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Development of validated stability indicating assay method for the simultaneous estimation of hydrochlorothiazide Amlodipine besylate and Losartan potassium in combine dosage form

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Abstract

A Stability indicating Reverse-Phase liquid chromatographic method for the simultaneous estimation of HCTZ, LOSA and AMLO was developed. The chromatographic assay involves the use of SUPELCO LC-8-DB column (15 cm x 4.6 mm, 5 µm) with a simple mobile phase composition of Buffer (monobasic Potassium Dihydrogen phosphate of 0.025 M having pH 3.7): Acetonitrile (60:40) at a flow rate of 1mL/min with U.V detection at wavelength of 232 nm. The method showed good linearity in the concentration range of 4-40 µg/mL for HCTZ and 2-22 µg/mL for AMLO and 15-150 µg/mL for LOSA. The proposed method was also successfully applied to 20 tablets of marketed formulation (Trilopace). The developed method was successfully validated as per the ICH guidelines for following parameters. Accuracy, precision, ruggedness, robustness, system suitability tests, etc. The RSD for system precision was found to be 0.89-0.49 for HCTZ, AMLO, and LOSA and for method precision 1.0-1.4 for HCTZ, AMLO, and LOSA. The average percentage recoveries 99.75, 99.88, 98.93 for HCTZ, AMLO, LOSA which was in good agreement with labeled amount of Pharmaceutical formulation. The stability indicating capacity was tested by accelerated degradation of marketed formulation in acidic (0.1 N HCL), basic (0.1 N NaOH), , Oxidative (3% H_2O_2), Thermal (80^0C)

Keywords: LOSA, HCTZ, AMLO, Stability Indicating, Force degradation, Assay method

1. Introduction

The technique HPLC is so called because of its improved performance over the classical column chromatography. The technique basically involves the use of porous material as a stationary phase and the liquid mobile phase is pumped into the column under high pressure. The development of this technique is attributed to the small particle size of stationary phase. As the particle size is small the resistance to the flow of mobile phase is very high that is the reason why the high pressure is recommended.[1, 2] The stability indicating assays are defined as validated quantitative analytical methods that can detect the changes with time in the chemical, physical, or microbiological properties of the drug substance and drug product, and that are specific so that the contents of active ingredient, degradation products, and other components of interest can be accurately measured without interference. Stress testing is the main tool that is use to predict stability problems, develop analytical methods, and identify degradation product and pathways. Stress testing is likely to be carried out on single batch of the drug substance. It should include the effect of temperature in 10ºC increments (Eg.50ºC, 60ºC etc). Above that for accelerated testing, humidity (Eg. 75% RH or greater) where appropriate oxidation and photolysis on the drug substance. The testing should also evaluate the susceptibility of the drug substance to hydrolysis across a wide range of pH values when in solution or suspension. Photostability testing should be an integral part of stress testing. The review of literature[9-13, 17-19] suggested that very few stability indicating assay method for the above combination has been reported so the present work is undertaken with following objectives The present work is undertaken with an objective to develop economical, simple, accurate, precise and reproducible stability indicating assay method for estimation of these drugs in their combined dosage form. Amlodipine besylate [Figure 1] chemically is 3-ethyl 5-methyl 2-[(2-aminoethoxy) methyl]-4- (2-chlorophenyl)-6- methyl-1,4-dihydropyridine-3,5-dicarboxylate. It is a white to off-white crystalline powder and Solubility Slightly soluble in water, freely soluble in Methanol, sparingly soluble in ethanol. While the losartan potassium [figure 2] chemically is (2-butyl-4-chloro-1-{[2'-(1*H*-tetrazol-5-yl) biphenyl-4-yl]methyl}-1*H*-imidazol-5-yl)methanol. It is white to off-white free flowing crystalline powder and it is freely soluble in water, soluble in alcohols, and slightly soluble acetonitrile and methyl ethyl ketone.

While hydrochlorothiazide is [Figure 3] chemically is 6-chloro-1,1-dioxo-3,4-dihydro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide and it is white crystalline powder and it is slightly soluble in Water methanol and Soluble in Acetone[14-16, 5].

Figure 1: Chemical structure of amlodipine besylte

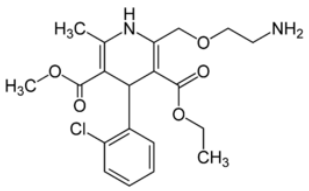

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Figure 2: Chemical structure of losartan potassium

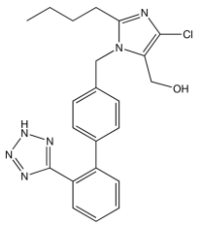
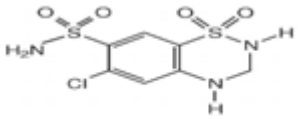


Figure 3: chemical structure of hydrochlorothiazide



2. Experimentals

Chemicals and reagents: The gift sample of Hydrochlorothiazide, Amlodipine besylate and Losartan Potassium was provided by ZIM Laboratories, Nagpur. The tablet formulation of Hydrochlorothiazide, Amlodipine besylate and Losartan Potassium was purchased from the local market. All the chemicals used of HPLC Grade (Merk Ltd., Mumbai) and double distilled water was used for mobile phase preparation.

Instrument: An HPLC system of shimadzu with pump- LC 2010 CHT and Detector- PDA with Software- LC Solution (Shimadzu) with column of SUPELCO LC-8-DB (15 cm x 4.6 mm, 5 µm) and isocratic elution is performed using Buffer (monobasic Potassium Dihydrogen phosphate of 0.025 M having pH 3.7) : Acetonitrile (60: 40) as mobile phase. At flow rate of 1 ml/min at detection wavelength of 232 nm.

Preparation of Mobile Phase: Prepare a mixture of Acetonitrile of HPLC grade and Buffer in 40:60 ratio respectively. This was selected as common solvent for preparation of stock solution and further dilutions from stock solutions were made in the same mixture of Acetonitrile and Buffer in the ratio 40:60.

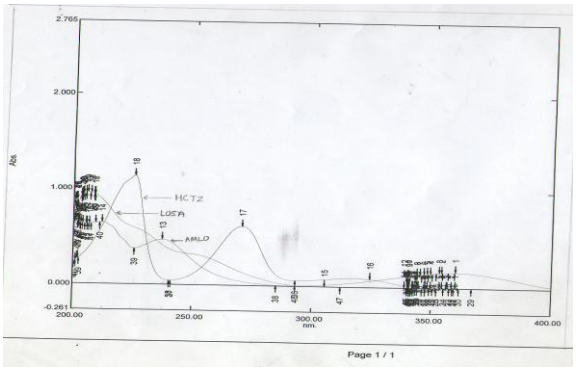
Preparation of Buffer: Dissolve 3.4 gms of monobasic Potassium Dihydrogen phosphate in 1000 ml of HPLC water, and adjust pH to 3.7 with Ortho Phosphoric Acid.

Preparation of Standard Solution: Weigh accurately 31.25 mg of Hydrochlorothiazide, 17.36 mg of Amlodipine Besylate equivalent to Amlodipine 12.5 mg and 125 mg of Losartan Potassium working standard in a 100ml volumetric flask and dissolve by sonication in sufficient mobile phase then make up the volume by mobile phase.

Preparation of working standard solution: Dilute 5.0 ml of this solution to 50 ml with mobile phase to get the working concentration of amlodipine besylate, losartan potassium and hydrochlorothiazide.

Selection of detection wavelength: from the overlain spectra the detection wavelength selected was 232 nm.

Figure 4: overlain spectra of LOSA, HCTZ, and AMLO



Linearity study: From the standard stock solution sufficient volume of aliquots were transferred to 10 ml volumetric flask to get the concentration in range of 4-40µg/ml for HCTZ, 2-22 µg/ml for AMLO and 15-150 µg/ml for LOSA. Volume of 20µl of each sample was injected with the help of Hamilton Syringe. All measurements were repeated five times for each concentration and calibration curve was constructed by plotting the peak area versus the drug concentration.

Analysis of Marketed formulation: Weighed accurately 20 tablets and determine the average weight then crushed the tablets into fine powder. Transfer powder equivalent to 125 mg Losartan Potassium in 100 ml volumetric flask and dissolve by sonication for 10 minutes and make up the volume to 100 ml with mobile phase stir the solution for 30 minutes. Centrifuge and dilute 5.0 ml of this solution to 50.0 ml with mobile phase. Equal volume (20µL) of standard and sample solution was injected separately after equilibrium of stationary phase. The chromatograms were recorded and the response i.e. peak area of major peaks were measured. The content of Hydrochlorothiazide, Amlodipine Besylate and Losartan Potassium were calculated by comparing a sample peak with that of standard.

Method validation[6]:

1. Accuracy: It was done by recovery study using standard addition method at 80%, 100% and 120% level; known amount of AMLO, HCTZ and LOSA standard was added to preanalysed sample and subjected to the proposed HPLC method. The percent recovery was then calculated by using following formula

$$\% \text{ Recovery} = \frac{E_w - B}{C} \times 100$$

Where, E_w = Total drug estimated (mg)

B= Amount of drug contributed by preanalysed capsule powder (mg)

C= Weight of pure drug added (mg). **2**.

Precision:

2.1 System precision: System Precision was determined by taking five replicate injections of Standard solution into HPLC system. Weigh accurately 31.25 mg of Hydrochlorothiazide, 17.36 mg of Amlodipine Besylate equivalent to Amlodipine

12.5 mg and 125 mg of Losartan Potassium working standard in a 100ml volumetric flask and dissolve by sonication in sufficient mobile phase then make up the volume by mobile phase. Dilute 5.0 ml of this solution to 50 ml with mobile phase i.e; 31.25 mcg/ml of Hydrochlorothiazide 17.36 mcg/ml of Amlodipine Besylate and 125 mcg/ml of Losartan Potassium.

2.2 Method precision: Method Precision was determined by six sample solution of Hydrochlorothiazide Amlodipine Besylate and Losartan Potassium Tablets were prepared and analyzed on the same day by HPLC method.

2.3 Intermediate precision (Ruggedness): Six sample solution of HCTZ, AMLO and LOSA Tablets was prepared as per described method and analyzed by different analyst using same make of different HPLC column. The percentage label claim of HCTZ, AMLO and LOSA in HCTZ, AMLO and LOSA tablet was calculated and reported along with the standard deviation (sample) and % RSD of the six samples.

3. Specificity: Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present in the formulation.

Placebo Interference study: The solution of analytical placebo (containing all the excipients except HCTZ, AMLO and LOSA) was prepared according to the sample preparation procedure and injected in to HPLC. To identify the interference by these excipients, Standard solution and Marketed formulation of HCTZ, AMLO and LOSA were analyzed by the developed method.

Placebo Preparation: Weigh accurately 571.68 mg of Placebo in a 100ml volumetric flask and dissolve by sonication in sufficient mobile phase then make up the volume by mobile phase. Dilute 5 ml of this solution to 50 ml with mobile phase and mix. Filter the solution through 0.45µm filter and inject the clear filtrate.

Standard solution preparation: Weigh accurately 31.25 mg of HCTZ, 17.36 mg of AMLO equivalent to 12.5 mg of AMLO and 125 mg of LOSA working standard in a 100ml volumetric flask and dissolve by sonication in sufficient mobile phase then make up the volume by mobile phase. Dilute 5.0 ml of this solution to 50 ml with mobile phase i.e 31.25 mcg/ml of Hydrochlorothiazide 17.36 mcg/ml of AMLO and 125 mcg/ml of Losartan Potassium.

Sample solution preparation: Weighed accurately 20 tablets and determine the average weight then crushed the tablets into fine powder. Transfer powder equivalent to 125 mg Losartan Potassium in 100 ml volumetric flask and dissolve by sonication for 10 minutes and make up the volume to 100 ml with mobile phase stir the solution for 30 minutes. Centrifuge and dilute 5.0 ml of this solution to 50.0 ml with mobile phase and mix. Filter the solution through 0.45µm filter and inject the clear filtrate.

4. Linearity and Range: For Drug release determination the concentration of Hydrochlorothiazide, Amlodipine Besylate and Losartan Potassium is 31.25 mcg/ml, 17.36 mcg/ml and 125 mcg/ml respectively. So the working Range of analytes was set between 18 mcg/ml to 43 mcg/ml for Hydrochlorothiazide, 10 mcg/ml to 24 mcg/ml for Amlodipine Besylate and 75 mcg/ml to 175 mcg/ml for Losartan Potassium to show the Linearity standard solutions 18,25,31,37 and 43 mcg/ml for Hydrochlorothiazide, 10,13,17,20 and 24 mcg/ml for Amlodipine Besylate and 75, 100, 125, 150 and 175 mcg/ml for Losartan Potassium corresponding to approximately 60 % to 140 % of the test concentration where prepared as per described method.

5. Robustness: The tablet sample of HCTZ, AMLO and LOSA was analyzed using proposed method after a deliberate change in detection wavelength for estimation by ±2 nm.

6. Stability of Analytical Solution: Prepare standard and sample solutions as per the method and Inject one standard and sample preparation initially at 0 hr and after specified time-intervals i.e after 4hrs, 8 hrs and 12 hrs Monitor the pattern of chromatogram at the pre-determined intervals and compare it against the initial pattern. Calculate the assay at each time interval. The stability of analytical solution is checked for Drugs up to 12 hrs**.**

7. System Suitability Study: Weigh accurately 31.25 mg of Hydrochlorothiazide, 17.36 mg of Amlodipine Besilate equivalent to Amlodipine 12.5 mg and 125 mg of Losartan Potassium working standard in a 100ml volumetric flask and dissolve by sonication in sufficient mobile phase then make up the volume by mobile phase. Dilute 5.0 ml of this solution to 50 ml with mobile phase.

Force Degradation Studies[4]:

Preparation of Sample Solution Stock: Weighed accurately 20 tablets and determine the average weight then crushed the tablets into fine powder. Transfer powder equivalent to 125 mg Losartan Potassium in 100 ml volumetric flask and dissolve by sonication for 10 minutes and make up the volume to 100 ml with mobile phase stir the solution for 30 minutes. Centrifuge and filter the solution through 0.45 µm membrane filterate dilute 5.0 ml of this solution to 50.0 ml with mobile phase.

1. Acid Degradation: 5 ml of sample stock solution was pipette out in 50 ml Volumetric flask and it was subjected to acid stress degradation by treating the sample with 5 ml of 0.1 N HCL and the solution was kept at room temperature for 8 hour. Neutralize with 0.1 N NAOH and make up the volume to 50 ml with mobile phase and injected into the HPLC system.

2. Base Degradation: 5 ml of sample stock solution was pipette out in 50 ml Volumetric flask and it was subjected to Alkali stress degradation by treating the sample with 5 ml of 0.1 N NAOH and the solution was kept at room temperature for 8 hour. Neutralize with 0.1 N HCL and make up the volume to 50 ml with mobile phase and injected into the HPLC system.

3. Oxidative Degradation: 5 ml of sample stock solution was pipette out in 50 ml Volumetric flask

and it was subjected to Peroxide stress degradation by treating the sample with 5 ml of 3% H_2O_2 and the solution was kept at room temperature in dark area for 8 hour and make up the volume to 50 ml with mobile phase and injected into the HPLC system.

4. Thermal Degradation: 5 ml of sample stock solution was pipette out in 50 ml Volumetric flask, make up the volume to 50 ml with mobile phase and then the solution was subjected to Thermal stress degradation by heating the sample on boiling water bath at 80ºc for 8 hours and injected into the HPLC system.

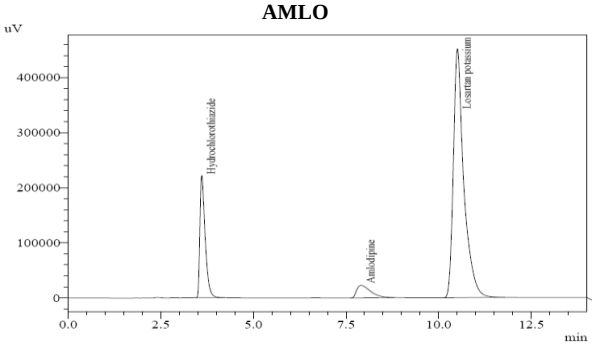
5. Photo Degradation: Photo degradation studies were carried out on solid dosage form (fine Tablet powder). The sample in a petri plate was spread as a thin layer (1 mm) and exposed to direct UV radiation for 24 hours in UV chamber. The withdrawn samples were dissolving and then diluted with mobile phase as per sample preparation.

3. Results and Discussion

HPLC method development and optimization: The finally optimized chromatographic conditions are.

Column	SUPELCO LC-8-DB column (15 cm x 4.6 mm, 5 µm)
Mobile Phase	Buffer (monobasic Potassium Dihydrogen phosphate of 0.025 M having pH 3.7) : Acetonitrile (60: 40)
Detection wavelength	232 nm
Injection volume	20 µl
Flow rate	1 ml/min
Temperature	30°C

Figure 5: Optimized chromatogram of HCTZ, LOSA and AMLO



2. Linearity

Table 1: Results of Linearity studies of LOSA, HCTZ and AMLO

Sr. No.	Concentration in µg/ml			Peak Area		
	HCTZ	AMLO	LOSA	HCTZ	AMLO	LOSA
1	4	2	15	271651	74553	1094766
2	8	4	30	543422	148106	2289533
3	12	6	45	825361	223659	3284299
4	16	10	60	1087011	288213	4279066
5	20	12	75	1353750	372766	5473832
6	24	14	90	1578502	446319	6668599
7	28	16	105	1903233	521872	7663365
8	32	18	120	2248012	595426	8658132
9	36	20	135	2485754	670979	9852898
10	40	22	150	2817505	747827	10847665
Y						
Correlative Coefficient (r^2)						NLT: 0.995

Figure 5: Calibration curve of HCTZ

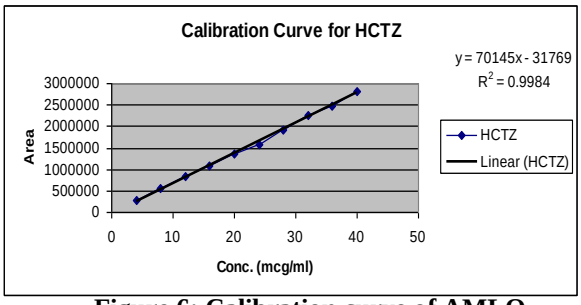


Figure 6: Calibration curve of AMLO

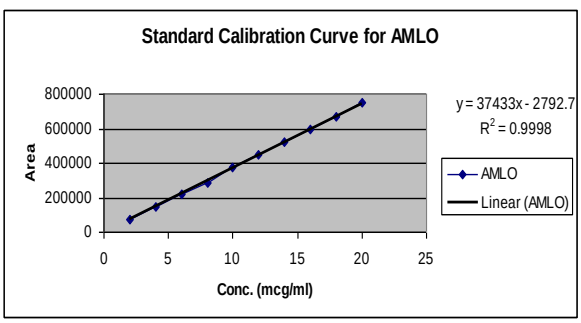


Figure 7: Calibration curve of LOSA

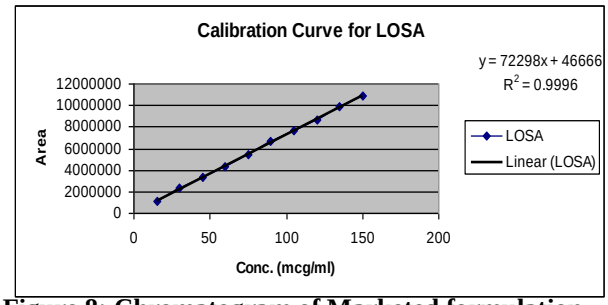
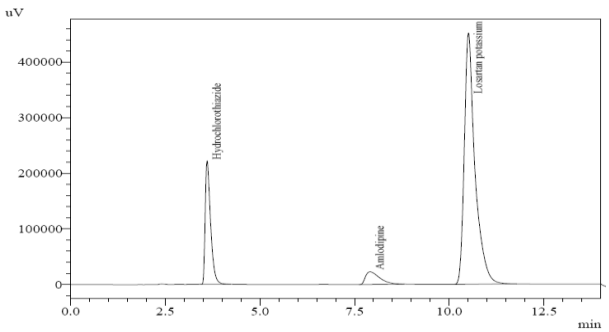


Figure 8: Chromatogram of Marketed formulation



Analysis of Marketed Formulation: The marketed formulation was Trilopace manufactured by sun pharmaceuticals.

Content:

1) Hydrochlorothiazide : 12.5 mg
2) Amlodipine Besylate : 5 mg
3) Losartan Potassium : 50 mg

Average weight : 292.2mg

Table 2: Results of marketed formulation analysis

Sr. No	Weight of std.(mg)			Weight of sample (mg)	Peak area of standard			Peak area of sample			% Label claim		
	HCTZ	AMLO	LOSA		HCTZ	AMLO	LOSA	HCTZ	AMLO	LOSA	HCTZ	AMLO	LOSA
1	32	17.5	125.9	738.7	2171510	596426	8778331	2118090	590519	8660513	98.27	98.69	98.56
2				736.0				2111385	588360	8648859	98.19	99.39	98.07
3				730.8				2125548	584203	8617588	98.62	98.69	98.41

Method Validation:

1. Accuracy:

Table 3: Recovery studies of HCTZ

% Level of Standard	Weight of Working Standard (mg) added its dilution (ml)	Area at 232 nm	Standard Recovered (mg)	% Recover	Mean Recover	% RSD
80	25.0/100/5/50	1713040	25.1175	100.47	99.75	0.6227
		1703150				
		1723146				
100	31.3/100/5/50	2141300	31.1028	99.37		
		2101299				
		2121301				
`120	37.6/100/5/50	2569560	37.3819	99.42		
		2529438				
		2549558				

Table 4: Recovery studies of AMLO

% Level of Standard	Weight of Working Standard (mg) added its dilution (ml)	Area at 232 nm	Standard Recovered (mg)	% Recover	Mean Recover	% RSD
80	13.9/100/5/50	472415	13.9105	100.07	99.88	0.3589
		475321				
		477252				
100	17.4/100/5/50	590519	17.3091	99.47		
		591810				
		590807				
120	20.9/100/5/50	708622	20.9230	100.11		
		717360				
		717358				

Table 5: Recovery studies of LOSA

% Level of Standard	Weight of Working Standard (mg) added and its dilution (ml)	Area at 232 nm	Standard Recovered (mg)	% Recover	Mean Recover	% RSD
80	100/100/5/50	6928410	98.9399	98.93	98.93	0.0058
		6917269				
		6937271				
100	125/100/5/50	8660513	123.6754	98.94		
		8654189				
		8664088				
120	150/100/5/50	10392615	148.4099	98.93		
		10389897				
		10391908				

2. Precision:

2.1 System precision: The results indicate that there is no variation in area counts of five consecutive injections. The RSD values are less than 2.0 % and are well within the limits.

Table 6: System precision data of HCTZ, AMLO and LOSA

Injection No.	Area at 232 nm for Hydrochlorothiazide	Area at 232 nm for Amlodipine Besylate	Area at 232 nm for Losartan Potassium	Limit
1	2191200	597904	8834082	
2	2189471	600107	8815351	
3	2174004	596944	8758132	
4	2151882	592328	873672	NMT 2.0 %
5	2150994	594847	8748020	
Mean	2171510	596426	8778331	
+_ SD	19511	2970	43564	
% RSD	0.898	0.498	0.496	

2.2 Method Precision: Six sample weights of Hydrochlorothiazide, Amlodipine Besylate and Losartan Potassium Tablets by same analyst in same laboratories on same day. The analysis results are ranging from 99.07 to 99.34 for Hydrochlorothiazide, 100.97 % to 101.14 % for Amlodipine Besylate and 100.05 % to 100.15 % for Losartan Potassium. The % deviation from mean Value for six samples is less than 2.0 %.

Table 7: Results of method precision

Sr. No.	Sample Weight (g)	Area at 232			% Assay			% Deviation Form Mean Assay Value		
		HCTZ	AMLO	LOSA	HCTZ	AMLO	LOSA	HCTZ	AMLO	LOSA
1	0.7315	2102187	593360	8698271	98.50	100.14	98.27	-0.021	-0.43	+1.69
2	0.7313	2099371	592104	8684515	98.39	99.96	99.11	-0.1	-0.25	+0.85
3	0.7311	2124008	600844	8808122	99.57	99.65	100.55	-1.28	+0.06	-0.59
4	0.7317	2101872	593338	8706062	98.45	100.11	99.30	-0.16	-0.4	+0.66
5	0.7319	2060974	583857	8547020	96.51	98.47	97.46	+1.78	+1.24	+2.5
6	0.7322	2100738	592951	8692276	98.33	99.98	99.08	-0.04	-0.27	+0.88
Mean					98.29	99.71	99.96			
± SD					0.9887	0.6358	1.040			
% RSD					1.0059	0.6376	1.0402	NMT 2.0 %		

2.3 Intermediate Precision (Ruggedness): The result are ranging from 98.46 % to 101.21 % for Hydrochlorothiazide, from 99.51 % to 99.98 % for Amlodipine Besylate and from 98.53 % to 99.17 % for Losartan Potassium the % deviation from mean assay value for six samples are not more than 2.0 %.

Table 8: Results of Intermediate precision

Sr. No.	Sample Weight (g)	Area at 232			% Assay			% Deviation Form Mean Assay Value		
		HCTZ	AMLO	LOSA	HCTZ	AMLO	LOSA	HCTZ	AMLO	LOSA
1	0.7310	2116832	589218	8630050	99.25	99.51	98.53	+0.27	+0.29	+0.43
2	0.7308	2099349	591821	8660513	98.46	99.98	98.91	+1.06	-0.18	+0.05
3	0.7315	2108090	590519	8690976	98.77	99.66	99.16	+0.75	+0.14	-0.20
4	0.7320	2131877	592201	8698271	99.82	99.88	99.17	-0.3	-0.08	-0.21
5	0.7318	2127310	591321	8650361	99.63	99.76	98.65	-0.11	+0.04	+0.31
6	0.7321	2161800	593361	8715188	101.21	100.06	99.35	-1.69	-0.26	-0.39
Mean					99.52	99.80	98.96			
+- SD					0.9713	0.2056	0.3224	NMT 2.0 %		
% RSD					0.9759	0.2059	0.3258			

3. Specificity: For the Chromatographic method does not show interference of excipients in chromatogram at the λ-max of the analytes or at the detection wavelength of subject analytes. In this case of HCTZ, AMLO and LOSA the detection wavelength is 232 nm.

Figure 9: Chromatogram of Placebo shows there are no peaks at the retention time of HCTZ, AMLO and LOSA in the chromatograph indicates that there is no placebo interference

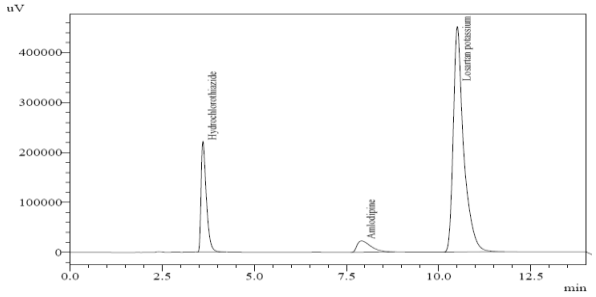


Figure 10: Chromatogram of HCTZ, LOSA and AMLO working standard

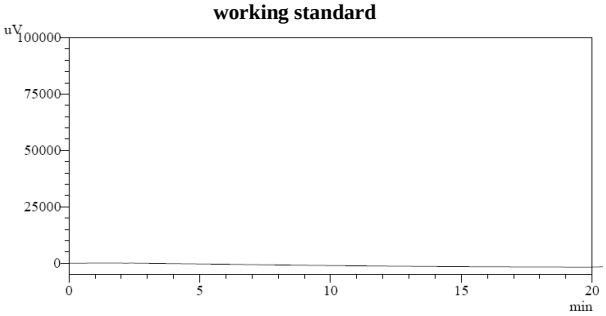
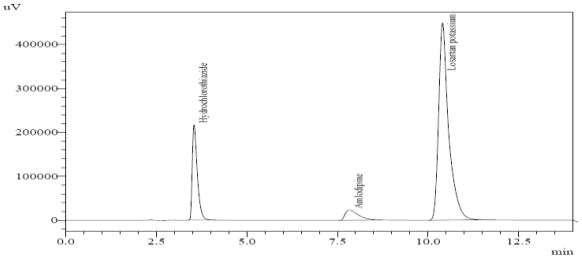


Figure 11: Chromatogram of Marketed formulation



4. Linearity and Range:

Table 9: Linearity and range study of HCTZ

Sr. No.	Weight of Working Standard (mg) added and its dilution (ml)	Concentration (mcg/ml)	Mean Area Count At 232 nm	Limit
1	31.30/100/3/50	18.78	1312999	NMT:0.995
2	31.30/100/4/50	25.04	1737298	
3	31.30/100/5/50	31.30	2181399	
4	31.30/100/6/50	37.56	2604798	
5	31.30/100/7/50	43.82	3141098	
Y			70301 X	
Correlation Coefficient (r^2)			0.9971	

Figure 12: Calibration curve of HCTZ

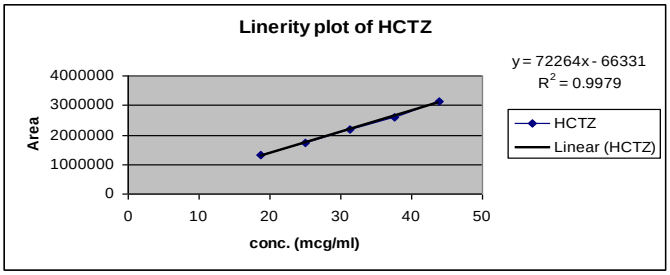


Table 10: Linearity and range study of AMLO

Sr. No.	Weight of Working Standard (mg) added and its dilution (ml)	Concentration (mcg/ml)	Mean Area Count At 232 nm	Limit
1	17.4/100/3/50	10.4	367397	**NMT:0.995**
2	17.4/100/4/50	13.92	477496	
3	17.4/100/5/50	17.40	598596	
4	17.4/100/6/50	20.88	724695	
5	17.4/100/7/50	24.36	843794	
Y			**34610 X**	
Correlation Coefficient (r^2)			**0.9995**	

Figure 13: Calibration curve of AMLO

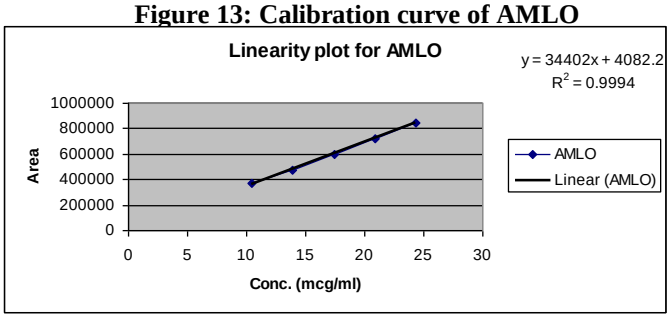
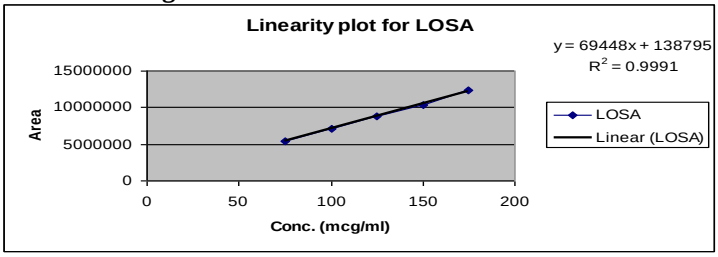


Table 11: Linearity and range study of LOSA

Sr. No.	Weight of Working Standard (mg) added and its dilution (ml)	Concentration (mcg/ml)	Mean Area Count At 232 nm	Limit
1	125/ 100/ 3/ 50	75	5366988	**NMT:0.995**
2	125/ 100/ 4/ 50	100	7124984	
3	125/ 100/ 5/ 50	125	8778981	
4	125/ 100/ 6/ 50	150	10434990	
5	125/ 100/ 7/ 50	175	12392973	
Y			**70476 X**	
Correlation Coefficient (r^2)			**0.9988**	

Figure 14: Calibration curve of LOSA



5. Robustness:

Table 12: Results of Robustness study

Sr. No.	Change in wavelength (± 2 nm)	% Estimation		
		HCTZ	AMLO	LOSA
1	230	98.06	98.51	98.20
2	232	98.36	98.92	98.35
3	234	98.12	98.23	98.28
Mean		98.18	98.55	98.28
±S.D.		0.1587	0.3470	0.0750
%R.S.D.		0.1617	0.3521	0.0763

6. Stability of Analytical solution:

Table 13: Results of stability evaluation for HCTZ

Time (h)	Peak Area of Standard Solution	Peak Area of Sample Solution	% Estimation
0 hrs	2172004	2117080	98.20
4 hrs	2170010	2116900	98.29
8 hrs	2168471	2136765	99.28
12 hrs	2155320	2158520	100.60
Mean			99.09
±S.D.			1.1178
%R.S.D.			1.1280

Table 15: Results of stability evaluation of LOSA

Time (h)	Peak Area of Standard Solution	Peak Area of Sample Solution	% Estimation
Initial	8776321	8660412	98.59
4 Hrs	8774092	8660269	98.61
8 Hrs	8770221	8689649	98.99
12 Hrs	8761532	8638718	98.51
Mean			98.68
±S.D.			0.2143
%R.S.D.			0.2172

Table 14: Results of stability evaluation

Time (h)	Peak Area of Standard Solution	Peak Area of Sample Solution	% Estimation
Initial	596528	590518	98.68
4 Hrs	596489	590764	98.56
8 Hrs	596121	589980	99.68
12 Hrs	595734	588620	100.88
Mean			99.45
±S.D.			1.0774
%R.S.D.			1.0834

Table 16: Results of system suitability for HCTZ

Sr. No.	Area Reproducibility	Retention Time	Tailing Factor (Asymmetry)	Theoretical Plates	Resolution
1	2189472	3.602	1.801	2995.527	-
2	2174882	3.578	1.805	2972.766	-
3	2162726	3.558	1.807	2963.596	-
4	2155432	3.546	1.806	2963.114	-
5	2160294	3.554	1.797	3013.302	-
Mean	2168561	3.567	1.803	2981.661	-
% RSD	0.6322	0.642	0.232	0.739	-
Limit	NMT 2.0 %	NMT 1 %	NMT 2	NMT 2000	NLT 1.5

Table 17: Results of system suitability for AMLO

Sr. No.	Area Reproducibility	Retention Time	Tailing Factor (Asymmetry)	Theoretical Plates	Resolution
1	597906	7.905	1.078	2166.912	13.585
2	596468	7.886	1.069	2181.686	13.607
3	593140	7.842	1.064	2164.146	13.631
4	592081	7.828	1.055	2211.021	13.700
5	591552	7.821	1.069	2207.671	13.700
Mean	594229	7.856	1.067	2186.287	13.645
% RSD	0.4722	0.469	0.787	1.011	0.389
Limit	NMT 2.0 %	NMT 1 %	NMT 2	NMT 2000	NLT 1.5

Table 18: Results of system suitability for LOSA

Sr. No.	Area Reproducibility	Retention Time	Tailing Factor (Asymmetry)	Theoretical Plates	Resolution
1	8834084	10.504	1.613	7959.575	7.217
2	8812217	10.478	1.613	7980.377	7.247
3	8773530	10.432	1.612	7976.077	7.266
4	8750822	10.405	1.619	7966.598	7.265
5	8751663	10.406	1.618	8004.281	7.294
Mean	8784463	10.445	1.615	8778.331	7.258
% RSD	0.4244	0.424	0.202	0.496	0.392
Limit	NMT 2.0 %	NMT 1 %	NMT 2	NMT 2000	NLT 1.5

System Suitability Parameters:

1. Relative standard deviation of the area of analytes peaks in standard chromatograms should not be more than 2.0 %.
2. Theoretical plates of analytes peak in STD chromatograms should not be less than 2000.
3. Tailing Factor (Asymmetry) of analytes peaks in Standard Chromatograms should less than 2.

Force Degradation Studies:

1. Acid Degradation:

Figure 15: Chromatogram of Acid degradation.

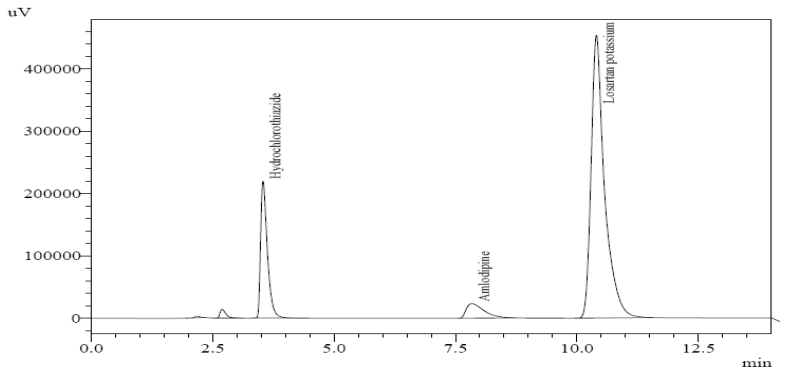


Table 19: Results of Acid degradation

Stress Condition	Drugs	% Degrdation	% Assay of Drugs after degradition
Acid degradation: 5 ml of 0.1N HCL at room temp. for 1 hr.	HCTZ	6.21	93.79
	AMLO	5.74	94.26
	LOSA	5.30	94.70

2. Base Degradation:

Figure 16: Chromatogram of Base Degradation

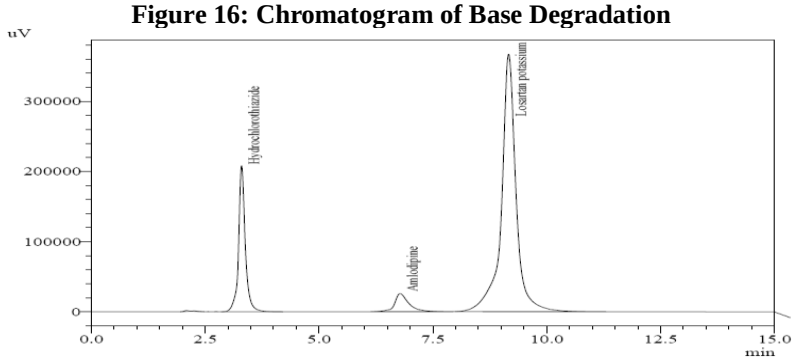


Table 20: Results of Base degradation

Stress Condition	Drugs	% Degrdation	% Assay of Drugs after degradition
Alkali degradation : 5 ml of 0.1N HCL at room temp. for 1 hr**.**	HCTZ	6.21	93.79
	AMLO	5.74	94.26
	LOSA	5.30	94.70

3. Oxidative Degradation:

Figure 17: Chromatogram of oxidative degradation

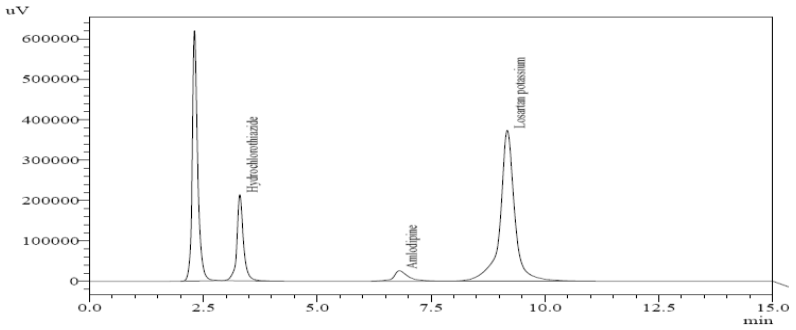


Table 21: Results of oxidative degradation

Stress Condition	Drugs	% Degrdation	% Assay of Drugs after degradition
Oxidative degradation: 5 ml of 3% H_2O_2 at room temperature for 1 hr.	HCTZ	8.24	91.76
	AMLO	7.76	92.24
	LOSA	5.68	94.32

4. Thermal Degradation:

Figure 18: Chromatogram of thermal degradation

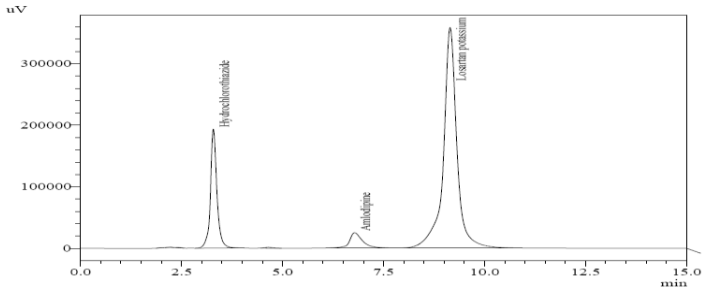


Table 22: Results of thermal degradation

Stress Condition	Drugs	% Degrdation	% Assay of Drugs after degradition
Thermal degradation 80^0c for 8 hrs	HCTZ	8.24	91.76
	AMLO	7.76	92.24
	LOSA	5.68	94.32

5. Photo Degradation:

Figure 19: Chromatogram of Photo degradation

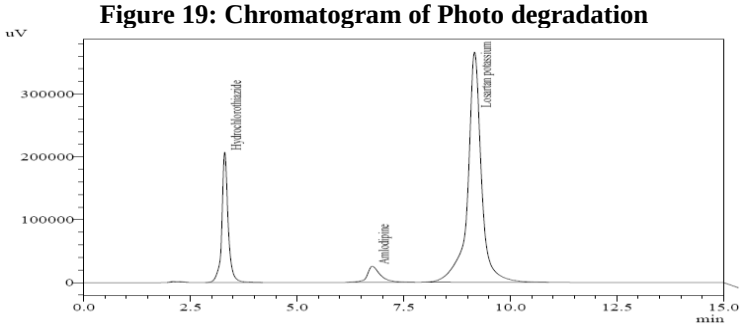


Table 23: Results of Photo degradation

Stress Condition	Drugs	% Degradation	% Assay of Drugs after degradition
Photo degradation: Solid dosage form exposed to direct UV radiation for 24 hours in UV chamber.	HCTZ	7.31	92.69
	AMLO	6.84	93.16
	LOSA	5.37	94.63

4. Conclusion

The proposed method was validated as Per the ICH Guidelines. The proposed method also showed the good resolution between HCTZ, AMLO and LOSA with run time of 10.5 min. The method is very simple and rapid and no where involves complicated sample preparation and mobile phase preparation. Also the proposed method showed good

specificity and selectivity in order to determine HCTZ, AMLO and LOSA in the presence of their degradation products. The linearity and reproducibility data of the drugs carried out by this method showed that no major interference is caused in the estimation of the drugs. Therefore the method can be use for routine quality control of these drugs.

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