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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF METOPROLOL AND AMLODIPINE IN TABLET DOSAGE FORM

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ABSTRACT

The goal of the current investigation was to simultaneously separate the anti-hypertensive agents, metoprolol and amlodipine and develop and validate an analytical method for simultaneous quantitative determination of metoprolol and amlodipine in tablet dosage form. The chromatographic separation was accomplished on Welchrom RP-C_{18} Column (250 mm X 4.6 mm; 5µm), Shimadzu LC-20AT Prominence Liquid Chromatograph and with a mixture of 10 mM Phosphate buffer (pH 3.0): acetonitrile (50:50, v/v). The flow rate was fixed at 1.0 mL/minute and the analysis was performed using Shimadzu SPD-20A Prominence UV-Visible detector at 235 nm. The anti-hypertensive agents, Metoprolol and Amlodipine were separated within 6 minutes. Metoprolol and Amlodipine showed retention times of 2.687 min and 3.797 min respectively. The calibration plots were linear over the concentration range of 5-25 µg/mL for Metoprolol (r^2=0.9999) and 1-5 µg/mL for amlodipine (r^2=0.9999). The method was correctly validated for important parameters such as accuracy, precision, linearity and specificity. The method was very sensitive with regard to limit of detection 0.125 µg/mL, 0.102 µg/mL and limit of quantitation 0.381 µg/mL, 0.311µg/mL respectively. The high recovery and low relative standard deviation was found to be suitable for the routine determination of Metoprolol and Amlodipine in bulk drug and combination of tablet dosage form.

KEYWORDS: Metoprolol, Amlodipine, Isocratic RP-HPLC, UV detection, Validation.

INTRODUCTION

Metoprolol succinate (MET) is considered as the prototype in cardio selective beta-adrenergic blockers. MET inhibit sympathetic activity by selective blockage of β_1-receptors causing reduction in BP. They usually utilized as first line treatment to reduce hypertension. MET is used to treat angina pectoris, acute myocardial infarction, ventricular tachycardia and the symptoms of alcohol withdrawal and can reduce the risk of repeated heart attacks and reduce the risk of death. MET reverses the effects of stress hormones so that it is normally recommended for anxiety treatment. Overdose of MET results in Bradycardia, hypotension, bronchospasm, cardiac failure etc. and not safe for asthmatics as it significantly precipitate asthma and contraindicated in nursing womens. MET is available in doses of 25 mg, 50 mg and 100 mg tablets and as IV injection in dose of 0.5 mg/mL. MET is chemically {2-hydroxy-3-[4-(2-methoxyethyl) phenoxy] propyl} (propane-2-yl) amine (Fig. 1).
Amlodipine besylate (AML) is a calcium channel blocker pertaining to the class of 1, 4-dihydropyridines. It can be used alone or in combination with other drugs like adrenergic blocking agents, ACE inhibitors or diuretics to reduce hypertension. The normal dose of AML is 5 mg/day. It can be hiked up to 10 mg/day depending on individual's response and severity of the hypertension. Main side effects of AML are slow heart beat, fainting, dizziness and shortness of breath etc., AML is chemically 3-ethyl-5-methyl-2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-6-methyl-3,5-pyridinedicarboxylate, monobenzenesulphonate (Fig. 2).

Few analytical methods have been reported in the literature for the simultaneous estimation of above said anti-hypertensive agents in biological fluids like plasma, blood and pharmaceutical dosage forms, with spectrophotometry (Sohan, 2012) (Kakde, 2008), HPTLC (Argekar, 2000), RP-HPLC (Boyaka, 2012) (R.J. Chandrabose, 2011) (Bhargavi Durga, 2011) (Ravisankar, 2013) with UV detection (Seema, 2013) and with Liquid Chromatography-Mass Spectrometry (Kallem, 2013). So well and up dated developed and validated analytical methods are quite essential for quality control of the drugs available in the market. So the proposed method provides fast separation with effective resolution, good peak shape, use of lesser sample volumes and buffer volumes, providing cost effective. The proposed established method was validated with respect to specificity, linearity, precision, accuracy, robustness, Limit of detection and Limit of quantitation subject to ICH Q2 (R1) guidelines 2005.

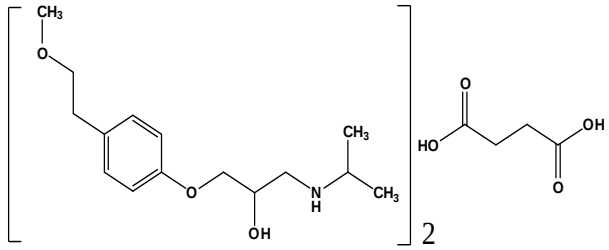

Figure.1.Structure Metoprolol succinate investigated in the present study

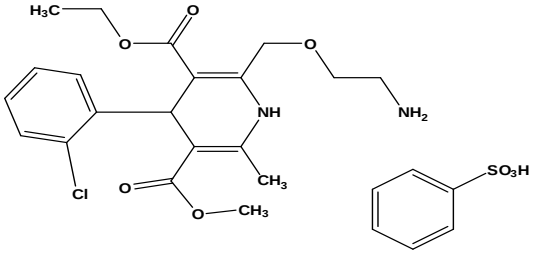

Figure.2.Structure Amlodipine besylate investigated in the present study.

MATERIALS AND METHODS

Chemicals and Reagents: Hetero Drugs Ltd., Hyderabad, Andhra Pradesh, India was kind enough and supplied the reference standards of MET and AML for this research work. All the chemicals used throughout the research work were of analytical grade. Potassium dihydrogen orthophosphate was bought from Rankem Ltd., Mumbai, India. Acetonitrile (HPLC grade) and triethylamine (HPLC grade) purchased from Merck Pharmaceuticals Private Ltd., Mumbai, India. O-Phosphoric acid was also purchased from Merck Specialties Private Ltd., Mumbai, India. Commercial tablets of METOLOR-AM consist of MET (25 mg) and AML (5 mg) was purchased from local market manufactured by Cipla Limited, Mumbai, India.

Instruments and Chromatographic conditions: Chromatographic separations were attained by using Shimadzu LC-20AT Prominence Liquid Chromatograph comprising a LC-20AT VP pump, Shimadzu SPD-20A Prominence UV-Vis detector and Welchrom C_{18} column (4.6 mm i.d. X 250 mm, 5 micron particle size). 20 µL of sample was introduced into the HPLC system. The HPLC system data acquisition was performed with "Spinchrom" software. Separations were executed on the reverse phase column comprising a mixture of 10 mM Phosphate buffer (pH adjusted to 3.0 using triethylamine) and Acetonitrile in ratio of 50:50 v/v as mobile phase. The mobile phase was set at a flow rate of 1 mL/minute and eluent was monitored at 235 nm. In addition, an electronic balance (Shimadzu TX223L), digital pH meter (Systronics model 802), a sonicator (spectra lab, model UCB 40) and UV-Visible Spectrophotometer (Systronics model 2203) were used in this present study.

Preparation of Reagents and Standards

Mobile phase: Precisely weighed and get it dissolved 6.056 g of potassium dihydrogen orthophosphate in 445 mL of HPLC grade water to obtain 10 mM Phosphate buffer. To this buffer 55 mL of 0.1 M phosphoric acid was poured and mixed well. The pH of the solution was then adjusted to 3.0 with triethylamine. The above prepared buffer and acetonitrile were mixed in the proportion of 50:50 v/v. The mobile phase was then duly filtered through 0.45 µm nylon membrane vacuum filtration and duly degassed by sonication.

Preparation of Standard Stock Solutions: A standard stock solution of the drug was prepared by mixing 25 mg of MET and 5 mg of AML in 100 mL calibrated flask having 60 mL mobile phase, then sonicated for about 10 minutes and brought up to 100 mL by pouring mobile phase and obtained the primary standard stock solution containing 250 µg/mL of MET and 50 µg/mL of AML.

Working standard solution: 1 mL of the above said stock solution was taken in 10 mL of calibrated flask and made up to 10 mL with mobile phase to obtain the working standard solution having 25 µg/mL of MET and 5 µg/mL of AML.

Preparation of sample solution: Twenty tablets of METOLAR-AM were correctly weighed and crushed it into smooth powder and the same powder equivalent to 25 mg of MET and 5 mg of AML was mixed with 100 mL mobile phase. The mixture was allowed to stand for 30 minutes with intermittent sonication to ensure total dissolution and filter through a 0.45 µm membrane filter. The said solution was pipetted out and dilute it with mobile phase to get a final concentration of 12.5 µg/mL of MET and 1.25 µg/mL of AML.

Selection of detection wavelength: The superimposed UV spectra of various diluted solutions of MET and AML in mobile phase were taken into account by using UV spectrophotometer. The isobestic point of maximum absorbance was observed at 235 nm and this wavelength was observed for detection of MET and AML which is detailed in Figure 3.

Method validation: The developed method of analysis was validated as per the ICH Q2 (R1) guidelines for the parameters like system suitability, specificity, linearity, precision, accuracy, robustness and system suitability, limit of detection and limit of quantitation.

System suitability: System suitability was assessed by six replicate analysis of the drugs at concentrations of 25 µg/mL of MET and 5 µg/mL of AML respectively. The % RSD of the peak areas and retention time for the MET and AML are within the limits of less than 2% which shows the system suitability. The system suitability parameters like resolution (NLT 2.0), tailing factor (NMT 1.5), theoretical plate count (NLT 3000) and % RSD for peak area of six replicate injections of standard (% RSD NMT 2.0) are within the limits. The results for system suitability are tabulated in Table 1.

Specificity: The specificity of the method was performed by separate injections of MET and AML standard and sample. The retention time of the MET did not interfere with the retention time of AML. The general excipients such as lactose anhydrous, microcrystalline cellulose, purified talc, hydroxyl propyl methyl cellulose (HPMC) and magnesium stearate have been added to the placebo solution and injected and tested. Figure 4 and Figure 5 shows the chromatograms of blank and drug matrix (synthetic mixture) pertaining to combination drug of MET and AML. The specificity results are tabulated in Table 2.

Linearity: Aliquots of primary working standard solution consisting MET and AML were diluted in such a way to get the eventual concentrations of MET and AML in the range of 5-25 µg/mL and 1-5 µg/mL respectively. The linearity graphs for the proposed assay methods were plotted over the concentration range. Method of least square analysis was carried out for getting the slope, intercept and correlation coefficient, regression data values. A calibration curve was plotted between concentration and peak area response and statistical analysis of the calibration curve was performed.

Precision: Precision was estimated by intra-day and inter-day study and carefully evaluated by carrying out the assay and analyse corresponding responses 6 times on the same day and on different days for the sample solution. The percent relative standard deviation (% RSD) was calculated.

Accuracy (Recovery studies): Accuracy studies were determined for both MET and AML at three different levels (80%, 100% and 120%) and the mixtures were analyzed in triplicate by the developed method. Known amount of standard MET and AML at 80%, 100% and 120% of pre determined sample was added to a pre quantified tablet sample.

Robustness: The robustness of the proposed method was evaluated by carrying out minute deliberate changes in flow rate (± 0.1 ml/min), detection wavelength (±5 nm) and Mobile phase composition (±2%). The effect of these variables on the developed method was determined.

Limit of detection and Limit of quantitation: Limit of Detection and Limit of Quantitation were calculated using following formula LOD = 3.3(SD)/S and LOQ = 10 (SD)/S, where SD = standard deviation of response (peak area) and S= slope of the calibration curve. The LOD and LOQ for the estimation of MET was found to be 0.125 µg/mL and 0.381 µg/mL and for the estimation of AML were 0.102 µg/mL and 0.311 µg/mL respectively.

Analysis of marketed formulation: The proposed validated method was successfully applied to determine the MET and AML in their tablet dosage form. 20 µL of sample solution was injected into liquid chromatograph. The assay was repeated for six times and the amount of the drug present per tablet was estimated from calibration equation. The mean % recovery was determined.

RESULTS AND DISCUSSION

For getting suitable mobile phase for the analysis of the selected drug combination, various mixtures of acetonitrile and phosphate buffer were tested. After some trials, it was found that the mixture of phosphate buffer (pH-3.0) and acetonitrile in a composition of 50:50, %v/v as mobile phase resulted in symmetric peak at 235nm in short runtime (6 min). The pH of buffer was corrected to 3.0 using triethylamine. Different column types and lengths were tried regarding other chromatographic parameters. C_{18} column with a 4.6 mm inner diameter, 250 mm length and 5 micron particle size was preferred. UV overlain spectra of these drugs showed that these drugs absorbed appreciably at 235 nm, so that this wavelength was chosen as the detection wave length (Figure 3). Flow rate used was set to 1 mL/min. chromatograms showed a peak of MET at retention time of 2.687 min and peak of AML at retention time of 3.797 min respectively. The calibration curve was obtained for a series of concentration

in the range of 5-25 µg/mL for MET and 1-5 µg/mL for AML respectively and it was found to be linear. The calibration data is shown in Table 3. The data of regression analysis of the calibration curve is presented in Table 4. The regression equation obtained from linearity plot for MET was Y = 130.44X+3.9793 with R^2=0.9999 and for AML was Y = 43.33X+0.8498 with R^2=0.9999 which shows that this method had good linearity. The representative chromatograms for calibration standards are shown from Fig. 6 to Fig. 10. The calibration plot for MET and AML were shown in Fig. 11 and Fig. 12 respectively. The proposed method was found to be precise for the determination MET and AML. The % RSD for the proposed method was found to be less than 2.0 which indicate the method's precision. Results of the precision study are represented in the Table 5. Recovery studies (Table 6) of the method was found to be good within the overall mean % recovery of the tablet dosage form. Robustness was done by small deliberate changes in the chromatographic conditions like mobile phase flow rate, λ_{max}, mobile phase composition. The developed method was found to be robust as there were no marked changes in the chromatograms. The Robustness results are shown in Table 7. The proposed validated method was successfully applied to determine the assay of METOLAR-AM tablet and results are presented in Table 8. The representative sample chromatogram is shown in Fig. 13. The assay results of different samples were found to be within the proposed limits. The results obtained for MET and AML was comparable with the corresponding labeled amounts Table 8. The mean assay value was found to be 99.772 ± 0.327 % for MET and 99.26 ± 0.493 % for AML.

Table.1.Optimized chromatographic conditions and system suitability parameters

Parameter	**Chromatographic conditions**	
Instrument	SHIMADZU LC-20AT Prominence liquid chromatograph	
Column	WELCHROM C_{18} Column (4.6 X 250mm, 5µm)	
Detector	SHIMADZU SPD-20A Prominence UV-Vis detector	
Diluents	10mM Phosphate Buffer(pH 3.0) : Acetonitrile (50:50, v/v)	
Mobile phase	10mM Phosphate Buffer(pH 3.0) : Acetonitrile (50:50, v/v)	
Flow rate	1mL/min.	
Detection wave length	UV at 235nm.	
Run time	5 minutes	
Column back pressure	155-158 kgf	
Temperature	Ambient temperature(25^{o}C)	
Injection Volume	20µL	
	Metoprolol succinate	**Amlodipine besylate**
Retention time (t_R)	2.687 min.	3.797 min.
Theoretical plates[th.pl] (Efficiency)	9520	9859
Resolution	-	10.504
Tailing factor (asymmetry)	1.067	1.053

Table.2.Specificity study

Name of the solution	**Retention time, (t_R)min.**
Mobile phase	No peaks
Placebo	No peaks
Solution containing a concentration of MET, 25µg/mL and AML, 5µg/mL.	Peaks at 2.687 min and 3.797 min for MET and AML respectively.

Table.3.Calibration data

Metoprolol		Amlodipine	
Concentration, µg/mL	**Peak area, mV.s.**	**Concentration, µg/mL**	**Peak area, mV.s.**
0	0	0	0
5	264.374	1	87.126
10	528.716	2	174.760
15	791.639	3	262.725
20	1050.324	4	347.885
25	1301.99	5	432.632

Table.4.Linear Regression Data

Parameter	MET	AML
Detection wavelength(λ_{max})	UV at 235 nm	UV at 235 nm
Linearity range (µg/mL)	5-25 µg/mL	1-5 µg/Ml
Regression equation (Y = aX + b)	Y = 130.44X + 3.9793	Y = 43.33X + 0.8498
Slope(a)	130.44	43.33
Intercept(b)	3.9793	0.8498
Standard error of slope (S_a)	0.594409359	0.16108
Standard error of intercept (S_b)	3.599327413	0.97540
Standard error of estimation (S_y)	4.973185502	1.3477
Regression coefficient (R^2)	0.9999	0.9999
% Relative standard deviation* i.e., Coefficient of variation(CV)	0.5691	0.4866
Percentage range of errors (Confidence limits)		
0.005 significance level	2.406107	2.083363
0.001 significance level	3.774152	3.267906

[#]**Average of 6 determinations; acceptance criteria < 2.0.**

Table.5. Results of precision study

PRECISION STUDY	**MET**	**AML**
	%RSD	%RSD
INTRA-DAY	0.25242	0.831
INTER-DAY	0.20335	0.873

[#] **Acceptance criteria < 2.0.**

Table.6.Recovery Data

Recovery level[#]	**MET**	**AML**
	Mean % Recovery ± SD	**Mean % Recovery ± SD**
80%	99.705 ± 0.460	99.316 ± 1.121
100%	100.456 ± 0.405	99.916 ± 1.156
120%	100.265 ± 0.425	100.340 ± 1.645

[#]**average of triplicate injections.**

Table 7: Robustness data

Parameter[a]	Used	Retention time (t_R), min.		Plate count[$]		Peak asymmetry[#]		Remarks
		MET	AML	MET	AML	MET	AML	
Flow rate (±0.2 mL/min)	0.8 mL/min	2.700	3.798	4273	10524	1.320	1.280	*Robust
	1.2 mL/min	2.521	3.368	3863	9971	1.294	1.263	*Robust
Detection wavelength (±5 nm)	240 nm	2.247	3.760	4012	10382	1.330	1.242	Robust
	230 nm	2.243	3.757	4027	10372	1.316	1.238	Robust
Mobile phase composition (±2 % v/v)	52:48,%v/v	2.125	3.473	3722	10112	1.288	1.288	*Robust
	48:52, %v/v	2.386	3.863	4129	10446	1.311	1.257	*Robust

Acceptance criteria (Limits): [#]Peak Asymmetry < 1.5, [$] Plate count > 3000, *significant change in Retention time.

Table.8.Assay results

Formulation	Labelled amount		Amount found		% Assay±SD*	
	MET	AML	MET	AML	MET	AML
Metolar-AM, Cipla Limited, Mumbai, India)	25 mg	5 mg	24.943 mg	4.9630 mg	99.772 ± 0.327 %	99.26 ± 0.493 %

***Average of 6 determinations; SD is standard deviation.

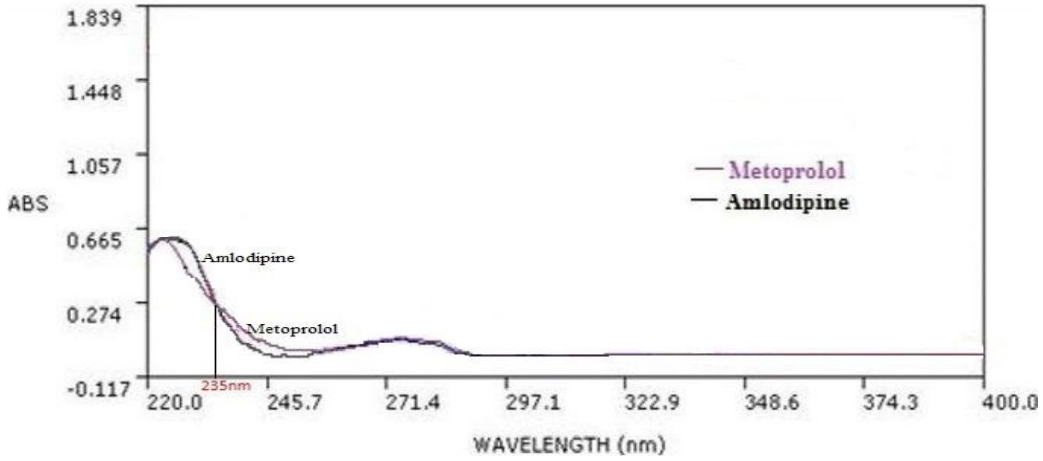

Figure.3.Overlain spectra of Metoprolol and Amlodipine

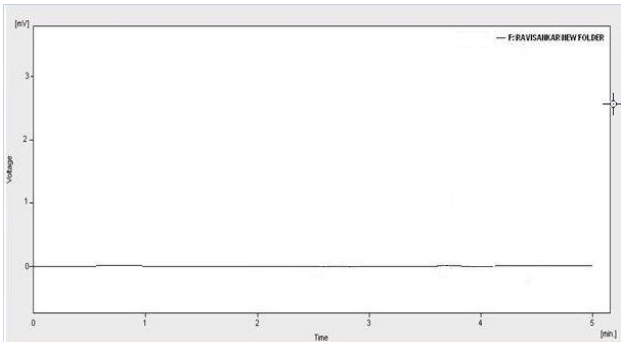

Figure.4.Chromatogram of blank solution (MET and AML).

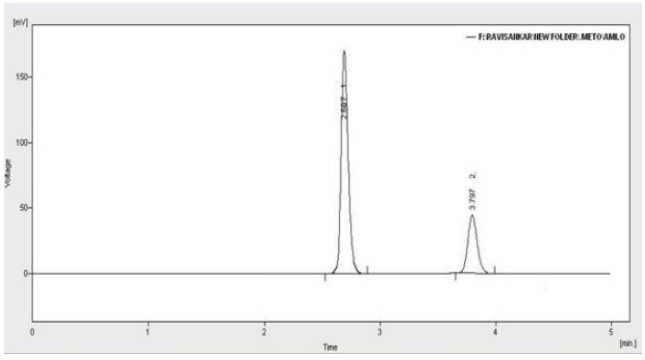

Figure.5.Chromatogram of MET and AML synthetic drug

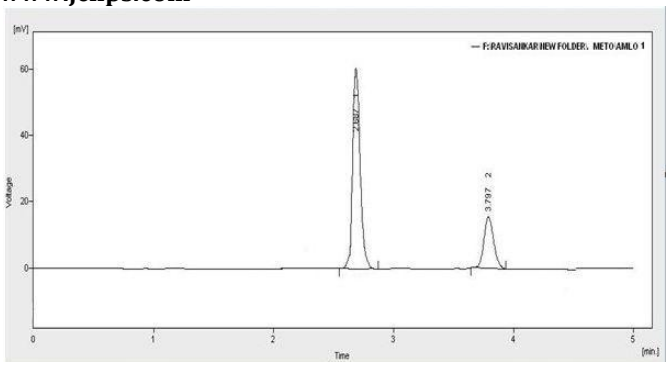

Figure.6.Standard chromatogram of Metoprolol succinate (5 µg/mL) and Amlodipine besylate (1 µg/mL)

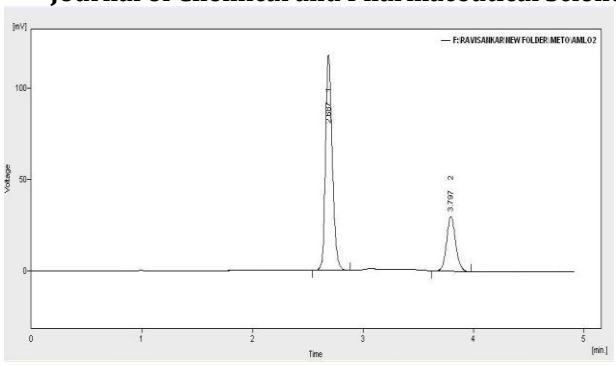

Figure.7.Standard chromatogram of Metoprolol succinate (10 µg/mL) and Amlodipine besylate (2 µg/mL)

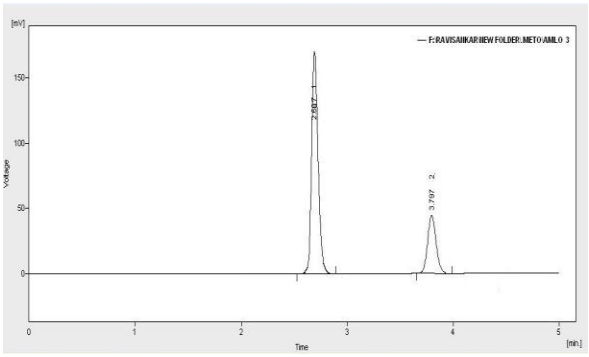

Figure.8.Standard chromatogram of Metoprolol succinate (15 µg/mL) and Amlodipine besylate (3 µg/mL)

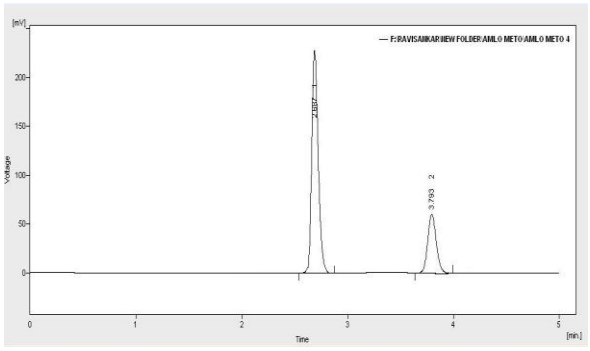

Figure.9.Standard chromatogram of Metoprolol succinate (20 µg/mL) and Amlodipine besilate (4 µg/mL)

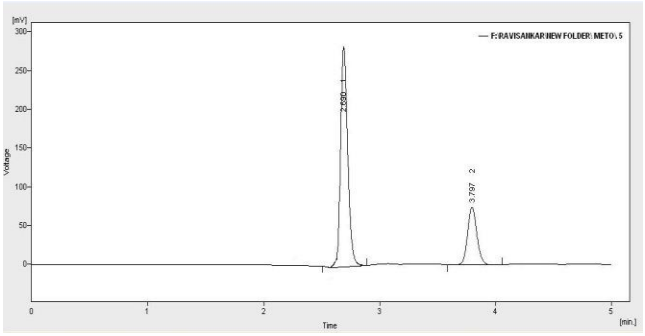

Figure.10.Standard chromatogram of metoprolol succinate (25 µg/mL) and Amlodipine besylate (5 µg/mL)

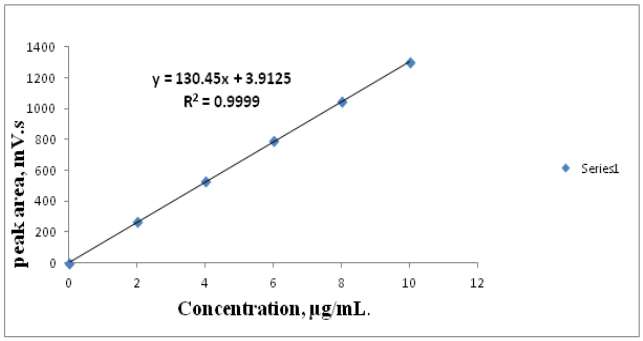

Figure.11.Calibration plot of Metoprolol

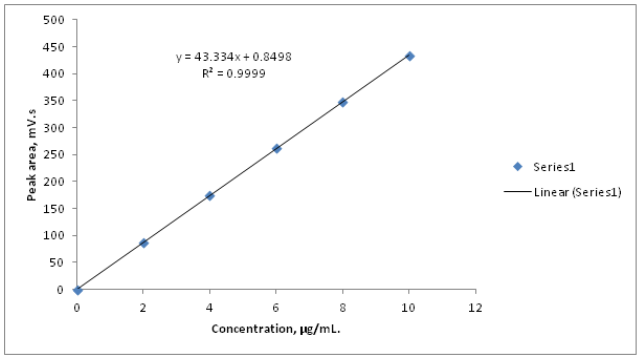

Figure.12.Calibration plot of Amlodipine besylate

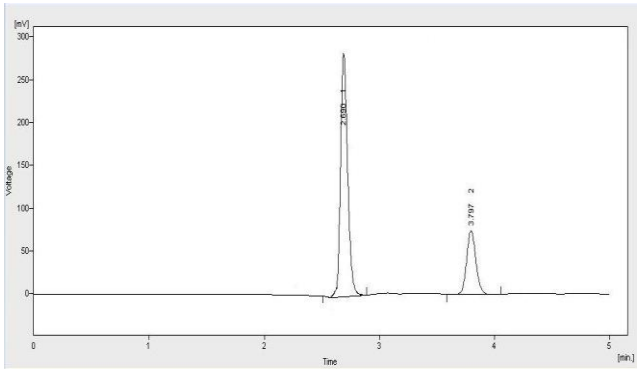

Figure.13.Chromatogram of market formulation (METOLAR-AM tablets)

CONCLUSION

The present proposed research study by the author describes the estimation of MET and AML available as combination tablet dosage forms and was carried out by utilizing RP-HPLC. The linearity of the proposed method was in the range of 5-25 µg/mL for MET and 1-5 µg/mL for AML respectively. The LOD and LOQ of MET were 0.125µg/mL and 0.381µg/mL and for the estimation of AML were 0.102 µg/mL and 0.311 µg/mL respectively. The above said antihypertensive agents of total runtime of 5 minutes with an elution window of 1.5 minutes were achieved. The developed RP-HPLC method for the quantification of MET and AML was found to be simple, specific, highly sensitive, fast, economical, precise and extremely accurate with robustness. The developed method has several advantages like decorous linearity, less retention times and less solvent consumption which makes the method more economical than the existing methods in practice. Therefore this method can be recommended for the routine analysis of MET and AML in quality control and clinical laboratories.

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