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Development and validation of RP-HPLC method for the simultaneous determination of azilsartan medoxomil and chlorthalidone in solid dosage form

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Abstract: The fixed dose combination of azilsartan medoxomil and chlorthalidone has been effectively used to treat hypertension. Development of a specific and accurate Reverse-Phase HPLC method for simultaneous estimation of AZL and CLR was main objective of this study that was used for simultaneous quantification of these drugs in human plasma. The method used 30:70 acetonitrile and water as mobile phase, thermo-scientific ODS hypersil C_{18} column (250 × 4.6 mm, 5µm),1.2mL/min flow rate analyzed at 230nm. The retention time was 1.61 and 4.12 for AZL and CLR respectively. All the validation parameters of proposed method were performed. Linearity was found 0.9991 R^2 for the concentration of 3-22µg/ml for CLR and 0.9997 for concentration 10-70µg/ml for AZL. The inter-day and intraday precision were found 0.37 and 0.20 for AZL and 0.83 and 0.34 for CLR. LOD of AZL and CLR was 0.010µg/mL and 0.016µg/ml while LOQ was 0.032µg/mL and 0.048µg/mL for AZL and CLR respectively. The method was found to be effective in AZL and CLR quantification for pharmacokinetic study in human blood plasma.

Keywords: Azilsartan medoxomil, chlorthalidone, RP-HPLC, validation, stress degradation.

INTRODUCTION

AZL and CLR as a combination used to treat hypertension was approved by Food and Drug Administration (FDA) in 2011. The chemical name of AZL is 2-ethoxy3- [[4- [2-(5-oxo-2H - 1, 2, 4-oxadiazol-3-yl) phenyl] phenyl] methyl] benzimidazole-4-carboxylic acid. (Naazneen *et al*., 2014) Physical properties of AZL are, it is a granular substance having off-white color and is particularly soluble in methanol, acetonitrile and insoluble in water (Bakera *et al.*, 2011). It is an antihypertensive prodrug (Kher *et al.*, 2020) having molecular weight around 606.62 and its empirical formula is $C_{30}H_{23}KN_4O_8$ (M. Gosavi, *et al*, 2020). Chemical name of CLR is 2-chloro-5-(l-hydroxy-3-oxo-l-isoindo-linyl) benzenesulfonamide. (Karnes and Cooper-DeHoff, 2009) and its empirical formula is $C_{14}H_{11}ClN_2O_4S$ (Sanap *et al.*, 2021). It is an odorless, yellowish white crystalline powder that completely insoluble in water, chloroform and ether and in alcohol it is soluble to some extent. It is completely soluble in acetonitrile and alkali hydroxide solutions (Kountz*et al.*, 2012).

The combination of CLR; a thiazide like diuretic and AZL; an angiotensin II receptor blocker, used to treat hypertension which cannot control by monotherapy. The recommended dosage of AZL and CLR combination is 40/25mg and 40/12.5 mg. (Kurtz *et al*., 2012) After the oral intake, the peak concentrations of CLR and AZL reached in plasma at 1 and 3 hours respectively. The absorption rate of CLR increased 47% when used in combination with AZL. The half-lives of AZL and CLR are approximately 12 and 45 hours respectively even when used in combination and any kind of food intake do not have any particular effect on its bioavailability. (Baker *et al*., 2014)

The pharmacokinetics of AZL and CLR remain the same even after co-administration of the drug. No drug-drug interactions have been found in the combination of AZL and CLR. (Ebeid *et al*., 2014)

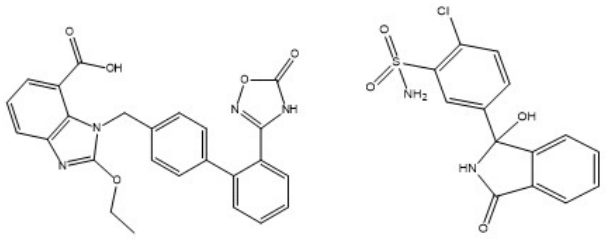

Fig. 1: Structure of AZL and Chlorthalidone

Literature survey revealed different analytical methods in previous researches for the simultaneous determination of AZL and CLR in solid dosage forms by RP-HPLC. The recent study is an attempt to develop a simple, accurate and precise, RP-HPLC method with stress degradation studies for the analysis of AZL and CLR in the pharmaceutical dosage form.

MATERIALS AND METHODS

Method development

Several trials have been conducted to assay an angiotensin receptor II blocker and thiazide as a reference. To observe

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the resolution and tailing factor, a known amount of the standard compound was added in each trial. The mobile phase consisted of acetonitrile and water in varying proportions, but a ratio of 70:30 of water and acetonitrile respectively was found to produce the best peak symmetry.

Selection of wavelength

Both standards of AZL and CLR had shown maximum absorption at 219 and 252 nm respectively. For the simultaneous estimation of AZL and CLR, a common wavelength must be selected. Therefore, the wavelength was optimized at 230nm.

Chromatographic conditions

Mobile phase used for this method was composed of deionized water and Acetonitrile in the ratio 70:30 pumped at 1.2mL/min flow rate for seven minutes and 20µL of the sample was injected in to mobile phase line passing through C18 column (250 × 4.6mm, 5µm) at room temperature. The eluents in the column were recorded using a UV detector, and the wavelength was optimized at 230nm for both drugs.

Method validation

After establishing chromatographic conditions, the developed method was validated according to the ICH guidelines. The validation parameters such as system suitability, linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, precision and specificity were performed.

System suitability

A working dilution was made from a standard stock solution according to the developed method. Six replicates of the sample were injected to test the method's accuracy. The system suitability parameters were recorded, including the theoretical plate count, resolution, tailing factor and area reproducibility. The percentage relative standard deviation (%RSD) was also reported.

Linearity

For the evaluation of the linearity range of the method, six linear dilutions were prepareed at different concentration levels. Linear concentrations of AZL were prepared ranging from 10-70µg/mL and 3.125-21.875 for CLR and calibration curve was constructed by plotting area against concentration from which linear equation, slope, regression coefficient R^2 and y-intercept were calculated.

Limit of detection (LOD)

Limit of detection was measured by consecutively diluting the standard stock solution of AZL and CLR. The concentration was determined as LOD whose sample peaks response were three times of the noise peak.

$$LOD = \frac{3.3\,\sigma}{S}$$

Limit of quantitation (LOQ)

Limit of quantitation was measured by consecutively diluting the standard stock solution of AZL and CLR. The concentration was determined as LOQ whose sample peaks response were ten times of the noise peak.

$$LOD = \frac{10\,\sigma}{S}$$

Precision

System precision was studied by injecting six replicates of standard sample. %RSD, plate count, and retention time were recorded.

Intraday precision was evaluated by injecting six replicates of each and the system response and % relative standard deviation was recoded.

Intermediate precision was assessed by injecting three replicates of a standard working solution on three different days using the same instrument and by having different analysts perform the study on the same instrument. Six replicate samples from the same batch were also injected into the system. % RSD was calculated for both sets of data.

Accuracy

Accuracy or recovery studies were carried out by spiking of the active ingredient in the known amount of placebo. Percentage recovery of AZL and CLR was calculated at three different concentration level 50%, 100% and 150%. 50% to 150% of the sample concentrations were prepared according the proposed method.

Robustness

Robustness of the developed method was calculated by varying chromatographic conditions. Flow rate was change to ±1mL/min and detection wavelength was varied to ±2nm. The sample that was injected for the purpose of assessing robustness was a standard test solution that had been prepared for the suitability studies.

Forced degradation studies

Force degradation studies were carried out by applying different stress conditions to the standard solutions of AZL and CLR. The standard sample was studied under six different stress conditions, which are;

Acid degradation

To the 1mL of standard stock solution 3mL of freshly prepared 0.1 N HCl was added and allow it to stand on sonicator bath at 60^{o}C for 30 minutes. The resultant solution was neutralized with 0.1 N NaOH and diluted to obtain 12.5µg/mL and 40µg/mL concentration of CLR and AZL respectively. 20µL volume was injected to the HPLC system. The chromatogram was recorded to evaluate the sample stability.

Alkaline degradation

To the 1 mL of standard stock solution 3mL of freshly prepared 0.1 N NaOH was added and allow it to stand on sonicator bath at 60°C for 30 minutes. The resultant solution was neutralized with 0.1 N HCl and diluted to obtain 12.5µg/mL and 40µg/mL concentration of CLR and AZL respectively. 20µL volume was injected to the HPLC system and the chromatograms were recorded to evaluate the stability of the sample.

Oxidative degradation

1mL stock solution of AZL and CLR was taken, to which 1mL of freshly prepared 3% H_2O_2 (hydrogen peroxide) was added and allow to stand at 60°C for 30mintues. After 30minutes the obtained solution was diluted to get the concentrations of 40µg/mL and 12.5µg/mL. 20µL of the diluted solution was injected to the system and the detector response and chromatogram were recorded to evaluate stbality of the sample.

Photo-stability

To assess the photostability of the drug, a 100µg/mL standard solution was placed in a beaker and exposed to sunlight for three hours. The resultant solution was diluted to obtain the concentration of 40µg/mL and 12.5µg/mL of AZL and CLR for further HPLC studies. 20µL of the prepared sample was injected and response of the detector and chromatograms were recoded.

Thermal degradation

The standard drug solution was kept in a petri-dish and placed in oven for three hours at 105ºC. The obtained sample was diluted to the concentration of 12.5µg/mL and 40µg/mL for HPLC study. 20µL sample was injected to system to record the chromatogram and detector's response for the further assessment of sample stability.

Degradation under UV light

To study the photo stability of the drug, 100µg/mL standard solution was placed in a beaker was placed in UV chamber for 2 hours. The resultant solution was diluted to obtain the concentration of 40µg/mL and 12.5µg/mL of AZL and CLR for further HPLC studies. 20µL of the prepared sample was injected and response of the detector and chromatograms were recoded.

STATISTICAL ANALYSIS

Microsoft Excel is used for analyzing obtained data.

RESULTS

Method optimization

A new RP-HPLC was developed and validated for simultaneous estimation of AZL and chlorthalidone. Over the previously reported methods, the developed method has many advantages. Analytical conditions had been selected on the basis of chemical nature of AZL and CLR. The selection of column (stationary phase) was based on peak shape, theoretical plates, method reproducibility resolution and back pressure. After the evaluation of all these parameters, C_{18} column (5µm, 250 × 4.6mm) was selected for analysis. Under isocratic conditions, preliminary trials were conducted using a mobile phase composed of 30% acetonitrile and 70% water (v/v), which was found to be the most suitable composition for analysis Trials on different flow rate were made but eventually flow rate for analysis was optimized at 1.2mL/min. The sample was tested on different wavelengths (as shown in fig. 2), and a wavelength of 230 nm was selected. The sample was evaluated at various wavelengths (as depicted in fig. 2) and it was determined that a wavelength of 230 nm produced the best results. The resulting chromatogram is shown in fig. 3.

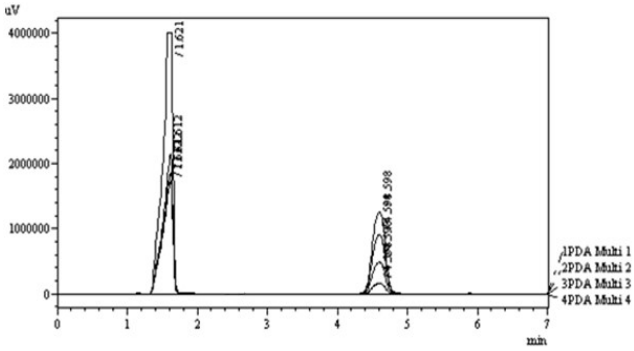

Fig. 2: Overlay chromatogram at different wavelengths

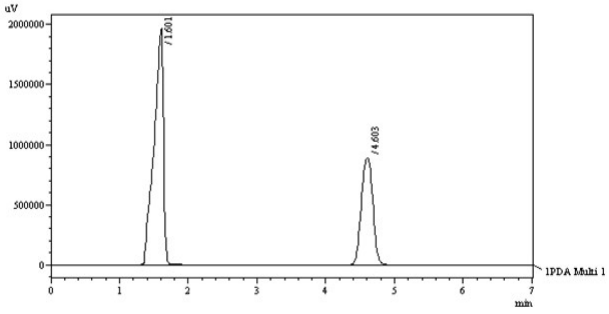

Fig. 3: Chromatogram of AZL and CLR

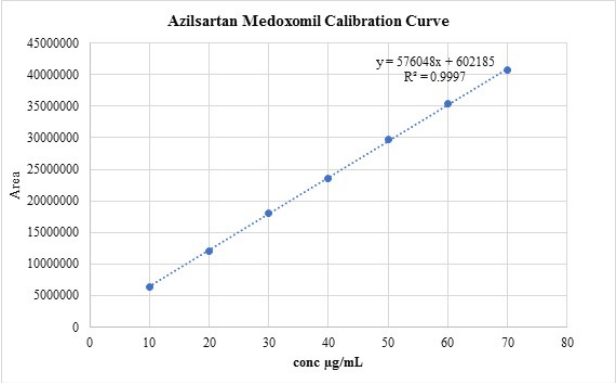

Fig. 4: Calibration curve of azilsartan medoxomil

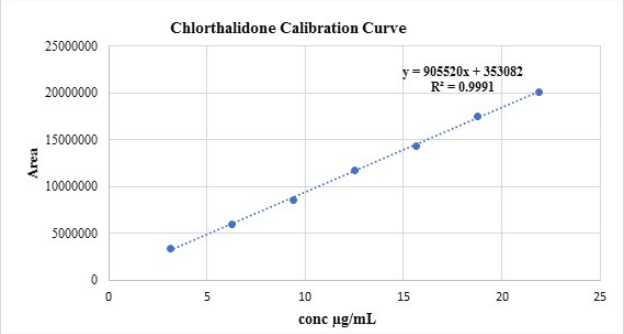

Fig. 5: Calibration curve of chlorthalidone

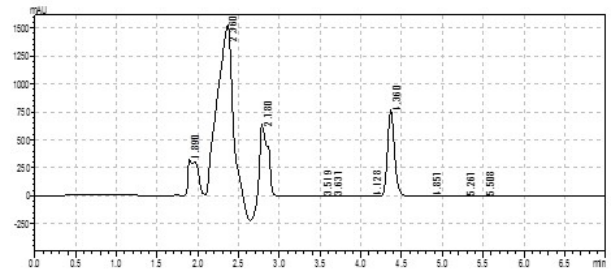

Fig. 6: Acid degradation Chromatogram

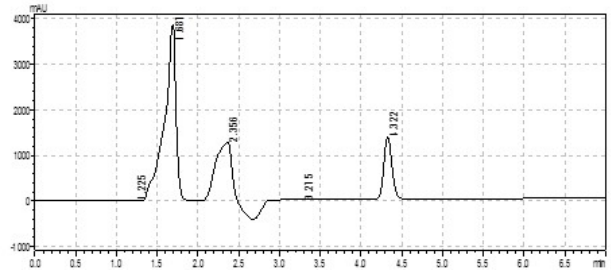

Fig. 7: Alkaline degradation Chromatogram

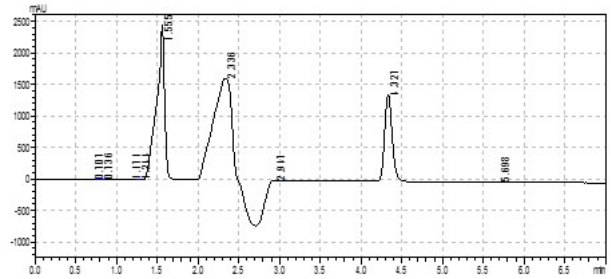

Fig. 8: Oxidative degradation Chromatogram

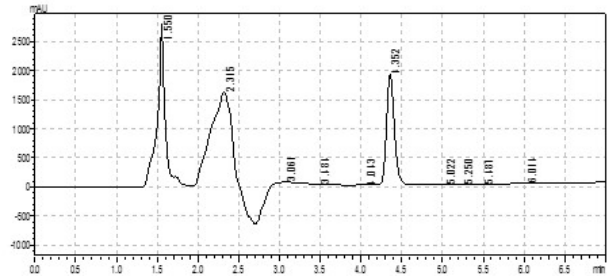

Fig. 9: UV degradation chromatogram

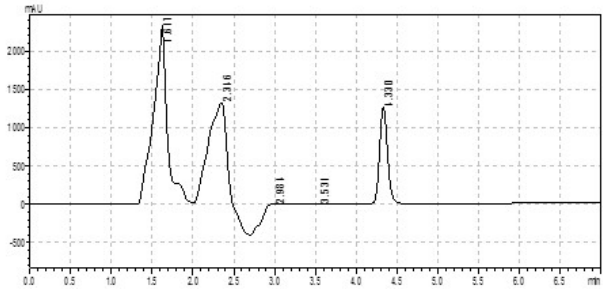

Fig. 10: Photolytic degradation Chromatogram

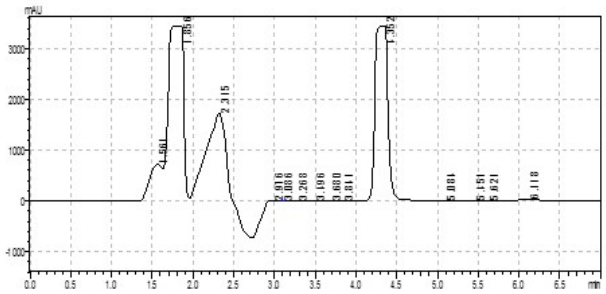

Fig. 11: Thermal degradation chromatogram

Table 1: Acceptance criteria for validation parameters

Parameters	Limits
Precision, system suitability	%RSD ≤ 2
Intermediate precision	%RSD ≤ 5
Correlation coefficient	$R^2 < 0.999$
Accuracy	Recovery 95-105%
Tailing factor (T)	≤ 2.0
Theoretical plates (N)	≥ 2000

System suitability

According to the developed method, working dilutions were prepared from the standard stock solution. Six replicates of the sample were injected into the system from the working solutions to evaluate the effectiveness of the method. In the system suitability study, several parameters were evaluated, including tailing factor, area reproducibility and theoretical plate count (as shown in table 3). The percentage standard deviation (%RSD) was recorded and found to be within the limits specified by the ICH guidelines.

Limit of detection (LOD)

The concentration was determined as LOD, based on a response of sample peaks was three times greater than the noise peak. The LOD for AZL was 0.010µg/mL and for CLR it was 0.016µg/mL.

Limit of quantitation (LOQ)

The concentration was determined as LOQ, based on a response of sample peaks was ten times greater than the noise peak. The LOQ for AZL was 0.032µg/mL and for CLR it was 0.048µg/mL.

Table 4: Linearity results of AZL and chlorthalidone

Levels	AZL	CLR	AZL	CLR
	Conc µg/mL	Conc µg/mL	Area	Area
25%	10	3.125	6356954	3370598
50%	20	6.25	11997013	5988381
75%	30	9.375	17979089	8558979
100%	40	12.5	23477011	11720393
125%	50	15.625	29708922	14371480
150%	60	18.75	35340516	17575113
175%	70	21.875	40649116	20119601
Mean			23644089	11672078
SD			12445743	6115635
Slope			576048	905520
Correlation coefficient (R^2)			0.9997	0.9991
Y-intercept			602185	353082

Table 4: Results of system suitability

Drug	Retention time	Area	Column efficiency	[1]USP tailing	%RSD for replicate injections
Azilsaratanmedoxomil	1.62	23594330	5106	0.66	0.20
chlorthalidone	4.61	11507364	3492	0.62	0.34

Table 4: Accuracy results of azilsartan medoxomil

Levels	Amount added µg/mL	Amount recovered µg/mL	%age recovery	Mean recovery	%RSD
50%	20	19.9	99.5	101.8	2.79
	20	20.2	101		
	20	21	105		
100%	40	39.2	98	99.57	1.51
	40	40.4	101		
	40	39.9	99.7		
150%	60	61.4	102.3	101.2	1.13
	60	60.8	101.3		
	60	60	100		

Table 4: Accuracy Results of Chlorthalidone

Levels	Amount added µg/mL	Amount recovered µg/mL	%age recovery	Mean recovery	%RSD
50%	6.25	5.80	100.07	98.89	0.19
	6.25	6.15	98.4		
	6.25	6.14	98.2		
100%	12.5	12.2	97.6	98.4	2.15
	12.5	12.1	96.8		
	12.5	12.6	100.8		
150%	18.75	17.9	95.4	98.06	2.49
	18.75	18.5	98.6		
	18.75	18.8	100.2		

Accuracy

The percentage recovery of AZL was found between 99.57-101.8 (table 4) and CLR was 98.06-100.07 (table 5). The accuracy of proposed method had been indicated because the %age recovery was found within the acceptance limit, given by ICH which is 100 ± 2%.

Precision

Intraday

The results of intraday precision, determined by six replicate injections, were found to be within limits. The intraday precision for AZL was 0.37% RSD and for CLR it was 0.83% RSD. These results are summarized in table 6.

Table 9: Intraday precision of AZL and CLR

S No.	Area	
	AZL	CLR
1	22833158	11685099
2	22977011	11490393
3	22988822	11587780
4	23843993	11501846
5	22767040	11466642
6	23938194	12012520
Mean	23224703	11624047
SD	86665	97353
% RSD	0.37	0.83

Table 9: System precision of AZL

Sr. No.	R. T	Area	Plate Count	Tailing factor
1	1.629	23643993	452.116	0.662
2	1.628	23537871	450.106	0.66
3	1.612	23667940	518.216	0.674
4	1.612	23539158	514.714	0.669
5	1.623	23578822	534.656	0.669
6	1.621	23598194	536.233	0.667
Mean		23594330		
STD		48942		
% RSD		0.20		

Table 9: System precision of CLR

Sr. No.	R.t	Area	Plate count	Tailing factor
1	4.635	11476992	4050.114	0
2	4.634	11519863	4051.014	0.82
3	4.603	11459928	3535.643	1.007
4	4.598	11585099	3085.532	0.961
5	4.623	11489780	3214.714	0.98
6	4.619	11512520	3015.455	0
Mean		11507364		
SD		40223		
% RSD		0.34		

Table 9: Intermediate precision results of AZL and CLR

Sr. No.	Day 1		Day 2		Day 3	
	AZL	CLR	AZL	CLR	AZL	CLR
1	23843993	11501846	23543184	11872520	21953014	11933907
2	22767040	11466642	23633158	12085899	23820648	11297725
3	23938194	12012520	21978822	11787780	22934464	12005008
Mean	23516409	11660336	23051721	11915400	22902709	11745547
SD	650680	305508	930247	153616	934222	389451
% RSD	2.76	2.62	4.03	1.28	4.07	3.31

Table 9: Intermediate precision results with different analyst

S No.	AZL		CLR	
	Area		Area	
	Analyst I	Analyst II	Analyst I	Analyst II
	AZL	AZL	CLR	CLR
1	20669518	20933158	11685099	11611992
2	20907271	20878822	11687780	11653563
3	20935033	20643184	11772520	11559928
4	20843993	20833158	11785099	11501846
5	20767040	20978822	11687780	11666642
6	20798194	20677011	11720393	11512520
Mean	20820175	20824026	11723112	11584415
SD	97313	136583	45239	70554
% RSD	0.46	0.65	0.38	0.60

Table 14: Robustness results for AZL and CLR

Parameters	Flow rate						Wavelength					
	1mL/min		1.2mL/min		1.3mL/min		228nm		230nm		232nm	
	AZL	CLR	AZL	CLR	AZL	CLR	AZL	CLR	AZL	CLR	AZL	CLR
USP tailing	0.61	0.98	0.69	0.67	0.63	0.99	0.66	0.69	0.66	0.87	0.62	0.60
Pate count	5032	3559	5244	3458	5014	3386	5218	3598	5409	3298	5098	3120
%RSD	0.31	0.64	0.44	0.26	0.41	0.76	0.79	0.49	0.44	0.26	0.20	0.40

Table 14: Forced degradation results of AZL

Stress conditions	% Assay	% Decomposition	RT	USP plate count	USP tailing factor
Controlled sample	99.7	--	1.601	5618	0.674
Acid degradation	86.79	12.91	2.360	5212	0.781
Alkaline degradation	90.97	8.73	1.654	6031	0.942
Oxidative degradation	95.07	4.63	1.555	5259	0.829
UV degradation	87.91	11.79	1.550	4923	0.981
Photolytic degradation	93.18	6.52	1.617	5448	0.992
Dry heat degradation	92.63	7.07	1.856	5645	0.891

Table 14: Forced degradation results of CLR

Stress conditions	% Assay	% Decomposition	RT	USP plate count	USP tailing factor
Controlled sample	100.8	--	4.603	3535	1.007
Acid degradation	80.04	20.76	4.360	3244	0.911
Alkali degradation	93.53	7.27	4.317	4283	0.879
Oxidative degradation	84.18	16.62	4.327	4040	0.980
UV degradation	82.2	18.6	4.352	3927	0.971
Photolytic degradation	98.99	1.81	4.330	3121	1.001
Dry heat degradation	92.51	8.29	4.352	4432	0.948

Table 14: AZL recovery from human plasma

S No	Amount Added µg/ml	amount recovered µg/ml	%age recovery	mean recovery	%RSD
1	40	39.82	99.5	99.4	0.15
2	40	39.84	99.6		
3	40	39.73	99.3		

Table 14: CLR recovery from human plasma

S No	Amount Added µg/ml	amount recovered µg/ml	%age recovery	mean recovery	% RSD
1	12.5	9.74	77.9	77.4	2.707
2	12.5	9.39	75.1		
3	12.5	9.90	79.2		

DISCUSSION

Results of method validation

Linearity

The linearity range of AZL was determined to be 10-70 µg/ml, and for CLR it was established to be 3.125-21.875 µg/ml. The linear regression equation for AZL was calculated as y = 576048x + 602185, with a high correlation coefficient (R^2) of 0.9997. Similarly, the regression equation for CLR was derived as y = 905520x + 353082, with a correlation coefficient (R^2) of 0.9991. The calibration data for both AZL and CLR is displayed in table 1 and the corresponding calibration curves are presented in figs. 4 and 5. The linearity data summarized in table 2.

System precision

%RSD of AZL was 0.20 and for CLR 0.34 and found to be within the acceptance criteria which is less than 2.0%. The results of system precision were summarized in table 7 and 8.

Intermediate precision

The intermediate precision of the AZL and CLR combination was determined using three replicate injections on the HPLC system on three separate days,

with different analysts analyzing the samples on the same day. The % RSD was found to be within the limit. The results are shown in tables 9 and 10, respectively.

Robustness

Robustness of the developed method was evaluated by varying chromatographic conditions. Flow rate was changed by ±1mL/min and detection wavelength was varied to ±2nm. The method remained unaffected by these minor variations, as evidenced by %RSD values that remained within the acceptance limit. The results of the robustness study were shown in table 11.

Results of forced degradation studies

In acidic conditions, AZL and CLR degraded to 12.91% and 20.96% respectively (fig. 6). In these stress condition, two degradant peaks appeared at the retention time of 1.89 and 2.78 min. In alkaline conditions, the percentage decomposition of AZL was 8.73% and % decomposition of CLR was 7.27% (fig. 7). In basic stress conditions, the appearance of one major peak and two minor peaks was noticed on the chromatogram. The major peak was appeared at retention time of 2.356 min.

Under oxidative conditions, AZL and CLR were degraded to 4.63% and 16.62% respectively. One major and many minor peaks were appeared on the chromatogram and the major degradant peak was found at the retention time of 2.336 (fig. 8).

When the standard of AZL and CLR was exposed to UV (ultra violet) light their percentage decompositions was recorded 11.79% and 18.6% respectively. A major degradant peak was observed at a retention time of 2.315 minutes, and several minor degradant peaks were also observed on the chromatogram depicted in fig. 9.

Under photolytic degradation CLR and azilsaratan medoxomil was degraded to 1.81% and 6.25% respectively. One degradant peak was appeared at the chromatogram (fig. 10) with retention time of 2.984. During dry heat degradation, 7.07% and 8.29% of AZL and CLR was degraded. Several minor peaks and one major peak with retention time of 2.315 were appeared on chromatogram in fig. 11. All the degradation data had tabulated in table 12 and 13.

Stability studies

The stability of the combination drug solution in the diluting solvent was assessed. The standard solution was stored in tightly capped vials at room temperature for 48 hours, and the prepared solution was analyzed using the developed method. No significant changes in the area were observed within 24 to 48 hours. The standard solution of AZL and CLR was found to be stable for up to 24 hours at room temperature.

Protein binding study in human plasma

The protein binding percentage of AZL and CLR were found to be 99.4% and 77.4% respectively. The results of the study (shown in tables 14 and 15) indicate that CLR and AZL are moderate and highly protein-bound drugs, respectively.

CONCLUSION

The developed RP-HPLC method for the simultaneous estimation of AZL and CLR was reliable, specific and simple. The proposed method was validated as per the guidelines of ICH and found accurate, robust, precise rugged and stability indicating. RP-HPLC method was also able to detect AZL and CLR in placebo solution without excipient interference which proves that it can applicable for routine QC pharmaceutical analysis. The stability of AZL and CLR, as indicated by the data from stress degradation studies, allowed for their evaluation under various stress conditions as recommended by ICH. The developed method for the bioanalytical analysis of AZL and CLR in human plasma provides reproducible and consistent drug recoveries. This method is suitable for pharmacokinetic studies of the AZL and CLR combination and routine analysis.

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