International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304
Vol.7, No.1, pp 172-184, 2014-2015

RP-HPLC Method for the Simultaneous Estimation of Cilnidipine and Metoprolol Succinate in Bulk and Tablet dosage form in Biorelevant Media (FaSSIF)

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Abstract: A simple, rapid, and precise reverse phase high performance liquid chromatographic (RP-HPLC) method for simultaneous analysis of Cilnidipine (CLN) and Metoprolol Succinate (MTS) in a tablet dosage form and in Biorelevant media has been developed and validated. This method was performed with a Symmetry C_{18} (4.6 × 150mm, 5µm) column with 35:65 (v/v) 20mM potassium dihydrogen orthophosphate buffer : methanol as mobile phase at a flow rate of 1.0 ml/min. UV detection at 225 nm; CLN and MTS were eluted with retention times of 3.516 and 4.870min, respectively. The method was continued and validated accordance with ICH guidelines. Validation revealed the method is rapid, specific, accurate, precise, reliable, and reproducible. Calibration curve plots were linear over the concentration ranges 10-50µg/mL for CLN, and 25-125µg/mL for MTS. Limits of detection (LOD) were 0.015 and 0.0375µg/ml and limits of quantification (LOQ) were 0.05 and 0.125µg/mL for CLN and MTS respectively. Statistical analysis was proves the method is suitable for the analysis of CLN and MTS as a bulk, in tablet dosage form and in biorelevant media without any interference from the excipients. It was also proved study for degradation kinetics of three drugs. It may be extended for its estimation in plasma and other biological fluids.
Keywords: Cilnidipine (CLN), Metoprolol Succinate (MTS), RP-HPLC, Validation, FaSSIF.

Introduction

Cilnidipine (CLN) chemically 3-(2-methoxyethyl)-5-[(E)-3-phenylprop-2-enyl]-2,6-dimethyl-4-(3-nitro phenyl)-1,4-dihydropyridine-3,5-dicarboxylate (Figure 1). It is a calcium channel blocker. Cilnidipine is a novel calcium antagonist accompanied with L-type and N-type calcium channel blocking function [1]. Metoprolol (MTS) chemically (RS)-1-(Isopropylamino)-3-[4-(2-methoxyethyle)phenoxy] propane-2-ol (Figure 1). It is a selective β_1 receptor blocking agent used in the treatment of various diseases of the cardiovascular system, mostly hypertension. The active substance Metoprolol is employed either as Metoprolol succinate or as Metoprolol tartrate (where 100 mg metoprolol tartrate corresponds to 95 mg metoprolol succinate). The tartrate is an immediate-release and the succinate is an extended-release formulation [2].

In the scientific literature, analysis of CLN and MTS has been reported as individual ingredients and in combination with other compounds. Analytical methods have included estimation of CLN [3, 4], MTS [5] individually. And in two component formulations of CLN and MTS have been analyzed in combination [6, 7]. And CLN and MTS with other drugs individually have also been reported [8-14].

No other chromatographic methods are found for simultaneous analysis of CLN and MTS in a combined dosage form and in biorelevant media. The method described is rapid, economical, precise, and accurate and can be used for routine analysis of tablets. It was validated as per ICH guidelines [15-17].

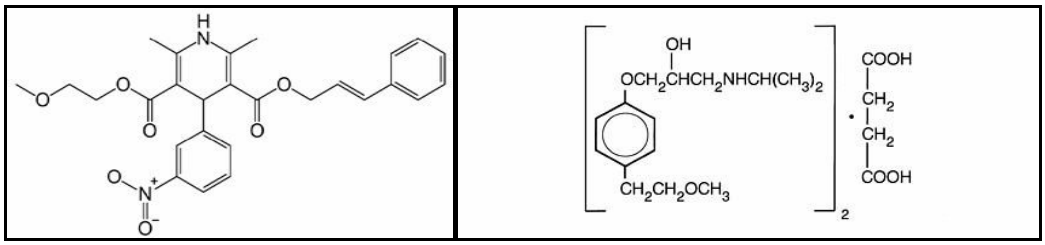

Fig. 1: Chemical structures of Cilnidipine (CLN) and Metoprolol Succinate (MTS)

Materials and Methods

1. Experimental

1.1. Materials and Methods

Pharmaceutical grade working standards Cilnidipine (CLN) and Metoprolol Succinate (MTS) were obtained from Hetero Labs, Jedcharla, India. All chemicals and reagents were HPLC grade and were purchased from Merck Chemicals, Mumbai, India.

1.2. Instrumentation

The analysis was performed using Waters-2695 (Modal Alliance) High Performance liquid chromatography, analytical balance (Mettler Toledo), PDA Detector (Standard cell) and data handling system (Empower 2), pH meter (lab India), Sonicator. The column used is Symmetry C_{18} (150×4.6mm, packed with 5µm) with the flow rate 1.0ml/min (isocratic).

1.3. Preparation of blank Fasted State Simulated Intestinal Fluid (FaSSIF)

Accurately weighed 1.74g of Sodium hydroxide pellets, 19.77g of Sodium dihydrogen orthophosphate, and 30.93g of Sodium chloride dissolve in 5 L of purified water and adjust the pH 6.5 exactly by using 1N Hydrochloric acid [19].

1.4. Preparation of FaSSIF

Accurately weighed 3.3g of sodium taurocholate dissolved in 500mL blank FaSSIF solution, added 11.8mL of a solution to 100mg/mL lecithin in methylene chloride, and forming an emulsion. The methylene chloride was eliminated under vacuum at 40°C. Then draw a vacuum for 15minutes at 250mbar and also followed by 15minutes at 100mbar. These results gave in a clear, micellar solution, having no perceptible odor for methylene chloride. After that it was cool to room temperature and adjusted the volume upto 2L with blank FaSSIF [19].

1.5. Preparation of Standard Stock solution

Accurately weighed 10 mg of CLN, and MTS working standard and separately transferred into a 10ml clean dry volumetric flasks, add about 7mL of biorelevant media (FaSSIF) to each volumetric flask and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Calibration standards at five levels were prepared by appropriately mixed and further diluted stock standard solutions in the concentration ranges from 10-50µg/mL for CLN and 25-125µg/mL for MTS. Samples in triple injections were made for each prepared concentration. Peak areas were plotted against the corresponding concentration to obtain the Linearity graphs.

1.6. Preparation of Standard solution

The above standard stock solution was containing 1000µg/mL of each CLN, and MTS in separate volumetric flasks. Then transferred the 0.3ml of CLN and 0.75ml of MTS of prepared standard stock solution into a clean 10ml volumetric flask and made upto the mark with diluent. And finally the standard solution concentrations were 30µg/mL and 75µg/mL of CLN, and MTS respectively.

1.7. Preparation of Test solution

For the analysis of a tablet dosage form, 20 tablets were weighed individually and their average mass was determined. Then, the tablets were crushed to a fine powder. The powder equivalent to 10mg of CLN and 25mg of MTS were transferred to a 10mL volumetric flask and dissolved in 10mL of biorelevant media (FaSSIF), sonication was done for 15 min with swirling. After sonication, the solution was filtered through a membrane filter paper (#0.45µ). From the above stock solution 0.3mL was transferred in to 10mL volumetric flask and made volume upto the mark with diluent, the final concentrations were 30µg/mL and 75µg/mL of CLN, and MTS respectively, then injected into the chromatographic system, and analyzed quantitatively. The analysis was repeated six times and the possibility of excipient interference with the analysis was examined.

1.8. Optimization of HPLC Method

The HPLC method was optimized and developed with a simultaneous method for CLN, and MTS. The mixed standard solution (30mg of CLN and 75mg of MTS) injected in HPLC by the followed chromatographic conditions. The chromatographic separation was achieved on a Symmetry C18 (4.6 x 150mm, 5µm). The isocratic mobile phase consisting of 20mM potassium dihydrogen orthophosphate and Methanol in the ratio of (35:65v/v) was used throughout the analysis and the pH 3.0 adjusted with orthophosphoric acid. The flow rate of the mobile phase was 1.0ml/min. Detection was monitored at wavelength of 225nm. The column temperature was kept at ambient and injection volume was 10µl.

1.9. Method validation

The method validation was done according to the ICH guidelines. The following validation characteristic parameters are accuracy, precision, linearity, and specificity, LOD, LOQ and robustness.

1.9.1. Linearity and range

Linearity of the method was studied by the injecting the mixed standard solutions with the concentration ranges from 10-50µg/ml for CLN and 25-125µg/ml for MTS levels of target concentrations were prepared and injected six times into the HPLC system keeping the constant injection volume. The peak areas were plotted against the concentrations to obtain the linearity graphs.

1.9.2. Precision

The precision of the optimized method was evaluated by carrying out six independent assays of test sample. %RSD of six assay values was calculated. Intermediate precision was carried out the samples by using another instrument and with different analyst.

1.9.3. Limit of Detection and Quantification

The LOD and LOQ procedures were performed on samples contain very lower concentrations of analytes under the ICH guidelines. By applying the visual evaluation method, LOD was expressed by establishing the lowest concentration at which the analyte can be detected. LOQ was considered as the lowest concentration of analytes that can be detected and quantified, with acceptable accuracy and precision.

1.9.4. Robustness

Robustness was studied by evaluating the effect of small variations in the chromatographic conditions. The conditions studied were flow rate altered by ±0.1ml/min, mobile phase composition with methanol ±5ml. These chromatographic variations are evaluated for resolution between CLN and MTS.

1.9.5. System suitability

The system suitability parameters with respect of tailing factor, theoretical plates, repeatability and resolution between CLN and MTS peaks were defined.

1.9.6. Specificity

The specificity of the analytical method is the ability of the method to estimate the analyte response in the presence of additional components such as impurities, degradation products and matrix [18]. The peak

purity of CLN and MTS were assessed by comparing the Retention time of standard CLN and MTS good correlation was obtained between the Retention time of standard and sample of CLN and MTS.

The specificity method was also evaluated to ensure that there were no interference products resulting from forced degradation studies.

1.9.6.1. Forced degradation study

Forced degradation or Stress testing of a drug substance will help to identify the degradation products, which can help to establish the intrinsic stability of the molecule.

All stress decomposition studies were performed at an initial drug concentration 30µg/mL of CLN and 75µg/mL of MTS.

The Stability indicating study of CLN and MTS were undergoes acid, alkali and oxidation degradation, photolysis and heat condition.

Placebo Interference: The placebo (in the present of excipients in tablet) sample were prepared as per the test method and analyzed in the HPLC. It expressed there is no additional peaks at the retention time of CLN and MTS in the chromatograph it indicates that there is no placebo interference.

Acid Degradation: Sample was treated with 3ml of 1N hydrochloric acid and kept for 10hrs. After 10hrs the solution was neutralized with 3ml of 1N sodium hydroxide, made the volume upto the mark with biorelevant media and analyzed using HPLC.

Alkali Degradation: Sample was treated with 3ml of 1N sodium hydroxide and kept for 10hr. After 10hr the solution was neutralized with 3ml of 1N hydrochloric acid, made the volume upto the mark with biorelevant media and analyzed using HPLC.

Oxidative Degradation: CLN and MTS solutions of 30 and 75µg/ml were mixed with 3mL of 30%v/v aqueous hydrogen peroxide solution and kept for 10hrs. After 10hrs made the volume upto the mark with biorelevant media and analyzed using HPLC.

Photolytic Degradation: The **s**amples were kept under UV light for different time intervals (15mins – 7days) and made the volume upto the mark with biorelevant media and analyzed using HPLC.

Thermal Degradation: Samples were heated at 80^0 C for 15mins - 60mins and 220^0 C for 2-5mins and analyzed.

1.9.7. Accuracy

Accuracy was carried out by applying the method to drug sample (CLN and MTS combination of tablets) to which known amounts of CLN and MTS standard powder corresponding to 50, 100 and 150% of label claim was added, mixed and the powder was extracted and determined by the system in optimized mobile phase. The experiment was performed in triplicate and percentage recovery, % RSD was calculated.

1.9.8. Analysis of marketed formulation

The marketed formulation was assayed by above description. The peak areas were monitored at 225nm, and determination of sample concentrations were using by multilevel calibration developed on the same HPLC system under the same conditions using linear regression analyzed for CLN and MTS in the same way as described above.

Results and Discussion

The simultaneous estimation of CLN and MTS was done by RP-HPLC and in the optimized method the mobile phase consists of buffer (350 volumes of phosphate buffer and 650 volumes of Methanol and the pH was adjusted to be 3.0. Then finally filtered using 0.45µ membrane filter paper and degassed in sonicator for 15 minutes. The detection is carried out using PDA detector at 225nm. The solutions are following at the constant flow rate of 1.0 ml/min.

The retention time for CLN and MTS was 3.516 and 4.870minutes respectively. Linearity ranges for CLN and MTS were 10-50µg/mL and 25-125µg/mL respectively and the results were found for in the acceptable as (R^2) = 0.9994 and 0.9991 for CLN and MTS respectively. LOD were 0.015 and 0.0375µg/ml and LOQ were 0.05 and 0.125µg/mL for CLN and MTS respectively. The all parameters value of RSD is less than 2.0% indicating the accuracy and precision of the method. The percentage recoveries were found 100.15-100.46% and 99.86-100.16 for CLN and MTS respectively.

1. Method Development and Optimization

The HPLC procedure was optimized with a view to develop a suitable LC method for the analysis of CLN and MTS in fixed dose for bulk and combined dosage form. It was found that 35:65 v/v (20mM) potassium dihydrogen orthophosphate buffer: methanol gave acceptable retention time (3.516 and 4.870min for CLN and MTS), plates, and good resolution for CLN and MTS at the flow rate of 1.0ml/min (**Table. 1**; **Fig. 2 & 3**).

Table No. 1: Optimized Chromatographic Conditions

Parameters	Method
Stationary phase (column)	Symmetry C18 (4.6 x 150mm, 5µm)
Mobile Phase	35:65v/v, (20mM Phosphate Buffer : Methanol)
pH	3.0 ± 0.02
Flow rate (ml/min)	1.0
Run time (minutes)	8.0
Column temperature (°C)	Ambient
Volume of injection loop (µl)	10
Detection wavelength (nm)	225
Drugs RT (min)	3.516 & 4.870

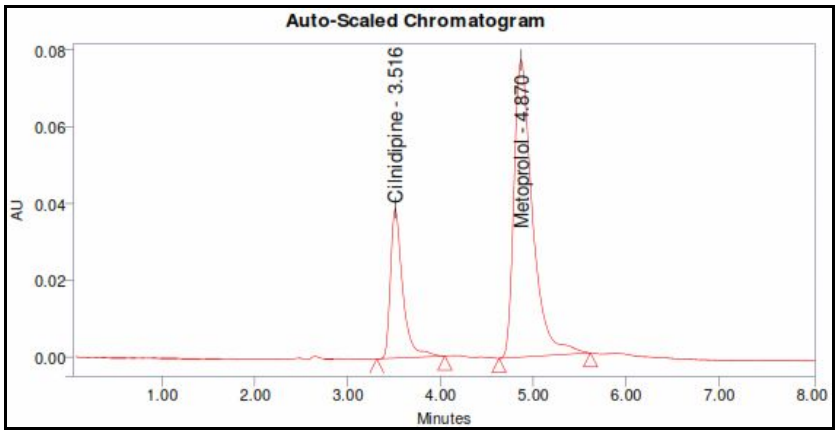

Fig. 2: Chromatogram of CLN and MTS at 225nm from bulk drug

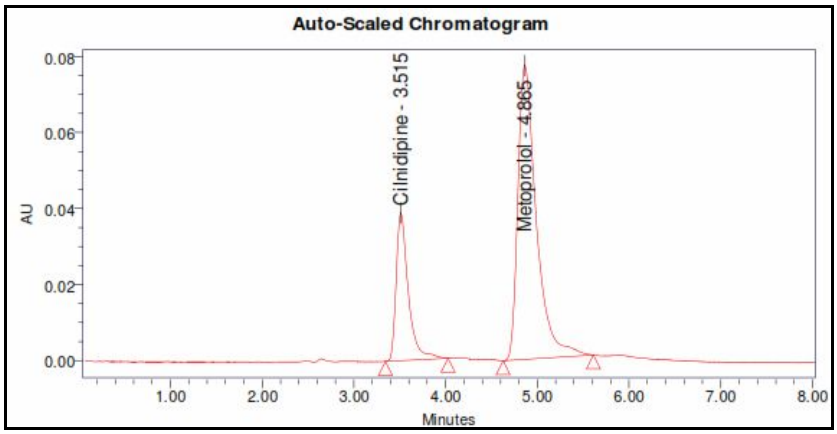

Fig. 3: Chromatogram of CLN and MTS at 225nm from pharmaceutical formulation (Cilacar + Met XL)

2. Validation of Developed method

2.1. Linearity

Linearity was evaluated by analysis of working standard solutions of CLN and MTS of five different concentrations. The range of linearity ranges from 10-50µg/ml for CLN and 25-125µg/ml for MTS (**Table. 2**). The result of correlation coefficients of CLN and MTS (R^2) = 0.9994 & 0.9991 respectively (**Fig. 4-6**). There was an excellent correlation between peak areas and concentrations of each drug.

Table 2: Data for linearity

Analyte	Concentration range (µg/mL)	Correlation Coefficient (R^2)	Slope	Intercept
CLN	10-50	0.9994	12079x	3689
MTS	25-125	0.9991	13123x	97697

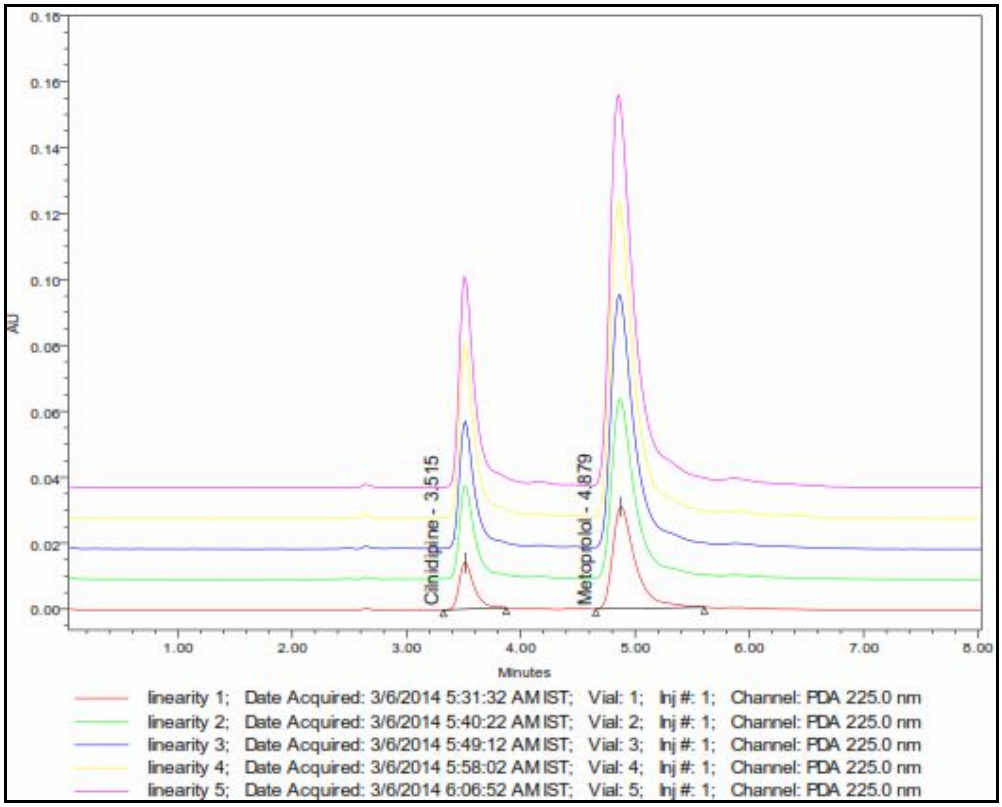

Fig. 4: Overlay linearity Chromatogram for CLN and MTS

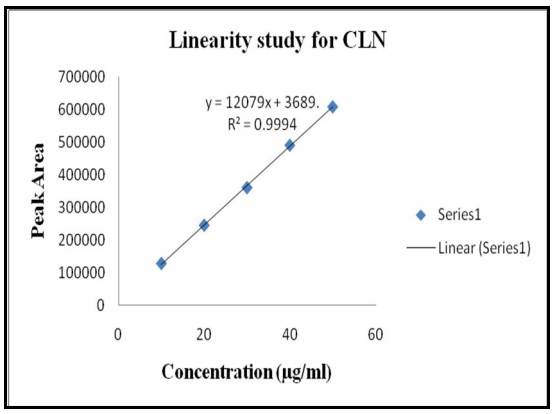

Fig. 5: Linearity Curve of Standard Cilnidipine (CLN)

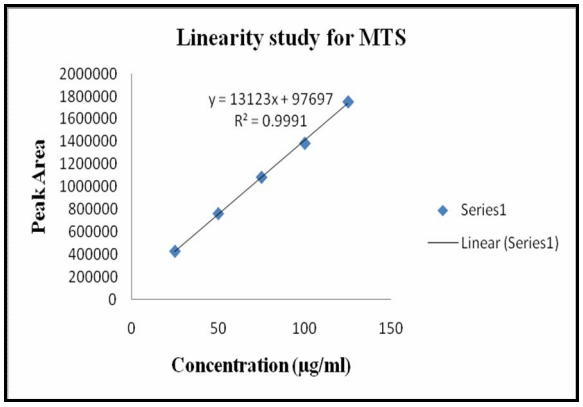

Fig. 6: Linearity Curve of Standard Metoprolol Succinate (MTS)

2.2. Precision

The results of precision method were evaluated by carrying out six independent test samples of CLN and MTS. The percentage of RSD of six sample peak area values was calculated. Different analyst from the same laboratory conditions analyzed the intermediate precision for the optimized method. The RSD values of intra-day and inter-day studies for CLN and MTS confirming good precision of the optimized method (**Table. 3**).

Table No. 3: Intra-day and inter-day Precision results of CLN and MTS from tablets

No. of Preparation	CLN		MTS	
	Intra-day precision	Inter-day precision	Intra-day precision	Inter-day precision
Pre-1	358728	359278	1077825	1078915
Pre-2	357258	358925	1078925	1079258
Pre-3	359268	359692	1075257	1076925
Pre-4	356825	358726	1076872	1077925
Pre-5	358926	359562	1075901	1076734
Mean	358201	359236.6	1076956	1077951.4
St. dev.	1086.792068	410.1204701	1469.5921	1137.09995
% RSD	0.303402857	0.114164445	0.136458	0.10548713

2.3. LOD and LOQ

The LOD and LOQ values were found to be 0.015 and 0.05µg/mL for CLN and 0.0375 and 0.125µg/mL for MTS (**Table. 5**).

2.4. Specificity

Injected the extracted solutions commonly used excipients were performed to demonstrate for the absence of interaction with the drugs. These results are expressed that there was no interference from the other excipients in the tablet formulation; therefore, confirm the method was specific.

2.5. System suitability

System suitability parameters such as the theoretical plates count, resolution, % RSD and peak tailing factors are determined (**Table. 5**).

Table No. 5: System suitability parameters for CLN and MTS

System suitability parameters	CLN	MTS
Retention time (min)	3.516	4.870
Repeatability of retention time; %R.S.D (n=5)	0.034	0.017
Repeatability of peak area; %R.S.D= (S.D./Mean)×100	0.319	0.128
Resolution (Rs)	-	4.52
Tailing factor (asymmetric factor)	1.48	1.56
USP plate count	3834	12111
LOD (µg/mL)	0.015	0.0375
LOQ (µg/mL)	0.05	0.125

2.6. Robustness

To ensure the insensitivity of the optimized RP-HPLC method to small alteration in the experimental conditions. The conditions studied were flow rate altered by ±0.1ml/min, mobile phase composition with methanol ±5ml. These chromatographic variations are evaluated for resolution between CLN and MTS (**Table. 6**).

Table No. 6: Robustness study for analytical method validation of CLN and MTS tablets

Parameters		Adjusted to	Mean Area [a]	Mean RT	SD	% RSD
CLN	Flow Rate As per method 1.0ml/min	0.9 ml/min	397724.33	3.92	489.69	0.12
		1.1ml/min	335048.50	3.22	1748.28	0.52
	Mobile Phase (35:65) (Buffer:Methanol)	40:60	327269.83	3.86	824.87	0.25
		30:70	342643.50	3.05	1657.58	0.48
MTS	Flow Rate As per method 1.0ml/min	0.9 ml/min	1220151	5.43	1727.16	0.14
		1.1ml/min	998100.5	4.42	1162.88	0.12
	Mobile Phase (35:65) (Buffer:Methanol)	40:60	988318.67	5.34	1243.75	0.13
		30:70	994944.5	3.93	2311.45	0.23

[a] **= 5 *Replicates***

2.7. Solution stability studies

Three different concentrations of CLN (30µg/mL) and MTS (75µg/mL) were prepared from the sample solution and stored at room temperature for 24 hrs. Then injected into the HPLC system and the additional peaks were not found in the chromatograms so, it was indicating the stability of CLN and MTS tablet in the solution (**Table. 7**).

Table No. 7: Solution stability study for analytical method validation of CLN and MTS tablets

Name	*Replicate (n = 5)*	*Initial*	*After 3 hrs*	*After 6 hrs*	*After 12 hrs*	*After 24 hrs*
CLN	Mean	358698.4	358127.4	358341.2	358155.4	356796.6
	SD	1023.571	784.3467	1188.667	739.3655	1236.244
	% RSD	0.285357	0.219013	0.331714	0.206437	0.346484
MTS	Mean	1079623	1079627	1077201	1074076	1069934
	SD	1680.88	1758.929	2432.105	2892.491	3946.006
	% RSD	0.155691	0.16292	0.22578	0.2693	0.368808

2.8. Recovery studies

Good recoveries of the CLN and MTS were obtained at different added concentrations for the tablets (**Table. 8**).

Tablet No. 8: Accuracy Results of CLN and MTS from tablets

Brand Name	Analyte	Recovery levels	Actual Conc. (µg/mL)	Added Conc. (µg/mL)	Theoretical Conc. (µg/mL)	Found Conc. (µg/mL)	% Recovery	% RSD	% Error [a]
Cilacar	CLN	**50 %**	10	5	15	15.07	100.46	0.029	0.46
		100 %	10	10	20	20.03	100.15	0.085	0.15
		150 %	10	15	25	25.08	100.32	0.129	0.32
Met XL	MTS	**50 %**	25	12.5	37.5	37.56	100.16	0.058	0.16
		100 %	25	25	50	49.97	99.94	0.086	-0.06
		150 %	25	37.5	62.5	62.41	99.86	0.129	-0.14

[a][found conc. – theoretical conc./theoretical conc.] x 100.

2.9. Analysis of a commercial formulation

Experimentally the results for the amount of CLN and MTS in tablets, expressed as a percentage of label claims were in good agreement with the label claims thereby suggesting that there is no interaction from the excipients which are commonly present in formulation of tablets.

2.10. Degradation study

Acid degradation study:

In acidic degradation study, sample was treated with 3ml of 1N hydrochloric acid and kept for 10hrs at 60ºC. After 10hrs the solution was neutralized with 3ml of 1N sodium hydroxide, made the volume upto the

mark with biorelevant media and analyzed using HPLC. The drug content was found to be degrading up to 4.160% in acidic condition (Figure 7 & 8, Table 9 & 10).

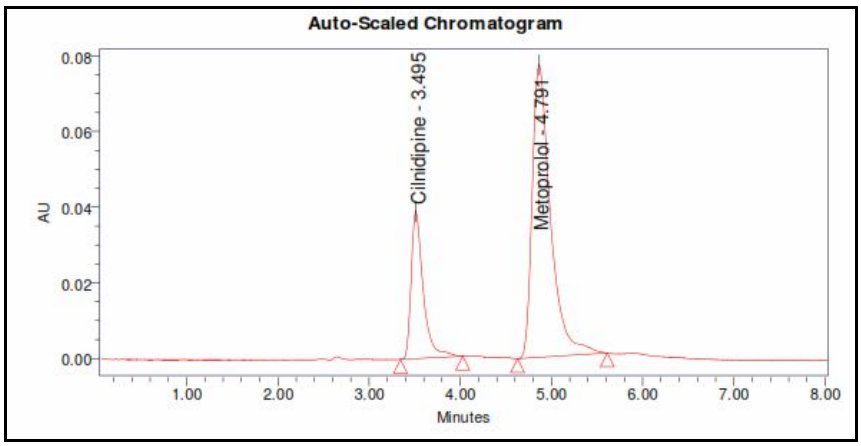

Figure 7: Chromatogram of acidic forced degradation of CLN and MTS

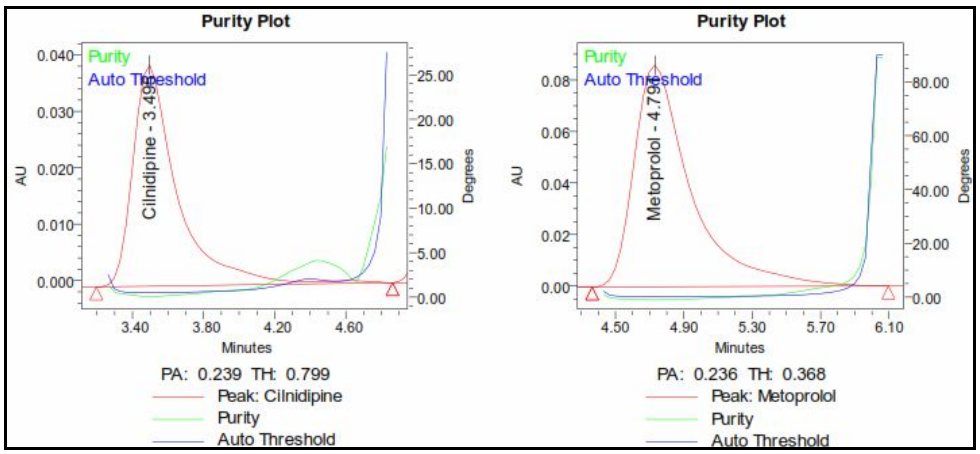

Figure 8: Purity Plots for CLN and MTS in acidic forced degradation

Alkaline degradation study:

Alkaline degradation study was performed by the sample was treated with 3ml of 1N sodium hydroxide and kept for 10hr. After 10hr the solution was neutralized with 3ml of 1N hydrochloric acid, made the volume upto the mark with biorelevant media and analyzed using HPLC. In alkali degradation, it was found that around 6.416% of the drug degraded (Figure 9 & 10, Table 9 & 10).

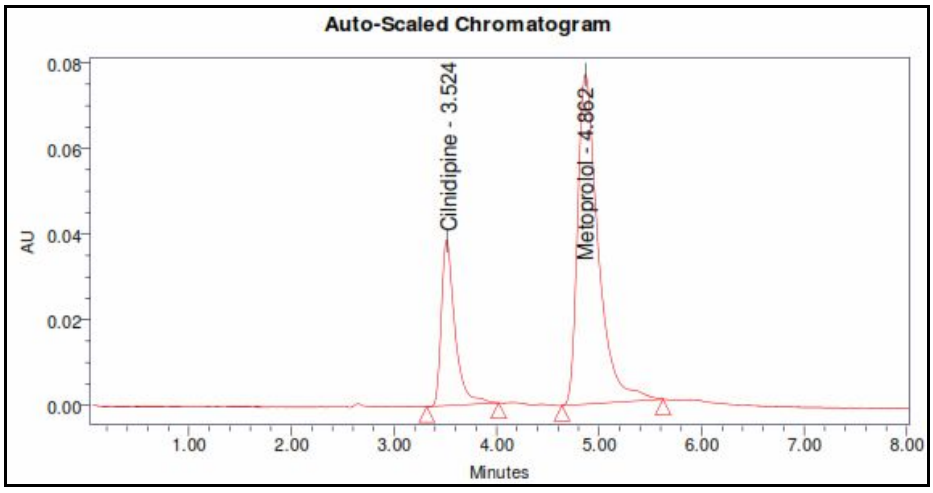

Figure 9: Chromatogram of alkali forced degradation of CLN and MTS

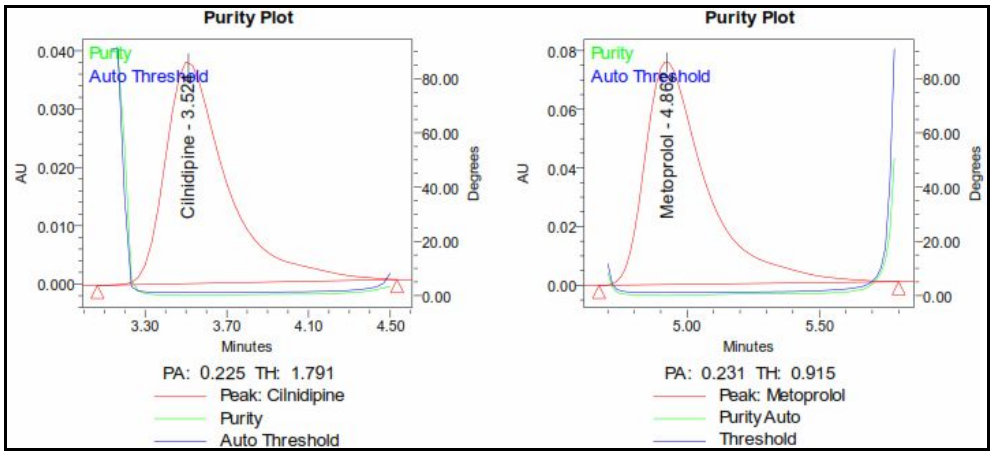

Figure 10: Purity Plots for CLN and MTS in alkali forced degradation

Oxidative degradation study:

Oxidation degradation study was performed by the sample solutions were mixed with 3mL of 30%v/v aqueous hydrogen peroxide solution and kept for 10hrs. After 10hrs made the volume upto the mark with biorelevant media and analyzed using HPLC. In oxidative degradation, it was found that around 2.672% of the drug degraded (Figure 11 & 12, Table 9 & 10).

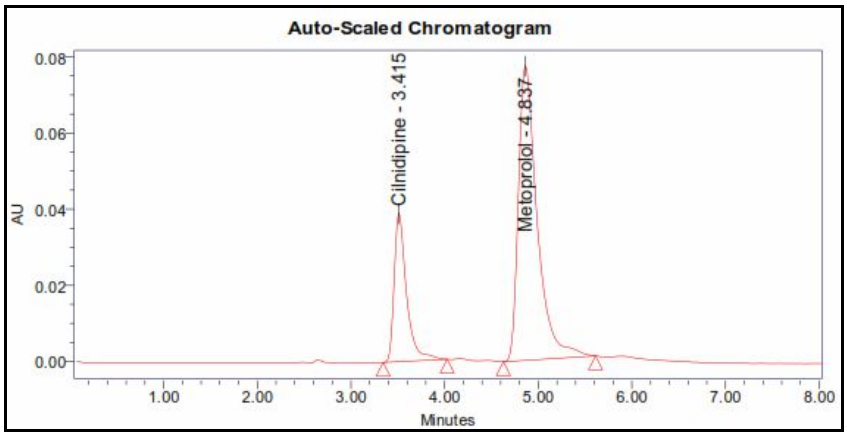

Figure 11: Chromatogram of oxidative forced degradation of CLN and MTS

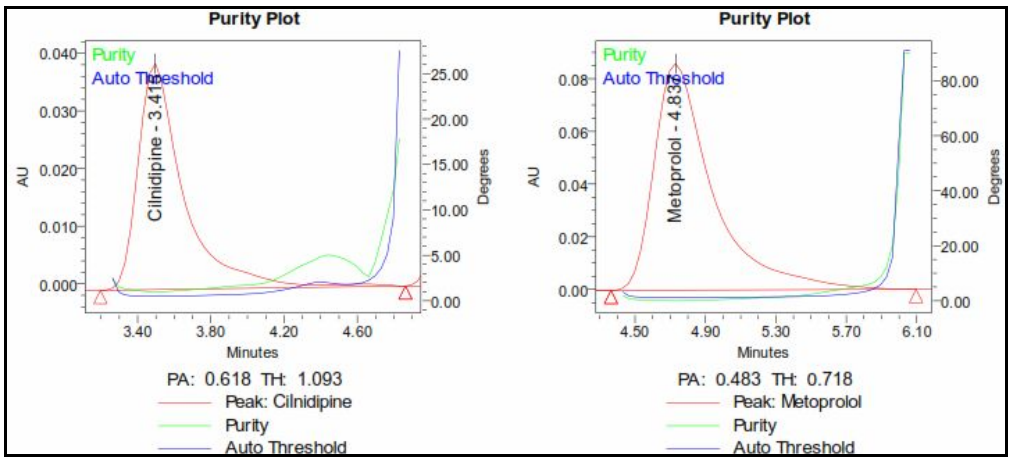

Figure 12: Purity Plots for CLN and MTS in oxidative forced degradation

Photolytic degradation study: Photolytic degradation study was performed by exposing the drug content in UV light for 15mins to 7days. There is 1.746% degradation observed in above specific photolytic condition (Figure 13 & 14, Table 9 & 10).

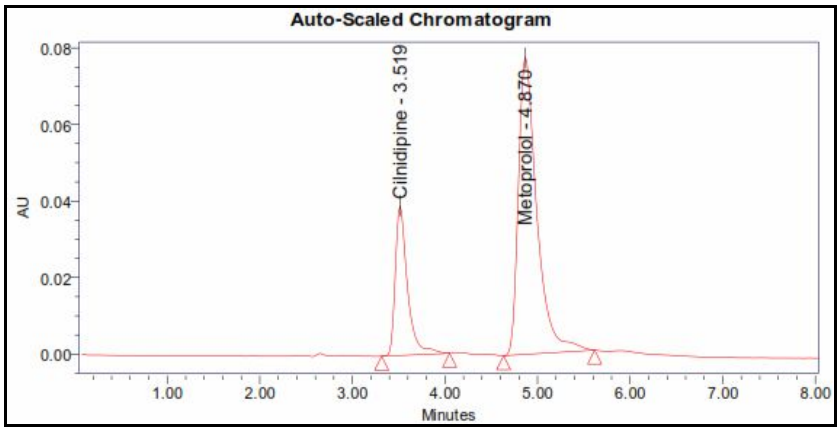

Figure 13: Chromatogram of UV-light degradation of CLN and MTS

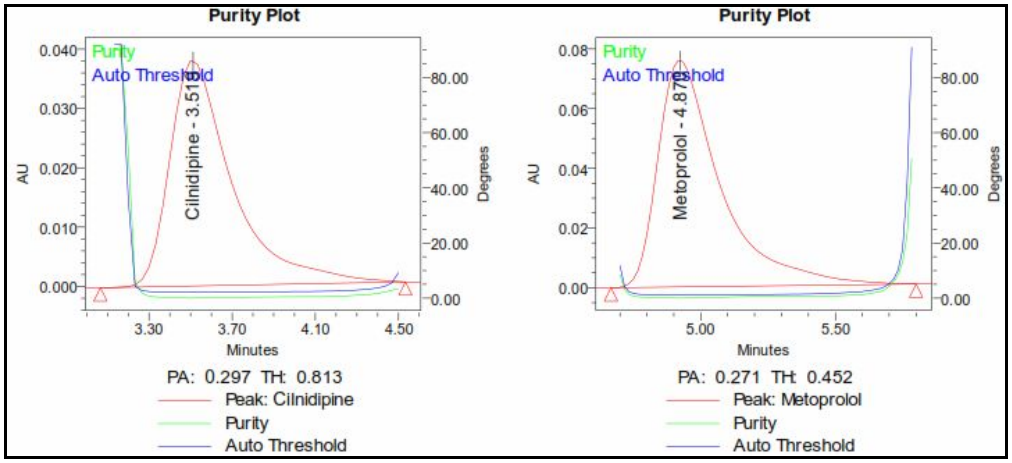

Figure 14: Purity Plots for CLN and MTS in UV-light degradation

Thermal degradation study:

Thermal degradation was performed by exposing solid drug at 80°C for 15mins to 60mins and at 220°C for 2-5mins. Resultant chromatogram of thermal degradation study (Figure 15 & 16, Table 9 & 10) indicate that drug is found to be slightly stable under thermal degradation condition. Only 4.894% drug content were degraded.

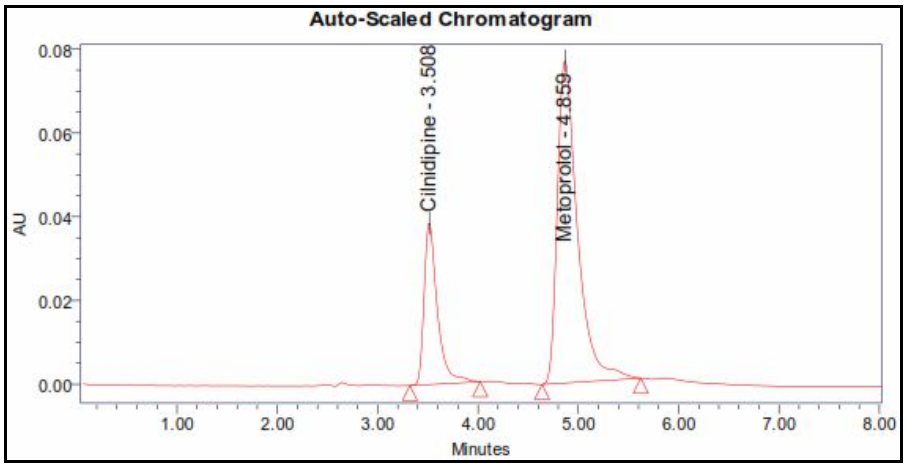

Figure 15: Chromatogram of thermal degradation of CLN and MTS

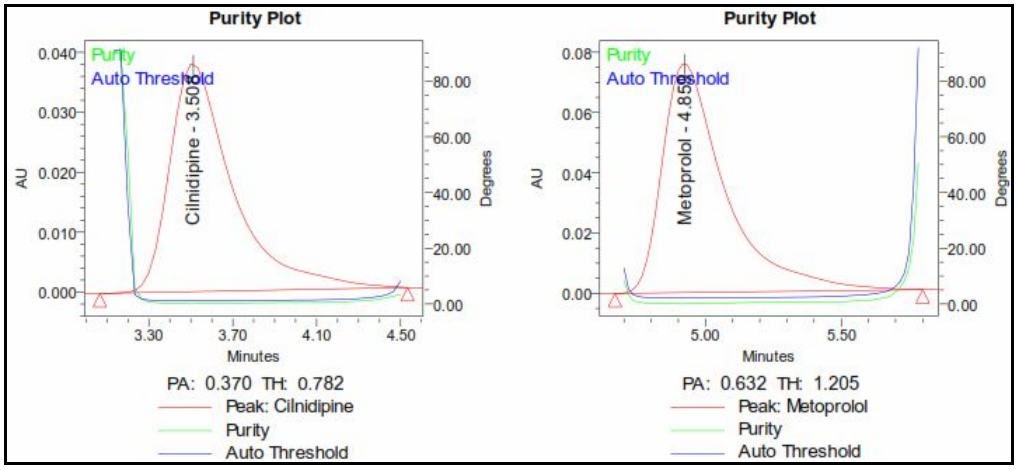

Figure 16: Purity Plots for CLN and MTS in thermal degradation

Table 9: Peak purity results of CLN and MTS

Stress	Purity Angle		Purity Threshold	
Condition	CLN	MTS	CLN	MTS
Acid Degradation	0.239	0.236	0.799	0.368
Alkali Degradation	0.225	0.231	1.791	0.915
Oxidative Degradation	0.618	0.483	1.093	0.718
Photolytic Degradation	0.297	0.271	0.813	0.452
Thermal Degradation	0.370	0.632	0.782	1.205

Table 10: Percentage of degradation of CLN and MTS

Drug Name		Acid	Alkali	Oxidative	Photolytic	Thermal
CLN	Std Area			357542		
	Sample Area	339158	329175	346158	351826	337258
	% of Degradation	5.141	7.933	3.183	1.598	5.673
MTS	Std Area			1079624		
	Sample Area	1045287	1026712	1056287	1059157	1035183
	% of Degradation	3.180	4.900	2.161	1.895	4.116
Average of % Degradation		4.160	6.416	2.672	1.746	4.894

Conclusion

A new RP-HPLC method described in this manuscript provides a simple, convenient and reproducible approach for the simultaneous estimation and quantification of Cilnidipine and Metoprolol Succinate in routine quality control analysis.

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