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Estimation of Telmisartan, Amlodipine and Chlorthalidone in Bulk and Fixed Dose Combination Using Stability Indicating High Performance Thin Layer Chromatography

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Keywords
Telmisartan (TEL); Amlodipine (AML); Chlorthalidone (CHLO); Stability Indicating Assay Method (SIAM); High-performance thin-layer chromatography (HPTLC); fixed dose combination (FDC); Validation (ICH Q2 R1).

ABSTRACT: A simple, accurate and precise stability-indicating high-performance thin-layer chromatography was developed and validated for the estimation of Telmisartan, Amlodipine and Chlorthalidone in bulk and pharmaceutical dosage form. Chromatographic separation was carried out on Merck TLC Aluminum plates, pre-coated with silica gel 60 F254 of size (20 cm × 10 cm) with 250 µm layer thickness with the mobile phase Chloroform: Toluene: Methanol: Glacial Acetic Acid (6:2:2:0.1 % V/V/V/V) at detection wavelength 254 nm for TEL, AML and CHLO. The Rf value for TEL, AML, and CHLO were found to be 0.64 ± 0.008, 0.25 ± 0.008 and 0.48 ± 0.01, respectively. The method was linear over the concentration ranges 400-4800 ng/band for TEL, 50-600 ng/band for AML and 125-1500 ng/band for CHLO. The LOD was found to be 19.98 ng/ band for TEL, 5.32 ng/ band for AML and 11.28 for CHLO. The LOQ was found to be 60.55 ng/ band for TEL, 16.12 ng/ band for AML and 34.18 ng/ band for CHLO. Under the forced degradation conditions, TEL degraded significantly under acidic and oxidative stress conditions, degraded moderately under alkaline, thermal and photolytic stress conditions; and showed negligible degradation under neutral hydrolysis condition. AML degraded significantly under acidic, alkaline and oxidative stress conditions, degraded moderately under thermal stress condition and degraded the least under neutral and photolytic stress conditions. CHLO degraded extensively under acid and alkaline stress conditions, degraded moderately under oxidative, thermal and photolytic stress conditions and degraded the least under neutral hydrolysis condition.

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INTRODUCTION

A fixed-dose combination of Telmisartan (40 mg), Amlodipine (5 mg), and Chlorthalidone (12.5 mg) is a very effective three –in –one pill with a minimum risk profile for cardiovascular events and longer duration of action for the treatment of essential hypertension. This FDC offers several advantages and is available under several brand names in India. Telmisartan {4' – [[4-methyl – 6- (1-methyl – 1 H – benzimidazol -2-yl) – 2- propyl – 1 H- benzimidazol -1- yl] methyl] biphenyl -2- carboxylic acid} **(Figure 1)** is an angiotensin II receptor antagonist which is used in the treatment of hypertension. Angiotensin II receptor blockers bind to angiotensin II type I receptors and inhibit its effect on vascular smooth muscle, which causes a reduction in arterial blood pressure [1]. It is official in BP [2], USP [3], EP [4] and IP [5]. A literature survey revealed that many methods are reported for the determination of TEL by spectrophotometry [6, 7], HPLC [8, 9], LC-MS/MS [10, 11], and HPTLC [12, 13].

Amlodipine {AML; 3- ethyl – 5-methyl (4RS)-2- [(2-aminoethoxy) methyl]-4- (2-chlorophenyl) -6- methyl -1, 4 -dihydropyridine -3, 5 –dicarboxylate sulfonate} **(Figure 2)** is official in BP [14], USP [15], IP [16] and EP [17]. It is one of the calcium channel blockers, which includes a nitrous oxide release from coronary micro vessels through a kinin-dependant mechanism and contribute positively to the therapeutic action of ACE inhibitors [18]. A literature survey revealed many methods for estimation of AML, via spectrophotometry [19, 20], HPLC [21, 22], HPTLC [23, 24], and LC-MS/MS [25, 26].

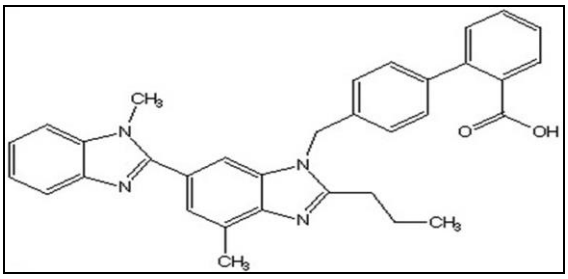

Figure 1: Structure of Telmisartan

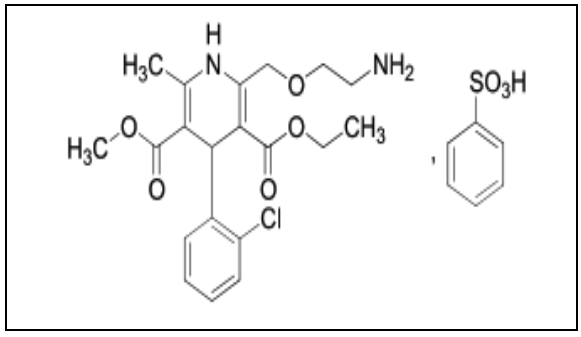

Figure 2: Structure of Amlodipine Besylate

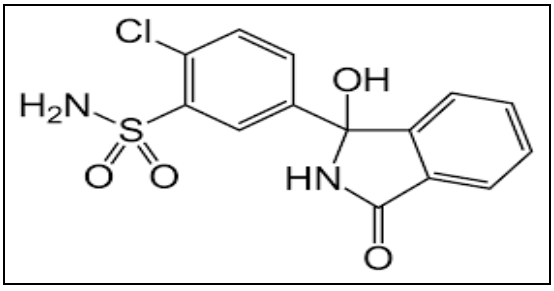

Figure 3: Structure of Chlorthalidone

Chlorthalidone is chemically a 2-chloro-5-(1-hydroxy-3-oxo-1-isoindolinyl) benzenesulfonamide **(Figure 3)** and has a pharmacological action like a thiazide-like diuretic. It inhibits Na+ K+ 2Cl- co-transport in the ascending loop of Henle. It is used as anti-hypertensive and in the treatment of other cardiovascular diseases [27]. It is official in BP [28], USP [29], IP [30] and EP [31]. Literature review reveals that many spectrophotometry [32], HPLC [33, 34], HPTLC [35, 36] and LC/MS-MS [37, 38] methods are reported for determination of CHLO.

The aim of the present work was to develop a stability-indicating HPTLC method for simultaneous estimation of TEL, AML, and CHLO in bulk and fixed-dose combination. It is pertinent to note that, all the published methods enabled the estimation of drugs in combination products containing two drugs only like AML and TEL tablets or TEL and CHLO tablets. Hence, the chromatography conditions for HPTLC were optimized, forced degradation conditions were applied, and the method was validated to establish selectivity for degradation products.

MATERIALS AND METHOD

Apparatus

Chromatographic separation of drugs was performed on Merck TLC aluminum plates, pre-coated with silica gel 60 F254 of size (20 cm × 10 cm) with 250 µm layer thickness. Samples were applied to the plates using Camag 100 µL sample syringe (Hamilton, Switzerland) with a Linomat 5 applicator (Camag, Switzerland). Plates were developed in a twin trough glass chamber and scanned by Camag TLC scanner 3, operated by winCATS planar chromatography manager software which used Deuterium lamp as a radiation source.

Chemicals and Reagents

The API of Telmisartan, Amlodipine Besylate and Chlorthalidone were provided as gift samples by Torrent Pharmaceuticals, West Coast Pharmaceuticals and IPCA Laboratories Ltd respectively. Tablets TELISTA TRIO 40 (Manufactured by Lupin Laboratories) was purchased from a local chemist store. All the solvents like Methanol, Acetonitrile, and Toluene were purchased from E. Merck Mumbai. All the chemical reagents were of analytical grade.

Preparation of Standard Stock Solution

The standard solution was prepared by weighing accurately 10 mg TEL, AML, and CHLO individually and transferred into a clean and dry 10 mL volumetric flask. Initially about 5 mL methanol was added to the flask respectively and sonicated. The volume was made up to the mark with the methanol to achieve 1000 µg/mL TEL, AML, and CHLO.

Preparation of working standard solution

From the above prepared stock solution pipette out 4 mL of TEL, 0.5 mL AML, and 1.25 mL CHLO solution and transferred into a clean and dry 10 mL volumetric flask, methanol was added up to the mark to get final concentration

400 µg/mL, 50 µg/mL and 125 µg/mL respectively, in mixture.

Preparation of test solution

Twenty TELISTA TRIO 40 tablets were accurately weighed, their mean weight was calculated and they were finely powdered. The powdered tablets equivalent to 40 mg TEL, 5 mg AML, and 12.5 mg CHLO were weighed and transferred into a 10 mL volumetric flask and the volume was adjusted to mark with methanol. The contents of the flask were sonicated for 30 min to dissolve the active ingredients completely. The solution was filtered through a Whatman filter paper no. 41. From this 1 mL aliquot was transferred into a 10 mL volumetric flask and the volume was made up with methanol. This test solution containing working concentrations of 400 µg/mL, 50 µg/ mL and 125 µg /mL, respectively, in the mixture, was analyzed for assay determination. 5 µL of this solution is used for assay determination which gave concentration of 2000 ng/ band of TEL, 250 ng/ band of AML, and 625 ng/band of CHLO.

Standardized Chromatography conditions

It was developed in pre-coated silica gel aluminum plate 60 F254 (20 cm × 10 cm with 0.25 mm thickness) pre-washed with methanol then dried at room temperature. Samples were applied at 6 mm wide bands with the application rate of 7 Sec/ µL. The plates were conditioned for 20 min in a pre-saturated twin trough glass chamber (20 cm × 10 cm) with the mobile phase of Chloroform: Toluene: Methanol: Glacial Acetic Acid (6:2:2:0.1 % V/V/V/V) and ascending development was performed till a distance of 90 mm from the point of application. Then the plates were dried with the help of air dryer at 50^0 C for 5 min and densitometry scanning was performed at 254 nm for all three drugs.

METHOD VALIDATION

The proposed method was validated as per ICH guidelines Q2 R1.

Linearity

The working standard solution containing 400 µg/ml TEL, 50 µg /ml AML, and 125 µg/ml CHLO was spotted on the TLC plate with injection volume 1-12 µl to get concentration in the range of 400-4800 ng/band, 50-600 ng/band and 125-1500 ng/band, respectively. Six replicates of each concentration were performed and then calibration plots were determined by linear least- squares regression. The TLC plate was developed on previously described mobile phase. The peak areas were plotted against corresponding concentrations to obtain the calibration graphs.

Precision

The reproducibility was determined by applying six replicates of test solution (2000 µg/ml TEL, 250 µg/ ml AML and 625 µg/ ml CHLO). The intra-day and inter-day precisions were determined by responses of six replicates on same and different days for the test concentration. The results were reported in terms of % RSD (Relative Standard Deviation).

Accuracy

Recovery study was carried out by the standard addition method where known amount of standard concentrations of 50%, 100% and 150% of the test solution were spiked in the test solution in triplicate. The recovered amount of drugs was estimated by substituting values in the regression equation. The % RSD of the recovery was calculated.

LOD and LOQ

The LOD and LOQ of the developed method were calculated from the calibration curve using equations, LOD= 3.3 ×σ/S and LOQ = 10 ×σ/S where σ is the standard deviation of y-intercept and S is the slope of the curve.

Robustness

By introducing small changes in the detection wavelength (±2 nm) and saturation time for the chamber before plate development (±2 min), the effects on the results were determined. One factor at a time was changed and the effect on the peak area of the drug was studied. The robustness of the method was ascertained on a single level for six replicates followed by, the % RSD was calculated.

Specificity

The specificity of the method was checked by the peak purity of the analyte and forced degradation studies.

FORCED DEGRADATION STUDIES

Force degradation studies were intended to ensure the effective separation of TEL, AML, and CHLO from their potential degradation products which are generated under different stress conditions like acid and alkaline hydrolysis, neutral hydrolysis, oxidative degradation, thermal and photolytic degradation.

Acid Hydrolysis

An accurately weighed 40 mg TEL, 5 mg AML, and 12.5 mg CHLO were transferred in a 10 ml volumetric flask individually and in combination. To this were added 5 ml methanol and 5 ml of 0.1 N HCl and kept at 80^0 C for 3 h. From that solution 1 ml was transferred into 10 ml volumetric flask, neutralized with 0.2 N NaOH and diluted to mark with

methanol. An aliquot of 10 µL of the resultant solution corresponding to 4000 ng/band TEL, 500 ng/ band AML, and 1250 ng/band CHLO was applied and developed on a TLC plate.

Alkaline Hydrolysis

An accurately weighed 40 mg TEL, 5 mg AML, and 12.5 mg CHLO were transferred in 10 ml volumetric flask individually and in combination. To this were added 5 ml methanol and 5 ml of 0.1 N NaOH and it was kept at 80^0 C for 3 h. From that solution 1 ml was transferred into 10 ml volumetric flask, neutralized with 0.1 N HCl and diluted to mark with methanol. An aliquot of 10 µL of the resultant solution corresponding to 4000 ng/band TEL, 500 ng/ band AML, and 1250 ng/band CHLO was applied to a TLC plate and developed.

Neutral Hydrolysis

An accurately weighed 40 mg TEL, 5mg AML, and 12.5 mg CHLO were transferred in 10 ml volumetric flask individually and in combination. To this were added 5 ml methanol and 5 ml of water and it was kept at 80^0 C for 3 h. From that solution 1 ml was transferred into 10 ml volumetric flask and diluted to mark with methanol. An aliquot of 10 µL of the resultant solution corresponding to 4000 ng/band TEL, 500 ng/ band AML, and 1250 ng/band CHLO was applied on a TLC plate and developed.

Oxidative Hydrolysis

An accurately weighed 40 mg TEL, 5 mg AML, and 12.5 mg CHLO were transferred in 10 ml volumetric flask individually and in combination. From that solution 1 ml was transferred into 10 ml volumetric flask and diluted to mark with methanol. An aliquot of 10 µL of the resultant solution corresponding to 4000 ng/band TEL, 500 ng/ band AML, and 1250 ng/band CHLO was applied on a TLC plate and developed.

Thermal Degradation

An accurately weighed quantity of 40 mg TEL, 5 mg AML and 12.5 mg CHLO were kept individually and in combination in a petridish. Those were kept at 80^0 C for 6 h. After that those were dissolved in 10 ml methanol. From this solution 1 ml was transferred into 10 ml volumetric flask and diluted to mark with the mobile phase. An aliquot of 10 µL of the resultant solution corresponding to 4000 ng/band TEL, 500 ng/ band AML, and 1250 ng/band CHLO was applied on a TLC plate and developed. The same conditions were applied to formulation and solutions were prepared with the above-mentioned concentrations according to the dilution scheme.

Photolytic Degradation

An accurately weighed quantity of 40 mg TEL, 5 mg AML, and 12.5 mg CHLO were kept individually and in combination in a petridish. It was exposed in photostability chamber (TH-90S, Thermo lab, Mumbai, India) in UV light at 254 nm for 24 h to get 200 watt-hours / m^2 intensity. Also, another set of petridish with same quantity of drugs was exposed to sunlight for 24 h. After that those were dissolved in 10 ml methanol. From this solution 1 ml was transferred into 10 ml volumetric flask and diluted to mark with the mobile phase. An aliquot of 10 µL of the resultant solution corresponding to 4000 ng/band TEL, 500 ng/ band AML and 1250 ng/band CHLO was applied on a TLC plate and developed. Same condition was applied to formulation and solutions were prepared with the above mentioned concentrations according to the dilution scheme.

RESULT AND DISCUSSION

Optimization of the chromatography conditions

For separation of TEL, AML, and CHLO individually and from their degradation peaks different mobile phases with different solvents in different ratio were tried like (1) Methanol: Ethyl Acetate: Ammonia (4:6:0.1 % V/V/V) (2) Tetrahydrofuran: Dichloroethane: Methanol: Ammonia (6:2:1.5:0.5 % V/V/V/V) (3) Chloroform: Toluene: Methanol: Acetic acid (6:2.5:1.5: 0.5 % V/V/V/V) (4) Ethyl acetate: Methanol: Toluene: Ammonia (6.5:2: 1: 0.5 % V/V/V/V). Finally, Chloroform: Toluene: Methanol: Glacial Acetic acid (6:2:2:0.1 % V/V/V/V) showed well resolved peaks with better peak shape. The drugs were resolved with Rf value of 0.64 ± 0.008, 0.25 ± 0.008 and 0.48 ± 0.01 for TEL, AML and CHLO, respectively. Determination of all three drugs was done at wavelength 254 nm. The spot appeared more compact and peak shape more symmetrical when the HPTLC plate were pre-treated with methanol and activated at 50^0 C for 5 min **(Figure 4).**

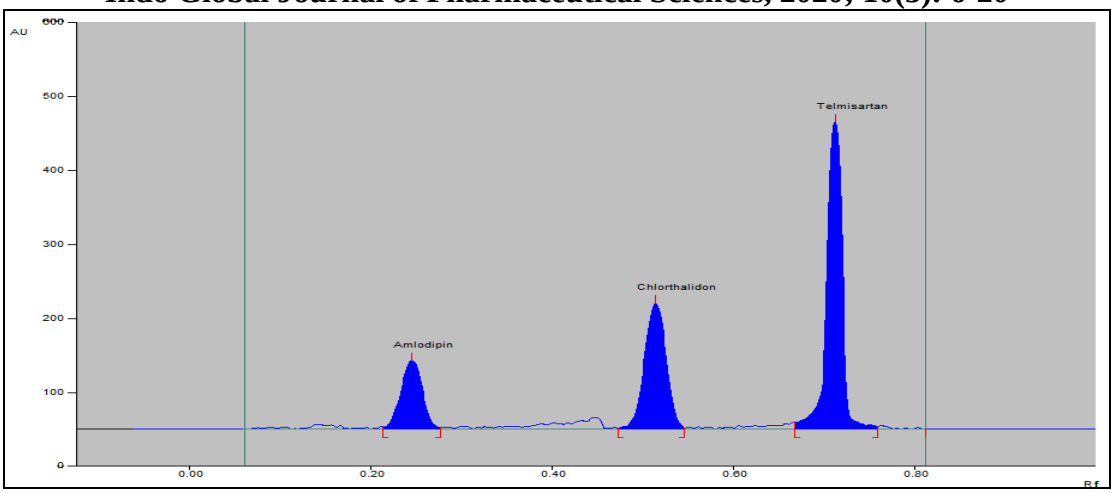

Figure 4: Standard Chromatogram of TEL (4000 ng/ band), AML (500 ng/ band) and CHLO (1250 ng/ band) at 254 nm

Table 1: Linearity range of TEL, AML and CHLO

Sr. No	TEL			AML			CHLO		
	Conc (ng/ band)	Peak Area* ± SD	%RSD	Conc (ng/ band)	Peak Area*±SD	%RSD	Conc (ng/ band)	Peak Area*±SD	%RSD
1	400	1753.167 ± 20.37	1.16	50	324.67 ± 5.57	1.72	125	785.833 ± 13.95	1.78
2	800	2649.167 ± 22.01	0.83	100	567.50 ± 4.85	0.85	250	1281.67 ± 17.28	1.35
3	1600	4264.5 ± 33.67	0.78	200	1022.00 ± 14.68	1.44	500	2190.00 ± 17.56	0.80
4	2400	5986.33 ± 9.092	0.15	300	1511.17 ± 8.40	0.56	750	3072.00 ± 18.95	0.62
5	3200	7680.33 ± 32.13	0.42	400	1967.33 ± 10.80	0.55	1000	4022.17 ± 20.29	0.50
6	4000	9198.83 ± 31.28	0.34	500	2496.33 ± 13.76	0.55	1250	4862.33 ± 20.49	0.42
7	4800	10686.70 ± 40.168	0.34	600	3013.17 ± 9.52	0.32	1500	5863.5 ± 17.60	0.30

*Average of six determinations

Table 2: Repeatability, Intraday and Interday Precision of TEL, AML and CHLO

Parameter	TEL			AML			CHLO		
	Conc (ng/ band)	Peak Area* ± SD	%RSD	Conc (ng/ band)	Peak Area*±SD	%RSD	Conc (ng/ band)	Peak Area*±SD	%RSD
Repeatability	2000	5095.83 ± 54.39	1.07	250	1302.67 ± 16.00	1.26	625	2632.00 ± 18.86	0.72
Intraday Precision	2000	5086.17 ± 30.21	0.59	250	1296.50 ± 15.25	1.18	625	2633.33 ± 66.57	0.73
Interday Precision	2000	5076.67 ± 39.59	0.78	250	1297.67 ± 16.40	1.20	625	2653.83 ± 45.46	1.71

* Average of six determinations

Table 3: Recovery study data of TEL, AML and CHLO

Drug	Amount of Test Solution (ng/ band)	Amount of Std added (ng/ band)	Peak area* ± SD	Amount recovered (ng/ band)	%Total Amount Recovered	Recovery (ng/band)	%RSD
TEL	2000	0	5097.33 ± 51.00	2002.89	100.14	0	1.24
	2000	1000	7159.33 ± 37.29	3010.62	101.06	1010.62	1.80
	2000	2000	9193.00 ± 78.88	4004.496	100.22	2004.496	1.92
	2000	3000	11124.33 ± 52.73	4948.56	98.23	2948.36	0.87
AML	250	0	1295.00 ± 17.35	252.78	101.11	0	1.41
	250	125	1895.67 ± 10.07	376.60	101.28	126.60	1.67
	250	250	2516.33 ± 08.33	504.55	101.82	254.55	0.67
	250	375	3119.67 ± 31.66	628.93	101.03	378.93	1.72
CHLO	625	0	2639.33 ± 33.12	626.903	100.31	0	1.44
	625	313	3790.66 ± 14.80	941.95	101.26	316.96	1025
	625	625	4898.67 ± 28.04	1245.15	99.22	620.15	1.24
	625	938	6120.00 ± 29.61	1579.36	101.74	954.36	0.85

* Average of three determinations

Table 4: Robustness study of TEL, AML and CHLO

Condition	Variation	%Assay ± SD*			% RSD		
		TEL	AML	CHLO	TEL	AML	CHLO
Detection Wavelengh (254±2 nm)	256 nm	99.87 ± 0.74	101.23 ± 1.24	100.06 ± 0.84	0.95	1.28	0.96
	252 nm	100.11± 1.33	101.74 ± 1.32	99.98 ± 0.83			
Chamber Saturation time (20 ± 2 min)	17 min	99.87 ± 0.74	101.23 ± 1.24	100.06 ± 0.84			
	13 min	99.64 ± 0.97	101.33 ± 1.33	101.33 ± 1.97			

* Average of six determinations

Table 5: Analysis of marketed formulation

Drug	Amount of Drug (mg)		%Label claimed ± SD*	% RSD
	Labelled	Estimated		
TEL	40	39.95	99.87 ± 0.74	0.74
AML	5	5.06	101.23 ± 1.26	1.24
CHLO	12.5	12.51	100.06 ± 0.84	0.84

* Average of six determinations

Table 6: Summary of validation parameters

Sr. No.	Parameter	TEL	AML	CHLO
1	Specificity	Specific	Specific	Specific
2	Rf value	0.64 ± 0.008	0.25 ± 0.008	0.48 ± 0.01
3	Linearity Change	400-4800 ng/ band	50-600 ng/ band	125-1500 ng/ band
4	Regression Line equation	y = 2.046x + 999	y = 4.8509x + 68.802	y = 3.6544x + 348.37
5	Correlation Coefficient	0.9993	0.9994	0.9997
6	Precision (%RSD)			
	Repeatability	1.07	1.26	0.72
	Intraday Precision	0.59	1.18	0.73
	Interday Precision	0.78	1.20	1.71
7	Accuracy (% Assay)	98.23-100.22	100.31-101.82	99.22-101.74
8	LOD(ng/band)	19.98 ng/ band	5.32 ng/ band	11.28 ng/ band
9	LOQ(ng/band)	60.55 ng/ band	16.12 ng/ band	34.18 ng/ band
10	Robust	Robust	Robust	Robust

Table 7: Summary of forced degradation study of TEL, AML and CHLO in mixture

Degradation condition	Rf Value of Analyte			No. of degradation peaks	Rf of Degradation Peak	%Degradation		
	TEL	AML	CHLO			TEL	AML	CHLO
0.1 N HCL at 80^0 C, 3 h	0.64	0.24	0.46	2	0.17, 0.62	15.00	19.98	32.34
0.1 N NaOH at 80^0 C, 3 h	0.64	0.25	0.45	2	0.15, 0.64	9.48	26.51	31.25
Water, 80^0 C, 3 h	0.64	0.25	0.44	1	0.55	1.54	7.58	3.48
3% H_2O_2, RT, 12 h	0.64	0.25	0.45	3	0.15 , 0.37, 0.55	19.28	24.26	16.27
Thermal at 80^0 C, 6 h	0.63	0.25	0.44	2	0.39, 0.55	11.11	15.80	11.43
UV light, 254 nm, 24 h	0.64	0.26	0.46	--	--	10.58	5.84	14.46
Sunlight, 24 h	0.65	0.24	0.43	--	----	9.15	7.45	13.54

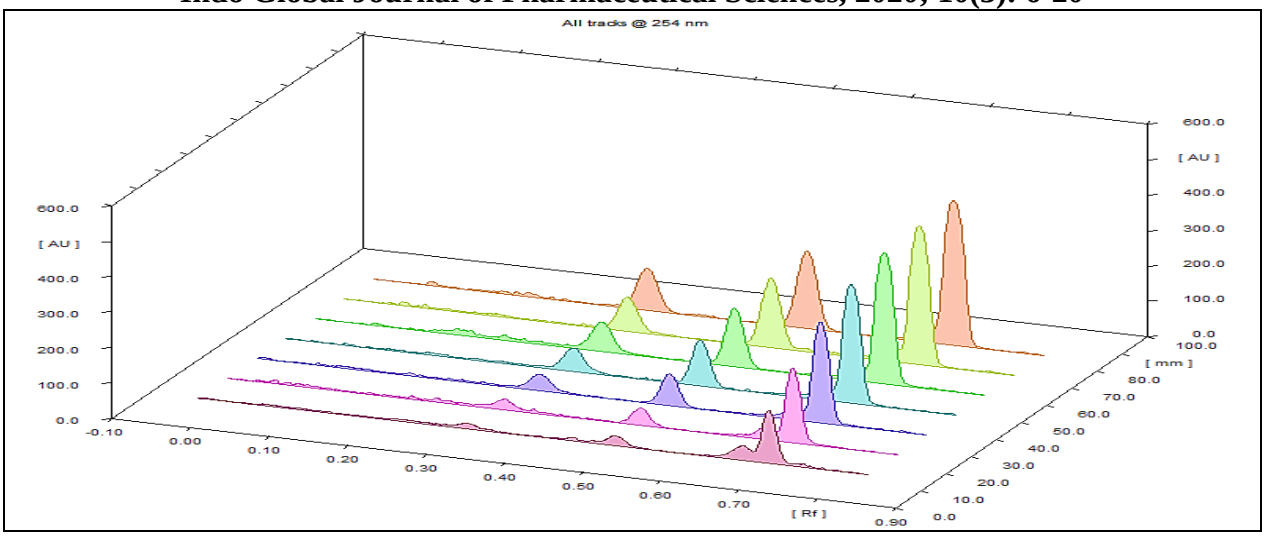

Figure 5: 3D overlain chromatogram of Linearity of TEL (400-4800 ng/band), AML (50-600 ng/band) and CHLO (125-1500 ng/band) at 254 nm

Table 8: Summary of forced degradation study in pharmaceutical dosage form

Degradation condition	Rf Value of Analyte			No. of degradation peaks	Rf of Degradation Peak	%Degradation		
	TEL	AML	CHLO			TEL	AML	CHLO
Thermal at 80^0 C, 6 h	0.68	0.25	0.48	1	0.60	12.49	15.18	11.34
UV light, 254 nm, 24 h	0.68	0.24	0.49	--	--	9.24	6.58	13.71
Sunlight, 24 h	0.67	0.24	0.48	--	--	11.36	7.63	12.82

Linearity

Aliquots of standard were applied in the concentration range of 400-4800 ng/band, 50-600 ng/band, and 125-1500 ng/band for TEL, AML, and CHLO respectively and plates were developed under above optimized condition. The calibration curve obtained by the least square regression analysis between average peak area and concentration showed a linear relationship with a correlation coefficient of 0.9992, 0.9994 and 0.9997 for TEL, AML and CHLO, respectively. The linear regression equations were y = 2.0462x + 999, y = 4.8509x + 68.802 and y = 3.6544x + 348.37 for TEL, AML and CHLO respectively **(Figure 5-8) (Table 1).**

Precision

The % RSD for reproducibility was found to be 1.07 for TEL, 1.26 for AML and 0.72 for CHLO. The % RSD of intra-day precision was found to be 0.59 for TEL, 1.18 for AML and 0.73 for CHLO. The %RSD of inter-day precision was found to be 0.78 for TEL, 1.20 for AML and 1.71 for CHLO. Hence, confirming precision of the developed method **(Table 2).**

Accuracy

The accuracy of the developed method was established by the standard addition method by adding known standard concentration solutions to the pre- analyzed samples. Recoveries were in between 101.04 – 101.82% of TEL, 99.22 – 101.74 % of AML and 98.28- 101.062% of CHLO which is in accordance with ICH guidelines which proves method to be accurate **(Table 3).**

LOD and LOQ

The LOD calculated by formulae was found to be 19.98 ng/ band for TEL, 5.32 ng/ band for AML and 11.28 for CHLO. The LOQ calculated by formulae was found to be 60.55 ng/ band for TEL, 16.12 ng/ band for AML and 34.18 ng/ band for CHLO.

Robustness

Slight change in the chromatography condition of the developed method like saturation time of chamber, detection wavelength did not affect the result significantly. The % RSD values were found below 2 indicated the method to be robust **(Table 4).**

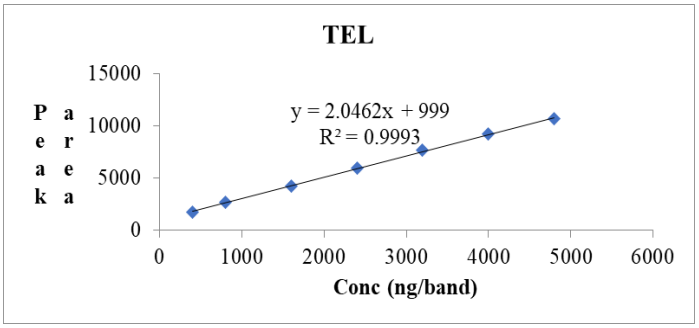

Figure 6: Calibration curve of Linearity of Telmisartan

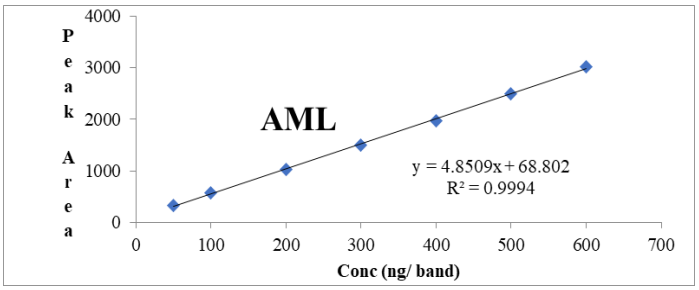

Figure 7: Calibration curve of Linearity of Amlodipine

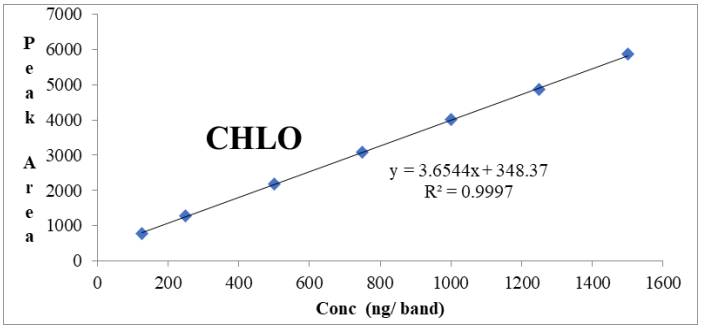

Figure 8: Calibration curve of Linearity of Chlorthalidone

Analysis of the marketed formulation

The developed method was applied to marketed tablet preparation. The assay results of TEL, AML and CHLO were found to be 99.87 ±0.74 %, 101.23 ± 1.26 % and 100.06 ± 0.84%, respectively of the labeled amount **(Figure 9) (Table 5 and 6).**

Specificity

A good correlation was obtained between standard mixture and sample spectra of TEL, AML and CHLO. The purity of all three analyte peaks was found considerably good as spectra of each peak in initial, middle and end position compared well. This confirmed the peak purity and identity. The method was specific respect to excipients present in the marketed formulation and potential degradation products as none of them interfered with the analyte peaks.

Forced degradation studies

The result of forced degradation studies are summarized in **Table no. 7 and 8** for mixture of APIs and in formulation. Under the optimized chromatography conditions, degradation products of analytes were well-resolved and the percent degradation was calculated by comparing peak area with standard preparation.

During stress degradation experiments, it was observed that AML degraded significantly under acidic, alkaline and oxidative stress conditions, degraded moderately under thermal stress condition and degraded the least under neutral and photolytic stress conditions. Overall, six degradation products were found in different stress conditions. Two reliable published papers indicate formation of dehydro derivative (i.e. Impurity D as per European Pharmacopoeia) under acid hydrolysis and oxidative stress conditions [39, 40]. In the co-relation with the above studies, the present study also found one degradation product corresponding to Rf 0.50 under acid hydrolysis and oxidative stress conditions. This can therefore be attributed to dehydro derivative. Further, it was reported that the formation of one degradation product might be due to an acetyl group under alkaline stress conditions [39]. The present study also found 1 degradation product under alkaline hydrolysis corresponding to Rf 0.15 which could be due to an acetyl group containing degradation product.

CHLO degraded extensively under acid and alkaline stress conditions, degraded moderately under oxidative, thermal and photolytic stress conditions and degraded the least under neutral hydrolysis condition. In all, three degradation products were formed in different forced degradation conditions. One research paper reported two hydrolysis products of CHLO as (4’ – chloro -3’ – sulfamoyl – 2- benzophenone carboxylic acid) and (2- chloro-5 – (1- methoxy -3- oxo-1- isoindolinyl) benzenesulfonamide) [41, 42]. The present study found one degradation product under acidic hydrolysis and two degradation products under alkaline hydrolysis corresponding to Rf 0.25 and 0.64 which can correlates to two hydrolysis products referred above. The oxidative degradation and acidic hydrolysis also found one of these degradation products at Rf 0.66.

TEL degraded significantly under acidic and oxidative stress conditions, degraded moderately under alkaline, thermal and photolytic stress conditions; and showed negligible degradation under neutral hydrolysis condition. In all, four degradation products were formed under different degradation conditions. One UHPLC-MS/MS study has reported two acid hydrolysis degradation products under stress conditions corresponding to m/z ration 529 (methyl ester) and 487 (with

cleavage of propyl side chain) [43]. The present study also revealed formation of 2 degradation products corresponding to Rf 0.55, 0.73 and this could be 2 hydrolysis products reported above. The drug is known to be stable under alkaline stress condition and is known to give high dissolution under alkaline condition. No degradation product has been reported for alkaline hydrolysis in the reference cited above and likewise, no degradation product was found in our study. In the same way, no degradation product has been reported in the thermal stress condition and no degradation product was found in our study. The published paper has reported three degradation products corresponding to m/z ratio of 547, 531, and 429. The present study revealed two degradation products under oxidative conditions which corroborate well with the reported study.

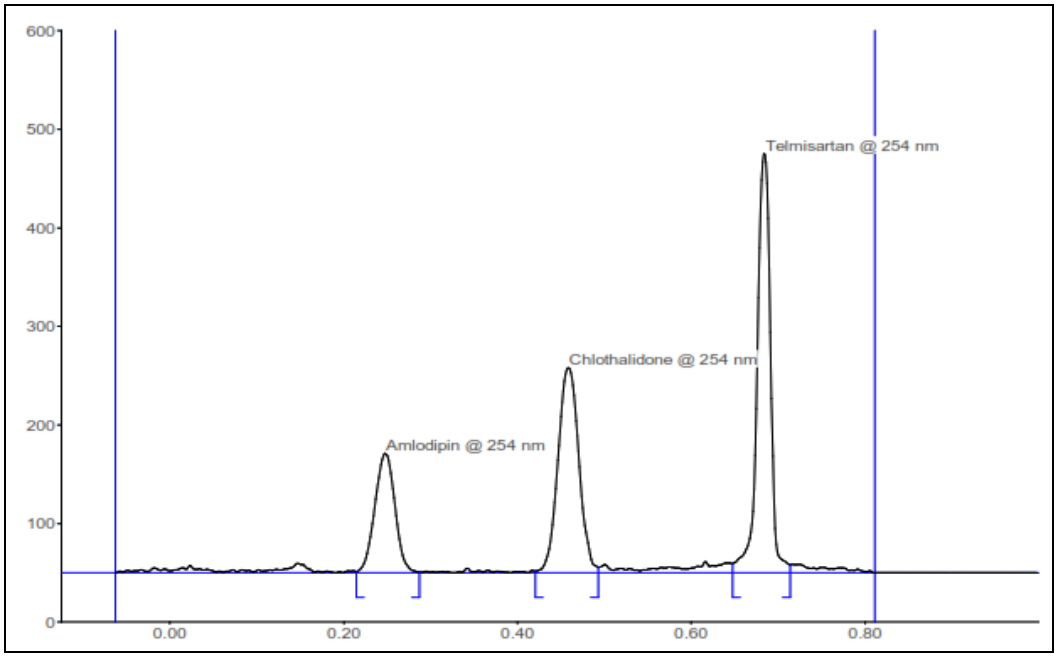

Figure 9: Chromatogram of test solution of TEL (4000 ng/band), AML (500 ng/ band) and CHLO (1250 ng/band)

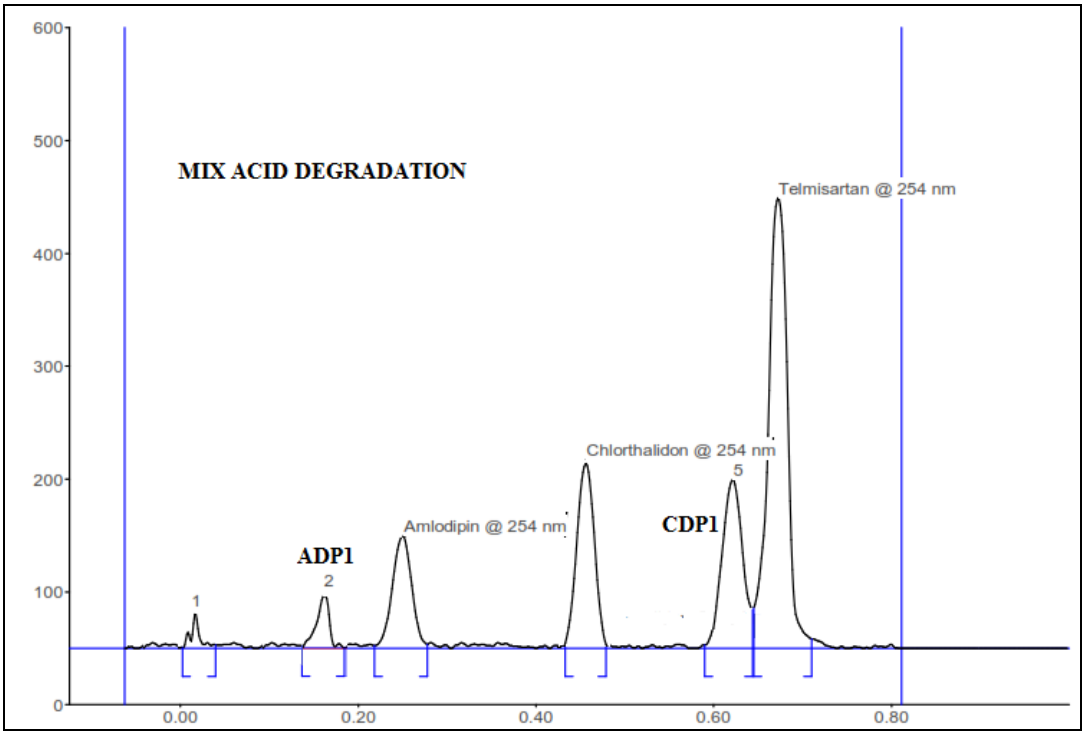

Figure 10: Chromatogram of acid hydrolysis of mixture of TEL, AML and CHLO

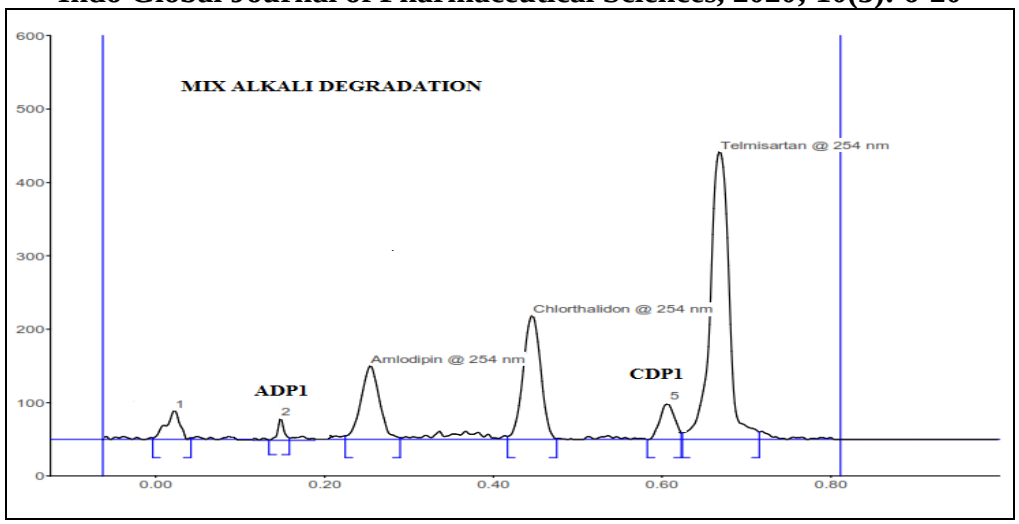

Figure 11: Chromatogram of alkali hydrolysis of mixture of TEL, AML and CHLO

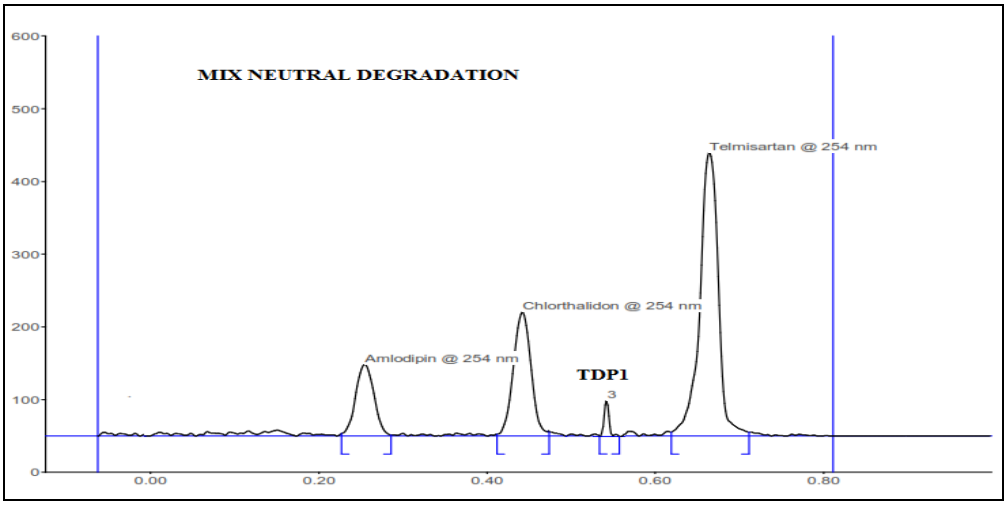

Figure 12: Chromatogram of neutral hydrolysis of mixture of TEL, AML and CHLO

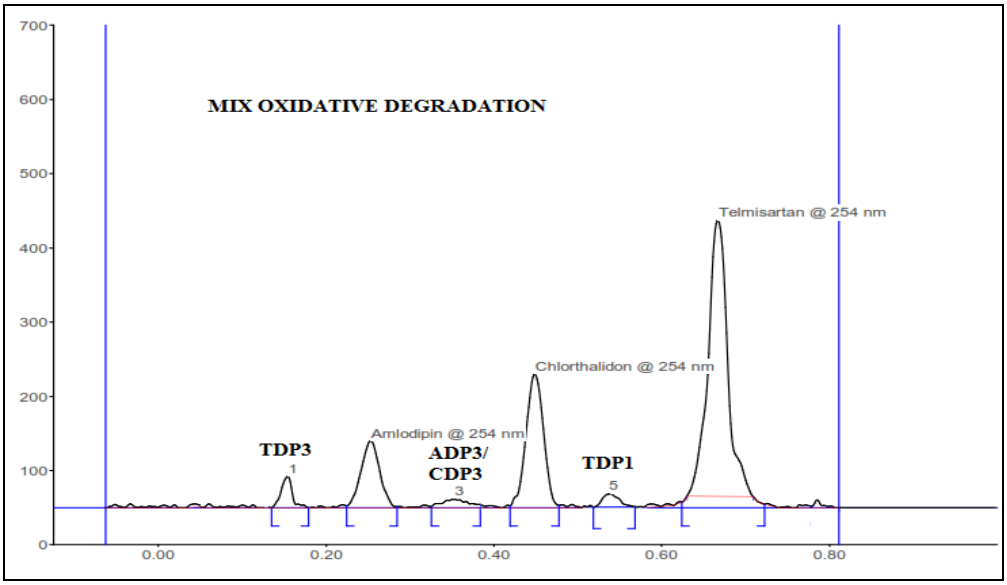

Figure 13: Chromatogram of oxidative degradation of mixture of TEL, AML and CHLO

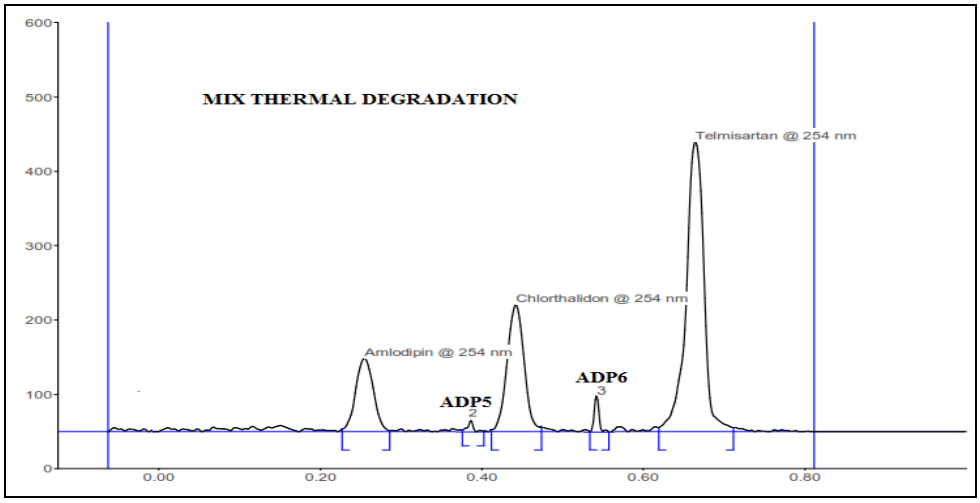

Figure 14: Chromatogram of thermal stress condition of mixture of TEL, AML and CHLO

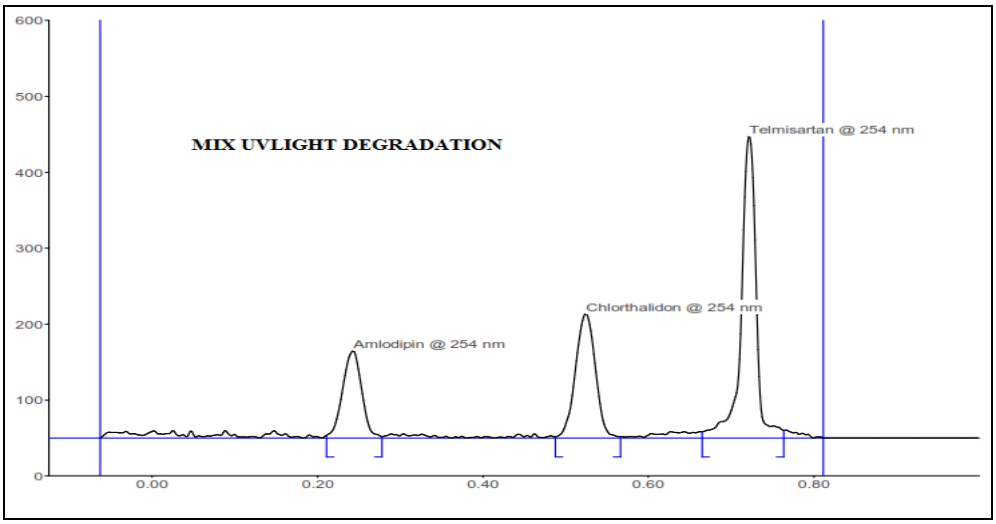

Figure 15: Chromatogram of Photolytic stress condition (UV Light) of mixture of TEL, AML and CHLO

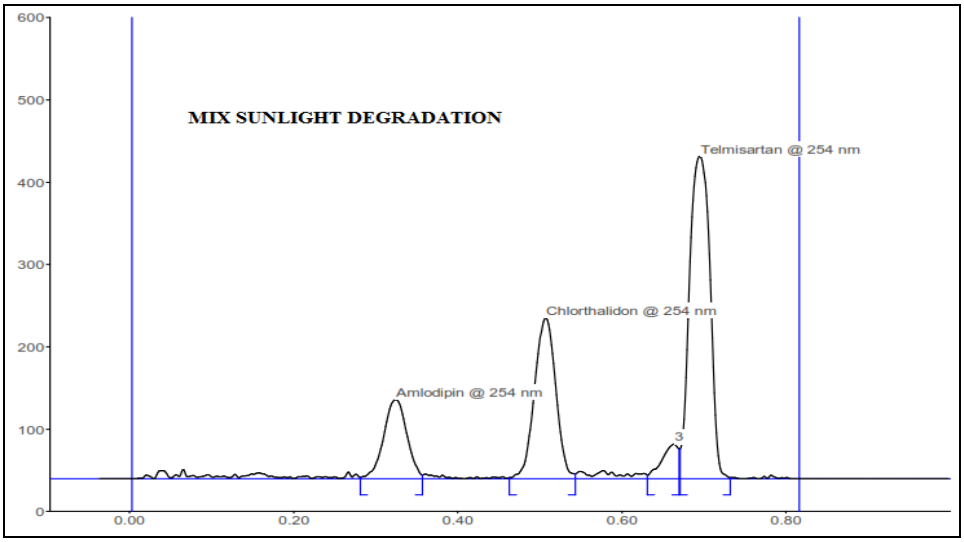

Figure 16: Chromatogram of Photolytic stress condition (Sun Light) of mixture of TEL, AML and CHLO

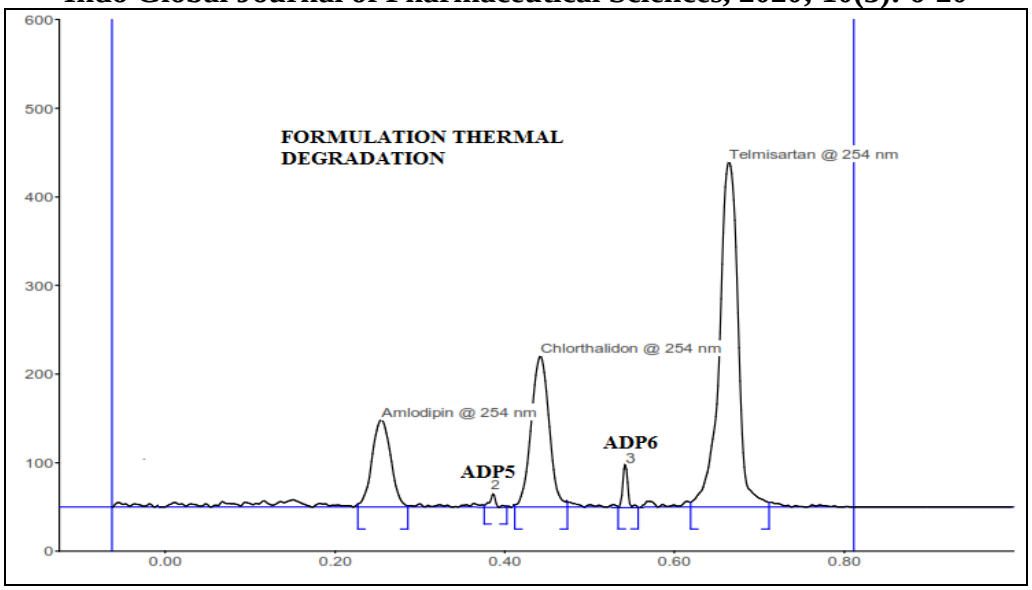

Figure 17: Chromatogram of thermal degradation of test solution of TEL, AML and CHLO

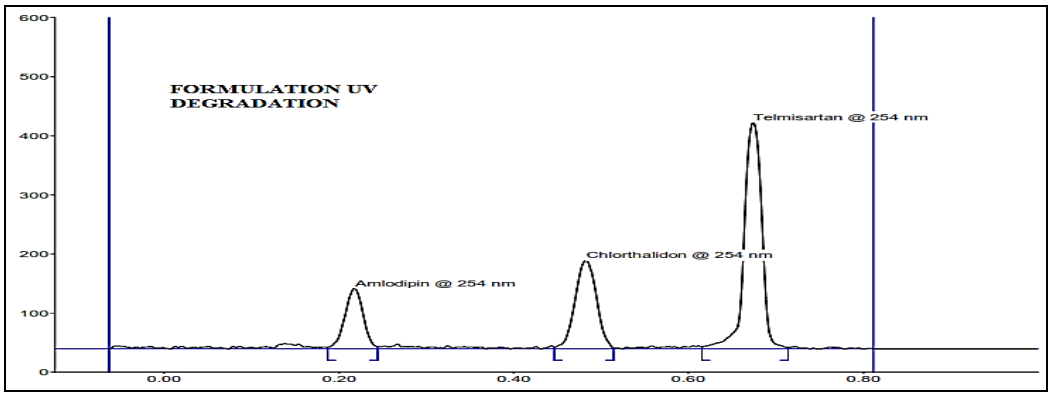

Figure 18: Chromatogram of Photolytic (UV Light) degradation of test solution of TEL, AML and CHLO

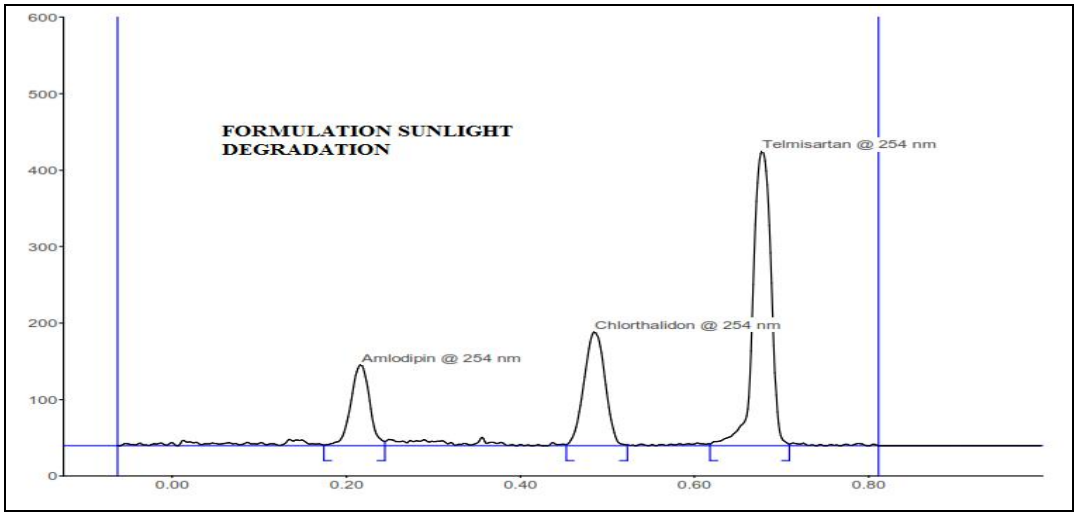

Figure 19: Chromatogram of Photolytic (Sun Light) degradation of test solution of TEL, AML and CHLO

The purity of the drug (analyte peak) was ascertained by analyzing the spectrum at peak start, peak end and peak apex position which showed no interference of any excipients or process impurities in analyte peak. The method is also deemed to be specific from potential degradation products as most of the degradation products are adequately resolved from all

three analyte peaks (with exception of peaks found at Rf of 0.5, 0.65 (AML); 0.25, 0.69 (CHLO); 0.55, 0.73, 0.61 (TEL). The impurities that could not be resolved in mixture of 3 drugs correspond to impurities found under acidic degradation, alkaline hydrolysis and oxidative degradation. These impurities are less likely to be formed in the tablet solid dosage form. The sample tablet was exposed to thermal and photolytic degradation conditions as per standard guidance and like individual drugs, no degradation products were found despite about 10 % degradation of drug content. Therefore, the method can be considered to be stability indicating for tablet solid dosage form **(Figure 10-19) (Table 7 & 8).**

CONCLUSION

The proposed HPTLC method is precise, specific, linear and accurate for the estimation of TEL, AML, and CHLO in pharmaceutical dosage form without interference from the excipients and potential degradation products in various stress conditions like acid hydrolysis, alkaline hydrolysis, neutral hydrolysis, oxidative, thermal, and photolytic stress conditions. All three drugs showed significant degradation under acidic, alkaline and oxidative stress condition with the exception of TEL under alkaline condition. All three drugs showed moderate degradation under thermal and photolytic stress conditions and the least degradation under neutral hydrolysis (elevated temperature and humidity). The results of stress testing were critically analyzed to establish correlation with degradation products reported in published literature. The developed method is validated as per ICH guidelines. The results showed the suitability of the developed method for degradation kinetic studies and stability studies of the fixed dose combination. A method can also be suitably applied for the estimation of FDC containing two drugs like TEL and CHLO; and AML with CHLO.

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DATA AVAILABILITY

Not declared.

CONFLICTS OF INTEREST

Nil

FUNDING SOURCE

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