	2495148			
Stdev	5729.1			
% RSD	0.23			

Data for Test preparations				
Set No.	Replicate	Area	Mean area	Weight of the sample
1	1	2556521	2558172	1535.9 mg
	2	2559823		
2	1	2524320	2518807	1526.8 mg
	2	2513293		
3	1	2506264	2504771	1517.7 mg
	2	2503277		
4	1	2538147	2541142	1529.7 mg
	2	2544136		
5	1	2500372	2505624	1525.5 mg
	2	2510875		
6	1	2490697	2492394	1514.6 mg
	2	2494090		

% Assay calculation for each set is as under:

For Set 1:-

$$\% \text{ Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{1115.28}{1155.36} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_2} x P_2$$

$$= \frac{2558172}{2495148} x \frac{51.5}{100} x \frac{5}{50} x \frac{1115.28}{1155.36} x \frac{200}{1535.9} x \frac{50}{5} x \frac{152.5}{10} x\ 99.2$$

$= 100.4$

For Set 2:-

$$\% \text{ Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{1115.28}{1155.36} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_2} x P_2$$

$$= \frac{2518807}{2495148} x \frac{51.5}{100} x \frac{5}{50} x \frac{1115.28}{1155.36} x \frac{200}{1526.8} x \frac{50}{5} x \frac{152.5}{10} x\ 99.2$$

$= 99.4$

For Set 3:-

$$\% \text{ Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{1115.28}{1155.36} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_2} x P_2$$

$$= \frac{2504771}{2495148} x \frac{51.5}{100} x \frac{5}{50} x \frac{1115.28}{1155.36} x \frac{200}{1517.7} x \frac{50}{5} x \frac{152.5}{10} x\ 99.2$$

$= 99.5$

For Set 4:-

$$\% \text{ Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{1115.28}{1155.36} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_2} x P_2$$

$$= \frac{2541142}{2495148} x \frac{51.5}{100} x \frac{5}{50} x \frac{1115.28}{1155.36} x \frac{200}{1529.7} x \frac{50}{5} x \frac{152.5}{10} x\ 99.2$$

$= 100.1$

For Set 5:-

$$\% \text{ Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{1115.28}{1155.36} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_2} x P_2$$

$$= \frac{2505624}{2495148} x \frac{51.5}{100} x \frac{5}{50} x \frac{1115.28}{1155.36} x \frac{200}{1525.5} x \frac{50}{5} x \frac{152.5}{10} x\ 99.2$$

$= 99.0$

For Set 6:-

$$\% \text{ Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{1115.28}{1155.36} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_2} x P_2$$

$$= \frac{2492394}{2495148} x \frac{51.5}{100} x \frac{5}{50} x \frac{1115.28}{1155.36} x \frac{200}{1514.6} x \frac{50}{5} x \frac{152.5}{10} x \, 99.2$$

$$= 99.2$$

Where by,

A_{T2} = Average area of Atorvastatin obtained in test preparation

A_{S2} = Average area of Atorvastatin obtained in standard preparation

W_3 = Weight taken of Atorvastatin calcium reference standard (mg)

W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

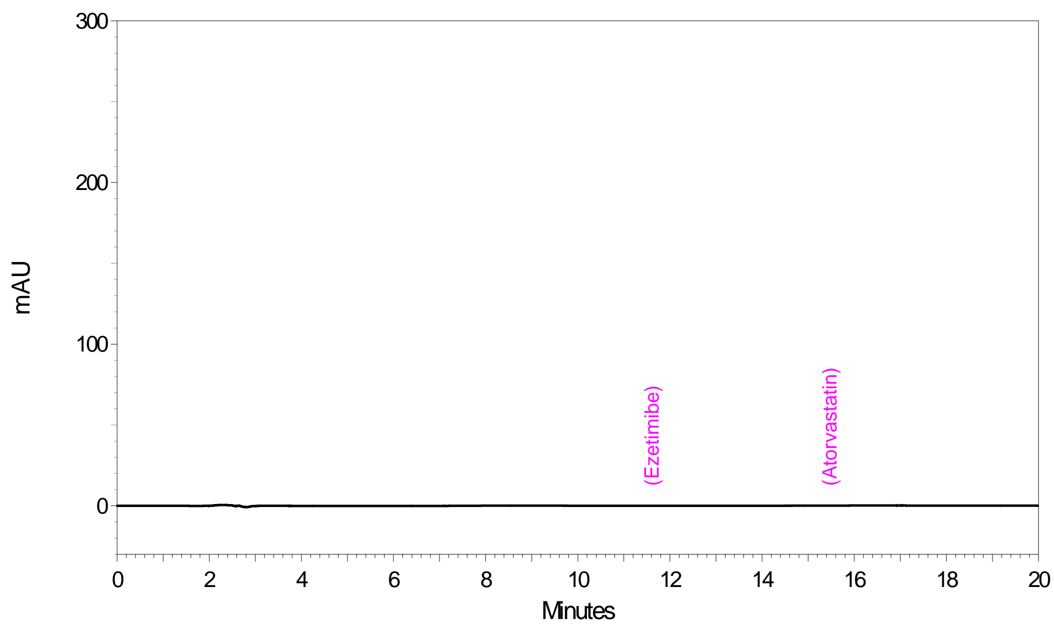
LC_2 = Label claim of Atorvastatin (mg)

P_2 = Potency of Atorvastatin calcium reference standard (%)

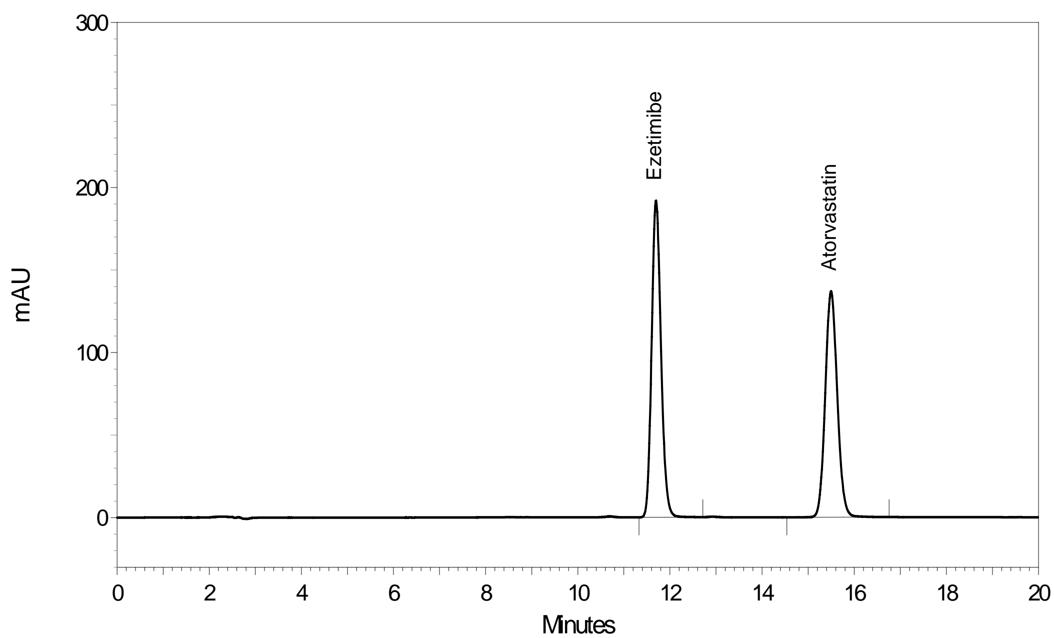
Table 4: Summary of Method Precision study

Analyte	Set No.	Assay (%)	Mean Assay (%)	Stdev	RSD (%)	95% Confidence Interval
Ezetimibe	1	101.6	101.4	0.41	0.40	0.43
	2	100.8				
	3	101.4				
	4	101.8				
	5	100.9				
	6	101.6				
Atorvastatin	1	100.4	99.6	0.54	0.54	0.57
	2	99.4				
	3	99.5				
	4	100.1				
	5	99.0				
	6	99.2				

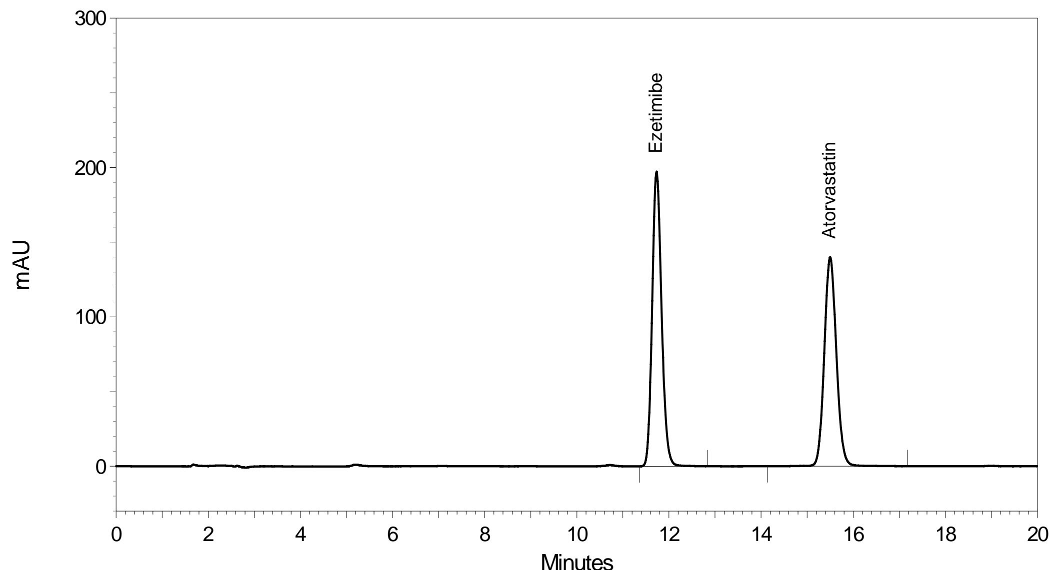
Chromatogram of blank preparation of method precision study:



Chromatogram of standard preparation of method precision study:



Prototype chromatogram of test preparation of method precision study:



Acceptance criteria:

%RSD of six replicate sets of method precision study for both analyte should be less than 2.0

Results:

%RSD of six replicate sets of method precision study is 0.40 for Ezetimibe and 0.54 for Atorvastatin.

Conclusion:

All the results are well with-in the acceptance criteria. Hence, the analytical method is found precise.

5.4.5 **System suitability study:**

A system suitability test for the chromatographic system was performed before each validation parameter. Five replicate injections of standard preparation were injected and asymmetry, theoretical plate and % RSD of peak area were determined for same.

Table 5: Summary for System suitability of method precision

System suitability parameters (In-house limit)	% RSD[a] (NMT[b] 2.0)	Theoretical plates (NLT[c] 5000)	Asymmetry (NMT[b] 2.0)	Resolution (NLT[c] 5)
Method Precision				
Atorvastatin	0.23	15267	1.14	--
Ezetimibe	0.07	16580	1.08	8.8
[a]Relative standard deviation [b]Not more than [c]Not less than				

Acceptance criteria:

1) RSD of peak area of five replicate standard preparations should be less than 2.0 %
2) Theoretical plates of the analyte peak should be more than 5000 for standard preparation.
3) Asymmetry of the analyte peak should be less than 2.0 for standard preparation.
4) Resolution of the both analyte peak should be more than 5.0 in standard preparation.

Result:

1) RSD of peak area of five replicate standard preparations is found less than 2.0 % for both analyte peak.
2) Theoretical plates of the both analyte peak is found more than 5000 in standard preparation.
3) Asymmetry of the both analyte peak is found less than 2.0 for standard preparation.
4) Resolution of the both analyte peak is found more than 5.0 for standard preparation.

Conclusion:

System suitability was found satisfactory during method precision. Hence, system is suitable for the same.

5.4.6 Reporting of deviation

The entire precision activity was performed in accordance with the acceptance criteria and no deviation was observed.

5.4.7 Discussion and conclusion

The observation and result obtained for precision and system suitability lies well with-in the acceptance criteria. Since, all the results are with-in the limit, the developed analytical method is considered as precise for assay quantification.

5.5 Determination method of Ezetimibe and Simvastatin

5.5.1 Objective:

To develop analytical method for the assay determination of Ezetimibe and Simvastatin from combination dosage form

5.5.2 Standard testing procedure:

Aim:

To determine assay of Ezetimibe and Simvastatin from its combination dosage form (tablet) through High performance liquid chromatography

Instrument:

High performance liquid chromatograph (Shimadzu; LC-10AT*vp*) equipped with PDA detector (SPD-M10A*vp*) and connected to multi-instrument data-acquisition and data-processing system (Class-*VP* 6.13 SP2)

Reagents:

Acetonitrile (HPLC grade)

Methanol (HPLC grade)

Orthophosphoric acid (GR grade)

Water (HPLC grade)

Chromatographic condition:

Mobile phase	:-	0.1% Orthophosphoric acid *(v/v)*:Acetonitrile (30:70, *v/v*)
Column	:-	Phenomenex Luna (2) C_{18}, 4.6 x 250 mm, 5 µm
Flow rate	:-	1.0 ml/min
Detection	:-	232 nm
Injection volume	:-	20 µl
Diluent	:-	Water:Acetonitrile:Methanol (40:50:10, *v/v*)

Blank preparation:

Use diluent as blank.

Standard preparation:

Stock solution: Weigh accurately about 50 mg Ezetimibe reference standard and 50 Simvastatin reference standard and transfer into 100 ml volumetric flask. Add 70 ml of diluent to dissolve the substance by sonication for one minute and then dilute to volume with diluent. The concentration obtained is 500 µg/ml of Ezetimibe and 500 µg/ml of Simvastatin.

Pipette out 5 ml of above standard stock solution and transfer into 50 ml volumetric flask and dilute to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe and 50 µg/ml of Simvastatin.

Test Preparation:

Weigh accurately 20 tablets and find out the average weight. Weigh accurately 10 tablets and transfer into 200 ml volumetric flask. Add 140 ml of diluent into the

flask and sonicate of 30 minutes with normal hand-shaking. Cool the flask to room temperature and dilute to volume with diluent. Filter 10 ml of this solution through 0.45 µm nylon syringe filter. The concentration obtained is 500 µg/ml of Ezetimibe and 500 µg/ml of Simvastatin.

Pipette out 5 ml of above test stock solution and transfer into 50 ml volumetric flask and dilute to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe and 50 µg/ml of Simvastatin.

System suitability:

- % RSD for replicate standard preparation should be less than 2.0 % for both analyte peaks.
- Asymmetry of the both analyte peaks should be less than 2.0 in standard preparation.
- Theoretical plates of the both analyte peaks should be more than 5000 in standard preparation.
- Resolution of the both analyte peak should be more than 20.0 in standard preparation.

Procedure:

- Inject the blank preparation and record the chromatogram.
- Inject five replicate of standard preparation and check for the system suitability and record the chromatograms.
- Inject test preparation in duplicate and record the chromatograms.
- Calculate the % assay of the sample.

Elution order:

1. Ezetimibe
2. Simvastatin

Calculation:

Calculate the % assay of Ezetimibe and Simvastatin in the sample using following formula.

For Ezetimibe:

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_1} x P_1$$

Where by,

A_{T1} = Average area of Ezetimibe obtained in test preparation

A_{S1} = Average area of Ezetimibe obtained in standard preparation

W_1 = Weight taken of Ezetimibe reference standard (mg)

W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

LC_1 = Label claim of Ezetimibe (mg)

P_1 = Potency of Ezetimibe reference standard (%)

For Simvastatin:

$$\% \text{ Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_2} x P_2$$

Where by,

A_{T2} = Average area of Simvastatin obtained in test preparation

A_{S2} = Average area of Simvastatin obtained in standard preparation

W_3 = Weight taken of Simvastatin reference standard (mg)

W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

LC_2 = Label claim of Simvastatin (mg)

P_2 = Potency of Simvastatin reference standard (%)

5.5.3 **Product Information:**

Name :- Ezetimibe and Simvastatin tablet

Label claim :- 10 mg for Ezetimibe ; 10 mg for Simvastatin

5.5.4 **Method precision study:**

Method precision of the analytical method was determined by analyzing six sets of sample preparation. Assay of all six replicate sample preparations was determined and mean % assay value, standard deviation, % relative standard deviation and 95% confidence interval for the same was calculated.

For method precision, blank, standard preparation and six sets of test preparations was prepared as per method as under:

Blank preparation:
Diluent was used as blank.

Standard preparation:
Stock solution: Weigh accurately about 49.1 mg Ezetimibe reference standard and 50.8 Simvastatin reference standard and transfer into 100 ml volumetric flask. Add 70 ml of diluent to dissolve the substance by sonication for one minute and then dilute to volume with diluent. The concentration obtained is 491 µg/ml of Ezetimibe and 508 µg/ml of Simvastatin.

Pipette out 5 ml of above standard stock solution and transfer into 50 ml volumetric flask and dilute to volume with diluent. The concentration obtained is 49.1 µg/ml of Ezetimibe and 50.8 µg/ml of Simvastatin.

Test Preparation (Set 1):
Test stock solution: 10 Tablets were accurately weighed (1941.4 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 500 µg/ml of Ezetimibe and 500 µg/ml of Simvastatin.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe and 50 µg/ml of Simvastatin.

Test Preparation (Set 2):

Test stock solution: 10 Tablets were accurately weighed (1934.7 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 500 µg/ml of Ezetimibe and 500 µg/ml of Simvastatin.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe and 50 µg/ml of Simvastatin.

Test Preparation (Set 3):

Test stock solution: 10 Tablets were accurately weighed (1957.3 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 500 µg/ml of Ezetimibe and 500 µg/ml of Simvastatin.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe and 50 µg/ml of Simvastatin.

Test Preparation (Set 4):

Test stock solution: 10 Tablets were accurately weighed (1944.2 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 500 µg/ml of Ezetimibe and 500 µg/ml of Simvastatin.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe and 50 µg/ml of Simvastatin.

Test Preparation (Set 5):

Test stock solution: 10 Tablets were accurately weighed (1945.9 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 500 µg/ml of Ezetimibe and 500 µg/ml of Simvastatin.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe and 50 µg/ml of Simvastatin.

Test Preparation (Set 6):

Test stock solution: 10 Tablets were accurately weighed (1952.1 mg) and transferred into 200 ml volumetric flask. 140 ml of diluent added into the volumetric flask. The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and

diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 μm nylon syringe filter. The concentration obtained is 500 μg/ml of Ezetimibe and 500 μg/ml of Simvastatin.

5 ml of above test stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50 μg/ml of Ezetimibe and 50 μg/ml of Simvastatin.

Average weight of tablets:

Average weight of tablets was performed on 20 tablets. 20 tablets are randomly selected and weighed (3890.0 mg) for the same. The average weight (194.5 mg) is calculated.

Chromatographic sequence for Method precision study is represented through Table 1.

Table 1: Sequence of Method precision study

Sr. No.	Description	Injection replicate
1	Blank	1
2	Standard preparation	5
3	Test Preparation (Set 1)	2
4	Test Preparation (Set 2)	2
5	Test Preparation (Set 3)	2
6	Test Preparation (Set 4)	2
7	Test Preparation (Set 5)	2
8	Test Preparation (Set 6)	2
9	Bracketing standard	1

<u>Observation, calculation and chromatograms:</u>

Table 2: Summary of method precision study for Ezetimibe

Observation				
Data for Standard preparation				
Replicate	Area		Standard weight	49.1 mg
1	2741870		Standard potency	99.8 %
2	2731024			
3	2754641			
4	2736033			
5	2756210			
Average	2743956			
Stdev	11165.7			
% RSD	0.41			
Data for Test preparations				
Set No.	Replicate	Area	Mean area	Weight of the sample
1	1	2817699	2822330	1941.4 mg
	2	2826961		
2	1	2825263	2824462	1934.7 mg
	2	2823660		
3	1	2822345	2824337	1957.3 mg
	2	2826329		
4	1	2823972	2825307	1944.2 mg
	2	2826641		
5	1	2825346	2822995	1945.9 mg
	2	2820643		
6	1	2823000	2824797	1952.1 mg
	2	2826594		

% Assay calculation for each set is as under:

For Set 1:-

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_1} x P_1$$

$$= \frac{2822330}{2743956} x \frac{49.1}{100} x \frac{5}{50} x \frac{200}{1941.4} x \frac{50}{5} x \frac{194.5}{10} x\, 99.8$$

$$= 101.0$$

For Set 2:-

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_1} x P_1$$

$$= \frac{2824462}{2743956} x \frac{49.1}{100} x \frac{5}{50} x \frac{200}{1934.7} x \frac{50}{5} x \frac{194.5}{10} x\, 99.8$$

$$= 101.4$$

For Set 3:-

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_1} x P_1$$

$$= \frac{2824337}{2743956} x \frac{49.1}{100} x \frac{5}{50} x \frac{200}{1957.3} x \frac{50}{5} x \frac{194.5}{10} x\, 99.8$$

$$= 100.2$$

For Set 4:-

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_1} x P_1$$

$$= \frac{2825307}{2743956} x \frac{49.1}{100} x \frac{5}{50} x \frac{200}{1944.2} x \frac{50}{5} x \frac{194.5}{10} x\, 99.8$$

$$= 101.0$$

For Set 5:-

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_1} x P_1$$

$$= \frac{2822995}{2743956} x \frac{49.1}{100} x \frac{5}{50} x \frac{200}{1945.9} x \frac{50}{5} x \frac{194.5}{10} x\, 99.8$$

$$= 100.8$$

For Set 6:-

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_1} x P_1$$

$$= \frac{2824797}{2743956} x \frac{49.1}{100} x \frac{5}{50} x \frac{200}{1952.1} x \frac{50}{5} x \frac{194.5}{10} x\, 99.8$$

$$= 100.5$$

Where by,

A_{T1} = Average area of Ezetimibe obtained in test preparation

A_{S1} = Average area of Ezetimibe obtained in standard preparation

W_1 = Weight taken of Ezetimibe reference standard (mg)

W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

LC_1 = Label claim of Ezetimibe (mg)

P_1 = Potency of Ezetimibe reference standard (%)

Table 3: Summary of method precision study for Simvastatin

Observation				
Data for Standard preparation				
Replicate	Area		Standard weight	50.8 mg
1	3241987		Standard potency	99.4 %
2	3276418			
3	3245604			
4	3246158			
5	3251826			
Average	3252399			
Stdev	13881.4			
% RSD	0.43			

Data for Test preparations				
Set No.	Replicate	Area	Mean area	Weight of the sample
1	1	3198957	3213795	1941.4 mg
	2	3228633		
2	1	3202822	3211832	1934.7 mg
	2	3220842		
3	1	3201042	3210063	1957.3 mg
	2	3219084		
4	1	3205122	3211184	1944.2 mg
	2	3217245		
5	1	3196393	3204149	1945.9 mg
	2	3211904		
6	1	3190909	3201912	1952.1 mg
	2	3212914		

% Assay calculation for each set is as under:

For Set 1:-

$$\% \text{ Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_2} x P_2$$

$$= \frac{3213795}{3252399} x \frac{50.8}{100} x \frac{5}{50} x \frac{200}{1941.4} x \frac{50}{5} x \frac{194.5}{10} x\ 99.4$$

$= 100.0$

For Set 2:-

$$\% \text{ Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_2} x P_2$$

$$= \frac{3211832}{3252399} x \frac{50.8}{100} x \frac{5}{50} x \frac{200}{1934.7} x \frac{50}{5} x \frac{194.5}{10} x\ 99.4$$

$= 100.3$

For Set 3:-

$$\% \text{ Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_2} x P_2$$

$$= \frac{3210063}{3252399} x \frac{50.8}{100} x \frac{5}{50} x \frac{200}{1957.3} x \frac{50}{5} x \frac{194.5}{10} x\ 99.4$$

$= 99.0$

For Set 4:-

$$\% \text{ Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_2} x P_2$$

$$= \frac{3211184}{3252399} x \frac{50.8}{100} x \frac{5}{50} x \frac{200}{1944.2} x \frac{50}{5} x \frac{194.5}{10} x\ 99.4$$

$= 99.8$

For Set 5:-

$$\% \text{ Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_2} x P_2$$

$$= \frac{3204149}{3252399} x \frac{50.8}{100} x \frac{5}{50} x \frac{200}{1945.9} x \frac{50}{5} x \frac{194.5}{10} x\ 99.4$$

$= 99.4$

For Set 6:-

$$\% \text{ Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{50}{5} x \frac{AW}{LC_2} x P_2$$

$$= \frac{3201912}{3252399} x \frac{50.8}{100} x \frac{5}{50} x \frac{200}{1952.1} x \frac{50}{5} x \frac{194.5}{10} x\ 99.4$$

$$= 99.1$$

Where by,

A_{T2} = Average area of Simvastatin obtained in test preparation

A_{S2} = Average area of Simvastatin obtained in standard preparation

W_3 = Weight taken of Simvastatin reference standard (mg)

W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

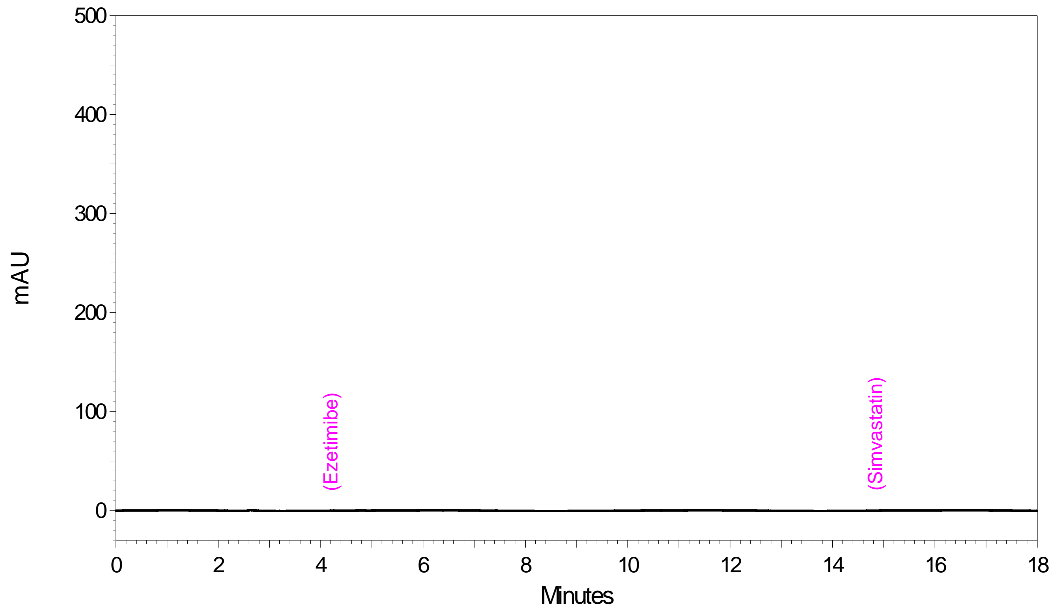
LC_2 = Label claim of Simvastatin (mg)

P_2 = Potency of Simvastatin reference standard (%)

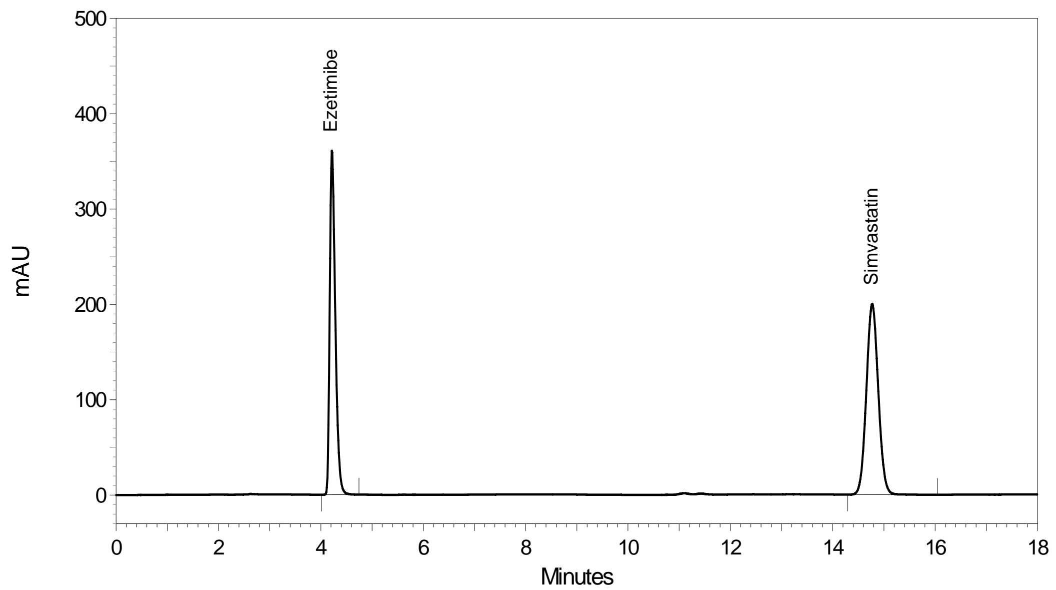
Table 4: Summary of Method Precision study

Analyte	Set No.	Assay (%)	Mean Assay (%)	Stdev	RSD (%)	95% Confidence Interval
Ezetimibe	1	101.0	100.8	0.42	0.42	0.44
	2	101.4				
	3	100.2				
	4	101.0				
	5	100.8				
	6	100.5				
Simvastatin	1	100.0	99.6	0.52	0.52	0.54
	2	100.3				
	3	99.0				
	4	99.8				
	5	99.4				
	6	99.1				

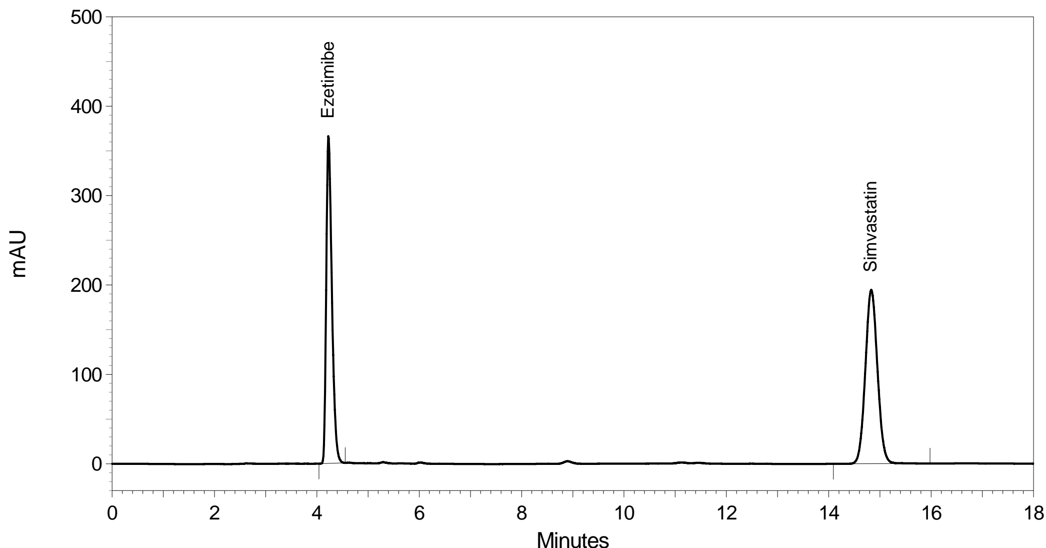
Chromatogram of blank preparation of method precision study:



Chromatogram of standard preparation of method precision study:



Prototype chromatogram of test preparation of method precision study:



Acceptance criteria:

%RSD of six replicate sets of method precision study for both analyte should be less than 2.0

Results:

%RSD of six replicate sets of method precision study is 0.42 for Ezetimibe and 0.52 for Simvastatin.

Conclusion:

All the results are well with-in the acceptance criteria. Hence, the analytical method is found precise.

5.5.5 **System suitability study:**

A system suitability test for the chromatographic system was performed before each validation parameter. Five replicate injections of standard preparation were injected and asymmetry, theoretical plate and % RSD of peak area were determined for same.

Table 5: Summary for System suitability of method precision

System suitability parameters (In-house limit)	% RSD[a] (NMT[b] 2.0)	Theoretical plates (NLT[c] 5000)	Asymmetry (NMT[b] 2.0)	Resolution (NLT[c] 20)
Method Precision				
Ezetimibe	0.41	6545	1.51	--
Simvastatin	0.43	18746	1.05	33.0

[a]Relative standard deviation
[b]Not more than
[c]Not less than

Acceptance criteria:

1) RSD of peak area of five replicate standard preparations should be less than 2.0 %
2) Theoretical plates of the analyte peak should be more than 5000 for standard preparation.
3) Asymmetry of the analyte peak should be less than 2.0 for standard preparation.
4) Resolution of the both analyte peak should be more than 20.0 in standard preparation.

Result:

1) RSD of peak area of five replicate standard preparations is found less than 2.0 % for both analyte peak.
2) Theoretical plates of the both analyte peak is found more than 5000 in standard preparation.
3) Asymmetry of the both analyte peak is found less than 2.0 for standard preparation.
4) Resolution of the both analyte peak is found more than 20.0 for standard preparation.

Conclusion:

System suitability was found satisfactory during method precision. Hence, system is suitable for the same.

5.5.6 **Reporting of deviation**

The entire precision activity was performed in accordance with the acceptance criteria and no deviation was observed.

5.5.7 **Discussion and conclusion**

The observation and result obtained for precision and system suitability lies well with-in the acceptance criteria. Since, all the results are with-in the limit, the developed analytical method is considered as precise for assay quantification.

5.6 Determination method of Ezetimibe, Atorvastatin and Fenofibrate

5.6.1 **Objective:**

To develop analytical method for the assay determination of Ezetimibe, Atorvastatin and Fenofibrate from combination dosage form

5.6.2 **Standard testing procedure:**

Aim:

To determine assay of Ezetimibe, Atorvastatin and Fenofibrate from its combination dosage form (tablet) through High performance liquid chromatography

Instrument:

High performance liquid chromatograph (Shimadzu; LC-10AT*vp*) equipped with PDA detector (SPD-M10A*vp*) and connected to multi-instrument data-acquisition and data-processing system (Class-*VP* 6.13 SP2)

Reagents:

Acetonitrile (HPLC grade)

Methanol (HPLC grade)

Orthophosphoric acid (GR grade)

Water (HPLC grade)

Chromatographic condition:

Mobile phase	:-	0.1% Orthophosphoric acid *(v/v)*:Acetonitrile (35:65, *v/v*)
Column	:-	Phenomenex Luna (2) C_{18}, 4.6 x 250 mm, 5 µm
Flow rate	:-	1.0 ml/min
Detection	:-	232 nm
Injection volume	:-	20 µl
Diluent	:-	Acetonitrile:Methanol (70:30, *v/v*)

Blank preparation:

Use diluent as blank.

Standard preparation:

Solution-A: Weigh accurately about 50 mg Ezetimibe reference standard and 51.8 Atorvastatin calcium reference standard (equivalent to 50 mg Atorvastatin) and transfer into 100 ml volumetric flask. Add 70 ml of diluent to dissolve the substance by sonication for one minute and then dilute to volume with diluent. The concentration obtained is 500 µg/ml of Ezetimibe and 500 µg/ml of Atorvastatin.

Weigh accurately about 50 mg Fenofibrate working standard and transfer into 50 ml volumetric flask. Add 35 ml of diluent to dissolve the substance by sonication for one minute. To this volumetric flask, add 5 ml of Solution-A and dilute to volume with diluent. The concentration obtained is 50 µg/ml of Ezetimibe, 50 µg/ml of Atorvastatin and 1000 µg/ml of Fenofibrate.

Test Preparation:

Weigh accurately 20 tablets and crush fine powder. Weigh accurately one average weight of powdered drug and transfer into 200 ml volumetric flask. Add 140 ml of diluent into the flask and sonicate of 30 minutes with normal hand-shaking. Cool the flask to room temperature and dilute to volume with diluent. Filter 10 ml of this solution through 0.45 µm nylon syringe filter. The concentration obtained is 50 µg/ml of Ezetimibe, 50 µg/ml of Atorvastatin and 1000 µg/ml of Fenofibrate.

System suitability:

- % RSD for replicate standard preparation should be less than 2.0 % for each analyte peak.
- Asymmetry of the each analyte peak should be less than 2.0 in standard preparation.
- Theoretical plates of the each analyte peak should be more than 4000 in standard preparation.
- Resolution of between the peaks of Ezetimibe and Atorvastatin should be more than 1.5 in standard preparation.

Procedure:

- Inject the blank preparation and record the chromatogram.
- Inject five replicate of standard preparation and check for the system suitability and record the chromatograms.
- Inject test preparation in duplicate and record the chromatograms.
- Calculate the % assay of the sample.

Elution order:

1. Ezetimibe
2. Atorvastatin
3. Fenofibrate

Calculation:

Calculate the % assay of Ezetimibe, Atorvastatin and Fenofibrate in the sample using following formula.

For Ezetimibe:

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{AW}{LC_1} x P_1$$

Where by,

A_{T1} = Average area of Ezetimibe obtained in test preparation

A_{S1} = Average area of Ezetimibe obtained in standard preparation

W_1 = Weight taken of Ezetimibe reference standard (mg)

W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

LC_1 = Label claim of Ezetimibe (mg)

P_1 = Potency of Ezetimibe reference standard (%)

For Atorvastatin:

$$\% \text{ Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{1115.28}{1155.36} x \frac{200}{W_2} x \frac{AW}{LC_2} x P_2$$

(Equivalency: 1155.36 mg of Atorvastatin calcium ≈ 1115.28 mg of Atorvastatin)

Where by,

A_{T2} = Average area of Atorvastatin obtained in test preparation

A_{S2} = Average area of Atorvastatin obtained in standard preparation

W_3 = Weight taken of Atorvastatin calcium reference standard (mg)

W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

LC_2 = Label claim of Atorvastatin (mg)

P_2 = Potency of Atorvastatin calcium reference standard (%)

For Fenofibrate:

$$\% \text{ Assay} = \frac{A_{T3}}{A_{S3}} x \frac{W_4}{50} x \frac{200}{W_2} x \frac{AW}{LC_3} x P_3$$

Where by,

A_{T3} = Average area of Fenofibrate obtained in test preparation

A_{S3} = Average area of Fenofibrate obtained in standard preparation

W_4 = Weight taken of Fenofibrate working standard (mg)

W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

LC_3 = Label claim of Fenofibrate (mg)

P_3 = Potency of Fenofibrate working standard (%)

5.6.3 **Product Information:**

Name :- Ezetimibe, Atorvastatin and Fenofibrate tablet

Label claim :- 10 mg for Ezetimibe ; 10 mg for Atorvastatin ; 200 mg for Fenofibrate

5.6.4 **Method precision study:**

Method precision of the analytical method was determined by analyzing six sets of sample preparation. Assay of all six replicate sample preparations was determined and mean % assay value, standard deviation, % relative standard deviation and 95% confidence interval for the same was calculated.

For method precision, blank, standard preparation and six sets of test preparations was prepared as per method as under:

Blank preparation:

Diluent was used as blank.

Standard preparation:

Solution-A: Accurately weighed about 50.8 mg Ezetimibe reference standard and

53.4 Atorvastatin calcium reference standard (equivalent to 51.5 mg Atorvastatin) were transferred into 100 ml volumetric flask. 70 ml of diluent was added into this volumetric flask to dissolve the substance by sonication of one minute and then diluted to volume with diluent. The concentration obtained is 508 µg/ml of Ezetimibe and 515 µg/ml of Atorvastatin.

Accurately weighed 50.9 mg Fenofibrate working standard was transferred into 50 ml volumetric flask. 35 ml of diluent was added into this volumetric flask to dissolve the substance by sonication of one minute. To this volumetric flask, 5 ml of Solution-A was added and diluted to volume with diluent. The concentration obtained is 50.8 µg/ml of Ezetimibe, 51.5 µg/ml of Atorvastatin and 1018 µg/ml of Fenofibrate.

Average weight of tablets:

Average weight of tablets was performed on 20 tablets. 20 tablets are randomly selected and weighed (8364.0 mg) for the same. The average weight (418.2 mg) is calculated.

Test Preparation (Set 1):

20 Tablets were accurately weighed (420.0 mg) and crushed to fine powder. Accurately weighed one average weight of powdered drug was transferred into 200 ml volumetric flask. 140 ml of diluent was added into the volumetric flask. The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 50 µg/ml of Ezetimibe, 50 µg/ml of Atorvastatin and 1000 µg/ml of Fenofibrate.

Test Preparation (Set 2):

20 Tablets were accurately weighed (417.8 mg) and crushed to fine powder. Accurately weighed one average weight of powdered drug was transferred into

200 ml volumetric flask. 140 ml of diluent was added into the volumetric flask. The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 50 µg/ml of Ezetimibe, 50 µg/ml of Atorvastatin and 1000 µg/ml of Fenofibrate.

Test Preparation (Set 3):

20 Tablets were accurately weighed (418.8 mg) and crushed to fine powder. Accurately weighed one average weight of powdered drug was transferred into 200 ml volumetric flask. 140 ml of diluent was added into the volumetric flask. The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 50 µg/ml of Ezetimibe, 50 µg/ml of Atorvastatin and 1000 µg/ml of Fenofibrate.

Test Preparation (Set 4):

20 Tablets were accurately weighed (418.4 mg) and crushed to fine powder. Accurately weighed one average weight of powdered drug was transferred into 200 ml volumetric flask. 140 ml of diluent was added into the volumetric flask. The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 50 µg/ml of Ezetimibe, 50 µg/ml of Atorvastatin and 1000 µg/ml of Fenofibrate.

Test Preparation (Set 5):

20 Tablets were accurately weighed (418.1 mg) and crushed to fine powder. Accurately weighed one average weight of powdered drug was transferred into 200 ml volumetric flask. 140 ml of diluent was added into the volumetric flask.

The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 50 µg/ml of Ezetimibe, 50 µg/ml of Atorvastatin and 1000 µg/ml of Fenofibrate.

Test Preparation (Set 6):

20 Tablets were accurately weighed (419.2 mg) and crushed to fine powder. Accurately weighed one average weight of powdered drug was transferred into 200 ml volumetric flask. 140 ml of diluent was added into the volumetric flask. The volumetric flask was proceed for sonication of 30 minutes with normal hand-shaking. Then, the flask was cooled to room temperature and diluted to volume with diluent. 10 ml of this solution was filtered through 0.45 µm nylon syringe filter. The concentration obtained is 50 µg/ml of Ezetimibe, 50 µg/ml of Atorvastatin and 1000 µg/ml of Fenofibrate.

Chromatographic sequence for Method precision study is represented through Table 1.

Table 1: Sequence of Method precision study:

Sr. No.	Description	Injection replicate
1	Blank	1
2	Standard preparation	5
3	Test Preparation (Set 1)	2
4	Test Preparation (Set 2)	2
5	Test Preparation (Set 3)	2
6	Test Preparation (Set 4)	2
7	Test Preparation (Set 5)	2
8	Test Preparation (Set 6)	2
9	Bracketing standard	1

Observation, calculation and chromatograms:

Table 2: Summary of method precision study for Ezetimibe

Observation				
Data for Standard preparation				
Replicate	Area		Standard weight	50.8 mg
1	2745425		Standard potency	99.8 %
2	2743143			
3	2769773			
4	2768061			
5	2769537			
Average	2759188			
Stdev	13644.93			
% RSD	0.49			

Data for Test preparations				
Set No.	Replicate	Area	Mean area	Weight of the sample
1	1	2704938	2704515	420.0 mg
	2	2704091		
2	1	2701923	2703029	417.8 mg
	2	2704134		
3	1	2704574	2703139	418.8 mg
	2	2701704		
4	1	2704426	2705352	418.4 mg
	2	2706277		
5	1	2701557	2701894	418.1 mg
	2	2702231		
6	1	2698838	2698856	419.2 mg
	2	2698874		

% Assay calculation for each set is as under:

For Set 1:-

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{AW}{LC_1} x P_1$$

$$= \frac{2704515}{2759188} x \frac{50.8}{100} x \frac{5}{50} x \frac{200}{420.0} x \frac{418.2}{10} x\, 99.8$$

$$= 99.0$$

For Set 2:-

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{AW}{LC_1} x P_1$$

$$= \frac{2703029}{2759188} x \frac{50.8}{100} x \frac{5}{50} x \frac{200}{417.8} x \frac{418.2}{10} x\, 99.8$$

$$= 99.4$$

For Set 3:-

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{AW}{LC_1} x P_1$$

$$= \frac{2703139}{2759188} x \frac{50.8}{100} x \frac{5}{50} x \frac{200}{418.8} x \frac{418.2}{10} x\, 99.8$$

$$= 99.2$$

For Set 4:-

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{AW}{LC_1} x P_1$$

$$= \frac{2705352}{2759188} x \frac{50.8}{100} x \frac{5}{50} x \frac{200}{418.4} x \frac{418.2}{10} x\, 99.8$$

$$= 99.4$$

For Set 5:-

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{AW}{LC_1} x P_1$$

$$= \frac{2701894}{2759188} x \frac{50.8}{100} x \frac{5}{50} x \frac{200}{418.1} x \frac{418.2}{10} x\, 99.8$$

$$= 99.3$$

For Set 6:-

$$\% \text{ Assay} = \frac{A_{T1}}{A_{S1}} x \frac{W_1}{100} x \frac{5}{50} x \frac{200}{W_2} x \frac{AW}{LC_1} x P_1$$

$$= \frac{2698856}{2759188} x \frac{50.8}{100} x \frac{5}{50} x \frac{200}{419.2} x \frac{418.2}{10} x\, 99.8$$

$$= 98.9$$

Where by,

A_{T1} = Average area of Ezetimibe obtained in test preparation

A_{S1} = Average area of Ezetimibe obtained in standard preparation

W_1 = Weight taken of Ezetimibe reference standard (mg)

W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

LC_1 = Label claim of Ezetimibe (mg)

P_1 = Potency of Ezetimibe reference standard (%)

Table 3: Summary of method precision study for Atorvastatin

Observation				
Data for Standard preparation				
Replicate	Area		Standard weight	53.4 mg
1	2632443		Standard potency	99.2 %
2	2598808			
3	2635844			
4	2607576			
5	2589781			
Average	2612890			
Stdev	20431.43			
% RSD	0.78			

Data for Test preparations				
Set No.	Replicate	Area	Mean area	Weight of the sample
1	1	2554859	2533264	420.0 mg
	2	2511668		
2	1	2538738	2525103	417.8 mg
	2	2511467		
3	1	2596386	2583168	418.8 mg
	2	2569949		
4	1	2541564	2527456	418.4 mg
	2	2513347		
5	1	2551512	2537450	418.1 mg
	2	2523388		
6	1	2532672	2524420	419.2 mg
	2	2516168		

% Assay calculation for each set is as under:

For Set 1:-

$$\% \text{ Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{1115.28}{1155.36} x \frac{200}{W_2} x \frac{AW}{LC_2} x P_2$$

$$= \frac{2533264}{2612890} x \frac{53.4}{100} x \frac{5}{50} x \frac{1115.28}{1155.36} x \frac{200}{420.0} x \frac{418.2}{10} x\ 99.2$$

$$= 98.7$$

For Set 2:-

$$\%\ \text{Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{1115.28}{1155.36} x \frac{200}{W_2} x \frac{AW}{LC_2} xP_2$$

$$= \frac{2525103}{2612890} x \frac{53.4}{100} x \frac{5}{50} x \frac{1115.28}{1155.36} x \frac{200}{417.8} x \frac{418.2}{10} x\ 99.2$$

$$= 98.9$$

For Set 3:-

$$\%\ \text{Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{1115.28}{1155.36} x \frac{200}{W_2} x \frac{AW}{LC_2} xP_2$$

$$= \frac{2583168}{2612890} x \frac{53.4}{100} x \frac{5}{50} x \frac{1115.28}{1155.36} x \frac{200}{418.8} x \frac{418.2}{10} x\ 99.2$$

$$= 101.0$$

For Set 4:-

$$\%\ \text{Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{1115.28}{1155.36} x \frac{200}{W_2} x \frac{AW}{LC_2} xP_2$$

$$= \frac{2527456}{2612890} x \frac{53.4}{100} x \frac{5}{50} x \frac{1115.28}{1155.36} x \frac{200}{418.4} x \frac{418.2}{10} x\ 99.2$$

$$= 98.9$$

For Set 5:-

$$\%\ \text{Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{1115.28}{1155.36} x \frac{200}{W_2} x \frac{AW}{LC_2} xP_2$$

$$= \frac{2537450}{2612890} x \frac{53.4}{100} x \frac{5}{50} x \frac{1115.28}{1155.36} x \frac{200}{418.1} x \frac{418.2}{10} x\ 99.2$$

$$= 99.3$$

For Set 6:-

$$\% \text{ Assay} = \frac{A_{T2}}{A_{S2}} x \frac{W_3}{100} x \frac{5}{50} x \frac{1115.28}{1155.36} x \frac{200}{W_2} x \frac{AW}{LC_2} x P_2$$

$$= \frac{2524420}{2612890} x \frac{53.4}{100} x \frac{5}{50} x \frac{1115.28}{1155.36} x \frac{200}{419.2} x \frac{418.2}{10} x\ 99.2$$

$$= 98.6$$

Where by,

A_{T2} = Average area of Atorvastatin obtained in test preparation

A_{S2} = Average area of Atorvastatin obtained in standard preparation

W_3 = Weight taken of Atorvastatin calcium reference standard (mg)

W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

LC_2 = Label claim of Atorvastatin (mg)

P_2 = Potency of Atorvastatin calcium reference standard (%)

Table 4: Summary of method precision study for Fenofibrate

Observation				
Data for Standard preparation				
Replicate	Area		Standard weight	50.9 mg
1	30775385		Standard potency	99.9 %
2	30755765			
3	31070302			
4	31033906			
5	31060293			
Average	30939130			
Stdev	159141.6			
% RSD	0.51			

Data for Test preparations				
Set No.	Replicate	Area	Mean area	Weight of the sample
1	1	30184035	30178407	420.0 mg
	2	30172779		
2	1	30197848	30181736	417.8 mg
	2	30165624		
3	1	30210659	30197384	418.8 mg
	2	30184109		
4	1	30228899	30211643	418.4 mg
	2	30194387		
5	1	30184139	30207992	418.1 mg
	2	30231844		
6	1	30182815	30171517	419.2 mg
	2	30160219		

% Assay calculation for each set is as under:

For Set 1:-

$$\% \text{ Assay} = \frac{A_{T3}}{A_{S3}} x \frac{W_4}{50} x \frac{200}{W_2} x \frac{AW}{LC_3} x P_3$$

$$= \frac{30178407}{30939130} \text{ x } \frac{50.9}{50} \text{ x } \frac{200}{420.0} \text{ x } \frac{418.2}{200} \text{ x } 99.9$$

$$= 98.8$$

For Set 2:-

$$\% \text{ Assay} = \frac{A_{T3}}{A_{S3}} \text{ x } \frac{W_4}{50} \text{ x } \frac{200}{W_2} \text{ x } \frac{AW}{LC_3} \text{ x} P_3$$

$$= \frac{30181736}{30939130} \text{ x } \frac{50.9}{50} \text{ x } \frac{200}{417.8} \text{ x } \frac{418.2}{200} \text{ x } 99.9$$

$$= 99.3$$

For Set 3:-

$$\% \text{ Assay} = \frac{A_{T3}}{A_{S3}} x \frac{W_4}{50} x \frac{200}{W_2} x \frac{AW}{LC_3} xP_3$$

$$= \frac{30197384}{30939130} \text{ x } \frac{50.9}{50} \text{ x } \frac{200}{418.8} \text{ x } \frac{418.2}{200} \text{ x } 99.9$$

$$= 99.1$$

For Set 4:-

$$\% \text{ Assay} = \frac{A_{T3}}{A_{S3}} x \frac{W_4}{50} x \frac{200}{W_2} x \frac{AW}{LC_3} xP_3$$

$$= \frac{30211643}{30939130} \text{ x } \frac{50.9}{50} \text{ x } \frac{200}{418.4} \text{ x } \frac{418.2}{200} \text{ x } 99.9$$

$$= 99.3$$

For Set 5:-

$$\% \text{ Assay} = \frac{A_{T3}}{A_{S3}} x \frac{W_4}{50} x \frac{200}{W_2} x \frac{AW}{LC_3} xP_3$$

$$= \frac{30207992}{30939130} \text{ x } \frac{50.9}{50} \text{ x } \frac{200}{418.1} \text{ x } \frac{418.2}{200} \text{ x } 99.9$$

$$= 99.3$$

For Set 6:-

$$\% \text{ Assay} = \frac{A_{T3}}{A_{S3}} x \frac{W_4}{50} x \frac{200}{W_2} x \frac{AW}{LC_3} x P_3$$

$$= \frac{30171517}{30939130} \text{x} \frac{50.9}{50} \text{x} \frac{200}{419.2} \text{x} \frac{418.2}{200} \text{x } 99.9$$

$$= 98.9$$

Where by,

A_{T3} = Average area of Fenofibrate obtained in test preparation

A_{S3} = Average area of Fenofibrate obtained in standard preparation

W_4 = Weight taken of Fenofibrate working standard (mg)

W_2 = Weight taken of test sample (mg)

AW = Average weight of tablets (mg)

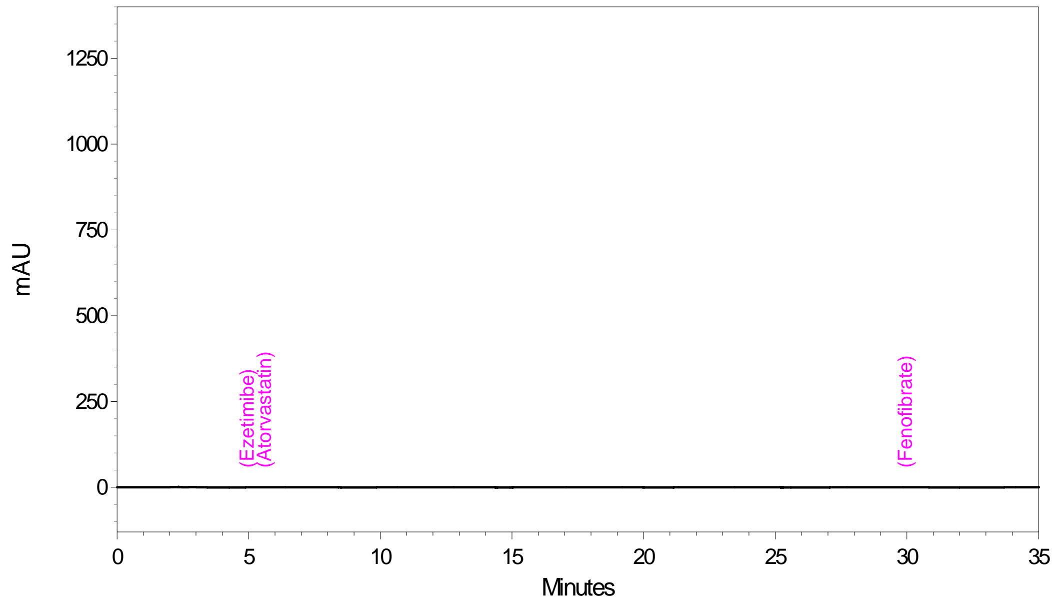
LC_3 = Label claim of Fenofibrate (mg)

P_3 = Potency of Fenofibrate working standard (%)

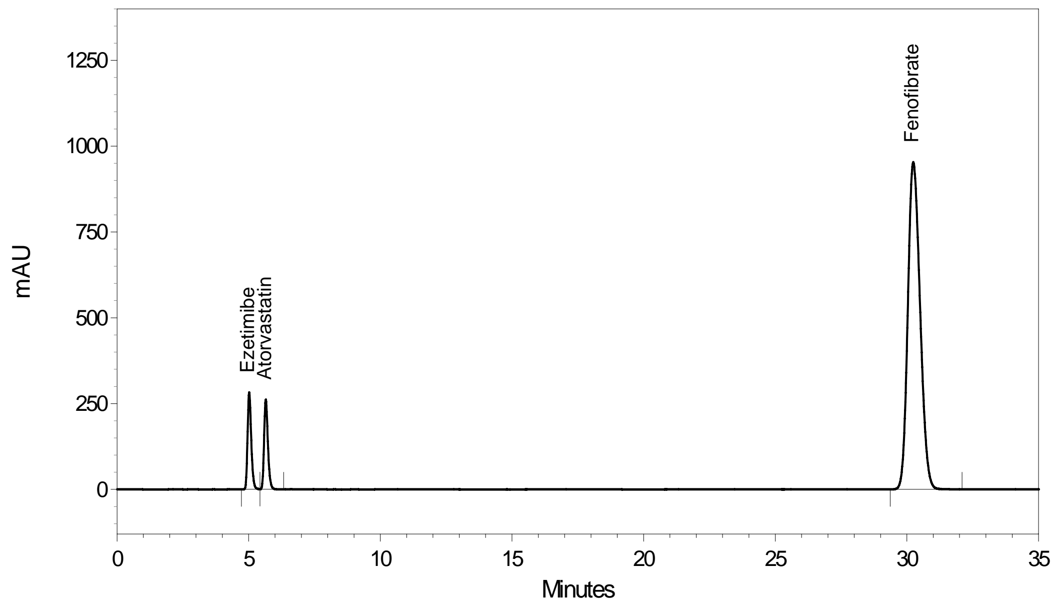
Table 5: Summary of Method Precision study

Analyte	Set No.	Assay (%)	Mean Assay (%)	Stdev	RSD (%)	95% Confidence Interval
Ezetimibe	1	99.0	99.2	0.21	0.21	0.22
	2	99.4				
	3	99.2				
	4	99.4				
	5	99.3				
	6	98.9				
Atorvastatin	1	98.7	99.2	0.90	0.91	0.94
	2	98.9				
	3	101.0				
	4	98.9				
	5	99.3				
	6	98.6				
Fenofibrate	1	98.8	99.1	0.22	0.22	0.23
	2	99.3				
	3	99.1				
	4	99.3				
	5	99.3				
	6	98.9				

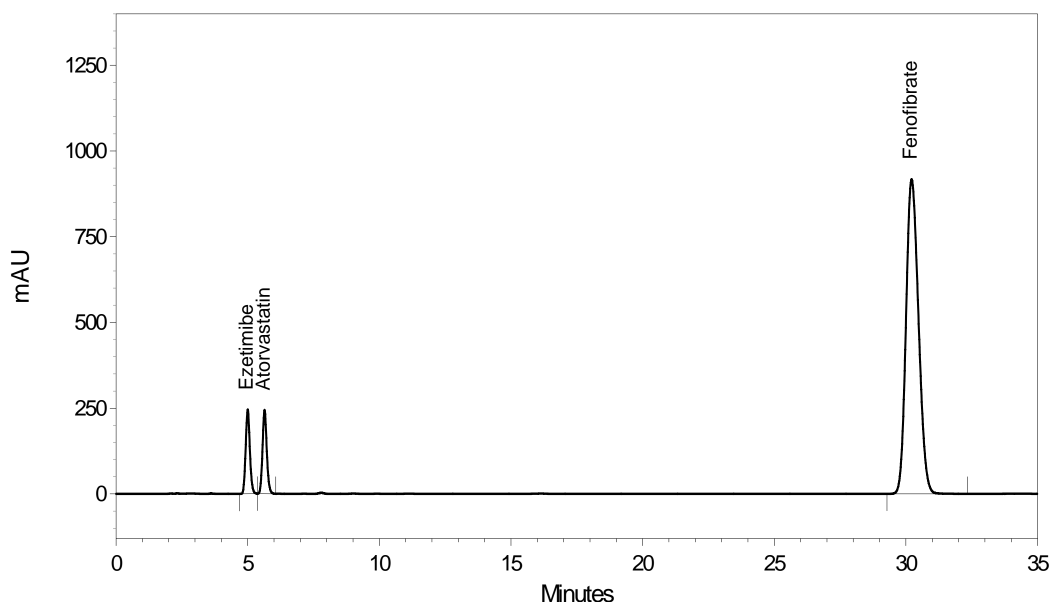
Chromatogram of blank preparation of method precision study:



Chromatogram of standard preparation of method precision study:



Prototype chromatogram of test preparation of method precision study:



Acceptance criteria:

%RSD of six replicate sets of method precision study for each analyte should be less than 2.0

Results:

%RSD of six replicate sets of method precision study is 0.21 for Ezetimibe, 0.91 for Atorvastatin and 0.21 for Fenofibrate.

Conclusion:

All the results are well with-in the acceptance criteria. Hence, the analytical method is found precise.

5.6.5 **System suitability study:**

A system suitability test for the chromatographic system was performed before each validation parameter. Five replicate injections of standard preparation were injected and asymmetry, theoretical plate and % RSD of peak area were determined for same.

Table 5: Summary for System suitability of method precision

System suitability parameters (In-house limit)	% RSD[a] (NMT[b] 2.0)	Theoretical plates (NLT[c] 4000)	Asymmetry (NMT[b] 2.0)	Resolution (NLT[c] 1.5)
Method Precision				
Ezetimibe	0.49	5831	1.31	--
Atorvastatin	0.78	7118	1.22	2.41
Fenofibrate	0.51	19648	1.19	43.48

[a]Relative standard deviation
[b]Not more than
[c]Not less than

Acceptance criteria:

1) % RSD for replicate standard preparation should be less than 2.0 % for each analyte peak.
2) Asymmetry of the each analyte peak should be less than 2.0 in standard preparation.
3) Theoretical plates of the each analyte peak should be more than 4000 in standard preparation.
4) Resolution of between the peaks of Ezetimibe and Atorvastatin should be more than 1.5 in standard preparation.

Result:

1) RSD of peak area of five replicate standard preparations is found less than 2.0 % for each analyte peak.
2) Theoretical plates of each analyte peak is found more than 4000 in standard preparation.
3) Asymmetry of the each analyte peak is found less than 2.0 for standard preparation.
4) Resolution of between the peaks of Ezetimibe and Atorvastatin is found more than 1.5 for standard preparation.

Conclusion:

System suitability was found satisfactory during method precision. Hence, system is suitable for the same.

5.6.6 **Reporting of deviation**

The entire precision activity was performed in accordance with the acceptance criteria and no deviation was observed.

5.6.7 **Discussion and conclusion**

The observation and result obtained for precision and system suitability lies well with-in the acceptance criteria. Since, all the results are with-in the limit, the developed analytical method is considered as precise for assay quantification.

6.0 Conclusion

The analytical methods are successfully developed and validated for assay and content uniformity quantification of Ezetimibe from its pharmaceutical dosage form. Further, analytical methods are developed for the assay quantification of Ezetimibe from its combined pharmaceutical dosage form with Statins and Fibrate. The aim of work is full-fill according to its objective with pre-defined acceptance criteria.

The developed method for dosage form of Ezetimibe can also use for dissolution determination of drug by deliberate fine tune. The developed methods for combination dosage forms of Ezetimibe can also use for content uniformity and dissolution determination of drugs for the same by deliberate fine tune.

7.0 References

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8.0 Publication:

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