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Original Article

Development and Validation of Stability-indicating HPLC Method for Estimation of Azilsartan in Pharmaceutical and Solid Lipid Nanoparticles

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ABSTRACT

Introduction: Azilsartan has been scientifically proven to be effective in treating hypertension. According to International Conference on Harmonization, the HPLC method was developed and validated to estimate the azilsartan in formulated solid lipid nanoparticles and marketed pharmaceutical formulations. **Objectives:** Develop and validate an HPLC method for analysing azilsartan in drugs and various formulations that demonstrated stability by ICH (International Conference on Harmonization) standards. **Materials and Methods:** The mobile phase uses methanol: phosphate buffer (0.1% orthophosphoric acid, pH 3.2) (70:30), having the chromatographic separator is an HPLC column C_{18} (4.6 mm X 250 mm) with a wavelength of 249 nm and a flow rate of 1 mL/min. **Results:** The developed method showed a correlation coefficient value is 0.999 and to be linear throughout a concentration range of 2-10 µg/mL. The proposed method was precise (percent RSD 2.0%), accurate (percent recovery 99-101%), and reliable. The detection and quantification limits for azilsartan were determined to be 0.01 µg/mL and 0.04 µg/mL, respectively. According to ICH criteria, the developed method was validated and a stress degradation study was conducted. The developed method was used to estimate azilsartan in solid lipid nanoparticles to determine the applicability of the developed method. **Conclusion:** A quick, accurate, simple and economical HPLC method was successfully developed and validated for the estimation of azilsartan in solid lipid nanoparticles and marketed formulation.

Keywords: Azilsartan, Solid Lipid Nanoparticle, HPLC, Method Development, Degradation Study.

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INTRODUCTION

Azilsartan is a class of angiotensin II receptor blockers used in the treatment of hypertension. Chemically, azilsartan is known as 2-ethoxy-3-[[4-[2-(5-oxo-4H-1,2,4-oxadiazol-3-yl) phenyl] phenyl] methyl] benzimidazole-4-carboxylic acid (Figure 1). It is a white, crystalline powder that is entirely not soluble in water, but readily soluble in methanol, Dimethyl Sulfoxide (DMSO), DMF and completely soluble in ethanoic acid.[1]

Azilsartan is an angiotensin II receptor blocker which dilates the blood vessels hence it is used in the treatment of high blood pressure.[2] Azilsartan can bind longer to the AT1 receptor, which helps to dilate the blood vessels and lower high blood pressure.[3]

On February 25, 2011, the US Food and Drug Administration approved an Edarbi tablet to treat adult hypertension. It is offered in quantities of 80 mg and 40 mg, with 80 mg once daily being the suggested dosage.[4]

Angiotensin II hormone has several crucial roles in the regulation of blood pressure, fluid-electrolyte balance, activation of the renin-angiotensin-aldosterone system, and the pathogenesis of hypertension. Vasoconstriction, aldosterone and vasopressin release, cellular proliferation, and other effects are regulated by activating the type 1 angiotensin receptor which is a member of a G protein-coupled receptor. Therefore, Azilsartan inhibits the binding of Angiotensin II to the AT1 receptor for a longer period, and as a result lower blood pressure. Hence, Angiotensin II type 1 blocker will be useful in the treatment of cardiovascular and renal illnesses.[5]

The literature review found that various analytical techniques have been reported to estimate azilsartan medoxomil in the pharmaceutical dosage form when combined with other drugs.[6]

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As per previously referred articles and literature review, the HPLC method has been published to estimate azilsartan medoxomil and cilnidipine in the pharmaceutical dosage form. According to Solanki *et al.*, the retention time for azilsartan medoxomil peak 1, azilsartan medoxomil peak 2, and cilnidipine were 2.16 min, 3.90 min, and 9.52 min respectively, with the mobile phase triethylamine: acetonitrile in the ratio of 40:60 with flow rate 1 mL/min. The recently published article almost used conventional dosage form to estimate azilsartan medoxomil either individually or in combination form.[7,8]

The proposed research study objective was to develop and validate an HPLC method for analysing azilsartan in bulk form that shows stability by ICH (International Conference on Harmonization) standards.[9,10] The relevancy of the developed method was analysed on azilsartan-loaded solid lipid nanoparticles and conventional dosage form. The forced degradation study, including acidic, basic, oxidative, heat, and photolytic conditions were studied to assure the suitability of the devised approach.

MATERIALS AND METHODS

Material and Reagent

Azilsartan (99%) was provided as a gift sample by CTX Lifescience Pvt. Ltd., Gujarat, India. Glyceryl Monostearate (GMS) was sponsored by Mohini Organics Pvt. Ltd., Mumbai. Throughout the study, HPLC analytical-grade methanol, Orthophosphoric Acid (OPA), and water were used. All additional ingredients were of pharmaceutical grade.

Instrumentation

The analysis was performed with the help of a High-Performance Liquid Chromatographic system (HPLC) (Agilent 1220 Infinity II) equipped with a Photodiode Array (PDA) detector. The instrument parameters were controlled using Open lan EZ Chrom chemstation edition software.

Chromatographic Conditions

Chromatographic analysis of azilsartan was performed with the help of a C_{18} column (4.6 mm X 250 mm), with an appropriate column temperature. The optimized mobile phase methanol: phosphate buffer [0.1% OPA, 70:30 v/v] of pH 3.2 was pumped at a flow rate of 1 mL/min. Before usage, a PVDF filter membrane (0.45 mm; Millex HV®, Millipore, USA) was used to filter the mobile phase. For the sample analysis, 249 nm wave lengths were used and the injection volume remained at 10 µL.

Buffer Preparation (0.1%OPA pH 3.2)

1 mL of concentrated orthophosphoric acid is put into a volumetric flask with a capacity of 1000 mL. Then the volumetric flask was filled with milli-Q water in an amount of about 900 mL and sonicated to degas and then finally made it up with water.[11]

Preparation of Sample

Azilsartan primary stock solutions were prepared using methanol at a concentration of 1 mg/mL. The standard solution of azilsartan was prepared with concentration ranges between 2-10 µg/mL from the stock solution. Before the HPLC analysis, all standard solutions were stored at a temperature of 4°C in amber-coloured volumetric flasks with light-resistant tightly fitting lids.[12,13]

Method Development

A method for analysing azilsartan was developed using various mobile phase ratios, OPA concentrations, pH values, flow rates, and column oven temperatures.

Method Validation

The new method was validated for the system appropriateness, linearity, limit of quantification, limit of detection, accuracy, precision, ruggedness, and robustness by the ICH guidelines.[14]

System Suitability

Five replicate injections of the standard preparation were used to test the method's system applicability. Reports included plate count, tailing factor, resolution, and %RSD.

Linearity

Linearity was estimated by injecting a concentration of azilsartan ranging from 2 to 10 µg/mL with a maximum wavelength of 249 nm. Firstly, the primary stock solution was prepared by adding 10 mg of the drug to 10 mL of methanol in a volumetric flask. To prepare the sample of the concentration ranging from 2 to 10 µg/mL an appropriate amount of sample was taken from the standard stock solution and volume make up 10mL with mobile phase.

Precision

Intraday precision also known as repeatability was developed by multiple sampling from a homogeneous mixture. Interday Precision, day-day precision, analyst-analyst precision, and intermediate precision are other names for it. Six samples were

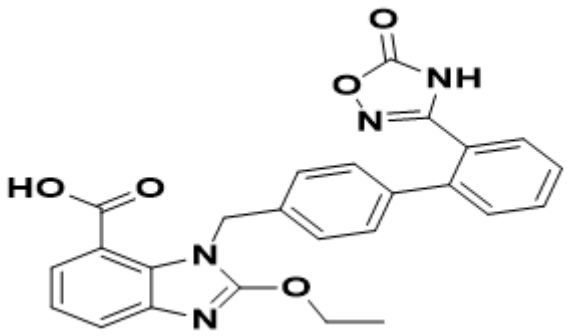

Figure 1: Structure of Azilsartan

made from a homogenous mixture and injected as a working solution. Relative standard deviation was used to express it.

Accuracy

A method's accuracy is measured by how closely the experimental value matches the concentration of the substance present in the matrix. Three concentrations of the sample solution-50%, 100%, and 150%-were produced and injected. The recovery percentage was determined and reported.

Sensitivity

LOD: The lowest drug concentration that can be detected at the detector level without the need for quantification is known as the LOD. S/N is a 3:1 ratio.

LOQ: Limit of quantification, or LOQ, refers to the lowest medication concentration that can be accurately and precisely measured. 10:1 S/N Ratio.

Robustness

Minor, intentional modifications were made to the procedure, such as the addition and deletion by ±2 in the parameters like flow rate, wavelength, and injection volume. The results were then reported as % Relative standard deviation.

Studies on Forced or Stress Deterioration

For the forced degradation study ICH guidelines suggested stress conditions such as acidic, basic, oxidative and photolysis, for single and mixture of drug solutions.[15] At zero-time drug solutions were kept under normal conditions and drug solutions were supposed to degrade for 1 hr.[16] 1 mL of the drug's stock solution was treated with 1 mL each of 1 N HCl and 1 N NaOH solutions in an acid-base degradation investigation. The above-mentioned solutions were sealed in volumetric flasks, which were then heated for 1 hr at 80°C. Both samples were neutralised before the examination. 1 mL of the drug mixture's stock solution was treated with 1 mL of a 30% Hydrogen peroxide (H_2O_2) solution as part of a study on oxidative degradation.[17] To study heat degradation, 1 mL of stock samples of the drug mixture was treated with 2 mL of methanol in a volumetric flask. The aforementioned liquids were placed in a water bath and heated for an hour at 80°C. 1 mL of the drug's stock solution was introduced in a clear volumetric flask (10 mL) and then it was made up to 10 mL with mobile phase, then the volumetric flask was sealed and placed in sunlight for 30 min to examine for photochemical degradation. The mobile phase was used to alter the final volume by up to 10 mL for each of the investigations listed above. Syringe filters (0.2 mm) were used for filtration, and the sample solution was then injected into an HPLC chromatographic system for additional analysis.[18]

Preparation of Solid Lipid Nanoparticle

The preparation of Azilsartan-loaded SLNs was done using hot homogenization and ultrasonic techniques.[19] Both strategies are widely used and easy to manage.[20,21] Weighing precisely 1% azilsartan, it was subsequently added to a lipid melted at approximately 70% of its melting point, producing a translucent solution and simultaneously aqueous phase was heated to the same temperature adding surfactant (Pluronic F 127) and maintaining the same temperature as the oil phase. Later, the oily and aqueous phases were homogenised in an ultra-turrax for 15 min at 12000 rpm. The primary emulsion was subjected to ultrasonic processing for 10 min. After cooling, the product was refrigerated.[22]

Azilsartan Estimation in Various Formulations Using a Developed Method

Several formulations containing Azilsartan were used for the estimation including formulated solid lipid nanoparticles and commercially available Azilsartan tablets. Azilsartan dilution was performed to reach a concentration of 10 µg/mL to identify its presence.[23]

RESULTS AND DISCUSSION

Optimization and Development of a Method

Different buffers and mobile phase compositions were used during the method development process for azilsartan, but the results with the mobile phase composition of methanol and 0.1% OPA buffer (70:30) with a flow rate of 1 mL/min were satisfactory. The injection volume was 10 µL, and the ideal wavelength was 249 nm. The resolution, tailing factor, and plate count are the system appropriateness factors that the aforementioned method satisfied. Azilsartan had satisfactory theoretical plates of 5079 and was eluted at 6.1 min. Azilsartan plate count, tailing factor, intraday and interday % relative standard deviation, and accuracy were all reported. A calibration plot was developed, and the linearity equation for azilsartan was found to be y=22152x+3162.4, with a correlation coefficient of 0.999. The azilsartan recovery rate was 99.85%. Azilsartan LOD and LOQ were found to be 0.01 µg/mL and 0.04 µg/mL, respectively. The robustness %RSD was found to be within acceptable limits. Azilsartan was discovered to have 100.08 percent of the medication total in the tablet after an assay was conducted.

Method Validation

The goal of analytical method validation is to make sure that the developed technique is appropriate and trustworthy for its intended use. The developed HPLC technique was validated for a variety of validation criteria, including system appropriateness, linearity, accuracy, precision, LOD, LOQ, robustness, and ruggedness, by ICH recommendations (Q2R1).[24,25]

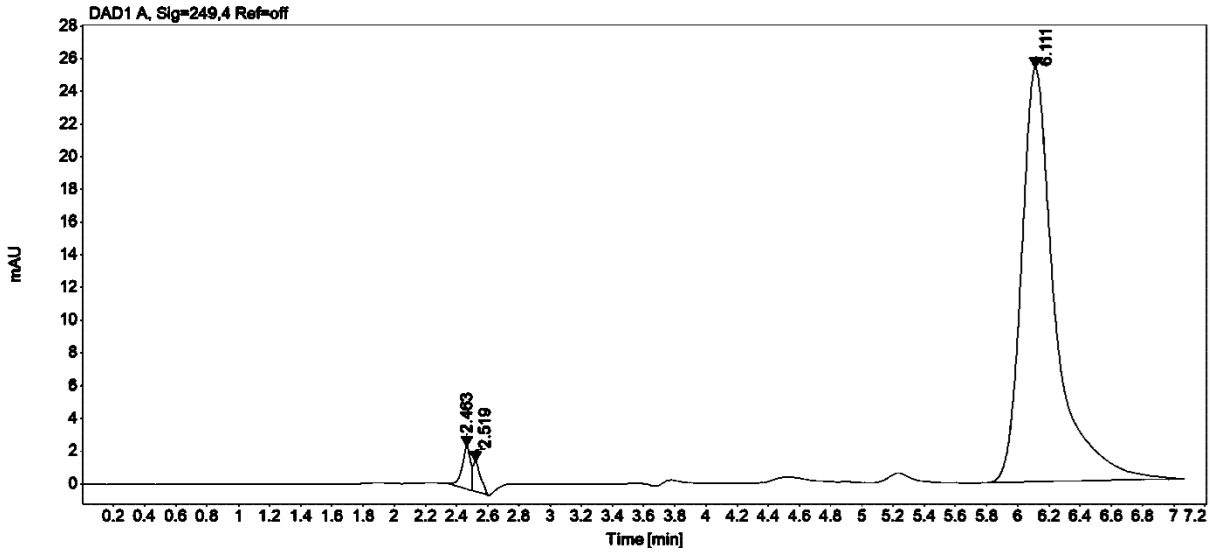

Figure 2: HPLC Chromatogram of Standard Azilsartan.

System suitability

It ensures the established method's validity and specificity. This test is intended to determine whether the developed method is repeatable and has sufficient resolution for chromatographic analysis. It is a crucial step in the method development process. Peak area, Retention Time (RT), theoretical plates, and tailing factor were among the characteristics whose %RSD was calculated (Table 1). The theoretical plates (n>2000), the percentage RSD of the peak area (<2), and the tailing factor (<2) were all within acceptable limits. This process guarantees that the specified HPLC technique is applicable and that the system suitability requirements are met. The results found for azilsartan were sharp, well separated, and with good resolution, as seen in (Figure 2). Results of the aforementioned parameters show that the chosen chromatographic technology is appropriate for further validating and analysing azilsartan.

Linearity

The capacity of the analytically designed technique to determine the outcome of the concentration of the sample analytes is in linear known as linearity. By assessing varied concentrations of azilsartan (2-10 µg/mL) at their maximum wavelength of 249 nm, the method linearity was examined. The calibration curve regression analysis data for azilsartan reveal a linear relationship over this concentration range (Figure 3). R^2>0.999 was discovered to be the correlation coefficient value indicating strong correlation and good linearity for the optimised approach (Table 2).

Limit of quantification (LOQ) and limit of detection (LOD)

LOD and LOQ calculations were done as:

$$LOD=3.3X\ \delta/s\quad LOQ=10X\ \delta/s$$

Where,

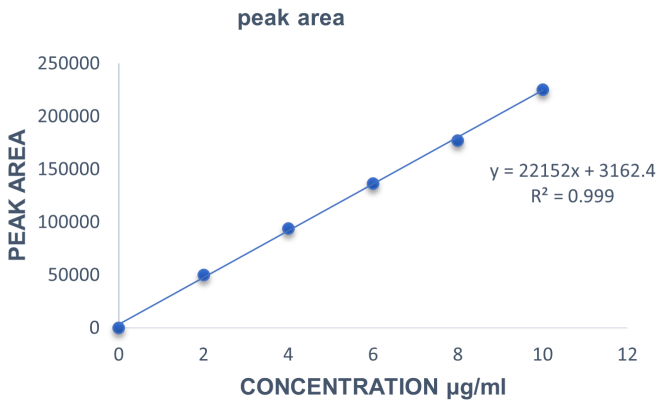

Figure 3: Linearity Curve.

δ=Variance of the y-intercept

s=Average calibration curve slope

The analyte's lowest detectable concentration is known as LOD and it gives a signal-to-noise ratio of three, whereas, the analyte's lowest quantifiable concentration is LOQ and it gives a signal-to-noise ratio of ten. LOD and LOQ for azilsartan in the current study were obtained to be 0.01 µg/mL and 0.04 µg/mL, respectively. The optimised approach was found to be sensitive to determining azilsartan entrapment efficiency in solid lipid nanoparticles based on the LOD and LOQ values.

Precision

Precision is a measure of the extent to which the proposed method readings differ from the number of readings obtained from several samplings of a similar sample using the specified analytical method. This study used intra-day and inter-day assays to evaluate the precision. Different analyte concentrations such as low, medium, and high (2,4 and 6 µg/mL) concentrations, were

analysed for repeatability (intra-day precision) and intermediate precision (inter-day accuracy) on the same day at various time intervals. For intra-day precision, the %RSD values ranged between 0.53 to 1.28%. The %RSD values ranged between 0.51 to 1.30% for inter-day precision. The %RSD values for both precision investigations were <2%, satisfying approval criteria and assuring the high precision of the designed approach (Table 3).

Accuracy

The accuracy of the analytical technique is determined by how closely the experimental result approaches the true value or a commonly accepted standard value. Following the evaluation of azilsartan sample solutions (2 µg/mL), a known quantity of azilsartan was withdrawn in triplicate injections at low, medium, and high dosages of a given concentration (50, 100, and 150%, respectively). The sample solutions were then further analysed using a developed method. Azilsartan's mean percentage recovery was determined to be between 99 to 101% (Table 4), resulting in a low percentage RSD and high recovery values, suggesting the good accuracy of the designed approach.

Ruggedness and Robustness

Intentional changes to the technical conditions, which provide a reliable and accurate assessment of medication components, were utilized to examine the system's robustness and ruggedness. Minor changes were made to the flow rate, mobile phase composition, and λ max of HPLC chromatography variables (Table 5). By using an analyte concentration of 10 µg/mL for the current investigation, it was found that retention time and % RSD values were in the range, demonstrating the reliability of the developed HPLC technology.

Studies of Forced or Stressed Deterioration

Table 6 summarises the findings of experiments on the forced deterioration of Analytes. It was found that in an acid degradation study, azilsartan degraded 9.69%. Azilsartan was degraded by 19.16% in the alkaline degradation study. Azilsartan is more likely to degrade in an alkaline environment since it has a weakly acidic nature. Azilsartan was found to degrade thermally at a rate of 3.29%. The results of thermal degradation indicate that while

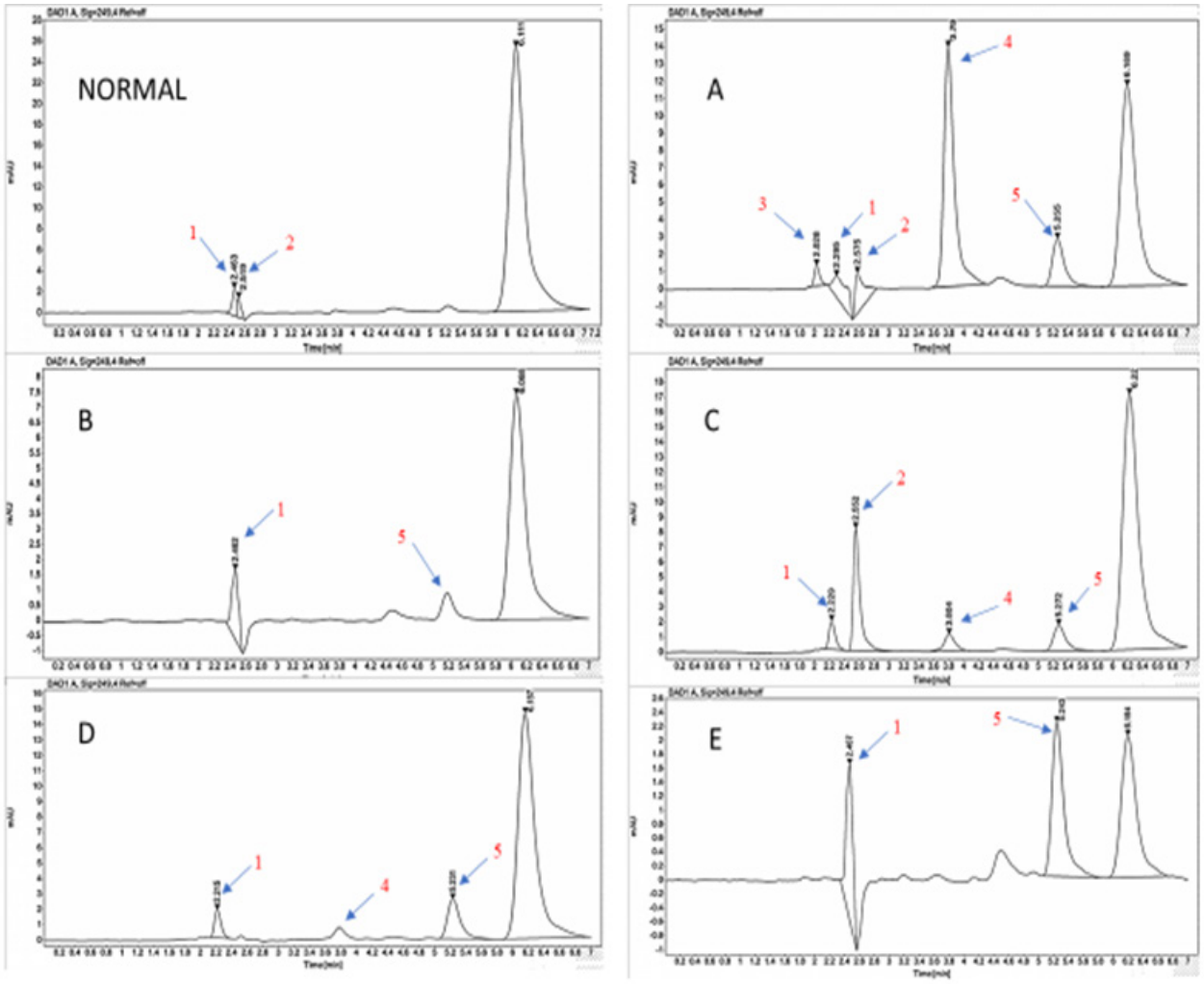

Figure 4: HPLC chromatograms of Azilsartan research of forced deterioration under typical, acidic (A), Basic (B), oxidative (C), thermal (D) and photosensitivity (E) conditions.

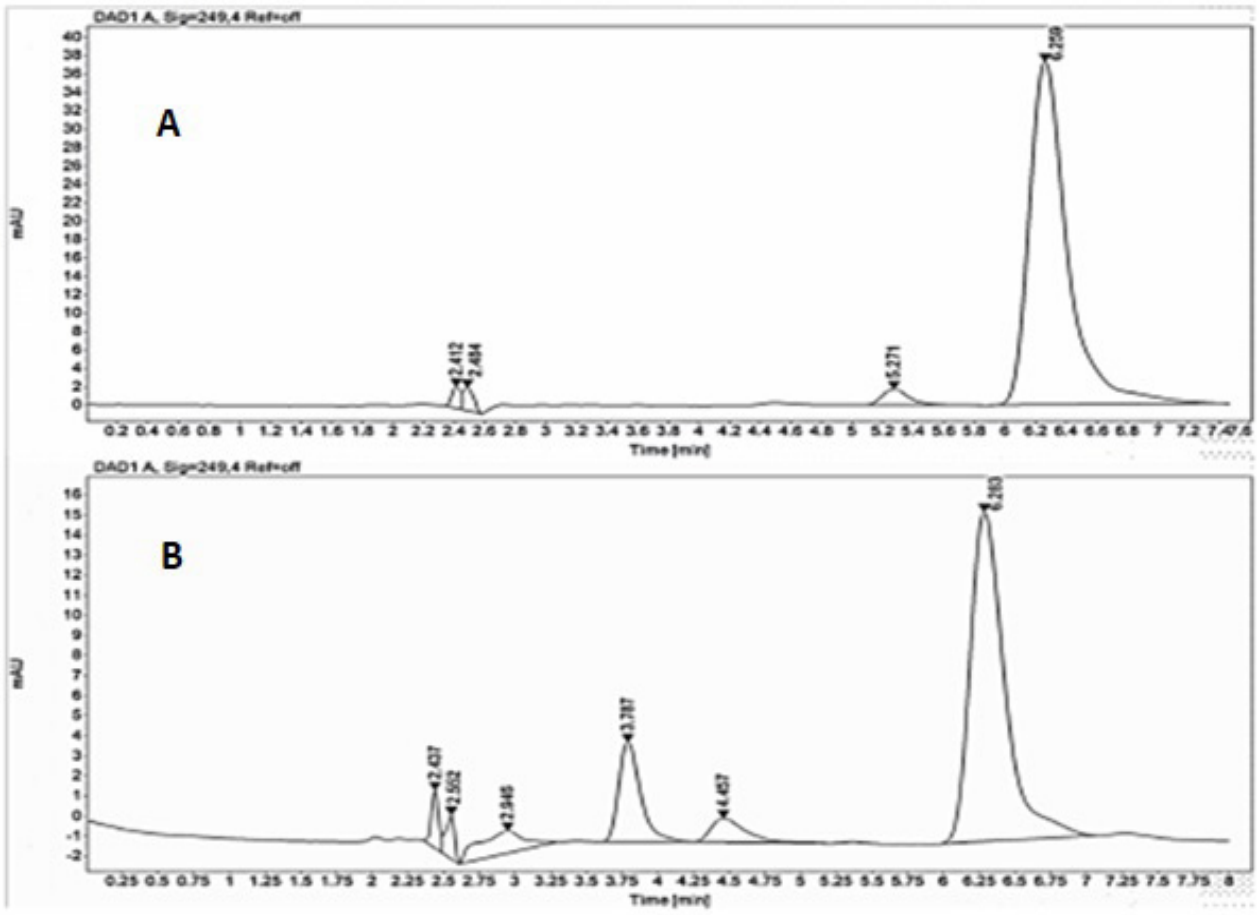

Figure 5: Azilsartan estimation in a different formulation, Azilsartan Tablet (Abel 40) (A), Azilsartan loaded solid lipid nanoparticle (B).

Table 1: Parameters for the Developed Azilsartan Method's System Suitability Research (N=6).

Parameter	Azilsartan		Acceptance criteria
	MEAN±SD	% RSD	
Retention Time	6.104±0.01	0.26	-
Peak Area	349.7681±2.30	0.66	-
Plate Count	5079±81	1.60	>2000
Tailing Factor	1.68±0.01	1.19	≤2

Table 2: Data from Linear Regression and the Method's Sensitivity Parameters.

Concentration range (µg/mL)	Slope	Intercept	Regression coefficient (R^2)	LOD (µg/mL)	LOQ (µg/mL)
0.20-10	22152	3162.4	0.999	0.01	0.04

LOD: Limit of Detection, LOQ: Limit of Quantification.

pharmaceuticals have high melting points, heating may have a minor impact on their stability behaviour. A minor peroxide peak was seen in 30% of the H2O2 degradation research. Under these conditions, azilsartan slowly oxidises with a 5.59% degradation rate. When Azilsartan was exposed to direct sunlight for 60 min, it was degraded by up to 32.92% under-regulated photolysis of stressful circumstances. The degraded peaks are displayed in Figure 4.

Characterization of Prepared Azilsartan Formulation

The results reveal that the formulated Azilsartan nanoformulation demonstrated a particle size of 503 nm with a Poly Dispersibility Index (PDI) value of 0.348 and a Zeta potential of -23.1 mv. PDI

Table 3: Intraday and Interday Precision of Azilsartan.

Azilsartan Concentration (µg/mL)	Intraday (*n*=3)	Interday (*n*=3) RSD (%)		
2	1.28	1.302	1.30	1.24
4	0.67	0.66	0.65	0.651
6	0.53	0.91	0.51	0.90

RSD: Relative Standard Deviation.

Table 4: Evaluation of Accuracy Based on Percent Recovery of Azilsartan.

Active content (µg/mL)	Level of added azilsartan (%)	Recovery (%)	RSD (%)
2	50	99	1.53
2	100	101	0.5
2	150	100	1.8

RSD: Relative Standard Deviation.

Table 5: Robustness and Ruggedness Evaluation of the Method for Azilsartan.

Parameter	Changes made	Retention time	%RSD	TF±SD	%RSD	Plate count±SD	RSD
Composition of Mobile Phase [Meoh: Phosphate Buffer (0.1% OPA)].	70:30	6.104±0.01	0.25	1.67±0.01	1.19	5079±81	1.60
	72:28	6.13±0.04	0.80	1.40±0.01	0.70	4089±8.62	0.21
	68:32	6.77±0.11	1.62	1.47±0.01	1.03	4177±18.14	0.43
Flow Rate	1 mL/min	6.104±0.01	0.25	1.67± 0.01	1.19	5079±81	1.60
	1.2 mL/min	6±0.09	1.59	1.63±0.02	1.54	3853±31.22	0.81
	0.8 mL/min	6.56±0.10	1.55	1.460±02	1.42	4480±10.06	0.22
Wavelength.	249 nm	6.104±0.01	0.25	1.67±0.01	1.19	5079±81	1.60
	251 nm	6.27±0.06	1.0	1.40±0.01	0.70	4177±18.14	0.43
	247 nm	6.112±0.06	0.10	1.47±0.01	1.10	3853±31.22	0.90

SD: Standard Deviation, RSD: Relative Standard Deviation, TF: Tailing Factor.

value obtained demonstrated narrow homogenous particle size distribution.

Estimation of Azilsartan in Various Formulations Using the Developed Method

The peak for azilsartan was identified in all formulations, as illustrated in Figure 5. The established approach was used to assess the amounts of azilsartan in several commercially available products, including azilsartan medoxomil tablets (azilsartan) and azilsartan solid lipid nanoparticles.

Comparison with Previously Published HPLC Procedures

According to previously published methods, the currently established HPLC method was used to do a comparative evaluation of mobile phase ratios, flow rate, wavelength and stability studies. When compared to previously published literature, the current method, which employs the mobile phase composition Methanol: 0.1% OPA (70:30 v/v), a flow rate of 1 mL/min, and a detection wavelength of 249 nm, is determined to be the most sensitive, inexpensive, and stable. The comparative data is displayed in (Table 7).

Table 6: Results of Forced Degradation for Azilsartan

Degradation of stress study	% Drugs degraded
Acidic (1 N HCl)	9.69±1.65
Basic (1 N NaOH)	19.16±1.08
Thermal	3.29±1.24
Oxidative	5.59±1.71
Photolytic	32.92±1.24

Table 7: Comparison between Previously Published Methods.

Sl. No.	Mobile Phase and Flow Rate	Wavelength	Column	Limitations	Application	References
1	Buffer (pH 3): Acetonitrile 55:45 Flow Rate-1.5 mL/min.	248 nm	C_{18}	The lowest limit of detection was unknown.	To estimate azilsartan from the solid dosage form	26
2	To estimate azilsartan from the solid dosage form	254 nm	C_{18}	The samples may not stable for a longer period.	The method used for the bioavailability and bioequivalence study.	4
3	Methanol: OPA Buffer (85:15) Flow Rate-1 mL/min	249 nm	C_{18}	The method was less sensitive.	More suitable for studying the stability of azilsartan medoxomil in bulk and the formulation.	13
Azilsartan and other drugs						
4	Triethylamine buffer: acetonitrile (40:60). Flow Rate-1 mL/min.	249 nm	C_{18}	Method was expensive.	Simultaneously estimate Azilsartan and clinidipine.	27
5	Simultaneously estimate Azilsartan and clinidipine.	260 nm	C_{18}	260 nm C18 230 nm ODS Column	The method is more suitable for the simultaneous estimation of drugs from the solid dosage form.	11
6	0.1%OPA: Acetonitrile (30:70) Flow Rate-1 mL/min.	230 nm	ODS Column	Lack of degradation study and less sensitive.	Azilsartan and chlorthalidone dose assessment simultaneously in a pharmaceutical dosage form.	12

CONCLUSION

An HPLC method was developed to selectively quantify azilsartan in developed solid lipid nanoparticles. The proposed approach was validated for numerous parameters that were within acceptable limits according to ICH guidelines. It was discovered to be simple, rapid, more sensitive, and effective. The developed approach was accurate and cost-effective. The developed approach demonstrated great accuracy, precision, and linearity with low LOD and LOQ values. It also has well-defined peaks and accurate drug measurement in solid lipid nanoparticles without excipient contamination. Azilsartan was discovered to be stable in thermal, acidic, and oxidative conditions. As a result, the established HPLC method is useful for routine azilsartan measurement in any formulation.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

AZN: Azilsartan; **ICH:** International Conference on Harmonization; **HPLC:** High-Performance Liquid Chromatography; **µg:** Microgram; **RSD:** Relative Standard Deviation; **nm:** Nanometre; **DMSO:** Dimethyl Sulfoxide; **DMF:** Dimethylformamide; **THF:** Tetrahydrofuran; **FDA:** Food and Drug Administrator; **H_2O:** Water; **BP:** Blood Pressure; **AII:** Angiotensin type II; **OPA:** Orthophosphoric Acid; **ACN:** Acetonitrile; **SLNs:** Solid Lipid Nanoparticles; **GMS:** Glyceryl Monostearate; **PDA:** Photodiode Array; **µL:** Microliter; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **H_2O_2:** Hydrogen Peroxide; **PDI:** Polydisperse Index; **ZP:** Zeta Potential; **SD:** Standard Deviation.

SUMMARY

Azilsartan is a class of angiotensin II receptor blockers used in the treatment of hypertension. To estimate the azilsartan from bulk drug and other formulations a simple, sharp and rapid HPLC method was developed. The proposed method shows linearity throughout a concentration range of 0.2-10 µg/mL and the method was precise (%RSD 2.0%), accurate (percent recovery 99-101%), and reliable. The detection and quantification limits for azilsartan were found to be 0.01 µg/mL and 0.04 µg/mL, respectively. In the degradation study, azilsartan was found to be stable in thermal,

acidic, and oxidative conditions. The proposed method can be used to quantify azilsartan in various formulations.

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