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Sustainable And Economical in-Vitro Bio Analytical Method Development and Validation of Azelnidipine in Rabbit Plasma Using RP-UPLC Follows ICH Guidelines M_{10}

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ABSTRACT
A simple, Accurate, precise method was developed for the simultaneous estimation of Azelnidipine in Rabbit plasma was developed and validated. By using Centrifugation technique, the sample preparation was prepared. Chromatogram run through Std ACQUITY UPLC HSS C18 Column, 1.8 µm, and 2.1 mm X 50 mm, Mobile phase containing Acetonitrile: Buffer Potassium dihydrogen Phosphate taken in the ratio 70:30 v/v was pumped through column at a flow rate of 0.3 ml/min. KH_2PO_4.in this method was buffer. For the separation of Azelnidipine Internal Standard [IS] used is Nicardipine. The Temperature was maintained at 30°C. Optimized wavelength selected was 257 nm. Retention time of Azelnidipine and Internal Standard were found to be 0.972 min and 0.603 min. The standard curve was linear (R2 >0.999) over the concentration range of 2 – 80 ng/ml. All the analytical validation parameters were determined as per ICH guidelines the bioanalytical method developed approach was selective, robust, and reliable, as accuracy, precision, recovery, and other validation parameters were all within the recommendations' limitations. The peaks produced for the drug of interest and the internal standard were well separated from one another without any plasma interferences, and the peaks were symmetrical with an adequate tailing factor. The method has the potential to be very beneficial in therapeutic drug monitoring (TDM), bioequivalence research, pharmacokinetics studies, toxicology, and biomedical investigations.
Key Words: *Azelnidipine, Internal Standard, Nicardipine, RP - UPLC, Bioanalysis, Rabbit Plasma.*

INTRODUCTION:

Bioanalytical techniques, employed for the quantitative determination of drugs and their metabolites in biological fluids and creates a specific procedure to enable a coalesce of interest to be identified and at the same time to be quantified in a matrix. A coalesce is measured by several procedures. The choice of analytical procedures involve many considerations, such as: concentration levels, chemical properties of the analyte, specimen matrix, cost of the analysis, experimental speed, quantitative or qualitative measurement, required precision and necessary equipment[2]. Bioanalytical method validation comprises all criteria determining data quality, such as selectivity, accuracy, precision, recovery, sensitivity, and stability.

Drug Analysis In Various Biological Media

Blood, urine, and faeces are the most commonly acquired samples for biopharmaceutical analysis, especially if the drug or metabolite is poorly absorbed or substantially eliminated in the bile. Saliva, breath, and tissue are examples of other media that can be used. The nature of the investigation heavily influences the selection of sampling media. In a clinical pharmacokinetic investigation, for example, medication levels necessitate the use of blood, urine, and saliva. A bioavailability study may necessitate drug level data in blood and/or urine, but a drug identification or drug addiction concern may only necessitate one type of biological sample.

The nature of the drug investigation heavily influences the selection of sample media. In a clinical pharmacokinetic study, for example, medication levels necessitate the use of blood, urine, and perhaps saliva. A bioavailability research may necessitate medication level measurements in blood or urine. The steps involved in estimating medicines in biological fluid are sample collection, sample treatment, separation of the compound of interest from the matrix, and analysis.

Bioanalysis can determine the therapeutic efficacy of a specific medicine. Bioanalysis is important in the pharmaceutical industry. The following steps are involved in bioanalysis.

- Biological fluid selection and collection

- Sample preparation -Analyte extraction from biological matrix.
- Analyte detection is accomplished through a variety of approaches.

UPLC systems are designed to operate at high pressures, utilizing smaller particles to enhance separation efficiency and reduce analysis time compared to traditional methods.

This advancement in UPLC technology allows for significant improvements in speed, resolution, and sensitivity, making it a valuable tool in various analytical applications.

Furthermore, UPLC's ability to maintain or even improve separation efficiency under high pressures further solidifies its role in modern analytical chemistry. The adoption of UPLC has revolutionized quality control processes in the pharmaceutical industry, enabling faster and more efficient analyses of complex formulations. This transformation has allowed for the seamless transition of quality control analyses from HPLC to UPLC systems, enhancing overall productivity and accuracy in pharmaceutical development. The continuous advancements in UPLC technology underscore its importance in meeting the growing demands of pharmaceutical analysis and development.

The recent advancements in UPLC technology have significantly contributed to the development of bioanalytical methods, allowing for faster and more accurate drug analysis in pharmaceutical research.

These advancements not only enhance the efficiency of drug analysis but also ensure that the methods meet the increasing demands for precision and reliability in pharmaceutical research. As a result, UPLC is becoming indispensable in the bioanalytical landscape, facilitating the rapid assessment of drug formulations and their effects.

Moreover, the transition from traditional HPLC to UPLC systems exemplifies the ongoing evolution of quality control methods in the pharmaceutical industry, addressing the need for enhanced analytical capabilities.

The integration of UPLC technology in pharmaceutical quality control not only enhances efficiency but also paves the way for the adoption of innovative analytical techniques that are crucial for modern drug development. For instance, the implementation of multi-attribute methods (MAM) and rapid microbial testing within UPLC frameworks exemplifies how advanced methodologies can further streamline the analysis of complex formulations while ensuring compliance with regulatory standards. Additionally, as the industry increasingly shifts towards personalized medicine, the ability of UPLC to provide rapid and precise analytical results will be vital in tailoring therapies to individual patient needs. This evolution signifies a broader trend in pharmaceutical analysis, where the convergence of advanced technologies not only improves operational efficiencies but also contributes to enhanced patient safety and drug efficacy, thereby solidifying UPLC's role as a cornerstone in contemporary pharmaceutical practices.

As UPLC continues to evolve, its integration with other analytical techniques is becoming increasingly prevalent, further enhancing its utility in pharmaceutical analysis. The combination of UPLC with mass spectrometry (MS), for instance, allows for the comprehensive characterization of complex drug formulations, providing insights into both qualitative and quantitative aspects of the compounds being analyzed. This hyphenation not only improves sensitivity and resolution but also facilitates the identification of metabolites and impurities, which is critical for drug safety assessments and regulatory compliance. Additionally, the adaptability of UPLC systems to accommodate various solvent compositions and gradients enhances their versatility across different applications, from environmental monitoring to food safety analysis, thereby broadening the scope of UPLC's impact in diverse scientific fields. As a result, the ongoing refinement of UPLC methodologies promises to further streamline analytical workflows, ultimately contributing to more efficient and effective drug development processes.

Azelnidipine is a dihydropyridine calcium channel blocker. It has a gradual onset of action and produces a long-lasting decrease in blood pressure, with only a small increase in heart rate, unlike some other calcium channel blockers. chemically called as 3-[1-(diphenylmethyl)azetidin-3-yl] 5-propan-2-yl 2-amino-6-methyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate.

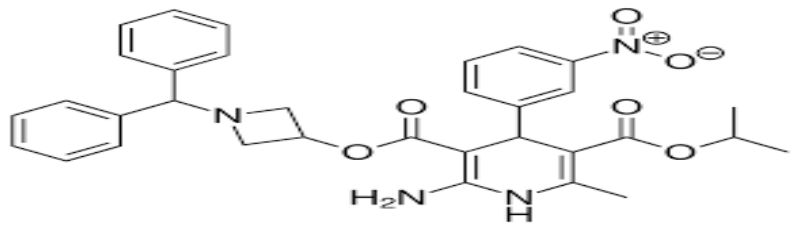

Figure1: Chemical Structure of Azelndipine

Experimental Work:

Materials:

Azelnidipine API and Internal Standard was obtained as a gift sample, From Akrivis Pharma pvt Ltd. K2 EDTA control plasm From Deccan Pathological labs, Hyderabad and Chemicals and Reagents Distilled water, Acetonitrile, Phosphate buffer, Methanol , Sodium dihydrogen phosphate, Ortho-phosphoric acid of Analytical Grade were used in the Project.

METHODOLOGY:

METHOD DEVELOPMENT

Based on drug solubility and P^{ka} Value following conditions has been used to develop the method estimation of Azelnidipine as per current ICH guidelines.

Optimization of the chromatographic conditions

For developing the method for the assay of Azelnidipine, a systematic study of the effect of various factors was undertaken by varying one parameter at a time and keeping all the other conditions constant. The following studies were conducted for this purpose. A high purity advance C18 column was chosen as the stationary phase for this study. The mobile phase and the flow rate in order to get sharp peaks and base line separation of the components, the author has carried out a number of experiments by varying the commonly used solvents, their compositions and flow rate. To effect ideal separation of the drug under isocratic conditions, mixtures of commonly used solvents like water, methanol and acetonitrile with or without buffers in different combinations were tested as mobile phases on a C18 stationary phase. A binary mixture of acetonitrile and KH_2PO_4 buffer in a ratio of 70:30 v/v was proved the most suitable of all the combinations since the chromatographic peaks obtained were well defined, resolved, and free from tailing. A mobile phase flow rate of 0.3 mL/min found to be suitable. The drug molecule was tuned on the UPLC for the detection of Azelnidipine and by injecting 2 ng/mL and 80 ng/ml concentration respectively. All the optimized system suitability parameters within the limits result.

Optimized method Chromatographic conditions

Table 1: Optimized method Chromatographic conditions

Mobile phase	Acetonitrile: KH_2PO_4 (70:30 v/v)
Flow rate	0.3 ml/min
Column	HSS C18 (2.1 x 50mm, 1.8µm)
Wave length	257 nm
Column temperature	30°C
Injection volume	0.50µL
Run time	2.0 min
Buffer	KH_2PO_4

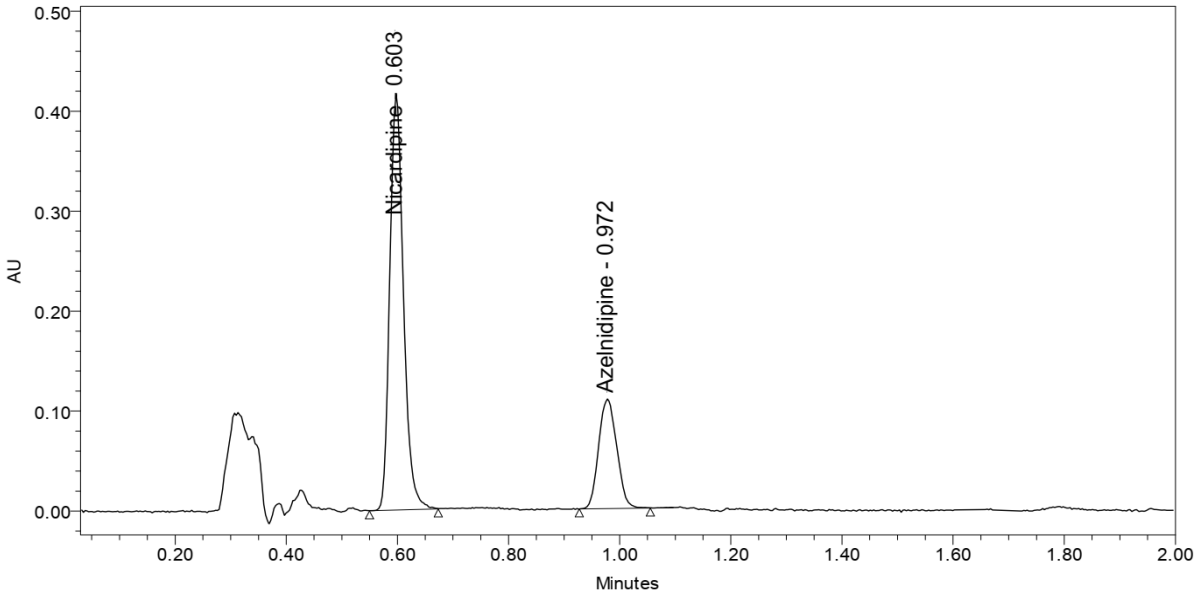

Figure 2 : Chromatogram of Optimized

Table 2 : Observation of Optimized Chromatogram

	Peak Name	RT	Area	USP Plate Count	USP Resolution	USP Tailing
1	Nicardipine	0.603	723338	2875.8		1.1
2	Azelnidipine	0.972	745587	3109.8	3.5	1.2

Observation: Azelnidipine and Internal Standard were eluted at 0.972 min, 0.603 min respectively with good resolution. Plate count and tailing factor was very satisfactory, so this method was optimized and to be validated. Drugs were eluted with good retention time, resolution; all the system suitable parameters like Plate count and Tailing factor were within the limits

METHOD VALIDATION

1) System suitability of Azelnidipine

This system suitability method intended to guarantee that the UPLC system is working in such a way that correct and reproducible data may be submitted to regulatory agencies with confidence. This procedure includes signal stability, carryover, and instrument response tests.

Table 3 : System Suitability of Azelnidipine

System Suitability						
Analyte	Azelnidipine	ISTD	Nicardipine			
Sample Name		Analyte Area	Analyte RT (min)	ISTD Area	ISTD RT (min)	Area Ratio
AQ MQC		572262	0.600	1822152	0.973	0.3141
AQ MQC		572813	0.601	1821906	0.977	0.3144
AQ MQC		573647	0.601	1826578	0.979	0.3141
AQ MQC		572309	0.602	1821121	0.981	0.3143
AQ MQC		572650	0.602	1829529	0.982	0.3130
AQ MQC		571412	0.603	1828777	0.982	0.3125
MEAN			**0.979**		**0.602**	**0.31371**
SD			**0.0035**		**0.0010**	**0.000787**
%CV			**0.36**		**0.17**	**0.25**

Discussion: plate count, tailing factor, resolution of Azelnidipine was According to ICH guidelines plate count should be more than 2000, tailing factor should be less than 2 and resolution must be more than 2. All the system suitable parameters were passed and were within the limits. The % CV of the retention time (RT) should be ≤ 2.00 %.

1) Auto sampler carryover of Azelnidipine

The carryover was tracked back to the injection valve and eradicated by converting from a partial loop injection to a full loop injection, which allowed more effective cleansing of the sample flow channel. The UPLC system's susceptibility to carryover was shown to be dependent on the detection method's absolute sensitivity and the mass of Analyte injected at the assay's lower limit of quantitation (LLOQ).

Table 4: Auto sampler carryover of Azelnidipine

Auto sampler Carryover				
Analyte	Azelnidipine		**ISTD**	Nicardipine
Sample ID	Peak Area		% Carryover	
	Drug	ISTD	Drug	ISTD
Unextracted samples				
RS	0	0	N/A	N/A
AQ ULOQ	1153189	1154519	**0.00**	**0.00**
RS	0	0		
AQ LLOQ	28648	28651	N/A	N/A
Extracted samples				
STD Blk	0	0	N/A	N/A
ULOQ	1142789	1145659	**0.00**	**0.00**
STD Blk	0	0		
LLOQ	28144	27989	N/A	N/A

Discussion: - The area obtained is less than 20 % of extracted LLOQ standard area to un-extracted area by injected of replicate manner.

Specificity and Screening of Biological Matrix

Specificity is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present

Table 5: Specificity and Screening of Biological Matrix of Azelnidipine

Specificity and Screening of Biological Matrix						
Analyte	Azelnidipine			**ISTD**	Nicardipine	
S.No.	**Sample ID**	**Response**		**% Interference**		**Pass/Fail**
		Drug	**ISTD**	**Drug**	**ISTD**	
1	STD Blk1	0	0	0.00	0.00	Pass
2	LLOQ1	28546	1859837			
3	STD Blk2	0	0	0.00	0.00	Pass
4	LLOQ2	28982	1850983			
5	STD Blk3	0	0	0.00	0.00	Pass
6	LLOQ3	28297	1859863			
7	STD Blk4	0	0	0.00	0.00	Pass
8	LLOQ4	28739	1854563			
9	STD Blk5	0	0	0.00	0.00	Pass
10	LLOQ5	28328	1857647			
11	STD Blk6	0	0	0.00	0.00	Pass
12	LLOQ6	28982	1857651			

Observation: We did not find and interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

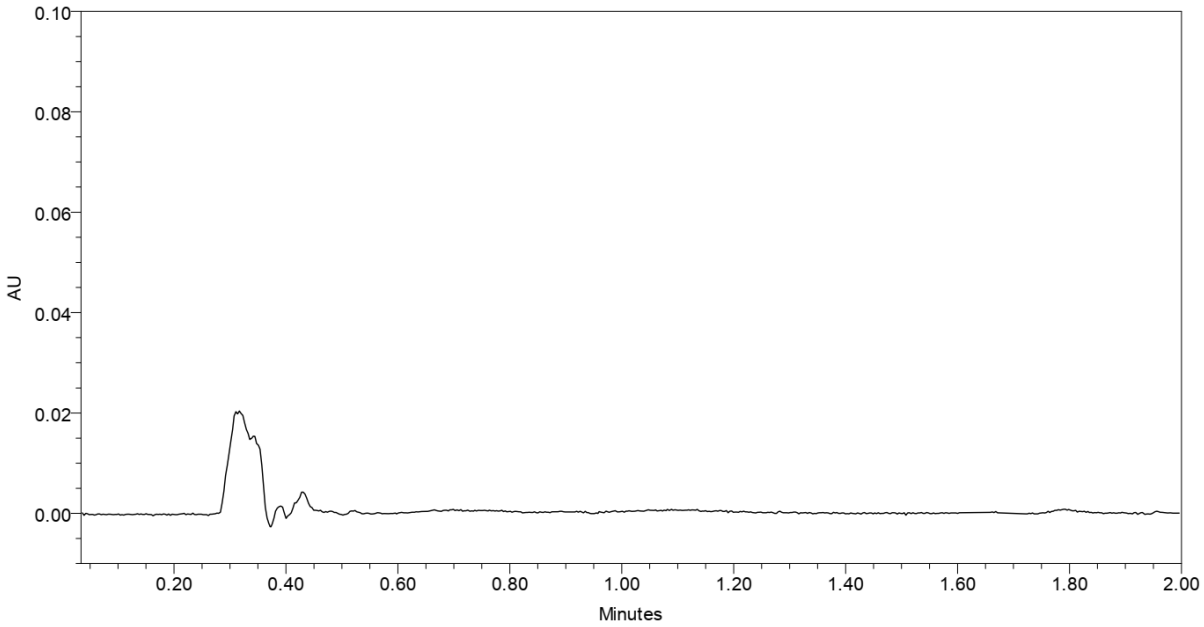

Figure 3 Representative Chromatogram of a Blank Plasma Sample

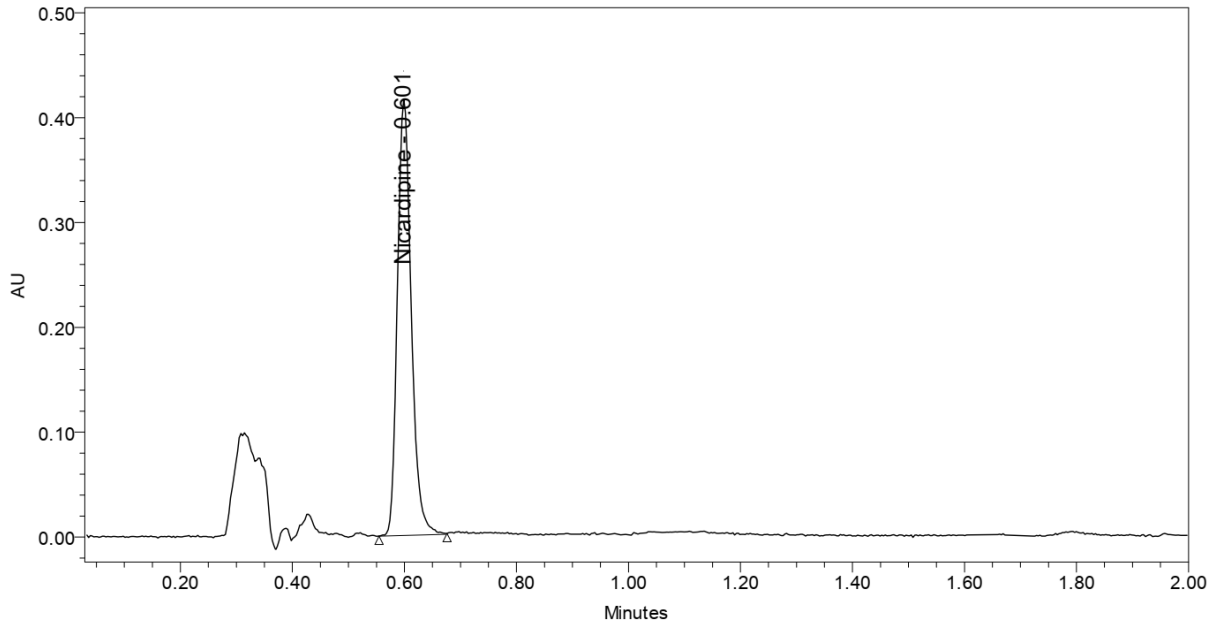

Figure 4 Representative Chromatogram of Blank Plasma with Internal Standard Sample

Discussion – The response areas obtained of analyte and internal standard are less than 20% and 5 % of LLoq Area. We did not find and interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific

Sensitivity

A sensitivity is defined as "the lowest analyte concentration that can be measured with acceptable accuracy and precision i.e., LLOQ

Table 6 : Sensitivity of Azelnidipine

Sensitivity			
Analyte	Azelnidipine	**ISTD**	Nicardipine
S No.			**LLOQ**
			Nominal Concentration (ng/mL)
			2.000
			Nominal Concentration Range (ng/mL)
			(1.600-2.400)

	Calculated Concentration (ng/mL)
1	1.954
2	2.039
3	1.989
4	2.074
5	1.926
6	1.944
n	6
Mean	1.9877
SD	0.05820
% CV	**2.93**
% Mean Accuracy	**99.38**

Discussion: The LLOQ concentration was found between 80 -120 % and % Coefficient of variation found to be 2.93% and mean of 6 injections was found to be 99.38% % within the acceptance limits. As the limit of Sensitivity % CV was less than "20%" the system Sensitivity was passed in this method.

1) Matrix factor evalution

Table 7 : Matrix factor evalution (absence of matrix factor)

Matrix Effect			
Analyte	Azelnidipine	**ISTD**	Nicardipine
S. No.	**Plasma Lot No.**	**HQC**	**LQC**
		Nominal Concentration (ng/mL)	
		64.000	6.000
		Nominal Concentration Range (ng/mL)	
		(54.400-73.600)	(5.100-6.900)
		Calculated Concentration (ng/mL)	
1	LOT1	65.092	**6.072**
		64.084	**6.006**
		64.490	**6.106**
2	LOT2	64.287	**6.014**
		64.020	**6.094**
		64.258	**6.018**
3	LOT3	63.953	**5.931**
		64.435	**6.010**
		64.254	**6.000**
4	LOT4	64.454	**5.815**
		63.539	**6.285**
		64.124	**6.129**
5	LOT5	64.191	**6.004**
		64.425	**5.926**
		65.014	**6.062**
6	LOT6	64.738	**6.021**
		64.369	**5.909**
		64.761	**6.001**
n		18	18
Mean		64.3604	6.0224
SD		0.37928	0.10053
% CV		**0.59**	**1.67**
% Mean Accuracy		**100.56**	**100.37**
No. of QC Failed		**0**	**0**

Discussion- The Evaluation of Matrix by injecting the QC samples of high and low concentrations in 6 lots the %Mean obtained was 100.56% and 100.37% of HQC and LOQ and % CV obtained are 0.59% and 1.67% of HQC and LOQ. As the limit of CV was less than "20%" the system Matrix was passed in this method.

Linearity:

Table 8: Linearity of Azelnidipine

Linearity								
Analyte	Azelnidipine						**ISTD**	Nicardipine
	STD1	**STD2**	**STD3**	**STD4**	**STD5**	**STD6**	**STD7**	**STD8**
	Nominal Concentration (ng/mL)							
	2.000	4.000	6.000	16.000	40.000	48.000	64.000	80.000
	Nominal Concentration Range (ng/mL)							
	(1.600-2.400)	(3.400-4.600)	(5.100-6.900)	(13.600-18.400)	(34.000-46.000)	(40.800-55.200)	(54.400-73.600)	(68.000-92.000)
	Back Calculated Concentration (ng/mL)							
	1.923	3.956	6.020	15.862	40.650	47.985	64.246	79.860
	1.996	3.865	6.032	16.058	39.620	47.956	64.654	80.860
	2.056	4.110	5.856	15.650	39.300	48.220	64.000	80.741
n	3	3	3	3	3	3	3	3
Mean	1.9917	3.9770	5.9693	15.8567	39.8567	48.0537	64.3000	80.4870
SD	0.06661	0.12384	0.09833	0.20405	0.70543	0.14478	0.33033	0.54625
%CV	**3.34**	**3.11**	**1.65**	**1.29**	**1.77**	**0.30**	**0.51**	**0.68**
% Mean Accuracy	**99.58**	**99.43**	**99.49**	**99.10**	**99.64**	**100.11**	**100.47**	**100.61**

Final Conc in ng/ml	**ISD(area)**	**Drug(area)**	**Area response ratio**
0	0	0	0
2	1832398	27295	0.015
4	1832152	57323	0.031
6	1836824	85642	0.047
16	1831366	233611	0.128
40	1839775	579526	0.315
48	1839023	686302	0.373
64	1834839	917706	0.500
80	1836826	1136201	0.619

Discussion: regression co-efficient value were

Parameter	**Azelnidipine**
Conc range (µg/mL)	2 – 80 ng/ml
Regression Equation	y = 0.0078x + 0.0012
Co-relation	0.9999

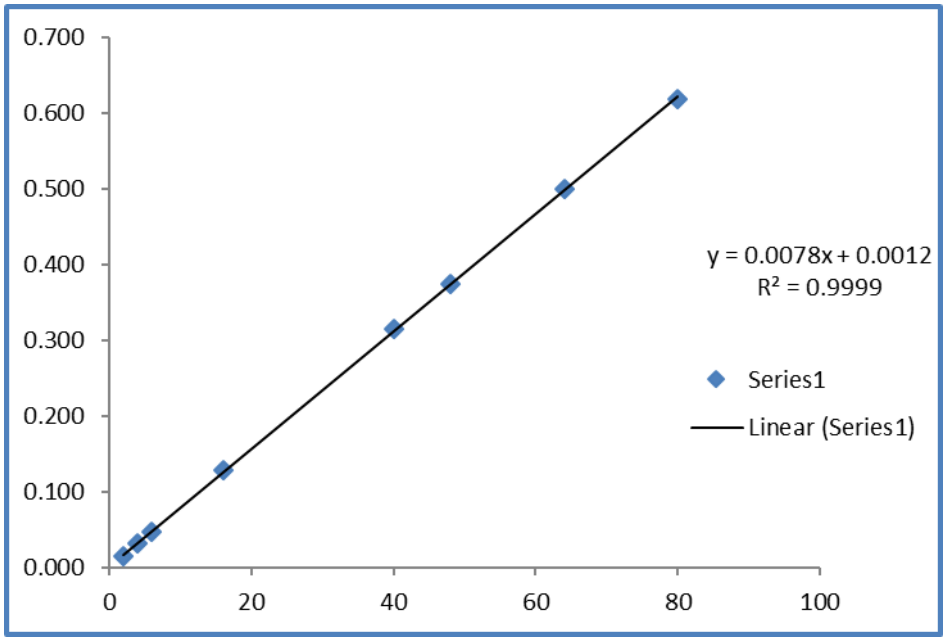

Figure 5 Representative Calibration Curve for Regression Analysis

Discussion :- Calibration was found to be linear over the concentration range of 2 to 80 ng /ml. The coefficient correlation (r^2) value was found consistently greater than 0.999 in all the cases. This indicating linearity of results and an excellent correlation between peak area ratios for each concentration of analytes.

Precision and accuracy (intra-day runs of Azelnidipine)

Table 9 : precision data for intra-day runs of Azelnidipine

Precision and Accuracy				
Analyte	Azelnidipine		Nicardipine	
	HQC	**MQC1**	**LQC**	**LLOQ QC**
	Nominal Concentration (ng/mL)			
	64.000	**40.000**	**6.000**	**2.000**
	Nominal Concentration Range (ng/mL)			
	(54.400-73.600)	(34.000-46.000)	(5.100-6.900)	(1.600-2.400)
	Back Calculated Concentration (ng/mL)			
	64.256	39.965	6.256	1.956
	64.020	39.954	6.259	2.065
	64.152	40.230	6.185	1.956
	64.025	39.658	6.153	2.156
	63.652	39.856	5.987	1.985
	63.562	39.652	5.320	1.952
n	**6**	**6**	**6**	**6**
Mean	**63.9445**	**39.8858**	**6.0266**	**2.0117**
SD	**0.27718**	**0.21764**	**0.36010**	**0.08264**
%CV	**0.43**	**0.55**	**5.98**	**4.11**

% Mean Accuracy	**99.91**	**99.71**	**100.44**	**100.58**
	64.250	40.020	6.156	1.958
	64.580	39.650	6.075	2.065
	64.650	39.652	5.856	1.865
	65.658	39.645	6.058	2.156
	63.650	39.352	5.956	2.052
	63.360	39.562	5.963	1.965
n	**6**	**6**	**6**	**6**
Mean	**64.3580**	**39.6468**	**6.0107**	**2.0102**
SD	**0.81651**	**0.21606**	**0.10647**	**0.10184**
%CV	**1.27**	**0.54**	**1.77**	**5.07**
% Mean Accuracy	**100.56**	**99.12**	**100.18**	**100.51**
	64.225	40.560	6.756	2.052
	64.632	40.456	5.675	1.965
	64.230	39.650	5.653	2.052
	64.620	39.350	6.036	2.084
	65.185	39.456	5.987	1.985
	65.100	39.456	5.698	1.856
n	**6**	**6**	**6**	**6**
Mean	**64.6653**	**39.8213**	**5.9675**	**1.9990**
SD	**0.41123**	**0.54164**	**0.42045**	**0.08324**
%CV	**0.64**	**1.36**	**7.05**	**4.16**
% Mean Accuracy	**101.04**	**99.55**	**99.46**	**99.95**
Between Batch Precision and Accuracy				
n	**18**	**18**	**18**	**18**
Mean	**64.3226**	**39.7847**	**6.0016**	**2.0069**
SD	**0.60065**	**0.35319**	**0.30680**	**0.08444**
%CV	**0.93**	**0.89**	**5.11**	**4.21**
% Mean Accuracy	**100.50**	**99.46**	**100.03**	**100.35**

Rugged Precision and Accuracy (inter-day runs of Azelnidipine)

Table 10: precision data for inter-day runs of Azelnidipine

Ruggedness Precision and Accuracy				
Analyte	Azelnidipine		**ISTD**	Nicardipine
	HQC	**MQC1**	**LQC**	**LLOQ QC**
	Nominal Concentration (ng/mL)			
	64.000	**40.000**	**6.000**	**2.000**
	Nominal Concentration Range (ng/mL)			
	(54.400-73.600)	(34.000-46.000)	(5.100-6.900)	(1.600-2.400)

	Calculated Concentration (ng/mL)			
Different Column	64.650	40.650	6.875	2.056
	64.658	39.568	5.786	2.065
	63.658	39.658	5.965	1.965
	64.658	39.856	5.856	2.116
	64.658	39.685	5.865	1.965
	65.230	40.356	5.875	1.956
n	**6**	**6**	**6**	**6**
Mean	**64.5853**	**39.9622**	**6.0370**	**2.0205**
SD	**0.50903**	**0.43913**	**0.41449**	**0.06735**
% CV	**0.79**	**1.10**	**6.87**	**3.33**
% Mean Accuracy	**100.91**	**99.91**	**100.62**	**101.03**
Different Analyst	64.690	39.657	6.050	1.756
	65.650	39.350	5.698	1.985
	64.650	39.652	5.980	2.163
	65.520	40.658	6.126	2.065
	65.320	40.250	5.956	2.095
	64.520	39.652	5.985	1.965
n	**6**	**6**	**6**	**6**
Mean	**65.0583**	**39.8698**	**5.9658**	**2.0048**
SD	**0.49475**	**0.48501**	**0.14501**	**0.14189**
% CV	**0.76**	**1.22**	**2.43**	**7.08**
% Mean Accuracy	**101.65**	**99.67**	**99.43**	**100.24**

Discussion: The intraday and inter day accuracy and precision was assessed by analysing six replicates at five different QC levels like LLOQ, LQC, MQC and HQC. Accuracy and precision method performance was evaluated by determined by six replicate analyses for Azelnidipine at four concentration levels, i.e., 2µg/ml(LLOQ), 6µg/ml (LQC), 40µg/ml (MQC) and 64µg/ml HQC.

The intra-day and inter day accuracy of plasma samples were assessed and excellent mean % accuracy was obtained with range varied from 99.46-100.03%, and 100.35% - 100.50% for intraday and 99.43% - 99.67% and 100.24% - 101.65% for inter day respectively. The precision (%CV) of the analytes and plasma samples were calculated and found to be 0.89%- 0.93% and 4.21% - 5.11% for intraday and 0.76% -1.22% and 2.43% - 7.08% for inter day respectively.

Recovery of Azelnidipine-

Table 11: Recovery of Azelnidipine

Recovery – Analyte						
Analyte	Azelnidipine					**ISTD**
S No.	**HQC**		**MQC1**		**LQC**	
	Unextracted Response	Extracted Response	Unextracted Response	Extracted Response	Unextracted Response	Extracted Response
1	929387	916384	578124	572167	85568	84546
2	929734	916573	577653	566724	85454	84658
3	928367	909970	577643	565409	86673	84487
4	919836	908884	588726	574986	86879	84376
5	918823	907445	585672	571934	85984	85339
6	929378	908263	579384	577634	85837	85497
n	6	6	6	6	6	6
Mean	925921	911253	581200	571476	86066	84817
SD	5136.04	4131.07	4788.16	4697.20	585.00	476.86
% CV	**0.55**	**0.45**	**0.82**	**0.82**	**0.68**	**0.56**
% Mean Recovery	**98.42**		**98.33**		**98.55**	

Overall % Mean Recovery	**98.431**
Overall SD	**0.1119**
Overall % CV	**0.11**

Recovery - Internal standard

Table 12 : Recovery of Nicardipine (IS)

Recovery - Internal standard		
Analyte	**ISTD**	Nicardipine
S.No.	Un extracted Area Ratio	Extracted Area Ratio
1	1893526	1822152
2	1829837	1821906
3	1829386	1826578
4	1825463	1821121
5	1830928	1829529
6	1830234	1828777
n	6	6
Mean	**1839895.7**	**1825010.5**
SD	**26343.54**	**3741.59**
% **CV**	**1.43**	**0.21**
% **Mean Recovery**	**99.19**	

Discussion: Recovery was determined by measuring the peak areas obtained from prepared plasma samples with those extracted blank plasma spiked with standards containing the same area with known amount of Azelnidipine . The overall % mean recovery for was found to be 99.19% at LQC, MQC and HQC levels and % CV ranged from 0.65% for IS, The results demonstrated that the bioanalytical method had good extraction efficiency. The results demonstrated that the bioanalytical method had good extraction efficiency.

Rugged Linearity:

Table 13 : Rugged Linearity of Azelnidipine

Ruggedness Linearity							
Analyte	Azelnidipine				**ISTD**	Nicardipine	
STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8
Nominal Concentration (ng/mL)							
2.000	4.000	6.000	16.000	40.000	48.000	64.000	80.000
Nominal Concentration Range (ng/mL)							
(1.600-2.400)	(3.400-4.600)	(5.100-6.900)	(13.600-18.400)	(34.000-46.000)	(40.800-55.200)	(54.400-73.600)	(68.000-92.000)
Calculated Concentration (ng/mL)							
Different Column							
2.056	4.310	6.050	16.600	39.650	48.658	64.256	81.650
Different Analyst							

1.950	4.250	5.980	16.630	40.300	47.950	63.650	80.680

Discussion: Linearity ruggedness is a measure for the susceptibility of a method to small changes that might occur during routine analysis, The calibration range is obtained by injecting 6 concentrations (2 ng/ml - 80ng/ml) of calibration standards not including blank and zero samples and establishing, The calibration curves were appeared linear and the coefficient of correlation was found to be 0.999 for Azelnidipine.

Reinjection Reproducibility

Table 14: Reinjection Reproducibility of Azelnidipine

Reinjection Reproducibility				
Analyte	Azelnidipine		**ISTD**	Nicardipine
	HQC	**MQC1**	**LQC**	**LLOQ QC**
	Nominal Concentration (ng/mL)			
	64.000	40.000	6.000	2.000
	Nominal Concentration Range (ng/mL)			
	(54.400-73.600)	(34.000-46.000)	(5.100-6.900)	(1.600-2.400)
	Calculated Concentration (ng/mL)			
	64.950	39.850	5.856	1.956
	64.054	40.250	5.956	1.956
	64.120	39.650	6.156	2.000
	64.658	38.650	6.056	2.132
	64.856	39.654	6.162	1.956
	64.520	39.745	6.030	1.976
n	6	6	6	6
Mean	64.5263	39.6332	6.0360	1.9960
SD	0.37249	0.53054	0.11791	0.06887
% CV	**0.58**	**1.34**	**1.95**	**3.45**
% Mean Accuracy	**100.82**	**99.08**	**100.60**	**99.80**

Discussion: - The % mean accuracy for LQC, MQC and HQC samples was found to be 100.60%, 99.08%, 100.82% and % Cv was found to be 1.95, 1.34, 0.58 and LLOQ was found 99.80 and % Cv was found to be 3.45. The results demonstrated that the bioanalytical method had good extraction efficiency.

Stabilities

Long-term stock solution stability

Table 15: stability of Azelnidipine (zero days)

DAY ZERO ASSESSMENT BATCH		
Analyte	**ISTD**	Nicardipine
S No.	**HQC**	**LQC**
	Nominal Concentration (ng/mL)	
	64.000	6.000
	Nominal Concentration Range (ng/mL)	
	(54.400-73.600)	(5.100-6.900)
	Calculated Concentration (ng/mL)	
1	64.652	6.160
2	64.580	6.250
3	64.580	6.160
4	64.465	6.005

5	64.745	5.986
6	64.580	6.074
n	6	6
Mean	64.6003	6.1058
SD	0.09290	0.10217
% CV	**0.14**	**1.67**
% Mean Accuracy	**100.94**	**101.76**

Discussion- In bench-top stability, six replicates of LQC & HQC samples (6 and 64 ng/ml) were analyzed for 9 hours at room temperature on the laboratory bench. The % mean stability was calculated and found to 101.76% for LQC and 100.94% for HQC respectively.

Matrix samples stability at -28±5 °C for 37 days

Table 16: Matrix samples stability at -28±5 °C for 37 days

Long Term Analyte Stability in Matrix				
Analyte Name	Azelnidipine	**Temperature**	-28	±5 ^{0}C
S No.	**HQC**		**LQC**	
	Nominal Concentration (ng/mL)			
	64.000	64.000	6.000	6.000
	Nominal Concentration Range (ng/mL)			
	(54.400-73.600)	(54.400-73.600)	(5.100-6.900)	(5.100-6.900)
	Calculated Concentration (ng/mL)			
	Comparison Samples	Stability Samples	Comparison Samples	Stability Samples
1	64.950	64.650	6.560	6.650
2	64.750	64.658	6.026	5.985
3	64.650	64.680	6.150	6.170
4	64.658	64.320	6.258	5.965
5	65.654	64.620	5.260	5.156
6	64.658	64.035	6.056	5.956
n	6	6	6	6
Mean	64.8867	64.4938	6.0517	5.9803
SD	0.39296	0.26179	0.43314	0.48266
% CV	**0.61**	**0.41**	**7.16**	**8.07**
%Mean Accuracy	**101.39**	**100.77**	**100.86**	**99.67**
% Mean Stability	**99.39**		**98.82**	

Matrix samples stability at -80±5 °C for 37days

Table 17: Matrix samples stability at -80±5 °C for 37 days

Long Term Analyte Stability in Matrix				
Analyte Name	Azelnidipine	**Temperature**	-80	±5 ^{0}C
S No.	**HQC**		**LQC**	
	Nominal Concentration (ng/mL)			
	64.000	64.000	6.000	6.000
	Nominal Concentration Range (ng/mL)			
	(54.400-73.600)	(54.400-73.600)	(5.100-6.900)	(5.100-6.900)
	Calculated Concentration (ng/mL)			
	Comparison Samples	Stability Samples	Comparison Samples	Stability Samples
1	64.65	64.33	5.956	5.874
2	64.52	64.31	5.956	5.852
3	64.65	64.06	6.056	6.033

4	64.25	64.36	5.856	5.856
5	64.04	64.05	6.095	6.053
6	64.52	64.65	5.952	5.925
n	6	6	6	6
Mean	64.4385	64.2922	5.9785	5.9321
SD	0.24622	0.22450	0.08521	0.08981
% CV	**0.38**	**0.35**	**1.43**	**1.51**
%Mean Accuracy	**100.69**	**100.46**	**99.64**	**98.87**
% Mean Stability	**99.77**		**99.22**	

Discussion: -Long-term stock solution stability for the Azelnidipine was determined at a concentration of LQC-HQC level after a storage period of 37 days at -28 °C & -80 °C in refrigerator. The % mean stability of the Azelnidipine was found to be 99.39% 98.82% at 28 ± 5°C and 99.77%, 99.22% at 80 ± 5°C respectively. Long term stock solution stability for the was determined at a concentration of LQC-HQC level after a storage period of 37 days at -28°C& -80°C in refrigerator.

Summary and Conclusion

A simple, Accurate, precise method was developed for the estimation of Azelnidipine in Rabbit plasma by using RP-UPLC was developed and validated. A simple, Accurate, precise method was developed for the simultaneous estimation of Azelnidipine in Rabbit plasma was developed and validated. By using Centrifugation technique, the sample preparation was prepared. Chromatogram run through Std ACQUITY UPLC HSS C18 Column, 1.8 µm, and 2.1 mm X 50 mm, Mobile phase containing Acetonitrile: Buffer Potassium dihydrogen Phosphate taken in the ratio 70:30 v/v was pumped through column at a flow rate of 0.3 ml/min. KH_2PO_4.in this method was buffer. For the separation of Azelnidipine Internal Standard [IS] used is Nicardipine. The Temperature was maintained at 30°C. Optimized wavelength selected was 257 nm. Retention time of Azelnidipine and Internal Standard were found to be 0.972 min and 0.603 min. The standard curve was linear (R2 >0.999) over the concentration range of 2 – 80 ng/ml. All the analytical validation parameters were determined as per ICH guidelines the bioanalytical method developed approach was selective, robust, and reliable, as accuracy, precision, recovery, and other validation parameters were all within the recommendations' limitations. The peaks produced for the drug of interest and the internal standard were well separated from one another without any plasma interferences, and the peaks were symmetrical with an adequate tailing factor. The method has the potential to be very beneficial in therapeutic drug monitoring (TDM), bioequivalence research, pharmacokinetics studies, toxicology, and biomedical investigations.

REFERENCES:

1.Lalit v sonawane, bhagwat n poul, sharad v usnale, pradeepkumar v waghmare and laxman h surwase , Bioanalytical Method Validation and Its Pharmaceutical Application, Pharmaceutical Analytical Acta,2014 vol.5,pg no:1-7.
2.Sachin, L.Darkunde, Rupali,N. Borhade, Bioanalytical Method Validation: A Quality Assurance Auditor View Point asian journal of pharmaceutical technology and innovation.2017.Vol.5. pgno:59-60
3.Tijare lk, rangari nt, mahajanun, A review on bioanalytical method development and validation, asian journal of pharmaceutical clinical research.2016 vol.9.pgno:1-5
4.Kirthi R. Shanmugam. A review on bioanalytical method development and
validation by RP – HPLC. Journal of Global Trends in Pharmaceutical Sciences.
2014;5(4) : 2265 - 2271
5.Kirthi R. Shanmugam. A review on bioanalytical method development and validation by RP – HPLC. Journal of Global Trends in Pharmaceutical Sciences. 2014;5(4) : 2265 - 2271
6.Richard R. Burgess. Protein precipitation techniques. Methods in Enzymology.2009; 463:331-341
7.www.mic.ucla.edu>ms_pr>proteomics
8.Method development and validation skills and tricks .2019.pgno:3
9.Pushpa Latha E, and Sailaja B, Bioanalytical Method Development and Validation by journal of medical and pharmaceutical innovation.2015 vol.1.pgno:1-9
10. Kirthi1, R. Shanmugam, M. Shanti Prathyusha , D. Jamal Basha, a review on bioanalytical method development and validation by rp - Journal of Global Trends in Pharmaceutical Sciences.2014 vol.5.
11. Gurdeep R.Chatwal , Sham K .Anand, Instrumental Methods of Chemical Analysis , Pg 2.566-2.638 (2007)
12. Nasal.A, Siluk.D, and Kaliszan.R. Chromatographic Retention Parameters in Medicinal Chemistry and Pharmacology, Pubmed, Vol.10, Issue 5 Pg no-381-426, March (2003)
13. Ashok Kumar, Lalith Kishore, navpreet Kaur , Anroop Nair. Method Development and Validation for Pharmaceutical Analysis. International Pharmaceutica Sciencia, Vol 2, Issue 3, Jul-Sep (2012)

14. Kaushal.C, Srivatsava.B, A Process of Method Development: A Chromatographic Approach. J Chem Pharm Res, Vol.2, Issue 2, 519-545, (2010)
15. Green JM. A Practicle guide to analytical method validation, Anal Chem (1996) 305A-309A
16. ICH, Validation of analytical procedures: Text and Methodology. International Conference on Harmonization, IFPMA , Geneva , (1996)
17. IUPAC. Compendium of Chemical Terminology, 2nd edn. (The Gold Bo). PAC69, 1137 (1997). Glossary of terms used in computational drug design (IUPAC Recommendations.
18. K. D. Tripathi, Essentials of Medical Pharmacology, 6th Edition, Jaypee brother's medical publishers (P) LTD, p-254-255.
19. Indian Pharmacopoeia, Indian Pharmacopoeial Commission, Controller of Publication, Government of India, Ministry of health and Family Welfare, Ghaziabad, India, 2 (2010) 1657-1658.
20. British Pharmacopoeia, The British Pharmacopoeial Commission, the stationary office, UK, London, 1408-1409 2 (2011).
21. https://go.drugbank.com/drugs/DB09230
22. Agrawal R., & Kotadiya R. (2024). AQbD-guided stability indicating HPLC method for azelnidipine and chlorthalidone fixed-dose combination tablet: a green approach. Journal of Taibah University for Science, 18(1).
23. Yamuna P & G Sammaiah (2023)., Method development and validation for the estimation of azelnidipine in bulk form and marketed pharmaceutical dosage form by using rp-hplc, International Journal of Allied Medical Sciences and Clinical Research, Vol. 11 No. 2 (2023): Volume -11 - Issue 2.
24. Singh A, Rajput A, Kureshi G, Carpenter G, Vashi J. An RP-HPLC Method Performance and Validation for Azelnidipine Measurment and Metoprolol Succinate Within a Synthetic Mixture. Pharmacophore. 2023;14(3):1-6
25. R. S. Sakhare et al. (2023), RP-HPLC Method Development and Validation for Simultaneous Estimation of Azelnidipine and Telmisartan in Pharmaceutical Dosage form by Qbd Approach, IJPSR, 14(12).5760-70. 2023.
26. D. Basava Chaitanya, M. Ajitha et al. (2022), Stability Indicating RP-HPLC Method Development and Validation for simultaneous estimation of Azelnidipine and Telmisartan in Bulk and Pharmaceutical Dosage Form. World J Pharm Sci 2022; 10(01): 121-127.
27. Manisha Panda, Vasudha Dadi, Srinivas Rao Yarraguntla, Vara Prasad Rao K. RP-HPLC Method for Determination of Azelnidipine and Telmisartan in Pharmaceutical Dosage Form. Research Journal of Pharmacy and Technology 2023; 16(2):509-3.
28. Hitanshi Darji, Zarna Dedania (2023), Simultaneous estimation of Azelnidipine and Metoprolol succinate with greenness assessment using HPLC and UV-spectrophotometric methods, Green Analytical Chemistry, Volume 7, December 2023.
29. Dr. Sushil D. Patil et al. (2021), Development and Validation of Stability Indicating RP-HPLC Method for Azelnidipine for bulk drug, Nat. Volatiles & Essent. Oils, 2021; 8(5): 11151-11157
30. D. Prabhakar et al. (2017), Method Development and Validation of Azelnidipine by RP-HPLC, International Journal of ChemTech Research, International Journal of ChemTech Research, 2017,10(10): 418-423.
31. Jayvadan K Patel, Nilam K Patel (2014), Validated Stability-Indicating RP-HPLC Method for the Simultaneous Determination of Azelnidipine and Olmesartan in Their Combined Dosage Form, Sci Pharm. 2014 Feb 27;82(3):541–554.