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Simultaneous Evaluation of Anti-Hypertensive agents by Using Stability Indicating RP-HPLC Method in Bulk and Tablets

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Abstract
The present article focuses on development of sensitive, simple, precise accurate and in expensive stability indicating RP-HPLC method using binary solvents for the simultaneous estimation of Amlodipine and Atenolol contents In Depisal a tablet. the separation was done with C_{18} water column come with the sizes 150×4.6mm&5µm with Acetonitrile&Na_2HPO_4 (P^H5.2) merger in ratio 50;50(v/v), as a mobile phase with 1.0ml /min flow in isocratic type. The detection was done at 228 nm accompanied by quantification. The minutes of retention time for Amlodipine and Atenolol were 2.786 and 2.106, respectively. The Amlodipine 2.5-7.5 µg/ml and Atenolol 25-75 µg/ml were reported for linear responses. The method was precise and robust with LOD of ALD 0.025 µG/ml And ATN 0.098 µg/ml, respectively. The LOQ for ALD and ATN Were 0.082 µg/ml and 0.327µg/ml, respectively. Drugs were subjected to stress conditions in acidic, alkaline, peroxide of 30 % potency, photolytic thermal and hydrolytic. The method for this simultaneous estimation was found to be accurate, precise with precise values of ATN and ALD were 0.336% and 0.098% respectively Assay percentiles (accuracy) for ATN&ALD were 98.63 % and 99.55%, respectively.

Keywords
Amlodipine, Atenolol, Stability indicating method, HPLC.

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INTRODUCTION
Amlodipine (3-O–ethyl-5-methyl-2-(2-aminoethoxy methyl)–4-(2-chlorophenyl)–6-methyl-1,4-dihydro pyridine-3, 5-dicarboxylate with an empirical formula $C_{20}H_{25}ClN_2O_5$ Belong to the class of periphery arterial vasodilator works specifically on vascular smooth muscle to lower blood pressure by lowering systemic vascular tolerance. ALD prevents calcium ions from accessing vascular smoother muscle including heart muscle which is needed for the vascular smooth muscle contraction [1]. ALD actively prevents calcium ion influx through cell membranes, it has the solubility in ethanol, dimethylformamide and DMSO and sparingly soluble in buffers of aqueous, type water and excreted 10% in urine in unchanged form. it belongs to the class of Dihydropyridine type with the mass of 408.9.[2-3]
Atenolol (ATN)chemically known as 2-(4-(2-hydroxy-3-propan -2-yl-amino) propoxy) phenyl) acetamide with an empirical formula $C_{14}H_{22}N_2O_3$ is a β- blocker, mostly affecting the heart because of elevated norepinephrine output from the periphery nervous system. ATN serves as a sympathetic innervations instigator preventing fluctuations in heart rate, electrical permeability, and contractility throughout the heart. In the near run, the reductions in contractility and frequency results in a decline in cardiac activity which is compensated for by raise in periphery vascular resistance. It is soluble in methanol, acetic acid & DMSO, slightly soluble in

ethanol, isopropanol, acetone, and dioxane, Insoluble in acetonitrile, chloroform& ethyl acetate it is excreted 85% via urine and 10% via fecal. It belongs to the class of phenylacetamide with the mass of 266.3. [4-5]

The mixture of amlodipine (ALD) and atenolol (ATN) significantly reduces blood pressure, (ALD+ATN) work together to render the heart more effective at circulating blood across the body

Literature survey showed that [6] developed a UV spectrophotometric method for the estimation of atenolol and Amlodipine in combined dosage form in 2006. Literature study showed that analytical techniques viz. [6-13] HPLC methods have been presented for simultaneous determination of amlodipine and atenolol. There is no article related to stability indicating RP-HPLC method using binary solvent system to quantify amlodipine and atenolol in bulk and tablets have ever been mentioned within literature referred. The primary goal of this study was to produce a low cost accurate reproducible stability indicating RP-HPLC method to assess the contents of amlodipine (ALD) and atenolol (ATN) in Depisal A Tablets according to the ICH guidelines.

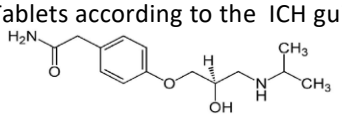
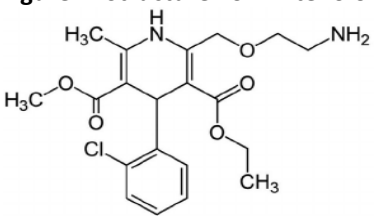


Figure: 1 Structure of Atenolol



Structure of Amlodipine

MATERIALS AND METHODS:

Chemicals and reagents All the chemicals utilized were HPLC grade acetonitrile, HCl, $NaHSO_4$, Na_2HPO_4, phosphoric acid. The water used in buffer preparation was freshly prepared from Milli-Q water and filtered using a nylon 0.45-micron membrane filter. The marketed formulation Amlodipine and Atenolol combination tablets i.e, Depisal-A tablets (5-50 mg) manufactured by Salveo life care, obtained from local drug store, Hyderabad.

Equipment

Waters Alliance make HPLC system, columns used for separation was waters C_{18} come up with sizes 150 × 4.6mm,5µm. Waters Alliance makes photodiode array detector.

Chromatographic conditions:

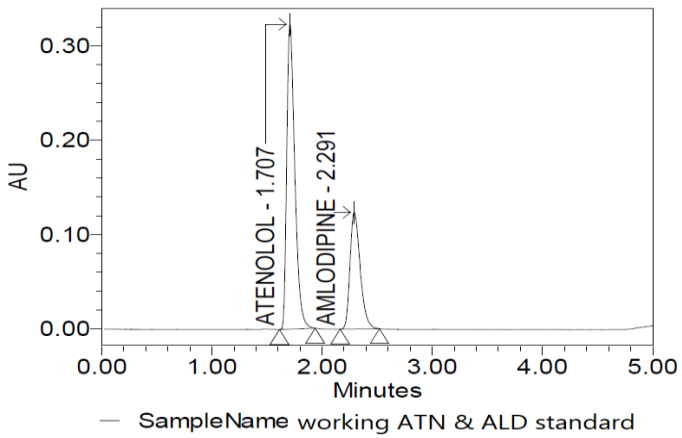
The optimized conditions for the simultaneous estimation of Amlodipine and Atenolol were performed using Waters C_{18} column (150× 4.6mm,5µm Particle size) as stationary phase at temperature of 25°C. The elution was completely isocratic and the mobile phase comprising of a blend of Na_2HPO_4 and Acetonitrile (50:50, v/v), P^H 5.2 at a flow rate of 1.0 ml /min. The elution was monitored at 228 nm: with run time of 5 min. The injection volume was 10 µl capacities. Before injecting a solution, the column was equilibrated using mobile phase for a minimum of 30 min through the system. The retention times for ALD and ATN under optimized chromatographic conditions were found to be 2.786 and 2.106 min, respectively.

Preparation of Analytical solutions:

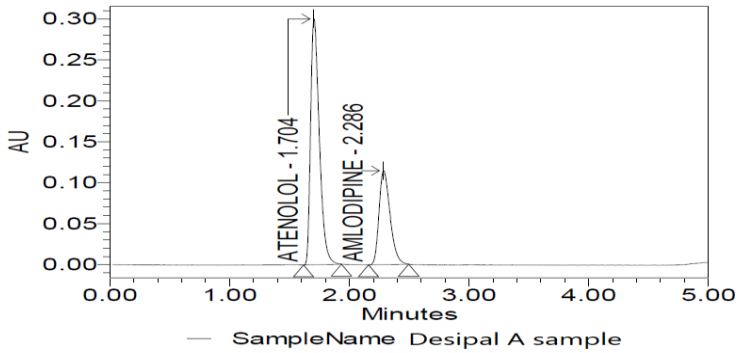
Preparation of mobile phase: It is done by merging 0.1 M Na_2HPO_4 (5.2 units P^H) and acetonitrile solvents in a 50:50 v/v blend. Diluent comprises of 0.1 M $NaHSO_4$ 5.2 P^H units and acetonitrile solvent in a 50:50 v/v blend.

Preparation of standard stock solution:

5mg of ALD& 50 mg of ATN is diluted in 100 ml of preferred diluents to form stock ALD& ATN solution (ALD-50µg /ml& ATN 500 µg/ml). 10 ml of stock ALD& ATN solution (ALD-50µg/ml& ATN-500µg/ml) is diluted in 100 ml of diluents to form a working ALD& ATN solution (5µg/ml& ATN-50µg/ml).



(FIGURE:2)



(FIGURE:3)

Preparation of sample solution (marketed formulation) Depisal A stock sample (ALD-50µg/ml&ATN-500 µg/ml) was formulated by suspending 128mg powdered Depisal A tablet dose (5mg ALD and 50mg ATN) in 100 ml of a diluents and ultrasonicate for twenty minutes

To the 10 ml of Depisal A stock sample (ALD-50 µg/ml &ATN -500µg/ml) add 100 ml of diluent to form a working Depisal A sample (ALD -5µg/ml &ATN- 50µg/ml).

Development and validation of HPLC method

The present research was investigated in order to obtain a new reliable, specific low-cost reverse phase chromatography methodology involving binary solvents was developed to assess the amlodipine (ALD) atenolol (ATN) contents in Depisal A tablets. The RP-HPLC method developed has been validated for parameters such as linearity, limit of detection (LOD), limit of quantification (LOQ), selectivity, accuracy, precision, robustness, and stability studies as per ICH guidelines. [14-15] Forced degradation studies were conducted to elucidate the characteristics of the active substance 's intrinsic stability.

Linearity

The linearity of the method developed was determined by linear regression analysis and is measured using least square method. A series of standard solutions of ALD and ATN were injected into the HPLC system at five different concentration levels i.e, 25-75 µg/ml (ATN) and 2.5-7.5µg/ml (ALD). Calibration curves of standard solutions were plotted against respective concentrations with their response ratios (ratios of the peak area of the analytes). Slope, intercept and correlation coefficient-R^2, were determined by applying linear regression equation.

Precision

Precision is achieved using six standard concentration replicates (ATN- 50µg/ml, and ALD-5µg/ml). Evaluated ALD and ATN solutions using the norms set in "conditions of ALD & ATN mixture assaying" division. Retention time and peak area were recorded and expressed as a mean and % RSD from the data collected.

Accuracy

Accuracy in terms of assay and percent recovery was evaluated for the newly developed method. It is performed by standard addition method at, 50,100,150% levels. Known amount of standard solution of drugs ATN & ALD were spiked to pre analyzed sample solutions injected into the HPLC system in triplicate and percentage recoveries were calculated using area observed for each level.

Sensitivity

The detection limit (LOD) and quantification limit (LOQ), as the quantities for which the signal- to –noise ratio were 3:1 and10:1, respectively. LOD&LOQ are calculated using standard deviation of the peak area and slope

Robustness

It aims to evaluate the effect of small deliberate variations/adjustments in variables such as flow rate (±2%), ratios of mobile phase, P^H (±2 units), temperature (±2°C), and shift in wavelength (±2 units).

Forced degradation study

According to ICH guidelines the forced degradation studies of ALD&ATN were attempted to elucidate the intrinsic stability characteristics of the active substance under stress conditions such as acid, alkaline, peroxide, thermal, photolytic, and hydrolytic conditions. for acidic degradation Depisal A stock sample of 10 ml size (ALD 50µg/ml& ATN -500µg/ml) was suspended with 0.1 N HCl of 10 ml size& allow it for ultrasonication upto 30 minutes. For alkali degradation suspend 10 ml of stock solution with 10 ml of 0.1 N NaOH and ultrasonicate for about 30 minutes. for peroxide degradation 10 ml of peroxide of 30% potency suspended with 10 ml of Depisal A stock solution column allow it for ultrasonication .for photolytic degradation Depisal A stock solution held open upto 6 hrs to temperature of 105°c .for hydrolytic degradation ,10 ml of depisal stock sample suspended with 10 ml of water ,ultrasonicate for 30 min. once the

degradation treatments are finished ,samples were cooled to room temperature and diluted with a diluent then injected into column for chromatographic analysis.

Procedure for Assay:

The chromatographic conditions that were optimized reported with steady baseline 10µl of standard and sample solutions were injected into the HPLC system separately and the chromatograms were recorded. The amount of drug in the sample determined from peak area of ALD & ATN.

Results and discussion:

The efforts made to develop a precise, reliable low cost and accurate method for the simultaneous estimation of ALD&ATN pure and tablet form by an isocratic RP-HPLC method. The absorption maximum of ALD&ATN was found to be 228 nm the same wavelength was selected for method development. By changing certain chromatographic conditions such as mobile phase composition, flow rate, P^H, temperature, an optimized method was developed a good peak symmetry for the ALD & ATN were attained with mobile phase consisting of Na_2HPO_4 and Acetonitrile 50:50v/v(P^H5.2) with the flow rate of 1.0 ml/min using Waters C_{18} Column(150×4.6 mm,5µm particle size) as a stationary phase ,with an injection volume of 10µl capacity. The retention time of 2.786 for ALD, 2.106 for ATN were eluted at lesser time compared to reported methods; none of the impurities were interfering with the assay.

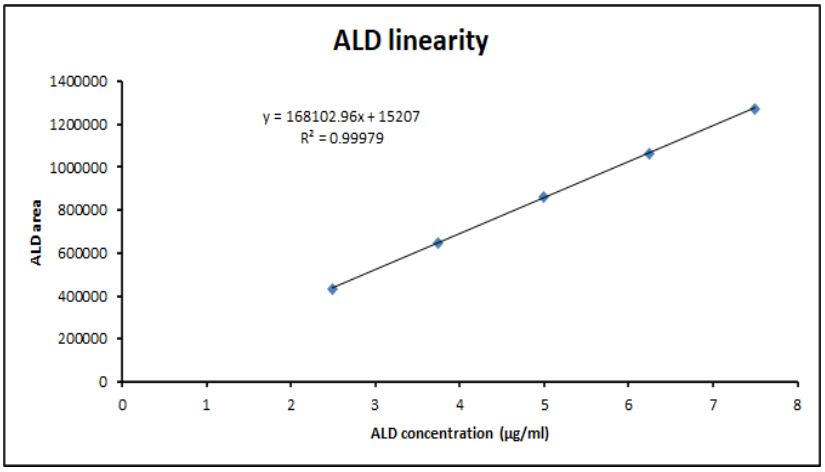
The method developed was validated according ICH guidelines, for linearity, specificity, accuracy, precision, robustness parameters such as USP plate count, for ATN and ALD was 8986 and 9368, USP tailing 1.46 and 1.35 ,retention time were 2.106 and 2.786 respectively, found to be within acceptable limits. There are no interferences of mobile phase or other excipients. developed method was specific& showed linear correlation at different concentration levels of 25-75µg/ml for ATN and 2.5-7.5 µg/ml 2.5-7.5µg/ml for ALD the calibration curve obtained by plotting peak area vs concentration given in figure.

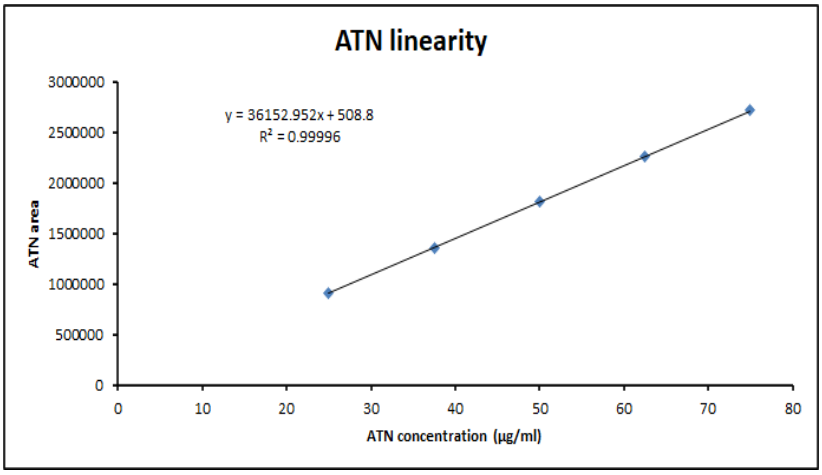
Table:1

\

S.NO	ATN linearity		ALD linearity	
	Response	µg/ml	Response	µg/ml
1	909100	25	430958	2.5
2	1349795	37.50	646792	3.75
3	1808750	50.00	862954	5
4	2259160	62.5	1065815	6.25
5	2713977	75	1272090	7.50

(FIGURE:4)





(FIGURE:5)

Was linear & correlation coefficient for ATN and ALD was found to be 0.99996 & 0.9997, with regression equation y= 36152.952x+508.8 and y=168102.96x+15207, respectively and the results are given in the table. Precision of the method was assessed from peak area determinations of six sample solutions replicates and %RSD for method precision is given in table, it was found to be ,0.336% (ATN) and 0.098% (ALD). The robustness studies revealed that the proposed method is repeatable, reliable, reproducible, and precise as %RSD was found to be less than 2% . The accuracy of optimized method was calculated by assay & recovery studies at threandncentration levels 50%, 100% and, 150%. The mean percentage recovery values for ATN and ALD were found to be 98.63% and 99.55%

ATN ACCURACY

Table:2

S.NO	µg/ml ATN considered	µg/ml ATN quantified	ATN Assay	
		ATN ACCURACY		
1	50	49.165	98.33	**Median assay**
2	50	49.155	98.31	98.63
3	50	49.61	99.22	**Standard deviance**
4	50	49.34	98.68	0.331
5	50	49.27	98.54	**Relative percentile standard**
6	50	49.34	98.68	**deviance:** 0.336

(Table:3)

S.NO	µg/ml ALD considered	µg/ml ALD quantified	ALD Assay	
			ALD ACCURACY	
1	5	4.983	99.65	**Median assay**
2	5	4.975	99.49	99.55
3	5	4.982	99.64	**Standard deviance**
4	5	4.973	99.46	0.097
5	5	4.982	99.63	**Relative percentile standard deviance:** 0.098
6	5	4.973	99.45	

(Table: 4)
ATN & ALD PRECISION

S.NO		Response
	ATN	
1	1800932	**Median response**
2	1800500	1806310
3	1817112	**Standard deviance**
4	1807265	6066.902
5	1804683	**Relative percentile standard deviance:** 0.336
6	1807366	
	ALD	
1	862928	**Median response**
2	861483	862076.3
3	862835	**Standard deviance**
4	861294	842.2664
5	862751	**Relative percentile standard deviance:** 0.098
6	861167	

(Table: 5) ATN recoveries

S.NO	µg/ml ATN considered	µg/ml ATN quantified	Recovered	
	50% supplementary			
1	24.750	24.83	100.34	**Median recovered** 100.38
2	24.750	24.84	100.36	
3	24.750	24.86	100.43	
	100% supplementary			
4	49.500	49.26	99.52	**Median recovered** 99.40
5	49.500	48.98	98.95	
6	49.500	49.37	99.74	
	150% supplementary			
7	74.250	74.04	99.72	**Median recovered** 99.65
8	74.250	74.00	99.66	
9	74.250	73.93	99.57	

(Table: 6) ALD recoveries

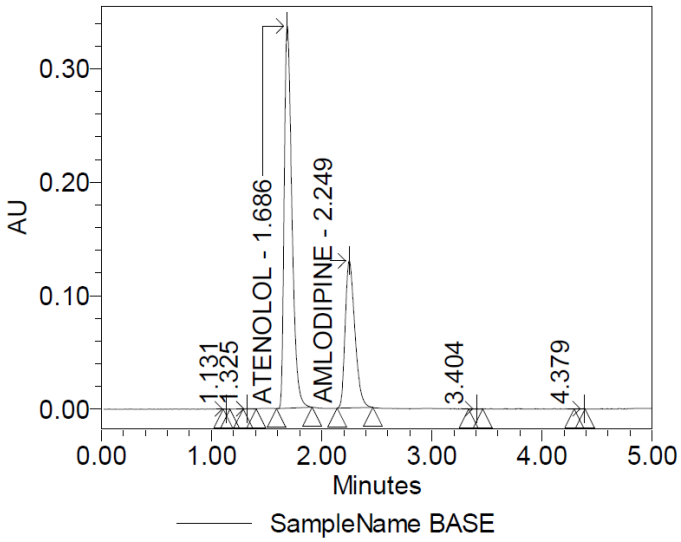
S.NO	µg/ml ALD considered	µg/ml ALD quantified	Recovered	
	50% supplementary			
1	2.500	2.49	99.42	**Median recovered** 99.34
2	2.500	2.48	99.33	
3	2.500	2.48	99.27	
	100% supplementary			
4	5.000	4.98	99.55	**Median recovered** 99.55
5	5.000	4.98	99.58	
6	5.000	4.98	99.51	
	150% supplementary			
7	7.500	7.37	98.24	**Median recovered** 98.02
8	7.500	7.37	98.22	
9	7.500	7.32	97.60	

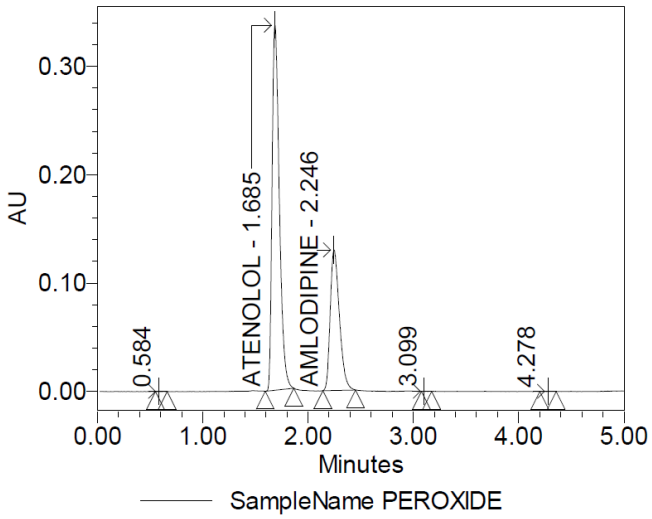
(Table :7) ATN & ALD Stabilities

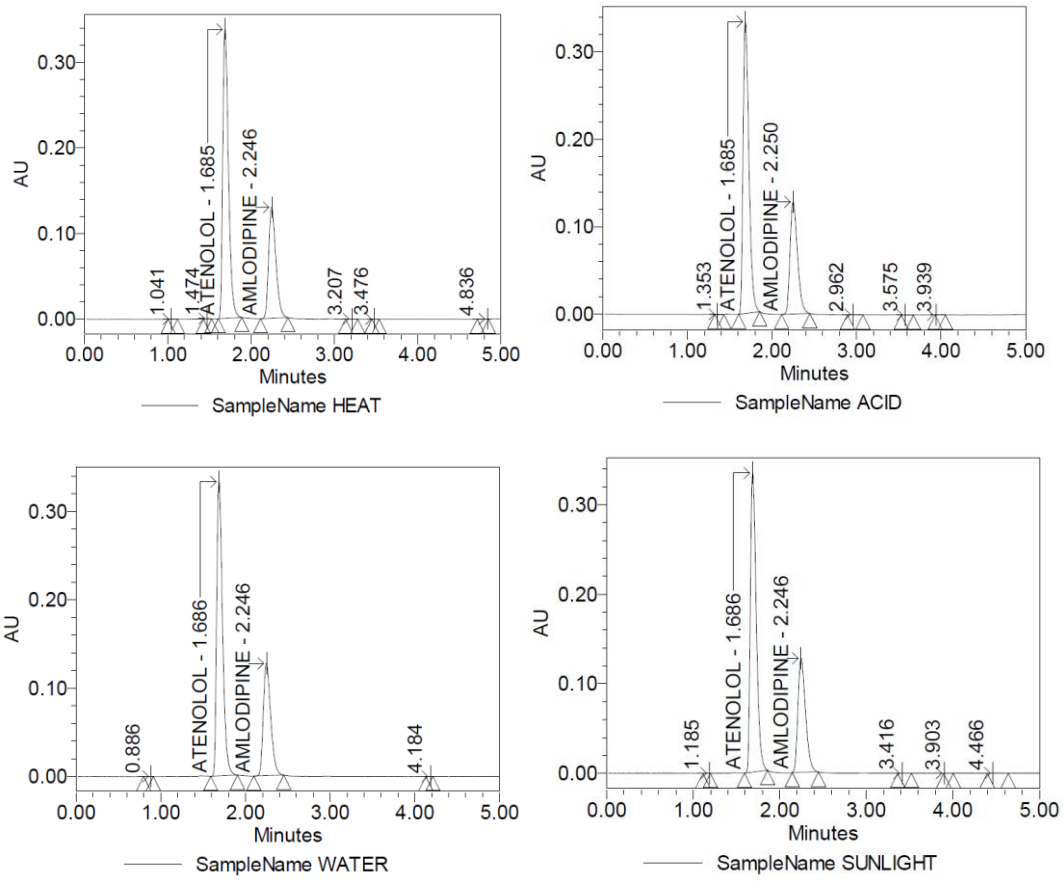
S.NO	Situation	ATN			ALD		
		Response	Assay (%)	Despoiled (%)	Response	Assay (%)	Despoiled (%)
1	0.1 N HCL	1654298	90.33	9.67	770998	89.04	10.96
2	0.1N NaOH	1711908	93.47	6.53	780322	90.11	9.89
3	PEROXIDE	1752833	95.71	4.29	799284	92.30	7.70
4	SUNLIGHT	1631569	89.09	10.91	792171	91.48	8.52
5	105^{O}C	1693609	92.47	7.53	820546	94.76	5.24
6	WATER	1815047	99.10	0.90	856304	98.89	1.11

(Table:8)

S.NO	SENSITIVITY DATA OF ALD AND ATN BY THE PROPOSED METHOD		
		ATN	ALD
1	LOD	0.098µg/ml	0.025µg/ml
2	LOQ	0.327µg/ml	0.082µg/ml







(FIGURE:6)

Chromatogram obtained after stress studies–alkali degradation, oxidative degradation thermal degradation, acid degradation, hydrolytic degradation, and photolytic degradation

(Table :9) Robustness data

	Variationsin	ALD				ATN			
	%Assay	RT	Area	USP plate count	USP tailing	RT	Area	USP plate count	USP tailing
1	+1ml/min	2.784	953867	9364	1.35	2.104	1971273	8961	1.46
2	-1ml/min	1.877	630143	9061	1.35	1.406	1315616	8789	1.48
3	-5% mobile phase	2.784	953867	9364	1.35	2.104	1971273	8981	1.46
4	**+5%**	2.054	700656	9101	1.36	1.536	1450873	8730	1.49
5	-2nm	2.296	945201	9001	1.34	1.713	2102425	8641	1.48
6	+2nm	2.293	757555	9003	1.33	1.709	1400934	8590	1.48
7	-2PH	2.276	771928	8956	1.34	1.699	1600932	8644	1.49
8	+2PH	2.276	790483	9031	1.35	1.700	1640500	8673	1.48
9	-2^{o} C	2.054	700656	9101	1.36	1.536	1450873	8730	1.49
10	+2^{o} C	2.481	854710`	9250	1.33	1.869	1783223	8920	1.48

The % recovery results shown that method was accurate limits were within the range of 98-102 %. Robustness studies with minor deliberate changes in parameters such as wavelength, p^H, buffer in mobile phase, flow rate, responses were within the limits of assay's plate count, were 8986 and 9368 and tailing factor was 1.46 & 1.35 respectively for (ATN) and (ALD) .small variations in these parameters did not alter the results that indicate the method is robust. LOD values for (ATN) & (ALD) 0.098µg/ml and 0.025 µg/ml, LOQ values 0.327µg/ml and 0.082 µg/ml, all the results obtained were within the limits.

CONCLUSION:

This project was to create a simple, precise, reliable, economical, and accurate method was developed RP-HPLC& analysis results of the established method were validated in parameters like linearity, accuracy, precision, robustness, LOD, LOQ, according to ICH guidelines results obtained were within acceptable limits. The developed method has many advantages, including reproducibility of findings, rapid interpretation, ease of sample preparation, and improved selectivity and sensitivity. The developed method can be used for routine research in the pharmaceutical industry for the bulk drug.

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