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DEVELOPMENT AND VALIDATION OF CHROMATOGRAPHIC AND SPECTROPHOTOMETRIC METHOD FOR SIMULTANEOUS ESTIMATION OF AMLODIPINE BESILATE AND BISOPROLOL FUMARATE IN TABLET DOSAGE FORM.

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ABSTRACT
A simple, rapid, precise and accurate stability-indicating RP-HPLC method were developed and validated for the simultaneous determination of Amlodipine Besilate and Bisoprolol Fumarate in pharmaceutical dosage form. Method include Agilent C18 (250mm * 4.6 mm, 5µm) column and Acetonitrile: Methanol: 50 mM Potassium Dihydrogen Phosphate (25:25:50% v/v/v) as mobile phase at 1.0 ml/min flow rate. Detection was carried out at 274nm. Rt was found to be 9.275 min for Amlodipine Besilate and 4.808 min for Bisoprolol Fumarate. For stability study drugs were subjected to acid hydrolysis, alkaline hydrolysis, oxidative degradation and thermal degradation. Amlodipine Besilate and Bisoprolol Fumarate both was highly susceptible to oxidative condition. Pharmaceutical dosage form was more stable than Active pharmaceutical ingredient. The linearity of the proposed method was investigated in the range of 100-500µg/ml (r2= 0.999) for Amlodipine Besilate and 100- 500µg/ml (r2= 0.999) for Bisoprolol Fumarate. The limit of detection were 4.0096µg/ml and 2.0388 µg/ml and the limit of quantification were 12.1504 µg/ml and 6.1738 µg/ml for Amlodipine Besilate and Bisoprolol Fumarate respectively.

KEYWORDS: Amlodipine Besilate, Bisoprolol Fumarate, Stability Indicating RP-HPLC method, Forced degradation Studies, Simultaneous Equation Method.

MATERIALS AND METHODS
Amlodipine Besilate (RS)-3-ethyl-5-methyl-2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)- 6-methyl-1,4-dihydropyridine-3,5-dicarboxylate. It is calcium channel blocker and used for Treatment of Hypertension. It is official in Indian pharmacopeia 2010. It is freely soluble in ethanol and methanol. Molecular weight of Amlodipine Besilate is 567.05 gm/mol and formula is $C_{26}H_{31}ClN_2O_8S$.

Bisoprolol Fumarate is chemically (RS)-1-{4-[(2-isopropoxy ethoxy) methyl] phenoxy}-3-(isopropylamino) propan-2-ol. B1-Adrenergic receptor antagonist. It is official in United States Pharmacopeia 29NF30. It is freely soluble in ethanol and methanol. Molecular weight of Amlodipine Besilate is 766.96 gm/mol and formula is $C_{22}H_{35}NO_8$.

Amlodipine Besilate is obtained from Zydus Cadila Healthcare ltd. Vadodara, and Bisoprolol Fumarate is obtained from Mangalam Drugs and Organics ltd. Vapi.

Instrumentation and Chromatographic method
The analysis of the drug was carried out on a Peak HPLC system equipped with a reverse phase Agilent C18 column, peak pump with auto sampler and a detector running on Class-VP Software. The mobile phase consists of Acetonitrile: Methanol: 50 mM Potassium Dihydrogen Phosphate (25:25:50 % v/v/v) and the flow rate were maintained at 1.0 ml/min. The mobile phase was freshly prepared and passed through nylon membrane filter of pore size of 0.45µm and it was degassed by sonicating for 10min. before it was used. The elution was monitored at wavelength of 274 nm with UV detector and the injection volume was 10µl.

Determination of maximum absorbance
The standard solutions of Amlodipine Besilate and Bisoprolol Fumarate were scanned in the range of 200-400 nm against mobile phase as blank. Isobestic point of Amlodipine Besilate and Bisoprolol Fumarate at 274nm. Thus the wavelength selected for the determination of Amlodipine Besilate and Bisoprolol Fumarate was

274nm.

Preparation of stock and standard solutions
Accurately weighed 100mg of Amlodipine Besilate and 100mg of Bisoprolol Fumarate were dissolved in 100 ml volumetric flask containing 100 ml of Methanol which is considered as stock solution. Working standard solution of Amlodipine Besilate and Bisoprolol Fumarate were prepared by making various dilutions of the drug solution from the stock solution. Five sets of the drug solution were prepared in the mobile phase containing 100-500µg/ml of Amlodipine Besilate and 100-500 µg/ml of Bisoprolol Fumarate. Each of this drug solution was injected into the column and the peak area and retention time was recorded.

Assay of marketed formulation (Brand name of tablet – Concor-AM 5)
Twenty tablets were weighed and average weight of a single tablet was calculated. Tablets were crushed and mixed using a mortar and pestle. Then drug sample equivalent to 100mg of Amlodipine Besilate and 100mg of Bisoprolol Fumarate were accurately weighed and transferred into a 100ml volumetric flask and mixed with known amount of methanol and the active pharmaceutical ingredients were extracted into the methanol followed by ultra- sonication and then filtered through a nylon membrane of pore size 0.45µm. The drug sample was diluted by adding methanol to obtain a stock solution of 100µg/ml of Amlodipine Besilate and 100 µg/ml of Bisoprolol Fumarate.

Method validation
The Proposed method was validated according to ICH guidelines. The parameters assessed were linearity, precision, accuracy, LOD and LOQ.

System Suitability
System suitability tests are an integral part of liquid chromatography. They are used to verify that resolution and reproducibility of chromatography system are adequate for the analysis to be done. System Suitability was performed on standard solution and system suitability parameters were calculated at the start of study for each parameter.

Linearity and Range
The linearity was determined at Three levels over the range of 100 - 500 µg/ml Amlodipine Besilate and 100-500 µg/ml Bisoprolol Fumarate. Peak area of above linearity solution preparations were taken at each concentration three times.

Accuracy
Recovery studies were carried out by addition of standard drug to the sample at 3 different concentration levels (80%, 100% and 120%) taking into consideration percentage purity of added bulk drug samples. These solutions were subjected to re-analysis by the proposed method and Results are calculated.

Precision Repeatability Study
Standard solutions of 200, 300, 400 µg/ml Amlodipine Besilate and 200, 300, 400 µg/ml Bisoprolol Fumarate were prepared and chromatograms were recorded. Area was measured of the same concentration solution three times and %RSD was calculated.

Intra-day precision
Mixed solutions containing 200, 300, 400 µg/ml Amlodipine Besilate and 200, 300, 400 µg/ml Bisoprolol Fumarate were analysed three times on the same day % R.S.D was calculated.

Inter-day precision
Mixed solutions containing 200, 300, 400 µg/ml Amlodipine Besilate and 200, 300, 400 µg/ml Bisoprolol Fumarate were analysed on three different days and % R.S.D was calculated.

Limit of Detection and Limits of Quantitation Limit of Detection (LOD)
From the linearity curve equation, the standard deviation (SD) of the intercepts (response) was calculated. The limit of detection (LOD) of the drug was calculated by using the following equation designated by International Conference on Harmonization (ICH) guideline:
LOD = 3.3× Intercept / Slope

Limit of Quantitation (LOQ)
The limit of quantitation (LOQ) of the drug was calculated by using the following equation designated by International Conference on Harmonization (ICH) guideline:
LOQ = 10 × Intercept / Slope

Robustness
The robustness of the method was established by making deliberate minor variations in the following method parameters

a) pH of mobile phase: ± 0.2
b) Flow rate : ± 0.2 ml/min
c) Change in the ratio of component in the mobile phase: ± 2%.

Stability studies
Stability Studies was carried out on the drug in order to check the stability of the drug by providing various stress conditions like acid, base, oxidation and thermal degradation compared with normal conditions. The purpose of force degradation method is to provide evidence that the analytical method is efficient in determination of drug substances in commercial drug product in the presence of its degradation products.

Acidic hydrolysis
Take 2 ml solution of Amlodipine Besilate 1000 µg/ml and Bisoprolol Fumarate 1000 µg/ml, 2 ml of 0.1M HCl was added. The solution was heated for 1 hr at 60°C and transferred to a 10ml volumetric flask, cooled, neutralized by 0.1M NaOH and diluted up to mark with

methanol to get final concentration 100 µg/ml of Amlodipine Besilate and 100 µg/ml of Bisoprolol Fumarate.

Alkaline hydrolysis

Take 2 ml solution of Amlodipine Besilate1000 µg/ml and Bisoprolol Fumarate 1000 µg/ml, 2 ml of 0.1M NaOH was added. The solution was heated for 1 hr at 60°C and transferred to a 10ml volumetric flask, cooled, neutralized by 0.1M HCl and diluted up to mark with methanol to get final concentration 100 µg/ml of Amlodipine Besilate and 100 µg/ml of Bisoprolol Fumarate.

Oxidative degradation

Take 2 ml solution of Amlodipine Besilate1000 µg/ml and Bisoprolol Fumarate 1000 µg/ml, 2 ml 3% H_2O_2 was added at room temperature for 4 hours at 60°C and transferred to a 10ml volumetric flask, cooled diluted up to mark with methanol to get final concentration 100 µg/ml of Amlodipine Besilate and 100 µg/ml of Bisoprolol Fumarate.

Thermal degradation

Take 2 ml solution of Amlodipine Besilate 1000 µg/ml and Bisoprolol Fumarate 1000 µg/ml, heat the solution for 2 hr at 80°C and transferred to a 10ml volumetric flask, cooled diluted up to mark with methanol to get final concentration 100 µg/ml of Amlodipine Besilate and 100 µg/ml of Bisorpolol Fumarate.

RESULT AND DISCUSSION

Method development

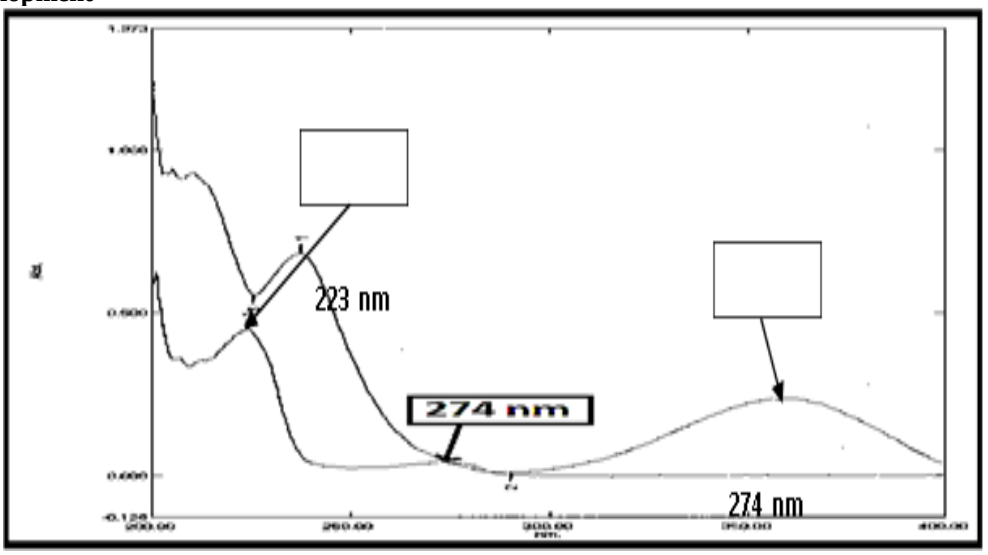

Figure 1 Determination of detection wavelength

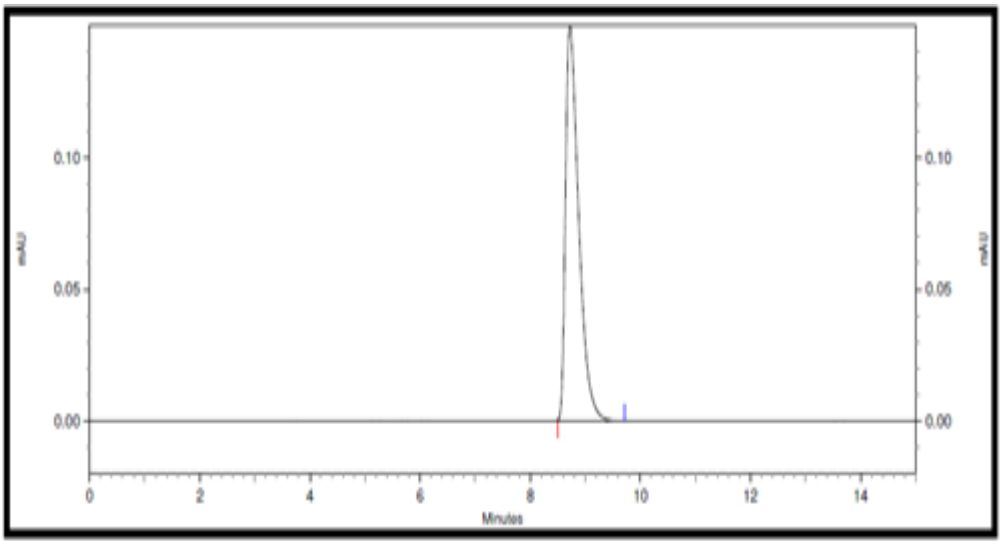

Figure 2 Chromatogram of Amlodipine Besilate

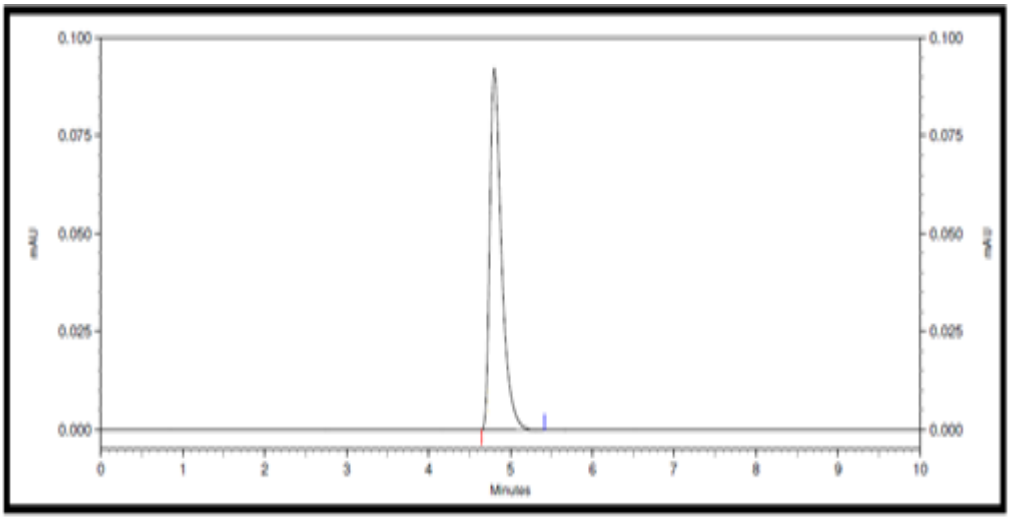

Figure 3 Chromatogram of Bisoprolol Fumarate

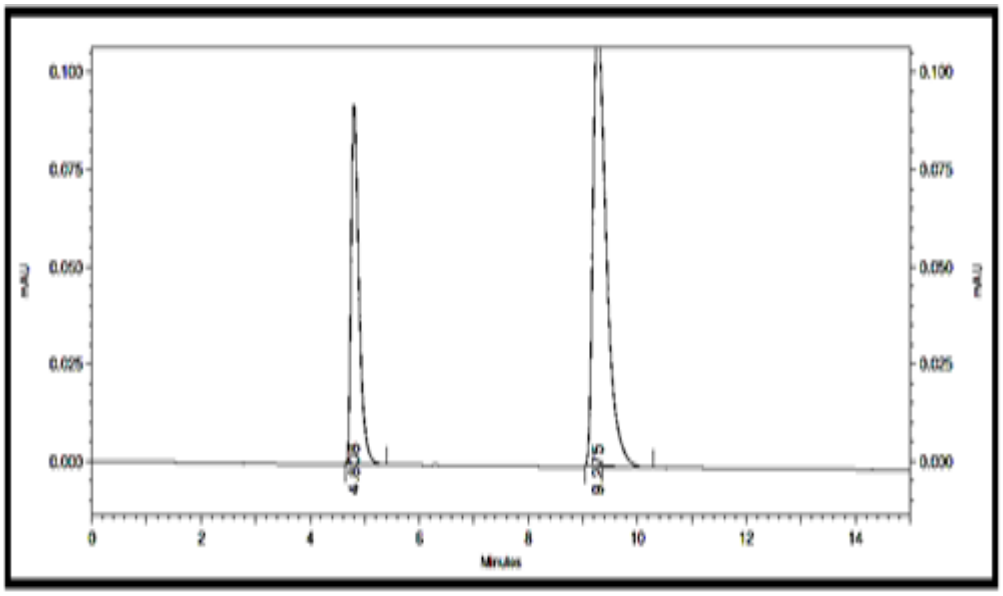

Figure 4 Chromatogram of formulation

Linearity

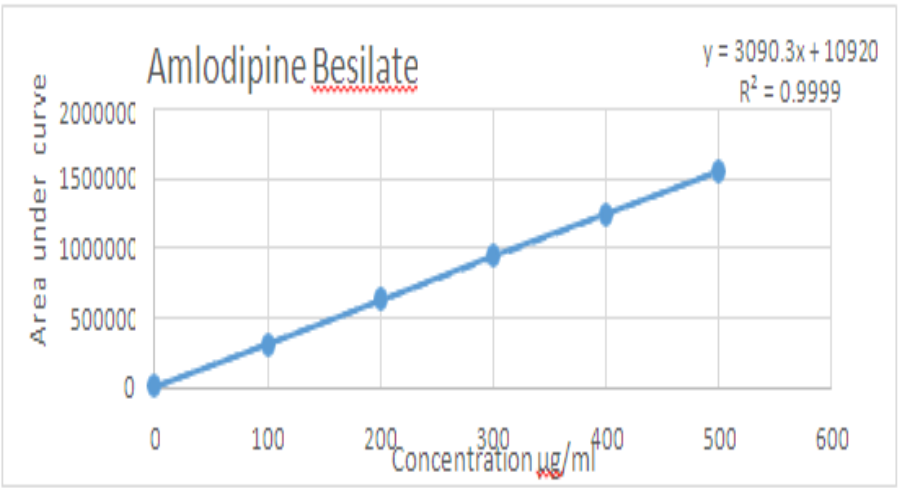

Figure 5 Calibration curve of Amlodipine Besilate

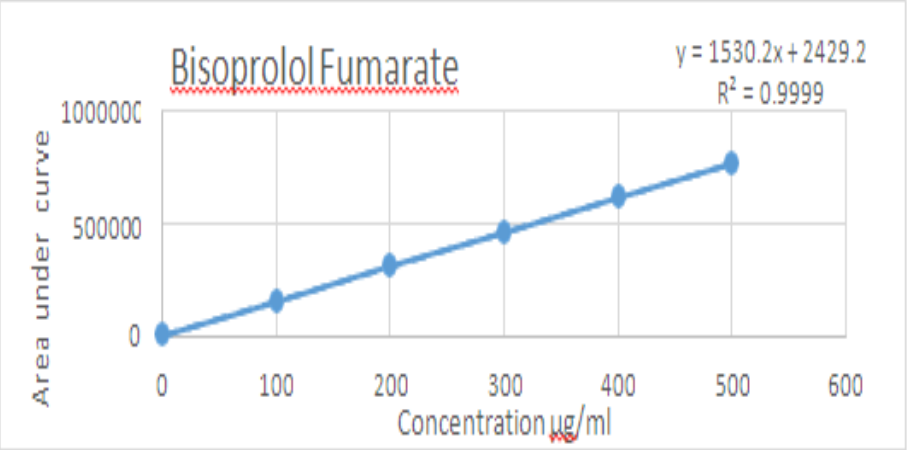

Figure 5 Calibration curve of Bisoprolol Fumarate

Table 1 System suitability parameters

SR. NO.	SYSTEM SUITABILITY PARAMETER	AMLODIPINE BESILATE	BISOPROLOL FUMARATE	SPECIFICATION AS PER IP AND USP 34 NF 29
1	Retention time (min)	9.275	4.808	-
2	Conc. (µg/ml)	300 µg/ml	300 µg/ml	-
3	Resolution (R)	4.4228		More than 1.5
4	Theoretical plate number (N)	4302.832	4770.076	Not less than 2000
5	Tailing factor (T)	1.243	1.124	Not greater than 2

Accuracy

Table 2 Recovery study of Amlodipine Besilate

% ADDED	TARGET CONC., (µg/ml)	SPIKED CONC., (µg/ml)	FINAL CONC., (µg/ml)	CONC., OBTAINED	% RECOVERY	SD	%RSD
80%	100	80	180	179.99	99.998	0.227	0.226
	100	80	180	179.99	99.699		
	100	80	180	180.00	100.00		
100%	100	100	200	199.99	99.599	0.253	0.253
	100	100	200	200.00	100.00		
	100	100	200	200.00	100.00		
120%	100	120	220	219.99	99.999	0.403	0.402
	100	120	220	220.00	100.00		
	100	120	220	219.99	99.299		

Table 3 Recovery study of Bisoprolol Fumarate

% ADDED	TARGET CONC., (µg/ml)	SPIKED CONC., (µg/ml)	FINAL CONC., (µg/ml)	CONC., OBTAINED	% RECOVERY	SD	%RSD
80%	100	80	180	180.0	100.0	0.207	0.204
	100	80	180	179.9	99.99		
	100	80	180	179.9	99.59		
100%	100	100	200	199.9	99.99	0.356	0.354
	100	100	200	200.0	100.0		
	100	100	200	199.9	99.39		
120%	100	120	220	220.0	100.0	0.257	0.253
	100	120	220	219.9	99.99		
	100	120	220	219.9	99.49		

Precision

Table 4 Repeatability study of both the drugs

Amlodipine Besilate			Bisoprolol Fumarate		
Conc. (µg/ml)	Area Mean ± S.D. (n=6)	% RSD	Conc. (µg/ml)	Area Mean ± S.D. (n=6)	% RSD
200	628592±1001.00	0.159245	200	312025.33±1003	0.321448
300	947392.33±1002.50	0.105816	300	460474±640.99	0.139202
400	1251998±5508.96	0.440013	400	615539.3±810.26	0.131635

Repeatability

Intra-day precision

Table 5 Intra-day precision of both the drugs

Amlodipine Besilate			Bisoprolol Fumarate		
Conc. (µg/ml)	Area Mean ± S.D. (n=3)	%RSD	Conc. (µg/ml)	Area Mean ± S.D. (n=3)	%RSD
200	628591.66±1000.5	0.1591	200	312024.66±1001.0	0.320808
300	947392±1002	0.1057	300	460509±609.18	0.132284
400	1251997.33±5508.6	0.4399	400	615406.33±1001.5	0.162738

Inter-day precision

Table 6 Inter-day precision of both the drugs

Amlodipine Besilate			Bisoprolol Fumarate		
Conc. (µg/ml)	Area Mean ± S.D. (n=3)	%RSD	Conc. (µg/ml)	Area Mean ± S.D. (n=3)	%RSD
200	628590.33±1001.5	0.1593	200	312025.33±1003.0	0.32144
300	947391.66±1001.5	0.1057	300	460540.66±575.32	0.12492
400	1251999.33±5506.36	0.4398	400	615406±1002	0.16281

Limit of Detection (LOD) Limit of Quantitation (LOQ)

Table 7 LOD and LOQ of both the drugs

Drugs	LOD (µg/ml)	LOQ (µg/ml)
Amlodipine Besilate	4.0096	12.1504
Bisoprolol Fumarate	2.0388	6.1738

Forced Degradation Studies

Table 8 Forced degradation studies of both the drugs

Stress Condition	% Degradation of API		% Degradation of pharmaceutical dosage form	
	AMLO	BISO	AMLO	BISO
Acid Hydrolysis	7.99	6.40	7.60	6.40
Alkaline Hydrolysis	7.49	8.79	7.50	8.80
Oxidative	16.99	17.99	17.00	18.00
Thermal	5.01	6.19	5.100038077	6.20

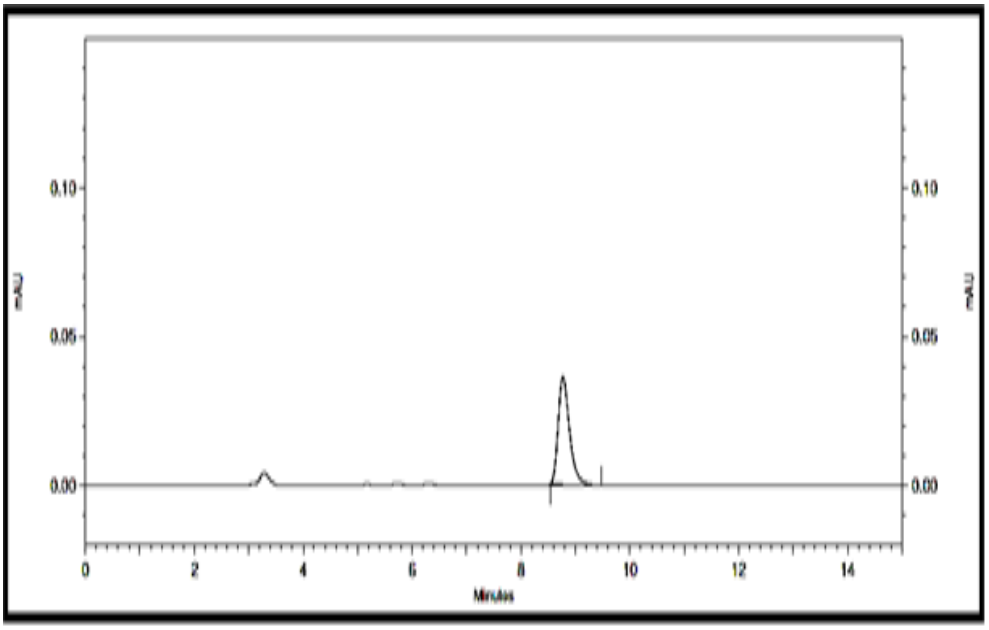

Figure 6 Acid Hydrolysis of Amlodipine Besilate

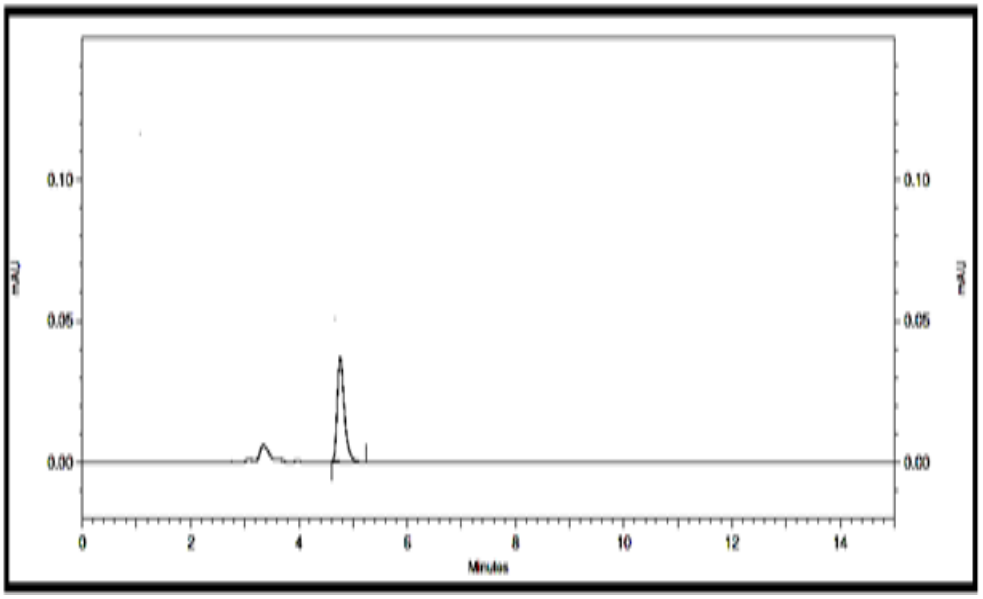

Figure 7 Acid Hydrolysis of Bisoprolol Fumarate

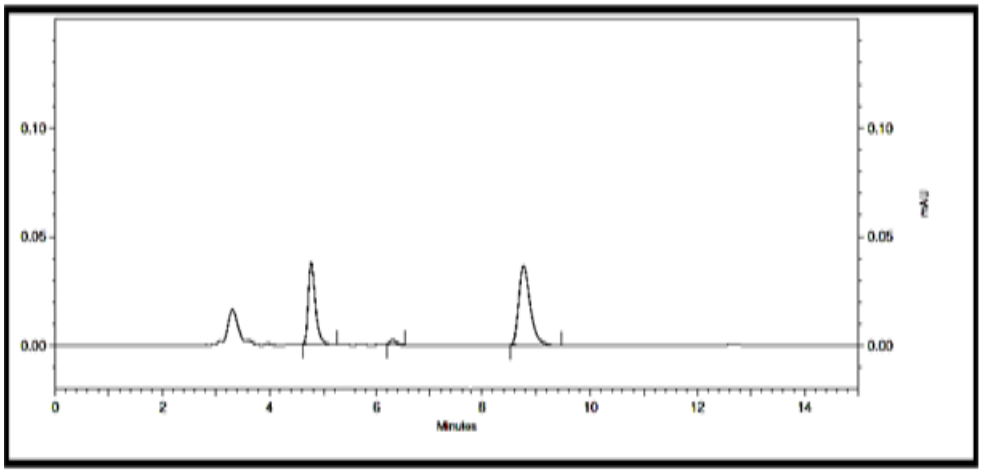

Figure 8 Acid Hydrolysis of formulation

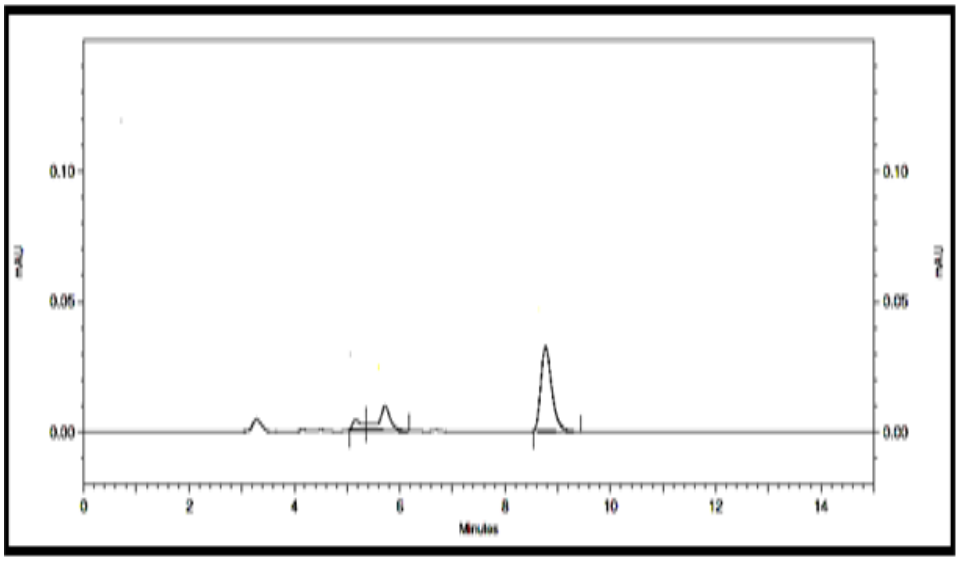

Figure 9 Alkali Hydrolysis of Amlodipine Besilate

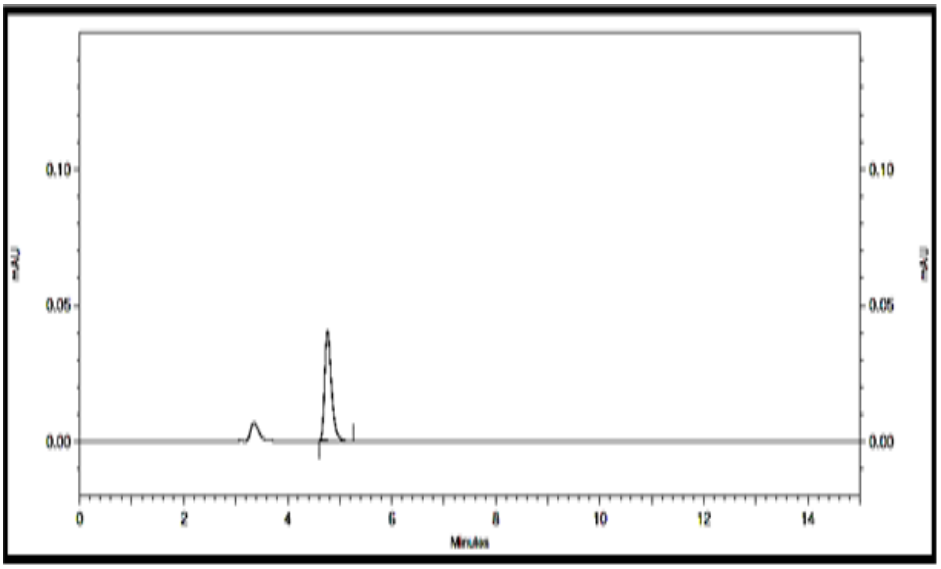

Figure 10 Alkali Hydrolysis of Bisoprolol Fumarate

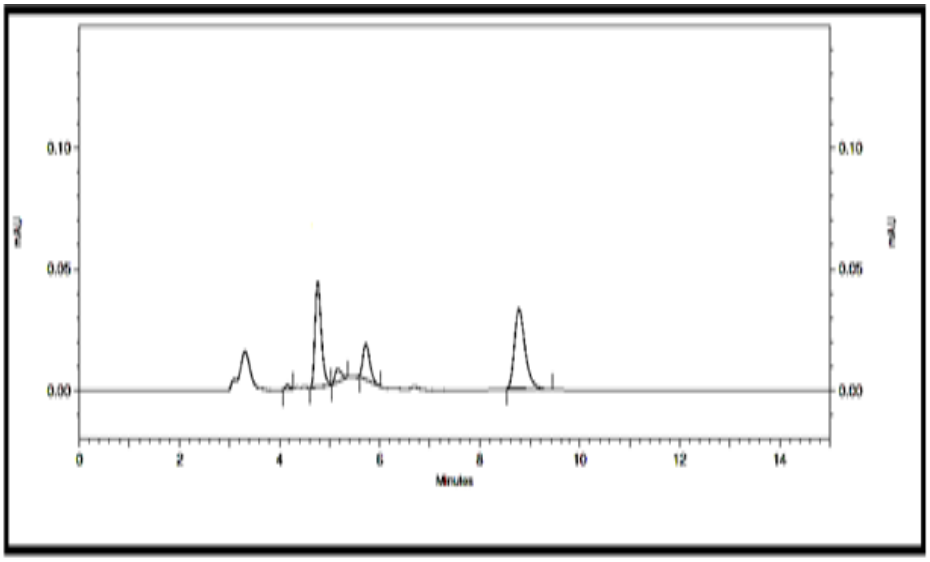

Figure 11 Alkali Hydrolysis of formulation

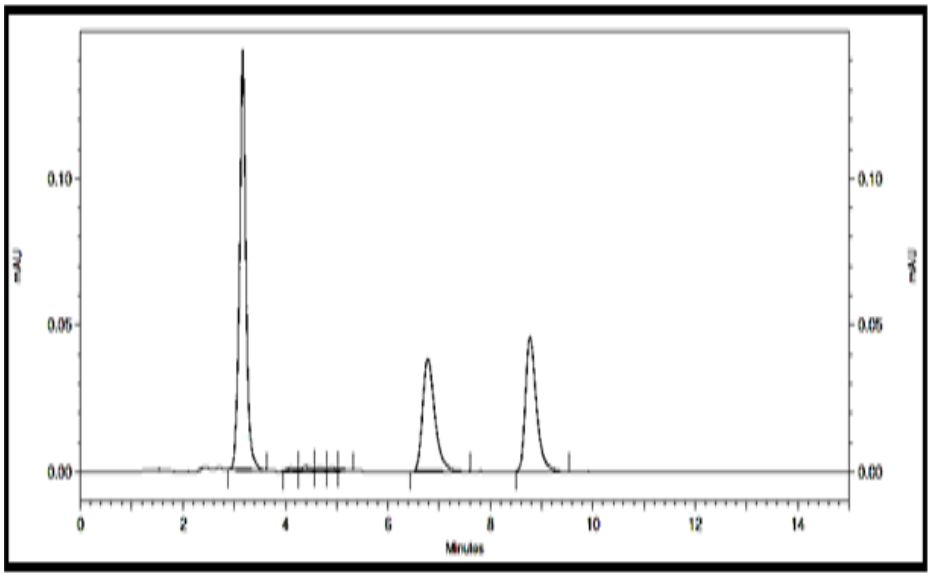

Figure 12 Oxidative Hydrolysis of Amlodipine Besilate

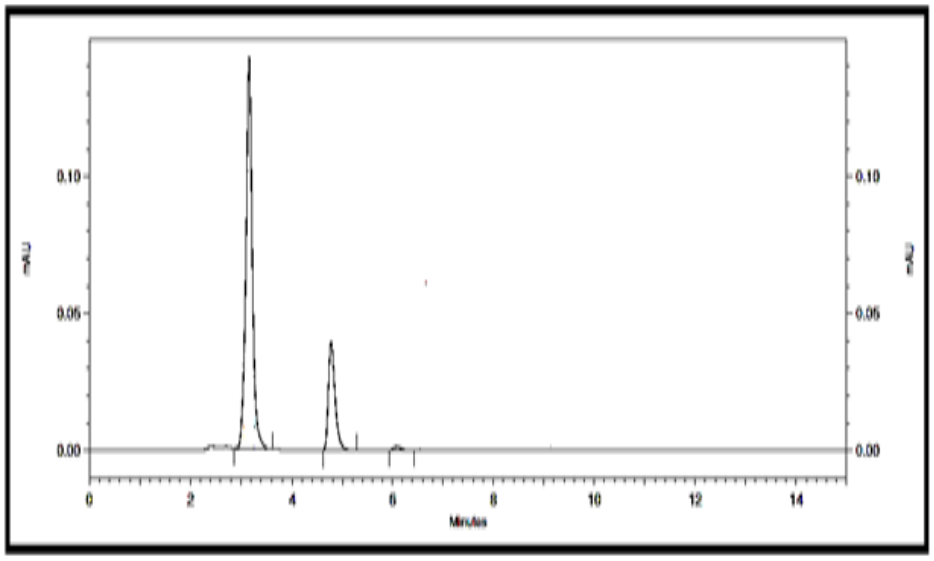

Figure 13 Oxidative Hydrolysis of Bisoprolol Fumarate

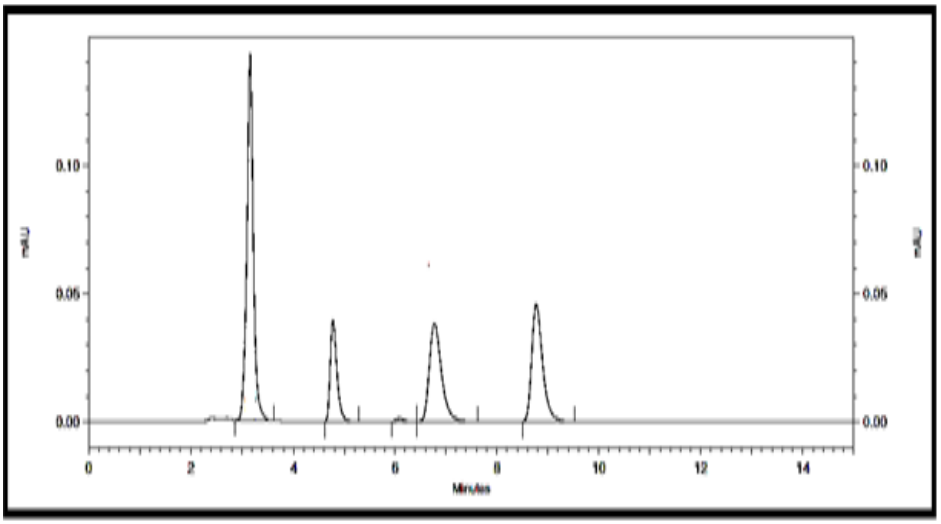

Figure 14 Oxidative Hydrolysis of Formulation

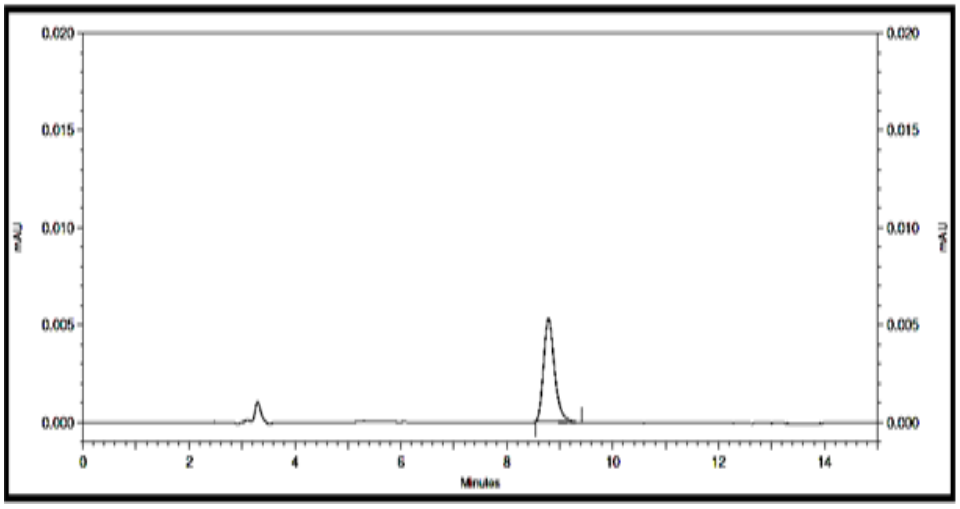

Figure 15 Thermal Degradation of Amlodipine Besilate

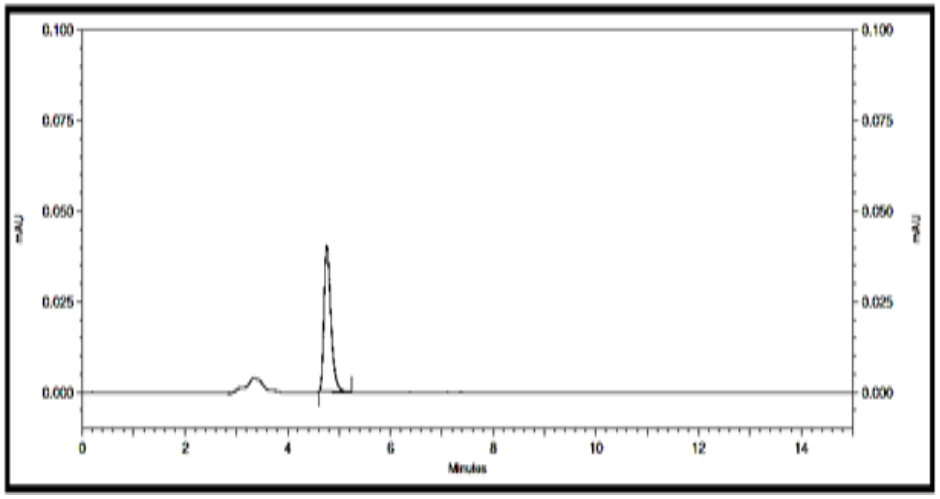

Figure 16 Thermal Degradation of Bisoprolol Fumarate

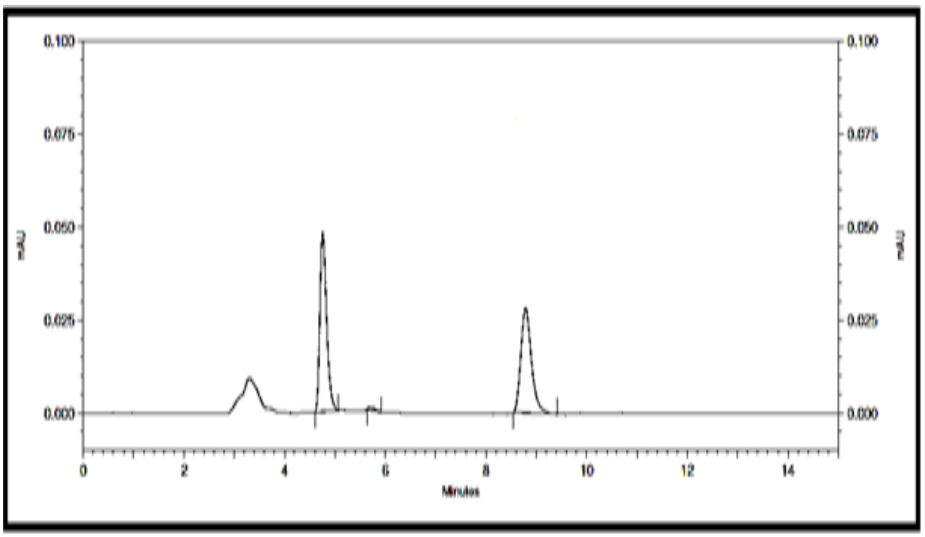

Figure 17 Thermal Degradation of Formulation

CONCLUSION

- From the Stability indicating RP – HPLC method, I conclude that Amlodipine Besilate was easily degrade in different stress condition while Bisoprolol Fumarate was slightly degrade in different stress condition. In oxidative degradation both of drugs are degraded at larger extent.
- Developed HPLC method can resolve all degradant

peak of both drug. No chromatographic interference from tablet excipients was found.

- It is concluded that the developed method is specific. The test parameters were also performed and were found to be within acceptable criteria. The method can be successfully employed for the simultaneous determination of Amlodipine Besilate and Bisoprolol Fumarate in pharmaceutical formulation.

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