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RESEARCH ARTICLE

Development and Validation of a Stability-Indicating RP-HPLC Method for the Simultaneous Estimation of Atorvastatin and Ezetimibe in Bulk and Pharmaceutical Dosage Forms

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Abstract: Simultaneous therapeutic management of hyperlipidemia and cardiovascular risk profiles frequently relies on combination of HMG-CoA reductase inhibitors and selective cholesterol absorption inhibitors. Analysis of such co-formulations requires robust, reliable, and stability-indicating methodologies. An isocratic reversed-phase high-performance liquid chromatography method was developed and validated for the concurrent quantitative determination of Atorvastatin and Ezetimibe in both active pharmaceutical ingredient forms and commercial tablet formulations. Chromatographic separation was achieved on a C18 stationary phase (250 mm × 4.6 mm, 5 µm) utilizing a mobile phase consisting of methanol and 0.1% formic acid in a 60:40 v/v ratio. The system operated at a flow rate of 0.8 mL/min with a column temperature maintained at 25∘C, and detection was performed at an isosbestic wavelength of 239 nm. The retention times for Atorvastatin and Ezetimibe were established at approximately 3.3 and 6.1 minutes, respectively. The analytical method exhibited excellent linearity over a concentration range of 5-25 µg/mL for both analytes, with correlation coefficients exceeding 0.999. Accuracy was confirmed by standard addition recovery studies, yielding recoveries between 98.35% and 102.06%. Forced degradation studies under hydrolytic, oxidative, photolytic, and thermal stress conditions indicated that the method effectively resolves degradation products from the active drug peaks. Ezetimibe showed extreme susceptibility to alkaline hydrolysis, achieving complete degradation, whereas Atorvastatin displayed moderate stability across all stress environments. The validated method meets all the validation criteria, proving its utility for routine pharmaceutical quality control and stability assessment.

Keywords: Atorvastatin; Ezetimibe; RP-HPLC; Stability-Indicating; Method Validation.

1. Introduction

Hyperlipidemia represents a critical, modifiable risk factor in the pathogenesis of atherosclerotic cardiovascular disease. Therapeutic strategies often involve aggressive lipid-lowering regimens to achieve low-density lipoprotein cholesterol target levels. Atorvastatin calcium, chemically designated as [R-(R*, R*)]-2-(4-fluorophenyl)-β, δ -dihydroxy-5-(1-methylethyl)-3-phenyl-4-(phenylamino) carbonyl-1H-pyrrole-1-heptanoic acid trihydrate, is a potent, synthetic HMG-CoA reductase inhibitor. It competitively inhibits the rate-limiting enzyme responsible for converting HMG-CoA to mevalonate, thereby reducing hepatic cholesterol synthesis.

To achieve synergistic lipid-lowering efficacy, Atorvastatin is frequently co-prescribed with Ezetimibe. Ezetimibe, chemically described as (3R,4S)-1-(4-fluorophenyl)-3-(3S)-3-(4-fluorophenyl)-3-hydroxypropyl-4-(4-hydroxyphenyl)azetidin-2-one, acts as a selective cholesterol absorption inhibitor. It targets the Niemann-Pick C1-Like 1 (NPC1L1) transporter protein in the brush border of the small intestine, inhibiting the uptake of dietary and biliary cholesterol.

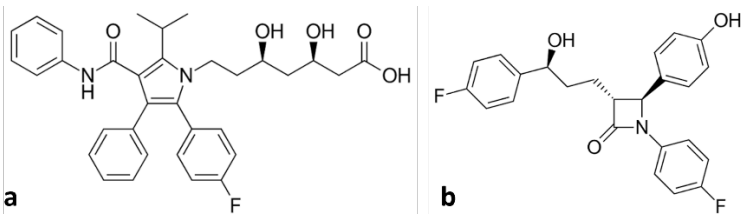

Figure 1 a. Atorvastatin b. Ezetimibe

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The combination of these two pharmacologically distinct compounds provides a dual mechanism of action, addressing both hepatic synthesis and intestinal absorption of cholesterol. Consequently, developing accurate, robust, and cost-effective analytical methods for their simultaneous estimation in commercial formulations is a vital objective for pharmaceutical quality assurance.

The quantitative analysis of multi-component dosage forms demands selective separation technologies to ensure clinical safety and efficacy. High-Performance Liquid Chromatography operating in reversed-phase mode (RP-HPLC) has emerged as the analytical standard for quality control, stability testing, and regulatory compliance due to its exceptional sensitivity, reproducibility, and resolution [1]. Developing a single, isocratic chromatographic run for compounds with different physico-chemical properties, such as partition coefficient (log P) values and dissociation constants (pK_a), presents a significant analytical challenge.

Atorvastatin possesses a polar carboxylic acid side chain with a pK_a of approximately 4.46, making its chromatographic retention highly sensitive to mobile phase pH. Ezetimibe, conversely, is a neutral-to-weakly-acidic molecule with a pK_a of 9.75, exhibiting a consistently hydrophobic character (log P ≈ 4.5) across a wide pH range. Previous studies have utilized various combinations of organic modifiers and buffered aqueous systems to resolve these compounds [2, 3]. While some methods employ complex gradient profiles, binary isocratic modes utilizing volatile organic acids like formic acid provide superior reproducibility and compatibility with modern detection techniques without requiring rigorous column re-equilibration steps [2]. The scientific literature highlights the continuous evolution of liquid chromatographic techniques for cardiovascular and lipid-lowering therapeutics. Comprehensive overviews of RP-HPLC method development strategies emphasize the systematic optimization of stationary phases, mobile phase chemistry, and detection wavelengths to guarantee reproducible analytical parameters [3]. Robust stability-indicating methods are particularly critical, as they must distinguish the active pharmaceutical ingredients from potential in situ degradation products formed under environmental stress conditions [3].

In lipid-lowering therapy, methods have been reported for the simultaneous analysis of Atorvastatin combined with nicotinic acid [4], fenofibrate [5], or irbesartan [6]. These methods underscore the critical role of validation parameters, including accuracy, precision, limit of detection, and limit of quantification, in maintaining pharmaceutical safety [7]. The literature also documents methods for evaluating dual-action statin and absorption inhibitor formulations. Studies on Simvastatin-Ezetimibe and Rosuvastatin-Ezetimibe combinations highlight the importance of wavelength selection and stationary phase selection in achieving well-resolved peaks [10, 11]. For instance, Bempedoic Acid and Ezetimibe co-formulations have been resolved using phosphate buffer systems, though such non-volatile salts limit compatibility with mass spectrometry and increase column wear [12].

Specific to the Atorvastatin-Ezetimibe combination, reported methods frequently rely on ammonium acetate buffers or high organic fractions to elute the highly hydrophobic Ezetimibe, which can lead to rapid elution and peak tailing for the more polar Atorvastatin [13, 14, 15]. The primary objective of this study was to develop, optimize, and validate a simple, volatile, buffer-free, and stability-indicating isocratic RP-HPLC method for the simultaneous quantification of Atorvastatin and Ezetimibe. This method avoids complex gradient setups and high-salt buffers by utilizing a methanol and 0.1% formic acid mobile phase, ensuring both analytical performance and operational simplicity.

2. Materials and Methods

2.1. Chemicals and Reagents

Atorvastatin calcium and Ezetimibe working standards (pure drug samples, >99.5% purity) were generously provided as gift samples by Reliable's Shree Industrial Training Centre and Laboratory (Jalgaon, India). Commercial combination tablets (Atorvastatin EZ, Steris Healthcare, India, labeled to contain 10 mg of Atorvastatin and 10 mg of Ezetimibe per tablet) were purchased from a local commercial pharmacy. Analytical grade methanol and formic acid (98-100%) were purchased from Merck India Ltd. (Mumbai, India). All solvents used for the mobile phase preparation were of HPLC grade. High-purity deionized water was prepared using a Millipore Milli-Q water purification system. Prior to chromatographic injection, all reagents, mobile phases, and standard solutions were filtered through a 0.45 µm membrane filter (Millipore, Bedford, MA, USA) and degassed using an ultrasonic bath for 15 minutes.

2.2. Instrumentation and Chromatographic Conditions

The liquid chromatographic system utilized for method development and validation was an Agilent Technologies 1100 series HPLC system. This setup consisted of an isocratic pump (Model G1310A IsoPump) coupled with a variable wavelength diode array detector (DAD, Model G1314B). Chromatographic data acquisition, peak integration, and instrument control were managed using Agilent ChemStation software. The pump unit featured a high-performance reciprocating double-piston design (HP-1100), ensuring constant flow delivery in the range of 0.001 to 5 mL/min with a pressure capacity up to 400 bar.

The stationary phase selected was a reversed-phase C18 analytical column with dimensions of 250 mm × 4.6 mm and a particle size of 5 µm (Agilent Zorbax ODS or equivalent). The optimized mobile phase consisted of a binary mixture of methanol and 0.1% aqueous formic acid in a ratio of 60:40 v/v. Chromatographic elution was performed under isocratic conditions at a flow rate of 0.8 mL/min. The column oven temperature was maintained at a constant 25°C throughout the analysis to stabilize retention behaviors. The sample injection volume was fixed at 20 µL using a high-precision autosampler loop. UV detection was conducted at an optimized wavelength of 239 nm to maximize peak response for both analytes.

2.3. Preparation of Standard and Working Solutions

2.3.1. Preparation of Standard Stock Solutions

Standard stock solutions of Atorvastatin (ATO) and Ezetimibe (EZE) were prepared individually. Accurately weighed portions of 5 mg of Atorvastatin reference standard and 5 mg of Ezetimibe reference standard were transferred into separate, clean, dry 10 mL volumetric flasks. Approximately 5 mL of HPLC-grade methanol was added to each flask, and the mixtures were sonicated for 10 minutes to ensure complete dissolution of the crystalline drug particles. Once dissolved, the volumes were adjusted to the 10 mL mark with methanol, yielding independent primary stock solutions with a final concentration of 500 µg/mL for each drug. These primary stock solutions were stored under dark conditions at 4∘C when not in use.

2.3.2. Preparation of Working Standard Solutions

Working standard solutions were prepared daily by diluting the primary stock solutions with the mobile phase (methanol: 0.1% formic acid, 60:40 v/v). Aliquots of the primary stock solutions were transferred into 10 mL volumetric flasks and diluted to volume to obtain mixed working standard solutions with concentrations ranging from 5 to 25 µg/mL for both analytes. These solutions were filtered through a 0.45 µm syringe filter prior to automated injection into the HPLC system.

2.4. Determination of Isosbestic Point

To determine the optimum detection wavelength for simultaneous estimation, the working standard solutions of Atorvastatin (10 µg/mL) and Ezetimibe (20 µg/mL) in methanol were scanned individually in the UV-visible wavelength range of 200 nm to 400 nm. A double-beam UV-Vis spectrophotometer was utilized, with pure methanol serving as the blank. The absorption spectra (λ_{max}) for both drugs were recorded and compared. Overlaying the individual absorption spectra revealed an intersection point where both drugs exhibited equivalent, stable absorbance relative to their molar concentration ratios. This intersection, known as the isosbestic point, was identified at 239 nm and selected as the detection wavelength for the subsequent liquid chromatographic method.

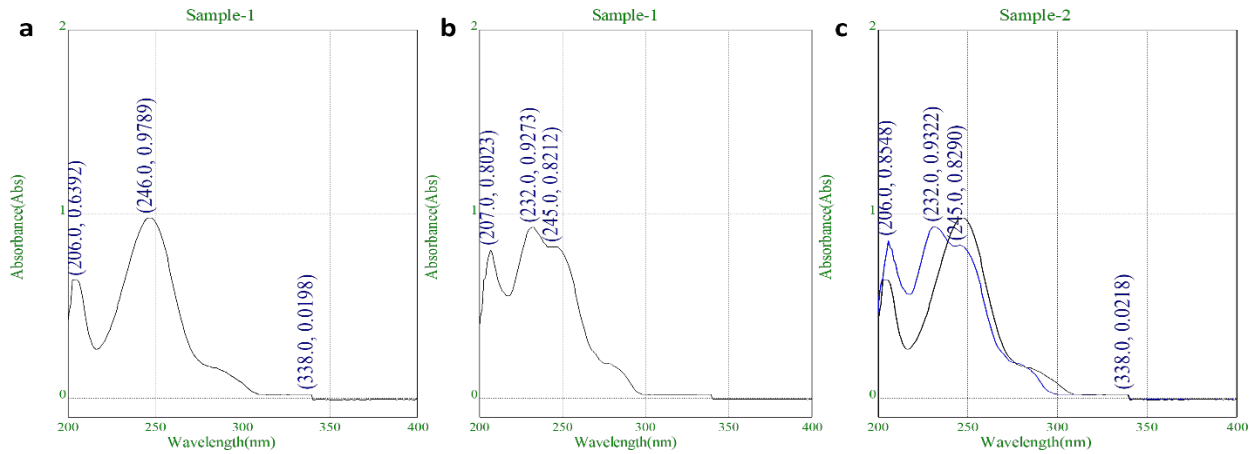

Figure 2. UV absorption spectrum of a. ATO (10 ug/mL) in methanol, b. EZE (20 ug/mL) in methanol and c. Isosbestic point of ATO and EZE at 239 nm

2.5. Method Development Trials

An experimental trial-and-error approach was conducted to optimize the chromatographic parameters. The primary objectives during method development were to achieve baseline resolution between Atorvastatin and Ezetimibe, maintain acceptable peak symmetry (asymmetry factor, A_s ≈ 0.9-1.2), obtain a high theoretical plate count (N > 2000), and minimize the total

chromatographic run time. The composition of the mobile phase was systematically varied by altering the organic modifier ratio (methanol) and adjusting the acidic modifier (0.1% formic acid). Additionally, flow rates were evaluated between 0.8 to 1.2 mL/min to balance backpressure and analysis time.

The organic phase selection is chemically critical. Methanol was selected over acetonitrile due to its protic nature, which aids in solvating the polar carboxylate functional groups on Atorvastatin, thereby reducing peak tailing. Formic acid (0.1%) maintains the aqueous phase pH at approximately 3.2, which is significantly below the pK_a of Atorvastatin (4.46). Under these conditions, the ionization of Atorvastatin's carboxylic acid group is suppressed, keeping the molecule in its highly retained, neutral form. This ensures adequate interaction with the C18 stationary phase and provides a clear separation from Ezetimibe.

Table 1. Method Development Trials for Atorvastatin and Ezetimibe

Sample label	Peak	Ret. Time (min)	Area (mAU·s)	Height (mAU)	Sym.	Width (min)	Plates	Resolution
Trial 1 (85% MeOH + 15% 0.1% FA, 1 mL/min)	ATO	2.668	358.36310	66.67805	0.49	0.0841	5574	1.96
	EZE	2.921	359.74783	83.59676	0.41	0.0671	10486	
Trial 2 (70% MeOH + 30% 0.1% FA, 0.8 mL/min)	ATO	3.245	451.12958	30.76632	1.19	0.1844	1715	4.39
	EZE	4.513	434.39948	39.61733	0.98	0.1553	4679	
Trial 3 (60% MeOH + 40% 0.1% FA, 0.8 mL/min)	ATO	3.378	476.60379	34.56373	1.38	0.1626	2391	8.30
	EZE	6.479	468.49042	25.79216	1.01	0.2766	3039	
	Unknown	10.589	20.22350	1.19648	0.94	0.2702	8511	8.83
Trial 4 (60% MeOH + 40% 0.1% FA, 0.8 mL/min, repeat)	ATO	3.315	524.59930	69.27912	0.96	0.0971	6461	12.84
	EZE	6.153	431.30298	38.37123	0.97	0.1626	7935	
Trial 5 (61% MeOH + 39% 0.1% FA, 0.9 mL/min)	ATO	2.928	175.27136	32.88293	0.90	0.0767	8080	11.88
	EZE	5.001	205.77498	24.39564	0.89	0.1283	8411	

2.6. Method Validation (as per ICH Guidelines)

The optimized chromatographic method was validated in accordance with the International Council for Harmonisation (ICH) Q2(R1) guidelines.

2.6.1. Linearity and Calibration Range

The linearity of the detector response was evaluated by analyzing mixed working standard solutions across a concentration range of 5-25 µg/mL at five distinct levels (5, 10, 15, 20, and 25 µg/mL) for both drugs. Each concentration level was injected in duplicate. Calibration curves were constructed by plotting the mean peak areas against the corresponding analyte concentrations. The regression equation was calculated using the least-squares linear regression method:

$$y = m \cdot x + c$$

where y represents the peak area, x represents the analyte concentration (µg/mL), m represents the slope of the calibration line, and c represents the y-intercept. The coefficient of determination (R^2) was used to evaluate the linearity of the method.

2.6.2. Accuracy

Method accuracy was verified through recovery studies utilizing the standard addition technique at three distinct levels: 80%, 100%, and 120%. To a pre-analyzed tablet formulation solution containing a nominal concentration of 5 µg/mL of both Atorvastatin and Ezetimibe, known quantities of pure active pharmaceutical ingredient (API) standards were added. Specifically, 4, 5, and 6 µg/mL of pure drug standards were added to represent the 80%, 100%, and 120% spike levels, respectively.

The spiked samples were prepared in duplicate, filtered, and analyzed under the optimized chromatographic conditions. The percentage of recovered drug was calculated using the following formula:

$$\% \text{ Recovery} = \frac{\text{Total concentration determined} - \text{Concentration initially present}}{\text{Concentration of pure standard added}} \times 100$$

2.6.3. Precision and Reproducibility

The precision of the analytical method was evaluated across three parameters: repeatability, intraday precision, and interday precision.

Repeatability (System Precision): Repeatability was assessed by performing six consecutive injections of a single mixed standard solution containing 15 µg/mL of Atorvastatin and 15 µg/mL of Ezetimibe. The relative standard deviation (%RSD) of the resulting peak areas was calculated to show instrument repeatability.

Intraday Precision: Intraday precision was evaluated by analyzing three distinct concentrations (5, 15, and 25 µg/mL) in duplicate at three different times on the same day.

Interday Precision: Interday precision was determined by analyzing the same three concentration levels (5, 15, and 25 µg/mL) on three consecutive days. The variability was expressed as the percentage relative standard deviation (%RSD) of the peak areas.

2.6.4. Sensitivity (LOD and LOQ)

The Limit of Detection (LOD) and Limit of Quantification (LOQ) define the sensitivity of the method. These values were calculated based on the standard deviation of the y-intercept (sigma) and the slope (S) obtained from the linearity calibration curves, using the following formulas:

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

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

2.6.5. Specificity, Ruggedness, and Robustness

Specificity: Specificity was determined by verifying that the active analytes could be resolved without interference from common tablet excipients (such as microcrystalline cellulose, lactose, starch, and magnesium stearate) or the mobile phase. Chromatograms of blank mobile phase, blank excipient solutions, standard API mixtures, and commercial formulations were compared to ensure peak purity.

Ruggedness: Ruggedness was evaluated by analyzing a mixed standard solution (15 µg/mL) under varying operational conditions, including analysis by two different analysts and on different instrument environments. The percentage relative standard deviation (%RSD) was calculated to confirm method ruggedness.

Robustness: Robustness was assessed by introducing small, deliberate variations in key chromatographic parameters. These modifications included adjusting the mobile phase ratio (from 60:40 v/v to 62:38 v/v) and shifting the detection wavelength (± 2 nm, corresponding to 238 nm and 240 nm). Mixed standard solutions (10 µg/mL) were analyzed under these altered conditions, and system suitability parameters were monitored.

2.7. Quantitative Assay of Commercial Formulations

The validated method was applied to the quantitative determination of Atorvastatin and Ezetimibe in commercial ATORVASTATIN EZ tablets. Twenty tablets were weighed individually, and the average tablet weight was calculated (26.32 mg per tablet). The tablets were then ground into a fine, homogeneous powder using a clean mortar and pestle.

A portion of the tablet powder equivalent to 5 mg of the active ingredients (13.16 mg of powder) was accurately weighed and transferred into a 10 mL volumetric flask. Approximately 5 mL of HPLC-grade methanol was added, and the sample was sonicated for 15 minutes to ensure extraction of the drugs from the excipient matrix.

The mixture was filtered through a 0.45 µm membrane filter to remove insoluble excipients. The filtrate was diluted to volume with methanol to prepare a stock solution of 500 µg/mL (Stock-II). From this solution, a 0.4 mL aliquot was transferred to a 10 mL volumetric flask and diluted to volume with the mobile phase, yielding a final concentration of 20 µg/mL for both analytes. This sample was analyzed under the optimized conditions, and the drug content was calculated by comparing peak areas against reference standards.

2.8. Stress Degradation Protocols (Stability-Indicating Studies)

Forced degradation studies were performed to evaluate the stability-indicating capacity of the method, in compliance with the ICH Q1A(R2) guidelines. Active pharmaceutical ingredient (API) standards were subjected to hydrolytic, oxidative, photolytic, and thermal stress conditions to generate degradation products and verify their baseline separation from the active drug peaks.

2.8.1. Acidic Hydrolysis

Acidic degradation was conducted by reacting a 0.3 mL aliquot of the mixed standard stock solution (500 µg/mL) with 2 mL of 0.1 N hydrochloric acid (HCl) in a 10 mL volumetric flask. The solution was maintained at room temperature. After a reaction period of 2 hours, the mixture was neutralized by adding 2 mL of 0.1 N sodium hydroxide (NaOH) to prevent further degradation. The sample was then diluted to volume with the mobile phase and filtered through a 0.45 µm syringe filter prior to HPLC analysis.

2.8.2. Alkaline Hydrolysis

Alkaline degradation was performed by reacting 0.3 mL of the standard stock solution with 2 mL of 0.1 N sodium hydroxide (NaOH). After a contact time of 2 hours at room temperature, the reaction was neutralized using 2 mL of 0.1 N hydrochloric acid (HCl). The solution was diluted to the 10 mL mark with the mobile phase, filtered, and analyzed.

2.8.3. Neutral Hydrolysis

Neutral hydrolytic degradation was evaluated by mixing 0.3 mL of the standard stock solution with 2 mL of high-purity deionized water. The solution was kept at room temperature for 2 hours, diluted to 10 mL with the mobile phase, filtered, and analyzed.

2.8.4. Oxidative Stress

Oxidative degradation was conducted by mixing 0.3 mL of the standard stock solution with 2 mL of a 3% hydrogen peroxide (H_2O_2) solution. The mixture was maintained at room temperature for 2 hours, diluted to 10 mL with the mobile phase, filtered, and analyzed to monitor oxidative degradation products.

2.8.5. Photolytic Stress

For photolytic degradation, approximately 50 mg of bulk drug powder (both Atorvastatin and Ezetimibe standards) was exposed to direct sunlight for 24 hours. Following exposure, a portion of the stressed powder was weighed and dissolved in methanol to prepare a stock solution. This solution was diluted with the mobile phase to achieve a final target concentration of 15 µg/mL for both analytes, filtered, and analyzed.

2.8.6. Thermal Stress

Thermal degradation was performed by exposing bulk drug powders to dry heat in a temperature-controlled hot air oven at 80∘C for a continuous period of 24 hours. After the exposure period, the powders were cooled, dissolved in methanol, and diluted with the mobile phase to a final concentration of 15 µg/mL before injection into the chromatographic system.

3. Results and Discussion

3.1. Optimization of Chromatographic Conditions

The chromatographic trials summarized in Table 1 indicate the systematic shift in selectivity and efficiency across different mobile phase configurations. Trial 1, utilizing a high concentration of organic modifier (85% methanol and 15% aqueous 0.1% formic acid) at a flow rate of 1.0 mL/min, resulted in rapid elution. Atorvastatin and Ezetimibe co-eluted close to the solvent front, with retention times of 2.668 and 2.921 minutes, respectively. Under these conditions, the column was highly saturated with the organic modifier, preventing partition-based selectivity, and leading to unacceptable peak asymmetry values of 0.49 for Atorvastatin and 0.41 for Ezetimibe.

To increase hydrophobic interactions with the stationary phase, Trial 2 decreased the organic fraction to 70%, maintaining a flow rate of 0.8 mL/min. Although this increased retention behavior (Atorvastatin retention time of 3.245 minutes and Ezetimibe retention time of 4.513 minutes), peak symmetry for Atorvastatin remained poor (1.19), and the overall chromatographic theoretical plate count (1715 plates for Atorvastatin) was far from analytical optimization. Trial 3, which tested a 60:40 v/v ratio of methanol to 0.1% formic acid, showed improved separation but introduced an unresolved, broad unknown peak at a late elution volume (10.589 minutes), indicating column overload or interference. Optimal selectivity was achieved during the replicate run of the 60:40 v/v configuration in Trial 4, operating at a flow rate of 0.8 mL/min. This system yielded symmetrical, sharp, and well-resolved peaks with an analytical retention time of 3.315 minutes for Atorvastatin and 6.153 minutes for Ezetimibe. The peak symmetry factors approached unity (0.96 and 0.97), and the system suitability parameters, including theoretical plate counts (6461 and 7935, respectively) and peak resolution (12.84), comfortably exceeded standard pharmacopeial thresholds. Trial 5 adjusted the composition slightly to 61:39 v/v at 0.9 mL/min, but this led to a compressed retention pattern and reduced resolution, confirming that Trial 4 represented the ideal, optimized isocratic chromatographic setup.

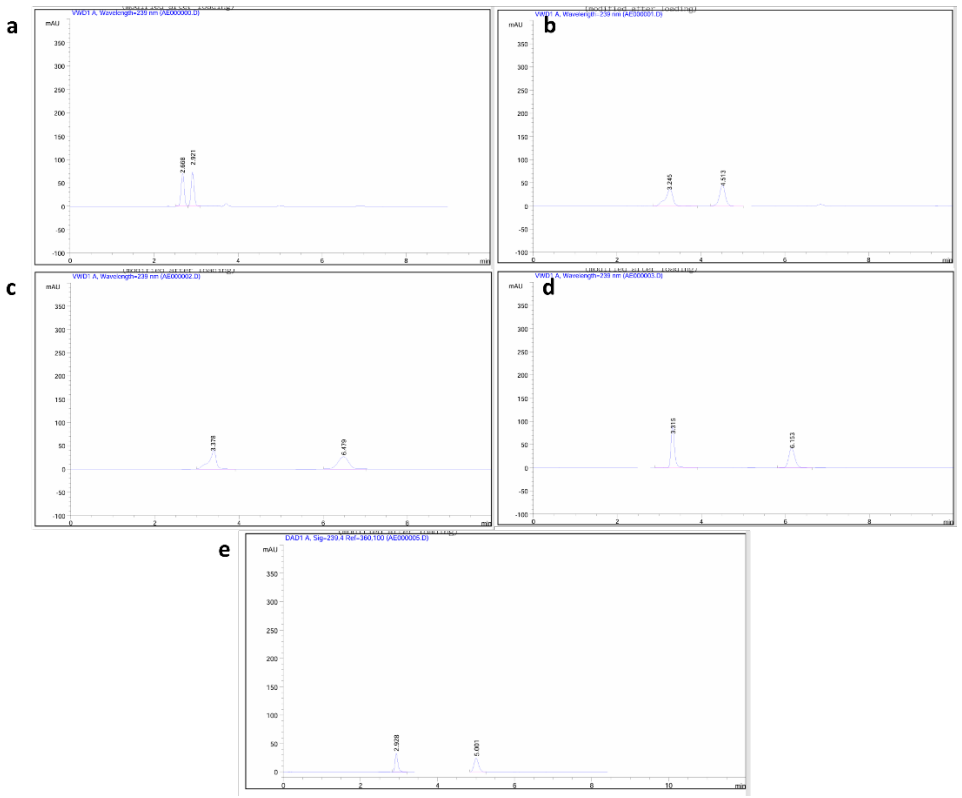

Figure 3. Chromatograms of method development trials. a. Trial 1, b. Trial 2, c. Trial 3. d. Trial 4, and e. Trial 5

3.2. Method Validation

Validation parameters were systematically evaluated in accordance with the International Council for Harmonisation (ICH) Q2(R1) guidelines. The validation results and corresponding system suitability criteria have been consolidated below in Table 2.

Table 2. Analytical Method Validation and System Suitability Parameters for Atorvastatin and Ezetimibe

Validation Parameter / System Metric	Atorvastatin (ATO)	Ezetimibe (EZE)
Linearity Range	5-25 µg/mL	5-25 µg/mL
Regression Equation	$y = 34.195 \cdot x + 10.352$	$y = 41.983 \cdot x + 4.119$
Correlation Coefficient (R^2)	0.9992	0.9996
Limit of Detection (LOD)	0.5951 µg/mL	0.3532 µg/mL
Limit of Quantification (LOQ)	1.8033 µg/mL	1.0702 µg/mL
System Repeatability (%RSD, n=6)	0.30%	0.48%
Intraday Precision (%RSD range, n=3)	0.11%-1.29%	0.11%-1.16%
Interday Precision (%RSD range, n=3)	0.08%-1.23%	0.12%-0.67%
Accuracy / % Recovery (Spike levels 80%-120%)	99.72%-100.83%	98.35%-102.06%
Ruggedness (%RSD, Analyst-to-Analyst)	0.58%	0.11%
Robustness (%RSD under Mobile Phase Shifts)	0.68%-0.89%	0.15%-0.33%
Robustness (%RSD under Wavelength Shifts)	0.18%-0.32%	0.37%-0.71%
Formulation Assay Recovery (% Mean ± SD)	101.01% ± 0.42%	101.01% ± 0.42%
Retention Time (t_R, optimized run)	3.315 min	6.153 min
Peak Symmetry Factor (A_s)	0.96	0.97
Chromatographic Theoretical Plates (N)	6461	7935
Peak Resolution (R_s)	—	12.84

3.2.1. Specificity

Specificity was showed by comparing the chromatograms of blank solvent mixtures, synthetic excipient blends, standard mixtures of Atorvastatin and Ezetimibe, and commercial dosage form extracts. No interfering peaks from excipients or mobile phase components were observed at the retention times of Atorvastatin (3.3 minutes) and Ezetimibe (6.1 minutes). The diode array detector confirmed peak purity, indicating that the baseline separation of both compounds was free from spectral co-elution.

3.2.2. Linearity and Range

Linearity was evaluated over a concentration range of 5 to 25 µg/mL for both analytes. Regression analysis of the calibration curves showed a highly linear relationship between analyte concentration and peak area response. For Atorvastatin, the mean peak area increased from 176.8875 mAU at 5 µg/mL to 864.4465 mAU.s at 25 µg/mL. Ezetimibe showed a corresponding linear increase from 208.6178 mAU.s to 1052.7176 mAU.s.

The linear regression equation obtained for Atorvastatin was y = 34.195 · x + 10.352 with a coefficient of determination (R^2) of 0.9992. For Ezetimibe, the regression equation was y = 41.983 ·x + 4.119 with an R^2 value of 0.9996. The relative standard deviations of the slope and intercept were within acceptable regulatory limits, confirming the stability and linearity of the detector response across the clinical range.

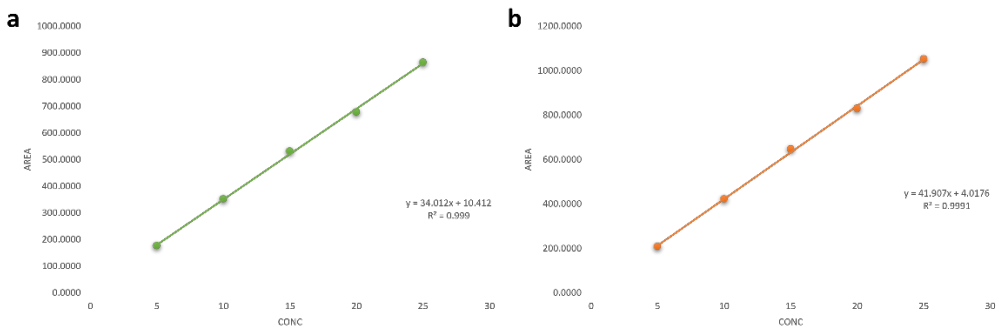

Figure 4. Calibration curve of a. ATO and b. EZE

3.2.3. Accuracy and Recovery

Method accuracy was verified using recovery studies at 80%, 100%, and 120% spike levels, where pure drug standards were added to a pre-analyzed tablet matrix containing 5 µg/mL of both analytes. The recovery values for Atorvastatin were 99.80%, 99.72%, and 100.83% at the 80%, 100%, and 120% levels, respectively. The corresponding recoveries for Ezetimibe were 98.35%, 101.29%, and 102.06%. The relative standard deviations (%RSD) for all recovery analyses were less than 0.75%, indicating that the method is highly accurate and free from systematic bias or interference from typical tablet formulation excipients

Table 3. Results of accuracy studies of ATO

Sample (% level)	Conc of marketed formulation (µg/mL)	Conc. of API added (µg/mL)	Absorbance (Area)	Amount Recovered (µg/mL)	% Recovery	Mean	SD	%RSD
80%	5	4	316.32	3.99	99.87	99.80	0.10	0.10
			316.124	3.99	99.72			
100%		5	349.92530	4.98	99.66	99.72	0.09	0.09
			350.14520	4.99	99.79			
120%		6	387.276	6.08	101.35	100.83	0.74	0.73
			385.1542	6.02	100.31			

Table 4. Results of accuracy studies of EZE

Sample (% level)	Conc of marketed formulation (µg/mL)	Conc. of API added (µg/mL)	Absorbance (Area)	Amount Recovered (µg/mL)	% Recovery	Mean	SD	%RSD
80%	5	4	378.55	3.94	98.47	98.35	0.17	0.17
			378.1554	3.93	98.23			
100%		5	425.10782	5.05	101.00	101.29	0.41	0.41
			426.32400	5.08	101.58			
120%		6	470.879	6.14	102.37	102.06	0.44	0.43
			469.3214	6.11	101.75			

3.2.4. Precision and Repeatability

Precision was evaluated across repeatability, intraday, and interday parameters. Repeatability, or system precision, was determined by performing six consecutive injections of a standard mixture containing 15 µg/mL of both drugs. The resulting relative standard deviation of the peak areas was 0.30% for Atorvastatin and 0.48% for Ezetimibe, confirming excellent instrumental consistency.

Table 5. Results of Intraday precision of Atorvastatin and Ezetimibe

Sample label	Inj. Vol.	Peak	Ret. Time (min)	Area (mAU·s)	Height (mAU)	Sym.	Width (min)	Plates	Resolution
5+5	01	ATO	3.018	178.30502	32.52728	0.90	0.0789	8106	12.99
		EZE	5.363	209.15778	23.74177	0.93	0.1333	8963	
	02	ATO	3.032	181.59796	32.91483	0.99	0.0789	8183	12.82
		EZE	5.401	212.60847	23.24601	0.94	0.1383	8445	
15+15	01	ATO	3.037	526.43384	101.10205	0.99	0.0778	8449	13.11
		EZE	5.412	646.90222	72.65061	0.93	0.1350	8903	
	02	ATO	3.038	534.90686	100.34533	0.99	0.0778	8452	13.08
		EZE	5.406	640.98633	71.88470	0.92	0.1350	8885	
25+25	01	ATO	3.014	883.33722	163.07043	0.90	0.0778	8320	12.77
		EZE	5.344	1057.87268	118.62193	0.91	0.1367	8471	
	02	ATO	3.030	882.00098	161.44124	0.99	0.0773	8503	12.77
		EZE	5.355	1056.22876	116.77399	0.92	0.1367	8505	

Intraday precision was determined by analyzing concentration levels of 5, 15, and 25 µg/mL on the same day. The recovery values remained close to 100%, with relative standard deviations ranging from 0.11% to 1.29% for Atorvastatin and from 0.11% to 1.16% for Ezetimibe.

Interday precision was evaluated using the same concentrations over three consecutive days. The relative standard deviation values ranged from 0.08% to 1.23% for Atorvastatin and from 0.12% to 0.67% for Ezetimibe. These consistently low variability metrics show that the method is highly precise for routine quality control testing.

Table 6. Results for Intraday Precision

Drug	Conc (µg/mL)	Mean Area	Amount Found (µg/mL)	% Amount Found	SD	%RSD
ATO	5	179.95	4.99	99.70	2.33	1.29
	15	535.67	15.44	100.85	1.08	0.20
	25	882.67	25.65	99.14	0.94	0.11
EZE	5	210.88	4.94	98.74	2.44	1.16
	15	643.94	15.27	101.82	4.18	0.65
	25	1057.05	25.13	100.53	1.16	0.11

3.2.5. Sensitivity (LOD and LOQ)

The sensitivity of the chromatographic method was calculated based on the standard deviation of the y-intercept (sigma) and the slope of the calibration curve (S). For Atorvastatin, the Limit of Detection (LOD) was established at 0.5951 µg/mL and the Limit of Quantification (LOQ) at 1.8033 µg/mL. Ezetimibe exhibited an LOD of 0.3532 µg/mL and an LOQ of 1.0702 µg/mL. These low values show that the method has sufficient sensitivity to identify and quantify both APIs at low concentrations.

3.2.6. Robustness and Ruggedness

Robustness was evaluated by introducing small, manual shifts in the organic modifier ratio (± 1%, equivalent to 62:38 v/v) and detection wavelength (± 2 nm, equivalent to 238 nm and 240 nm). For both conditions, system suitability metrics, including theoretical plate counts and peak resolution, remained stable, and the recovery values exhibited minimal variation with relative standard deviations below 0.90%.

Table 7. Results of Robustness Data for ATO and EZE

Condition	Drug	Retention Time (min)	Area (mAU·s)	Height (mAU)	Sym.	Width (min)	Plates	Resolution
Mobile Phase (60:40) 01	ATO	3.018	356.70703	66.31593	0.99	0.0778	8343	13.64
	EZE	5.547	426.20602	45.89959	0.92	0.1400	8697	
Mobile Phase (60:40) 02	ATO	3.019	361.21455	66.31593	0.99	0.0778	8343	13.64
	EZE	5.549	425.32144	45.89959	0.92	0.1400	8697	
Mobile Phase (62:38) 01	ATO	3.042	357.35449	65.56838	0.98	0.0778	8476	12.50
	EZE	5.217	428.57428	51.21484	0.94	0.1267	9397	
Mobile Phase (62:38) 02	ATO	3.043	354.13655	65.56838	0.98	0.0778	8476	12.50
	EZE	5.218	426.57428	51.21484	0.94	0.1267	9397	
Wavelength (238 nm) 01	ATO	3.015	351.72900	65.17086	0.99	0.0778	8325	12.87
	EZE	5.345	447.56528	50.04466	0.92	0.1350	8686	
Wavelength (240 nm) 01	ATO	3.015	365.04269	68.09072	0.99	0.0778	8325	12.87
	EZE	5.345	407.12491	45.54933	0.92	0.1350	8686	
Wavelength (238 nm) 02	ATO	3.017	350.14570	65.17086	0.99	0.0778	8325	12.87
	EZE	5.346	445.21322	50.04466	0.92	0.1350	8686	
Wavelength (240 nm) 02	ATO	3.017	364.12547	68.09072	0.99	0.0778	8325	12.87
	EZE	5.346	411.21544	45.54933	0.92	0.1350	8686	

Ruggedness was evaluated across variable operational environments, including testing by different analysts and on different high-performance liquid chromatography channels. The relative standard deviation values for Atorvastatin and Ezetimibe were 0.58% and 0.11%, respectively, confirming that the method provides reproducible results across different laboratory conditions.

Table 8. Results of Ruggedness of ATO and EZE

Drug	Conc (µg/mL)	Absorbance (Area)	Mean Area	Amount Found (µg/mL)	% Label Claim	SD	%RSD
ATO	15	525.89	523.74	15.09	100.62	3.03	0.58
		516.43		15.00	100.85		
EZE		640.22	640.73	15.20	101.31	0.71	0.11
		635.26		15.03	100.21		

3.3. Assay of Commercial Formulations

The validated chromatographic method was applied to the quantitative extraction and estimation of Atorvastatin and Ezetimibe in commercial ATORVASTATIN EZ tablets. Extracts of the tablets were prepared to a target concentration of 20 µg/mL for both analytes and analyzed under optimized isocratic conditions. The average recovery was 101.01% of the labeled claim for both drugs, with low standard deviations (0.42%) and an RSD of 0.41%. These results indicate that the method effective for the concurrent quantitative analysis of both compounds in dosage forms without excipient interference.

Table 9. Results of Assay of Commercial Formulations

Drug	Conc (µg/mL)	Area	Amount Found (µg/mL)	% Label Claim	Mean	SD	%RSD
ATOR	20.00	707.7271	30.21	100.71	101.01	0.42	0.41
		707.9080	30.39	101.30			
EZE		844.5560	30.21	100.71	101.01	0.42	0.41
		829.8060	30.39	101.30			

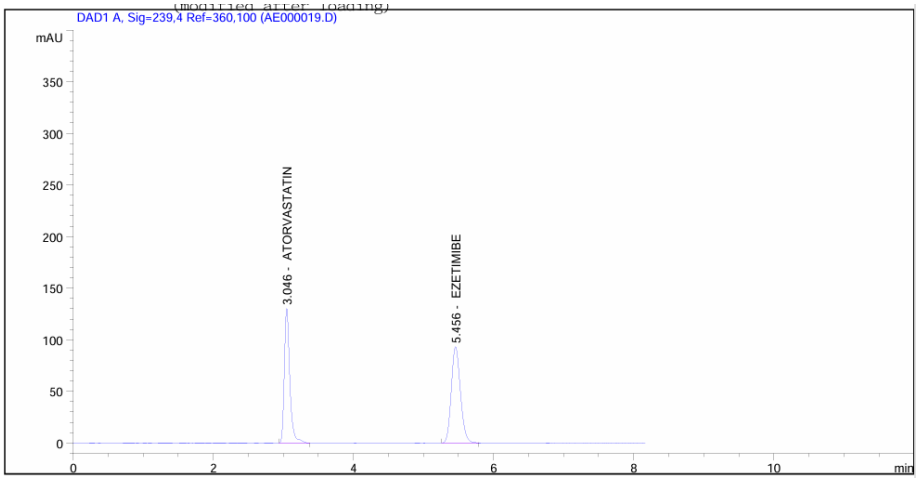

Figure 5. Chromatogram of assay study at 20 + 20 µg/mL

3.4. Forced Degradation Studies

3.4.1. Acidic Hydrolysis

Acidic degradation studies, conducted by reacting a standard mixture of the drugs with 2 mL of 0.1 N hydrochloric acid for 2 hours at room temperature, showed that both compounds are relatively stable under these conditions. The peak area for Atorvastatin decreased by 2.73%, with 97.27% of the active drug remaining. For Ezetimibe, the peak area decreased by 2.91%, leaving 97.09% of the active compound intact.

Analysis of the acidic sample resolved three distinct degradation peaks at retention times of 2.718 minutes (designated as DEG-01, 0.42%), 2.829 minutes (DEG-02, 0.14%), and 3.223 minutes (DEG-03, 0.83%). All degradation products were baseline-resolved from the parent peaks, confirming the selectivity of the method.

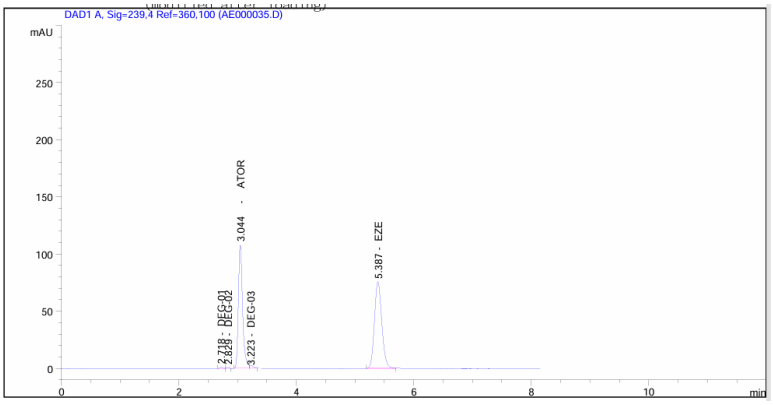

Figure 6. Chromatogram of forced degradation after 2 hours in 0.1 N HCl (Acidic condition)

3.4.2. Alkaline Hydrolysis

Under basic hydrolytic stress (0.1 N sodium hydroxide for 2 hours at room temperature), the target analytes exhibited highly contrasting stability profiles. Atorvastatin showed moderate stability, with 95.51% of the parent drug remaining, representing a degradation of 4.49%. In contrast, Ezetimibe underwent rapid and complete degradation, resulting in the total disappearance of its parent peak (100% degradation) under identical stress conditions.

This extreme susceptibility to alkaline hydrolysis is due to the unique chemical structure of Ezetimibe. The molecule contains a strained four-membered β-lactam ring (azetidin-2-one). In an alkaline environment, nucleophilic attack by hydroxide ions (OH-) occurs at the carbonyl carbon of the β-lactam ring. This nucleophilic addition opens the ring, breaking the cyclic amide bond and forming a highly polar acyclic amino acid derivative. This degradation product exhibits completely different retention behavior and elutes close to the solvent front, resulting in the complete loss of the original parent peak at 6.1 minutes.

Atorvastatin calcium lacks highly strained cyclic amide structures. Its primary functional sites are a heptanoic acid side chain and a pyrrole ring, which are less prone to basic nucleophilic cleavage. Consequently, Atorvastatin remains largely stable under basic conditions. Analysis of the basic sample resolved two major degradation peaks at retention times of 2.605 minutes (DEG-01, 6.29%) and 2.851 minutes (DEG-02, 72.99%). Both peaks were well resolved from the remaining Atorvastatin peak, confirming that the method can monitor stability even under severe basic stress.

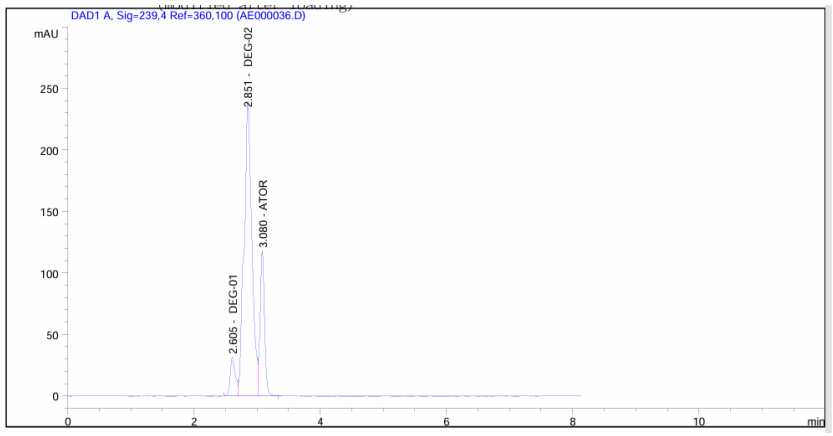

Figure 7. Chromatogram of forced degradation after 2 hours in 0.1 N NaOH (Basic condition)

3.4.3. Neutral, Oxidative, Photolytic, and Thermal Stress

Under neutral hydrolytic conditions, both analytes showed negligible degradation over a 2 hourS reaction period. Atorvastatin retained 99.84% of its initial area (0.16% degradation), while Ezetimibe retained 99.21% (0.79% degradation), showing excellent stability in neutral aqueous solutions.

Table 10. Forced Degradation Recovery for Atorvastatin and Ezetimibe

Stress Degradation Condition	Target Analyte	Baseline Peak Area (mAU.s)	Stressed Residual Area (mAU.s)	Analyte Recovery (%)	Degradation Fraction (%)
Acidic Hydrolysis (0.1 N HCl, 2 h)	ATO	529.6715	515.1934	97.27%	2.73%
	EZE	645.2972	626.4874	97.09%	2.91%
Alkaline Hydrolysis (0.1 N NaOH, 2 h)	ATO	529.6715	505.9093	95.51%	4.49%
	EZE	645.2972	0.0000	0.00%	100.00%
Neutral Hydrolysis (H_2O, 2 h)	ATO	529.6715	528.8390	99.84%	0.16%
	EZE	645.2972	640.2145	99.21%	0.79%
Oxidative Stress (3% H_2O_2, 2 h)	ATO	529.6715	515.9347	97.41%	2.59%
	EZE	645.2972	611.8719	94.82%	5.18%
Photolytic Exposure (UV sunlight, 24 h)	ATO	529.6715	527.7856	99.64%	0.36%
	EZE	645.2972	638.4512	98.94%	1.06%
Thermal Stress (Dry heat, 80°C, 24 h)	ATO	529.6715	528.1448	99.71%	0.29%
	EZE	645.2972	637.2145	98.75%	1.25%

Oxidative stress, using 3% hydrogen peroxide for 2 hours at room temperature, caused significant degradation in both molecules. Atorvastatin showed 2.59% degradation (97.41% remaining), while Ezetimibe degraded by 5.18% (94.82% remaining).

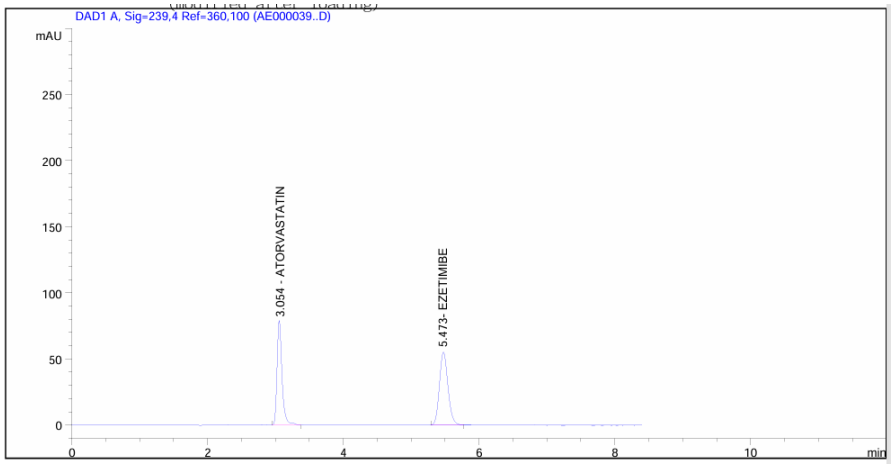

Figure 8. Chromatogram of forced degradation after 2 hours under neutral condition

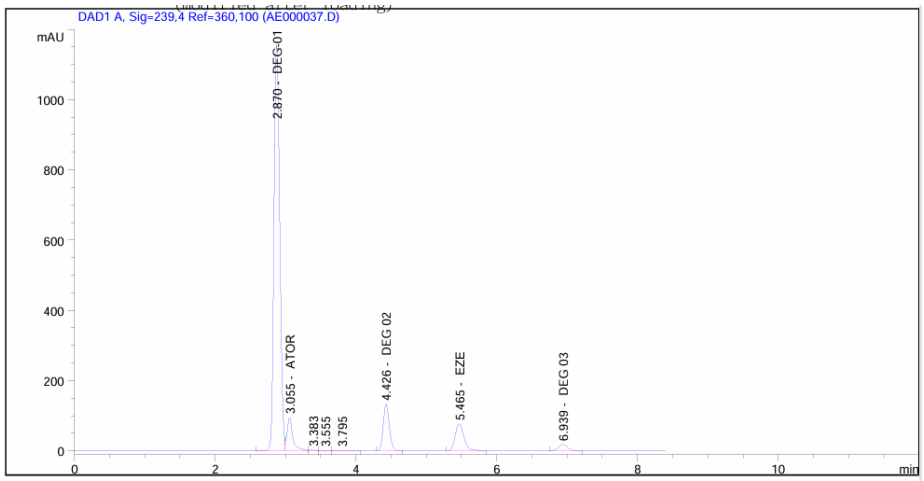

Figure 9. Chromatogram of forced degradation after 2 hours in 3% H_2O_2 (Oxidative condition)

The oxidative sample chromatogram showed three degradation peaks resolved from the parent compounds, eluting at 2.870 minutes (DEG-01, 75.02%), 4.426 minutes (DEG-02, 8.96%), and 6.939 minutes (DEG-03, 1.88%). Minor unknown peaks were also resolved at 3.383 minutes (0.19%), 3.555 minutes (0.15%), and 3.795 minutes (0.20%), indicating a complex oxidative pathway.

For photolytic degradation, bulk drug powders were exposed to direct UV sunlight for 24 hours. Under these conditions, Atorvastatin retained 99.64% of its initial area (0.36% degradation), and Ezetimibe retained 98.94% (1.06% degradation).

Similarly, thermal stress (80∘C dry heat for 24 hours) did not cause significant degradation, with Atorvastatin retaining 99.71% (0.29% degradation) and Ezetimibe retaining 98.75% (1.25% degradation). No significant degradation peaks were observed, confirming that both APIs are stable under photolytic and thermal conditions.

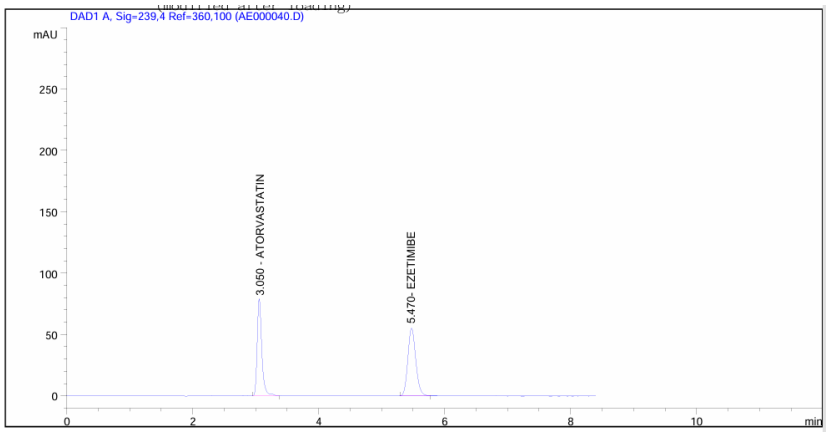

Figure 10. Chromatogram of photolytic degradation study at 15 + 15 µg/mL

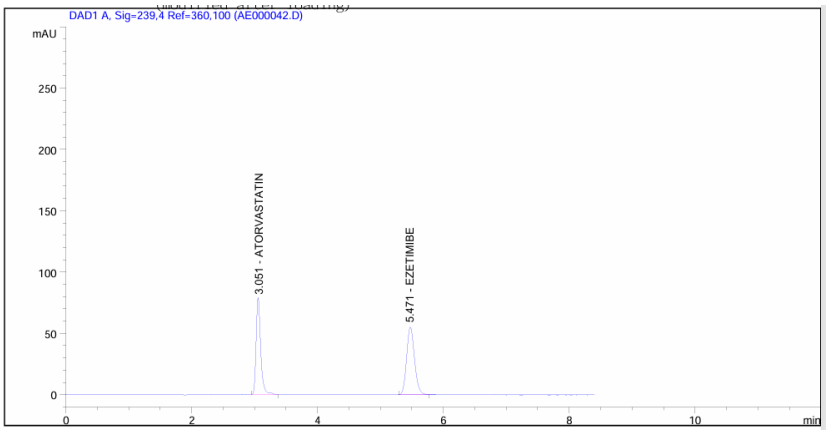

Figure 11. Chromatogram of Thermal degradation study at 15 + 15 µg/mL

4. Conclusion

An accurate, reproducible, and stability-indicating isocratic RP-HPLC method was developed and validated for the simultaneous quantitative estimation of Atorvastatin and Ezetimibe in active pharmaceutical ingredient matrices and commercial formulations. The chromatographic separation was optimized on a C18 stationary phase (250 mm times 4.6 mm, 5 µm) using a volatile, buffer-free mobile phase of methanol and 0.1% formic acid in a 60:40 v/v ratio. The method developed has a flow rate of 0.8 mL/min with column temperature control maintained at 25°C. UV detection was conducted at the isosbestic point of 239 nm to ensure equivalent and stable response sensitivity for both analytes, yielding retention times of approximately 3.3 and 6.1 minutes for Atorvastatin and Ezetimibe, respectively. Validation of the analytical method was conducted in accordance with ICH Q2(R1) guidelines. The detector response was highly linear over the concentration range of 5-25 µg/mL for both drugs, with correlation coefficients exceeding 0.999. The method's accuracy was confirmed by recovery values between 98.35% and 102.06% across three

concentration levels. Precision studies showed very low variability, with repeatable system injections yielding relative standard deviations below 0.50%. Forced degradation studies under hydrolytic, oxidative, photolytic, and thermal stress conditions showed the stability-indicating capability of the method. Degradation products formed under environmental stress were baseline-resolved from the parent drug peaks. The basic degradation pathway revealed that Ezetimibe undergoes rapid, complete hydrolysis due to the cleavage of its highly strained azetidin-2-one (β-lactam) ring, whereas Atorvastatin remained stable. This method avoids high-salt phosphate buffers, reducing column wear and ensuring compatibility with mass spectrometry, making it suitable for routine quality control and stability assessment in pharmaceutical development.

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