International Journal of Pharmaceutical Research and Applications
Volume 7, Issue 3 May-June 2022, pp: 1251-1262 www.ijprajournal.com ISSN: 2456-4494

Development and Validation of Analytical Method for Estimation of Azilsartan Medoxomil and Chlorthalidone in Api And Pharmaceutical Dosage Form

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Submitted: 26-05-2022 Revised: 03-06-2022 Accepted: 06-06-2022

ABSTRACT

OBJECTIVE: "Development and Validation of analytical method for estimation of Azilsartan Medoxomil and Chlorthalidone in api and Pharmaceutical Dosage Form".

EXPERIMENTAL WORK: First Order Derivative UV method was developed on two different detection wavelengths 249.55 nm and 283.49 nm for AZM and CTD respectively. HPTLC method was performed using pre-coated silica gel 60 F254 plates as stationary phase using mobile phase: Toluene: Ethanol: Acetonitrile (6: 0.5: 3.5, v/v/v) as mobile phase. The plates were scanned at 251 nm for estimation of Azilsartan Medoxomil and Chlorthalidone.

RESULTS AND DISCUSSIONS:AZM and CTD both show good solubility and considerable UV absorption in methanol. Thus, methanol was selected as solvent for the present work. From the zero order overlain UV spectra of AZM (10 µg/ml) and CTD (10 µg/ml), it was observed that the spectra are overlapping each other, demonstrating the complexity in measuring drugs by direct UV absorption measurement in a binary mixture. Hence, first order derivative method for the simultaneous estimation of AZM and CTD has been developed. The Simple Normal Phase HPTLC method permits the determination of each component in their mixture at the wavelengths corresponding to a maximum or minimum. The main advantage of this method is the chance of easy measurements in correspondence with peaks so it permits the use of the wavelength of the highest value of analytical signals (maximum or minimum).

CONCLUSION: First order derivative method shows easy measurements on the separate peaks, higher values of analytical signals and there was no need to work on zero cross over point. The proposed method does not need any mathematical calculations. The results demonstrate that the proposed First order derivative UV spectrophotometric method is simple, rapid and precise. Therefore, this method will be used for the simultaneous determination of AZM and CTD either bulk or in the marketed formulation. The method was validated for linearity, precision, accuracy and robustness, limit of detection and limit of quantification as per ICH parameter. The regression coefficient (r2) of Azilsartan medoxomil and Chlorthalidone were found to be 0.9971 and 0.9944, respectively. The mean percentage recovery was found to be 99.34 to 100.26 % for AZM while for CTD 99.07 to 100.85 % confirming the accuracy of the proposed method.

I. INTRODUCTION

Hypertension, also known as high or raised blood pressure, is a condition in which the blood vessels have persistently raised pressure. Blood is carried from the heart to all parts of the body in the vessels. Each time the heart beats, it pumps blood into the vessels. Blood pressure is created by the force of blood pushing against the walls of blood vessels (arteries) as it is pumped by the heart. The higher the pressure, the harder the heart has to pump. High blood pressure is a common condition in which the long-term force of the blood against your artery walls is high enough that it may eventually cause health problems, such as heart disease. Blood pressure is determined both by the amount of blood your heart pumps and the amount of resistance to blood flow in your arteries. The more blood your heart pumps and the narrower your arteries, the higher your blood pressure. [I]

Angiotensin II is a very potent chemical formed in the blood that causes muscles surrounding blood vessels to contract, thereby narrowing the vessels. This narrowing increases the pressure within the vessels and can cause high blood pressure (hypertension). Angiotensin II

International Journal of Pharmaceutical Research and Applications
Volume 7, Issue 3 May-June 2022, pp: 1251-1262 www.ijprajournal.com ISSN: 2456-4494

receptor blockers (ARBs) are medications that block the action of angiotensin II by preventing angiotensin II from binding to angiotensin II receptors on the muscles surrounding blood vessels. As a result, blood vessels enlarge (dilate) and blood pressure is reduced. Reduced blood pressure makes it easier for the heart to pump blood and can improve heart failure. In addition, the progression of kidney disease caused by the high blood pressure or diabetes is slowed. ARBs have effects that are similar to angiotensin converting enzyme (ACE) inhibitors, but ACE inhibitors act by preventing the formation of angiotensin II rather than by blocking the binding of angiotensin II to muscles on blood vessels. [II]

Diuretic, any drug that increases the flow of urine. Diuretics promote the removal from the body of excess water, salts, poisons, and accumulated metabolic products, such as urea. They serve to rid the body of excess fluid (edema) that accumulates in the tissues owing to various disease states. [III]

The number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing one. Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors. Under these conditions, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. Thus, it becomes necessary, to develop newer analytical methods for such drugs. [IV]

Principle of UV-Visible Spectroscopy is based on the absorption of ultraviolet light or visible light by chemical compounds, which results in the production of distinct spectra. Spectroscopy is based on the interaction between light and matter. [V]

HPTLC is a powerful analytical method equally suitable for qualitative and quantitative analytical tasks. HPTLC is playing an important role in today analytical world, not in competition to HPLC but as a complementary method. One of the most obvious orthogonal features of the two techniques is the primary use of reversed phases in HPLC versus unmodified silica gel in HPTLC, resulting in partition chromatography and adsorption chromatography respectively.[VI]

II. MATERIALS AND METHODS

CHEMICALS AND REAGENTS:

Azilsartan medoxomil (AZM) and Chlorthalidone (CTD) were obtained as a gift sample from CTX Lifesciences Pvt. Ltd., Sachin, Gujarat.

For UV method, All the reagent solutions (Methanol) used in the UV method were prepared. 5-30 and 10-60 µg/ml concentration of AZM and CTD respectively were prepared and used in the UV method. All the absorbances were taken on the 249.55 and 283.49 nm for AZM and CTD respectively.

For HPTLC method, All the reagent solutions (Toluene, ACN, Methanol) used in the HPTLC method were prepared for the mixture solution in the ratio of 6:0.5:3.5 %V/V/V. 50-300 and 100-600 ng/spot concentration of AZM and CTD respectively were prepared and used in the HPTLC method.

INSTRUMENTATION:

For UV method, Double beam UV-Visible Spectrophotometer (Model 1700, Shimadzu, Japan) having two matched quartz cells having 1 cm light path. UV-Probe 2.50 software, Shimadzu, Electronic analytical balance (AUW-220 D, Japan) Pipettes: 1, 2, 5, 10 ml, Volumetric flask: 10, 25, 50,100 ml used.

For HPTLC method, HPTLC System., TLC Scanner 3 (Camag), Flat bottom, Twin through developing chamber (10 x 10 cm2) (Camag), UV cabinet with wavelength (254 nm) UV lamp (Camag), Pre coated silica gel aluminum plate 60F254 (10 x 10 cm2 with 250 µm Thickness) were used.

SAMPLE PREPARATION:

For UV method, accurately weighed 10 mg of AZM and CTD were transferred separately into 10 ml volumetric flasks and dissolved in small volume of methanol. Then, the volume was diluted to the mark with methanol to get the final concentration of AZM and CTD (1000 µg/ml). 1 ml of each solution was transferred in 10 ml volumetric flask and volume was adjusted to the mark with methanol to get final concentration of 100 µg/ml of each drug. Further, 1ml pipette out from that solution and make up to 10 ml with methanol to get concentration of 10 µg/ml. Same procedure was followed for mixture solution, too.

For HPTLC method, accurately weighed 10 mg of AZM and CTD were transferred separately into 10 ml volumetric flasks and dissolved in small volume of methanol. Then, the

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volume was diluted to the mark with methanol to get the final concentration of AZM and CTD (1000 µg/ml). Pipette out 0.5 ml of AZM and 1 ml of CTD solution was transferred in 10 ml volumetric flask and volume was adjusted to the mark with methanol to get final concentration of 50 ng/spot of AZM and 100 ng/spot of CTD. Same procedure was followed for mixture solution, too.

III. RESULTS AND DISCUSSIONS

METHOD DEVELOPMENT:

For UV method, the first order derivative method permits the determination of each component in their mixture at the wavelengths corresponding to a maximum or minimum. The main advantage of this method is the chance of easy measurements in correspondence with peaks so it permits the use of the wavelength of the highest value of analytical signals (maximum or minimum). First order absorption overlay spectra showed considerable overlapping of peak of two drugs AZM and CTD. Therefore, zero order spectra of mixture solutions were transformed into first order derivative spectra. Different concentration of AZM (5,10,15,20,25,30 µg/ml) and CTD (10,20,30,40,50,60 µg/ml) were tested. Further ratio spectrum of both drugs was respectively converted to first order derivative for selection of optimum wavelength.

For HPTLC method, 1, 2, 3, 4, 5 and 6µl of standard stock solution of 50 µg/ml AZM and 1, 2, 3, 4, 5 and 6µl of standard stock solution of 100 µg/ml CTD were spotted on pre-coated silica gel GF 254 TLC column under nitrogen stream using Linomat V semi-automatic sample applicator. The plate was dried in the air and developed up to 80 mm using mixture of Toluene: Ethanol: Acetonitrile (6:0.5:3.5 v/v/v)as mobile phase in a twin trough chamber previously saturated with mobile phase for 30 minutes. The plate was removed from the chamber, dried in hot air oven and scanned and quantified at 251 nm in absorbance mode. The calibration curve was constructed by plotting area versus respective concentration (ng/spot).

SPECTRA OF UV METHOD

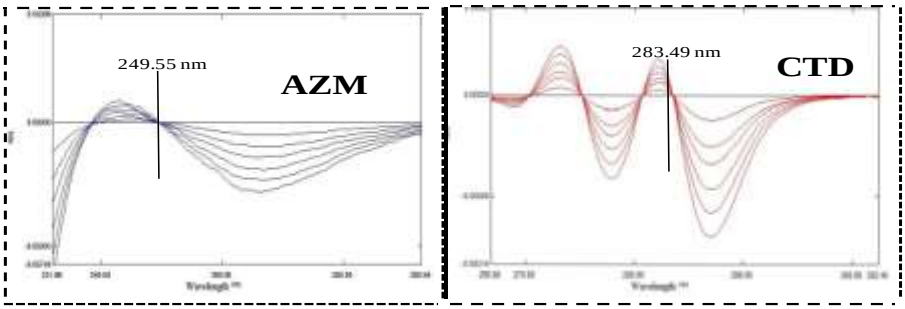

Figure: First order derivative spectra of AZM and CTD

CHROMATOGRAMS OF HPTLC METHOD

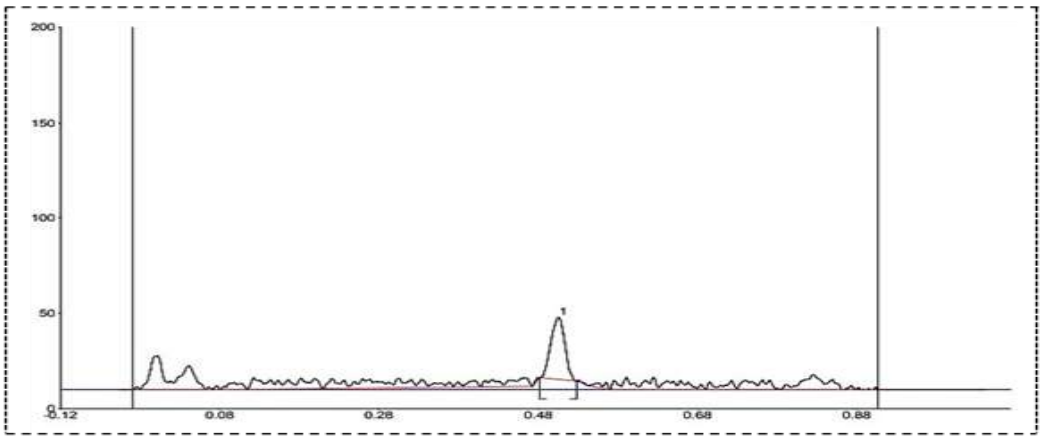

Figure: Chromatogram of AZM 100 ng/spot

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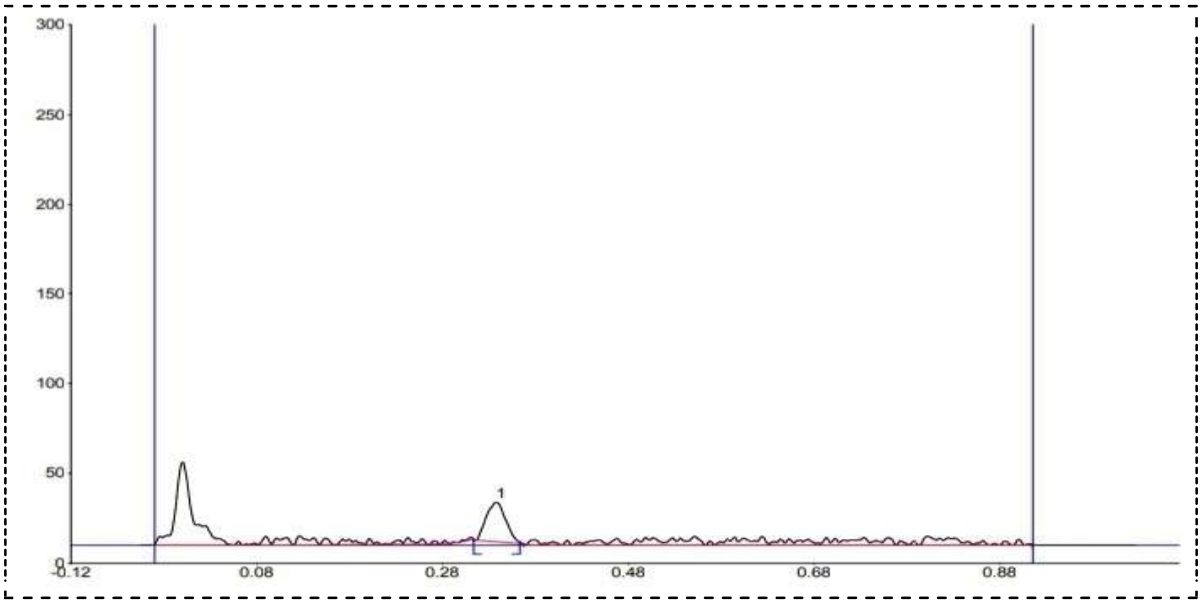

Figure: Chromatogram of CTD 200 ng/spot

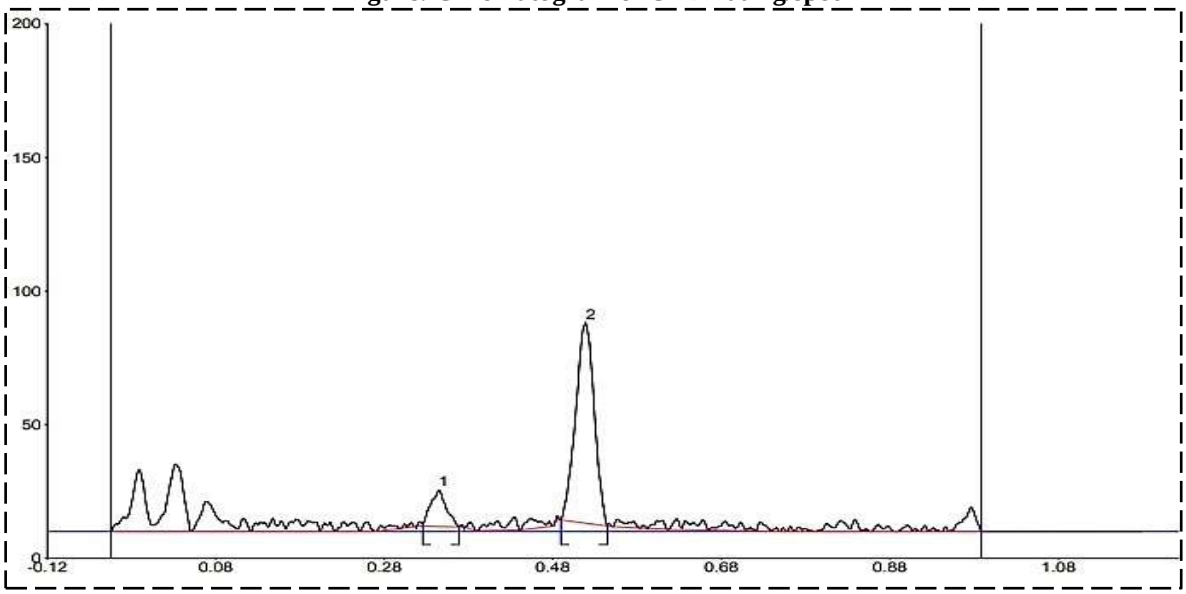

Figure: Chromatogram of AZM 100 ng/spot and CTD 200 ng/spot

VALIDATION PARAMETERS

LINEARITY:

For UV method, the linearity was calculated by ordinary linear regression analysis. The constructed calibration curve was linear over the concentration range of 5-30 µg/ml for AZM and 10- 60 µg/ml for CTD. The linear regression equation was y = -0.001x - 0.0008 for AZM and y = -0.0004x - 0.0001 for CTD with regression co-efficient of 0.9967 and 0.9999 respectively.The calibration curve when plotted, it was found to be linear over the concentration range 5-30 µg/ml for AZM with regression coefficient (R^2) 0.9967 at 249.55nm. The calibration curve when plotted, it was found to be linear over the concentration range 10-60 µg/ml for CTD with regression coefficient (R^2) 0.9999.

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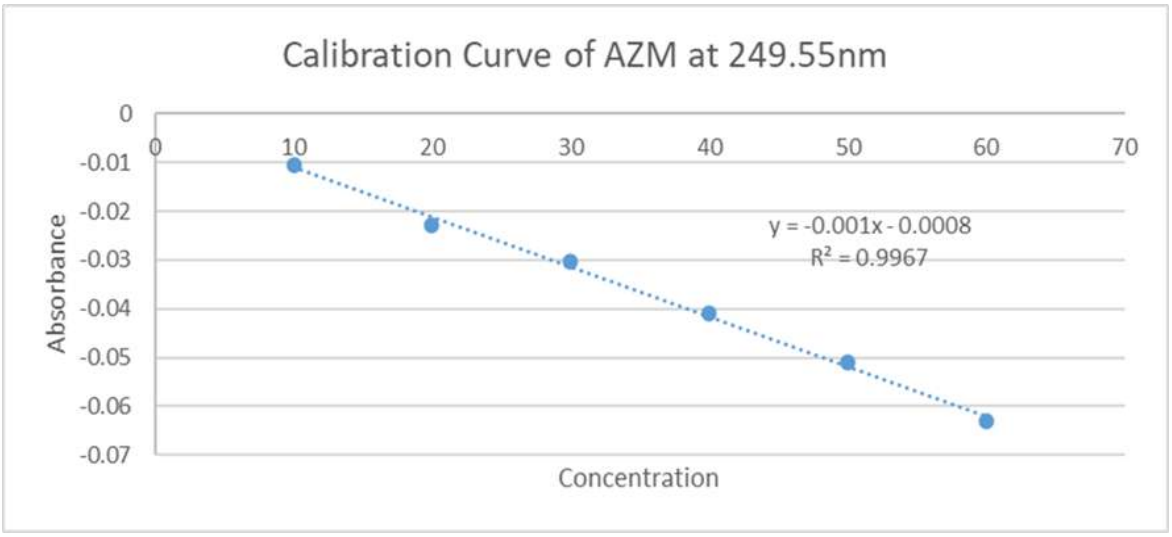

Figure: Calibration curve of AZM at 249.55 nm

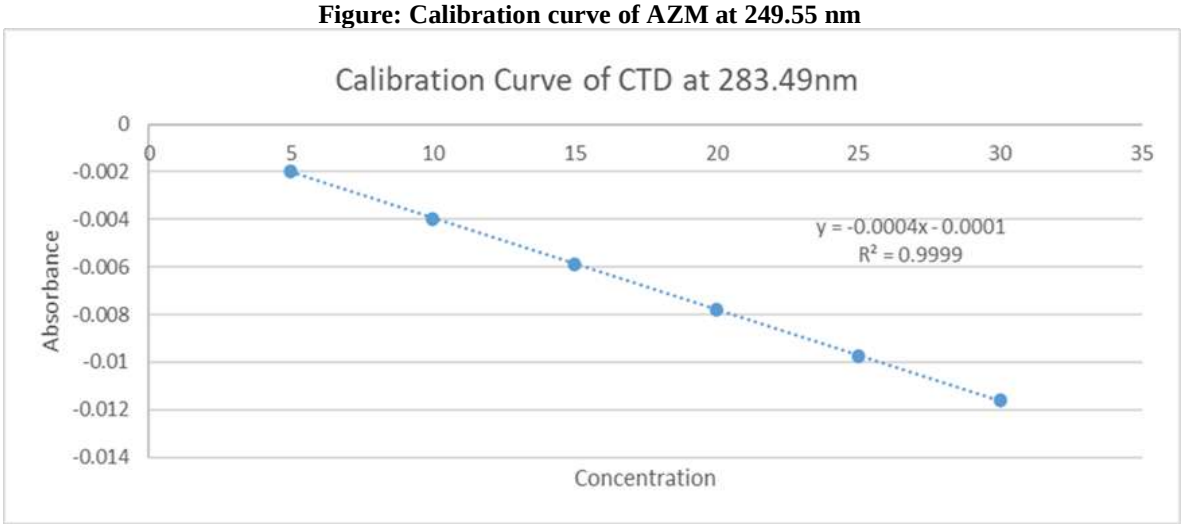

Figure: Calibration curve of CTD at 283.49 nm

For HPTLC method, the linearity was calculated by ordinary linear regression analysis. The constructed calibration curve was linear over the concentration range of 5-30 ng/spot for AZM and 10-60 ng/spot for CTD. The linear regression equation was y = 4.4999x + 21.687 for AZM and y = 1.715x - 23.64 for CTD with regression co-efficient of 0.9971 and 0.9944 respectively. The calibration curve when plotted, it was found to be linear over the concentration range 50-300 ng/spot for AZM with regression coefficient (R^2) 0.9971 at 251 nm. The linear regression data for the calibration curves (n=6), showed a good linear relationship over the concentration range 50-300 ng/spot. The calibration curve when plotted, it was found to be linear over the concentration range 100-600 ng/spot for CTD with regression coefficient (R^2) 0.9944. The linear regression data for the calibration curves (n=6), showed a good linear relationship over the concentration range 100-600 ng/spot.

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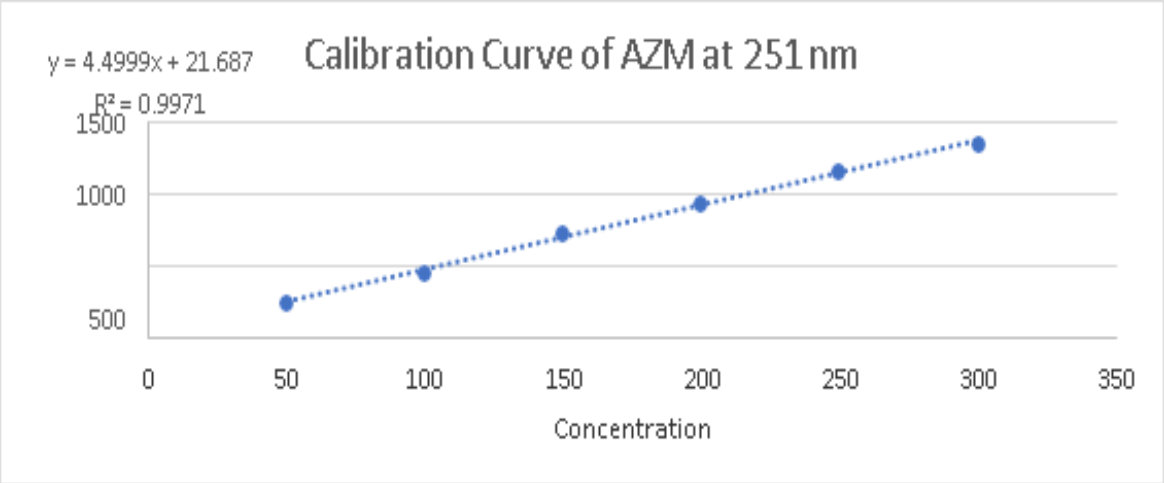

Figure: Calibration curve of AZM at 251 nm

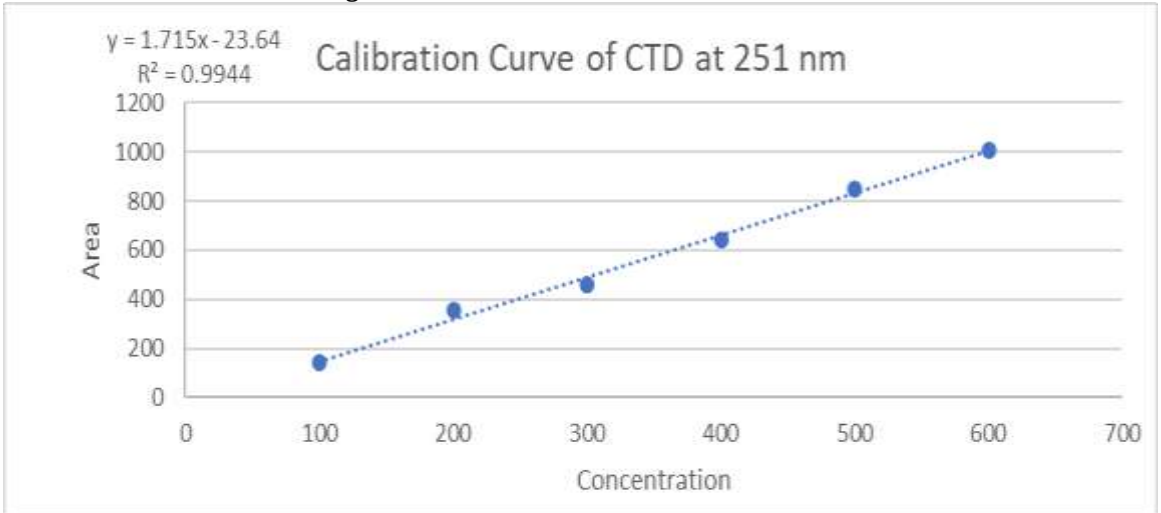

Figure: Calibration curve of CTD at 251 nm

LOD AND LOQ:

For UV method, LOD and LOQ were found to be 0.33680 and 0.20871 µg/ml for AZM as well as 1.02062 and 0.63245 µg/ml for CTD respectively indicating high sensitivity of the method.

For HPTLC method, Detection limit and quantification limit was calculated by the method as described in methodology section. The LOD and LOQ and were found to be 16.66 and 50 ng/spot, for AZM and 33.33 and 100 ng/spot for CTD respectively, which indicates the adequate sensitivity of the method.

PRECISION:

For UV method, The %RSD values for repeatability, intraday precision and interday precision of AZM and CTD respectively were found to be less than 2 indicating that the proposed method has excellent repeatability and reproducibility.

For HPTLC method, The %RSD values for repeatability, intraday precision and interday precision of AZM and CTD respectively were found to be less than 2 indicating that the proposed method has excellent repeatability and reproducibility.

ACCURACY:

For UV method, the recovery studies were carried out by adding known amount of standard to samples at 50, 100 and 150% level and analyzed by the proposed method, in triplicate. The percentage recovery was found between 99.33 to 100.33 % for AZM while for CTD 99.66 to 100.33 % confirming the accuracy of the proposed method.

For HPTLC method,the recovery studies

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were carried out by adding known amount of standard to samples at 50, 100 and 150% level and analyzed by the proposed method, in triplicate. The percentage recovery was found between 99.15 to 100.91 % for AZM while for CTD 99.18 to 100.52 % confirming the accuracy of the proposed method.

ROBUSTNESS:

For HPTLC method, The %RSD values for robustness of AZM and CTD was found to be less than 2 indicating that the proposed method has excellent repeatability and reproducibility.

Analysis of Marketed Formulation:

For UV method, the proposed method was applied for the first order derivative of AZM and CTD, in tablet dosage form. AZM and CTD were quantified using the proposed analytical method and the results are given. The percent recoveries of the amount of AZM and CTD in the tablet dosage form were found in acceptable range, there by suggesting that there is no interference from any of the excipient that normally present in tablet.

For HPTLC method, the proposed method was applied for the HPTLC of AZM and CTD, in tablet dosage form. AZM and CTD were quantified using the proposed analytical method and the results are given. The percent recoveries of the amount of AZM and CTD in the tablet dosage form were found in acceptable range, there by suggesting that there is no interference from any of the excipient that normally present in tablet.

Table: LINEARITY DATA OF AZM AND CTD BY UV AND HPTLC METHOD

Validation Parameters	**UV Method**			
	AZM		**CTD**	
Linearity	**Conc. (µg/ml)**	**%RSD^n**	**Conc. (µg/ml)**	**%RSD^n**
	5	0.9472	10	0.1767
	10	0.3696	20	0.0817
	15	0.3168	30	0.0615
	20	0.2403	40	0.0564
	25	0.2933	50	0.8014
	30	0.1612	60	0.1493

n= 6, mean of six replicates, %RSD = relative standard deviation

Validation Parameters	**HPTLC Method**			
	AZM		**CTD**	
Linearity	**Conc. (ng/spot)**	**%RSD^n**	**Conc. (ng/spot)**	**%RSD^n**
	50	1.0107	100	1.0382
	100	0.7728	200	0.7726
	150	0.4599	300	0.6313
	200	0.3395	400	0.5683
	250	0.2528	500	0.4776
	300	0.2360	600	0.3159

n= 6, mean of six replicates, %RSD = relative standard deviation

Table: PRECISION DATA OF AZM AND CTD BY UV AND HPTLC METHOD

REPEATABILITY OF AZM AND CTD: (UV METHOD)

Validation Parameter	**UV Method**		
Repeatability	**Conc.(µg/ml)**	**Mean^n ± SD**	**%RSD**
	10	1.4719E-05 ± 0.0039	0.3696
	20	1.8708E-05 ± 0.0228	0.0817

n = 6, SD = standard deviation, %RSD = relative standard deviation

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REPEATABILITY OF AZM AND CTD: (HPTLC METHOD)

Validation Parameter	HPTLC Method		
Repeatability	**Conc.(ng/spot)**	**Mean^n ± SD**	**%RSD**
	100	444.66 ± 3.4366	0.7728
	200	357.11 ± 2.7592	0.7726

n = 6, SD = standard deviation, %RSD = relative standard deviation

INTRADAY AND INTERDAY PRECISION OF AZM AND CTD: (UV AND HPTLC METHOD)

Validation Parameters	UV Method			
	AZM		**CTD**	
Intraday Precision	**Conc. (µg/ml)**	**%RSD^n**	**Conc. (µg/ml)**	**%RSD^n**
	5	1.2731	10	0.1965
	15	0.3530	30	0.0870
	30	0.2635	60	0.2245
Validation Parameters	**HPTLC Method**			
	AZM		**CTD**	
Intraday Precision	**Conc. (ng/spot)**	**%RSD^n**	**Conc. (ng/spot)**	**%RSD^n**
	50	0.7257	100	1.3976
	150	0.2619	300	0.4464
	300	0.1727	600	0.1734

n = 3 concentrations / 3 replicates, SD = standard deviation, %RSD = relative standard deviation

Validation Parameters	UV Method			
	AZM		**CTD**	
Interday Precision	**Conc. (µg/ml)**	**%RSD^n**	**Conc. (µg/ml)**	**%RSD^n**
	5	0.7740	10	0.1444
	15	0.2583	30	0.0329
	30	0.2168	60	0.0242
Validation Parameters	**HPTLC Method**			
	AZM		**CTD**	
Interday Precision	**Conc. (ng/spot)**	**%RSD^n**	**Conc. (ng/spot)**	**%RSD^n**
	50	0.5808	100	1.2775
	150	0.1650	300	0.3164
	300	0.1517	600	0.1238

n = 3 concentrations / 3 replicates, SD = standard deviation, %RSD = relative standard deviation

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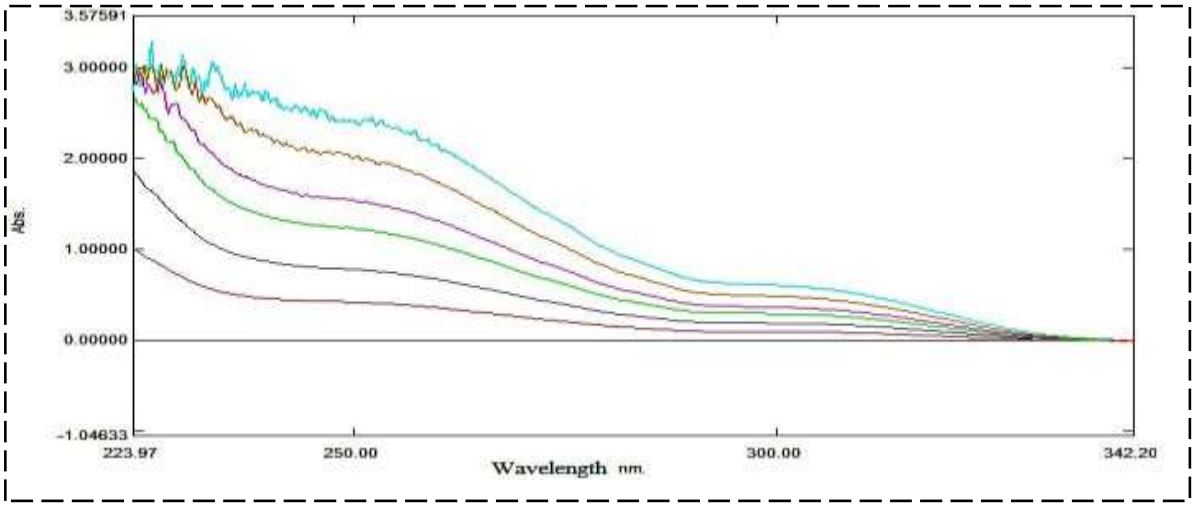

Figure: First Order Derivative UV Spectra of Mixture Solution

Table: ACCURACY DATA OF AZM AND CTD BY UV AND HPTLC METHOD

Validation Parameters	UV Method						
		AZM			CTD		
Accuracy	%	Total Conc.	Conc. Found (µg/ml)	%Recovery^{n} ± SD	Total Conc.	Conc. Found (µg/ml)	%Recovery^{n} ± SD
	50	15	15	100.33 ± 0.2753	30	30.1	100.33 ± 0.8504
		15	14.7		30	29.2	
		15	15.25		30	30.9	
	100	20	19.7	99.33 ± 0.3041	40	40.21	99.91 ± 0.1814
		20	19.75		40	39.99	
		20	20.25		40	39.85	
	150	25	24.6	99.88 ± 0.4821	50	49.2	99.66 ± 0.8736
		25	24.75		50	50.9	
		25	25.5		50	49.7	

n =3 concentrations / 3 replicates, S.D = standard deviation, %RSD= relative standard deviation

Validation Parameters	HPTLC Method						
		AZM			CTD		
Accuracy	%	Total Conc.	Conc. Found (ng/spot)	%Recovery^{n} ± SD	Total Conc.	Conc. Found (ng/spot)	%Recovery^{n} ± SD
	50	150	149.47	99.15 ± 0.5393	300	299.20	99.18 ± 0.9114
		150	150.38		300	301.01	
		150	149.42		300	299.90	
	100	200	200.80	100.91 ± 0.7801	400	401.07	100.52 ± 1.1251
		200	199.38		400	398.91	
		200	199.54		400	399.44	
	150	250	250.67	100.52 ± 0.9100	500	499.73	99.90 ± 1.8903
		250	249.00		500	501.07	
		250	249.20		500	497.34	

n =3 concentrations / 3 replicates, S.D = standard deviation, %RSD= relative standard deviation

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Table: ROBUSTNESS OF AZM AND CTD (HPTLC METHOD)

(1) Change in mobile phase ratio (Toluene: Ethanol: ACN; 6:0.5:3.5) (Toluene= 6%V ±0.5%V):

(2)

Drugs	Ratio (%v/%v/%v)	Rf	Mean^n	SD	RSD
AZM	5.5:0.5:3.5	0.53	727.01	4.6928	0.6455
	6.5:0.5:3.5	0.52	719.17	4.0496	0.5630
CTD	5.5:0.5:3.5	0.37	461.22	2.9814	0.6464
	6.5:0.5:3.5	0.37	455.64	2.7569	0.6050

Change in Chamber Saturation Time (30mins ±2mins):

Drugs	Sat. Time (min)	Rf	Mean^n	SD	RSD
AZM	28	0.52	725.41	4.0500	0.5583
	28	0.52	723.65	3.2163	0.4444
CTD	32	0.37	461.14	4.3743	0.9485
	32	0.37	457.63	2.1542	0.4707

(3) Change in Detection Wavelength (251 nm ± 2nm):

Drugs	Wavelength (nm)	Rf	Mean^n	SD	RSD
AZM	251	0.52	727.69	3.2338	0.4444
	249	0.52	725.25	2.7722	0.3822
CTD	251	0.37	461.97	4.7023	1.0178
	249	0.37	459.97	4.2620	0.9265

n =1 concentration / 3 replicates, S.D = standard deviation, %RSD= relative standard deviation

Table: ANALYSIS OF MARKETED FORMULATION (UV METHOD)

Drug	Label Claim (mg)	Label Claim Found (mg)	% Assay^n ± SD
AZM	40	39.89	99.75 ± 0.9609
CTD	12.5	12.47	99.44 ± 0.9317

n =1 concentration / 3 replicates, SD= standard deviation

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Table: ANALYSIS OF MARKETED FORMULATION (HPTLC METHOD)

Drug	Label Claim (mg)	Label Claim Found (mg)	% Assay^n ± SD
AZM	40	39.89	99.74 ± 0.4050
CTD	12.5	12.47	99.76 ± 0.9450

n =1 concentration / 3 replicates, SD= standard deviation

IV. CONCLUSION:

UV-Visible Spectrophotometry Method:
First order derivative method shows easy measurements on the separate peaks, higher values of analytical signals and there was no need to work on zero cross over point. The proposed method does not need any mathematical calculations. The results demonstrate that the proposed First order derivative UV spectrophotometric method is simple, rapid and precise. Therefore, this method will be used for the simultaneous determination of AZM and CTD either bulk or in the marketed formulation.
UV method was developed and validated based on ICH Q2 R1 guideline for the simultaneous estimation of Azilsartan medoxomil and Chlorthalidone in individual bulk and mixture. Concentration Range used was 5-30 µg/ml for AZM and 10-60 µg/ml for CTD. The common detection wavelength was selected as 249.55 nm and 283.49 nm.

The method was validated for linearity, precision, accuracy and robustness, limit of detection and limit of quantification as per ICH parameter. The regression coefficient (r^2) of Azilsartan medoxomil and Chlorthalidone were found to be 0.9967 and 0.9999, respectively. The mean percentage recovery was found to be 99.33 to 100.33 % for AZM while for CTD 99.66 to 100.33 % confirming the accuracy of the proposed method.

HPTLC Method: Proposed method is developed for the identification and quantification of Azilsartan medoxomil and Chlorthalidone in bulk and mixture. The developed method is simple, accurate, less time consuming, economical and sensitive when compared to other reported analytical methods. According to ICH guideline the method was found to be accurate, sensitive and precise. Statistical analysis proved that the method was repeatable and selective for the analysis of Azilsartan medoxomil and Chlorthalidone without any interferences. This method was successfully used in determination of individual and mixture.
HPTLC (High Performance Thin Layer Chromatography) method was developed and validated based on ICH Q2 R1 guideline for the simultaneous estimation of Azilsartan medoxomil and Chlorthalidone in individual bulk and mixture. Pre coated silica gel aluminum plate 60 GF254 was selected as the stationary phase and Toluene: Ethanol: Acetonitrile (6: 0.5: 3.5 %v/v/v) was used as developing mobile phase. Concentration Range used was 50-300 ng/spot and 100-600 ng/spot for AZM and CTD, respectively. The common detection wavelength was selected as 251 nm.

The method was validated for linearity, precision, accuracy and robustness, limit of detection and limit of quantification as per ICH parameter. The regression coefficient (r^2) of Azilsartan medoxomil and Chlorthalidone were found to be 0.9971 and 0.9944, respectively. The mean percentage recovery was found to be 99.34 to 100.26 % for AZM while for CTD 99.07 to 100.85 % confirming the accuracy of the proposed method.

V. ACKNOWLEDGEMENT:

The authors gratefully acknowledge CTX Lifescience Pvt. Ltd., for providing the gift sample of Azilsartan Medoxomil and Chlorthalidone for the research work.

STATISTICAL COMPARISON BETWEEN UV & HPTLC DEVELOPED METHODS:

UV and HPTLC was developed for Azilsartan Medoxomil and Chlorthalidone in its bulk and Marketed Formulation. To compare the proposed method by **F Test Two Sample for Variances Test**. F cal has been found to be less than F tab in Azilsartan Medoxomil and Chlorthalidone. Statistical comparison at 95% confidence interval is shown in Table.

International Journal of Pharmaceutical Research and Applications
Volume 7, Issue 3 May-June 2022, pp: 1251-1262 www.ijprajournal.com ISSN: 2456-4494

Table: STATISTICAL COMPARISON OF UV AND HPTLC DEVELOPED METHOD BY F TEST.

Sample No.	Azilsartan Medoxomil		Chlorthalidone	
	UV	HPTLC	UV	HPTLC
Mean of Assay[n]	99.452	100.002	99.528	99.85
SD	0.8159	0.2122	0.6745	0.7012
F cal	3.6866		1.5344	
F tab	9.2766		6.3882	

n =1 concentration / 5 replicates of 2 methods i.e., UV and HPTLC, S.D = standard deviation, F cal= F calculated, F tab= F tabulated.

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