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A Novel Analytical Method for Simultaneous Quantification of Bisoprolol and Cilnidipine by Reversed-Phase HPLC in Pure and Tablet Dosage Form

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Current study was intended to establish and validate an advanced, novel, stable, simple and systematic analytical procedure to determine Bisoprolol & Cilnidipine in pure drug & tablet by Reversed-Phase HPLC method. The partition of analytes was attained with a combination of reverse phase C18 column (Waters X Terra 150x4.6mm; 3.5μm) and a mobile phase made up of 60% acetonitrile and 40% 0.1% trifluoroacetic acid is utilized, at a flow rate of 1mL/min over an 8minute, isocratic flow and at 225nm isobestic point. With the best acceptable resolution the peaks of Bisoprolol and Cilnidipine was eluted at 3.129min and 6.925min respectively. The linearities of the analytes especially with this proposed method were 2.52 to 37.80μg/ml & 5.00 to 75.00μg/ml for Bisoprolol and Cilnidipine respectively. For the proposed method, all the validation parameters were tested as indicated by ICH guidelines. The %RSD values for system precision was 0.41% for BISO and 0.14% for CILN, %RSD values for method precision was 0.4% for BISO and 0.3% for CILN and %RSD values for intermediate precision was 0.4% for BISO and 0.3% for CILN respectively. As per the validation report, the results of induced degradation studies, stability, precision, accuracy and the remaining validation parameters, however, were all excellent and within the acceptance criteria. The comparative study results reveal that the established process was economic, fast, suitable, sensitive and stable technique for determination of Bisoprolol & Cilnidipine in pure & tablet formulation.

Keywords: Bisoprolol; Cilnidipine; RP-HPLC; Stability; Validation.

Bisoprolol (BISO) is a cardio protective drug, prescribed for managing high blood pressure and heart failure (Figure 1). Bisoprolol reduces cardiac workload by decreasing contractility and the need for oxygen through competitive inhibition of â1-adrenergic receptors.[1,2] Cilnidipine (CILN) is

a calcium channel blocker, used to treat high blood pressure (Figure 2). Cilnidipine acts by blocking N-type & L-type of calcium channels which in turn dilates arterioles and venules results in reduce in blood pressure in capillary level.[3,4] The clinical applications of BISO and CILN are lowers blood pressure, reducing the chances of heart problems such as heart attack and stroke. The combination of BISO and CILN is one of the best choices in the management of hypertension.[5]

Extremely a very few analytical methods was established for estimation of BISO and CILN by RP-HPLC either by alone, combination or by in combination with other analytes[6-9], which was less satisfactory levels of acceptance. The previous reported methods have lack of specificity and more retention times. The need for simultaneous quantification includes accuracy, cost-effectiveness and short run time. Hence the author aimed for development and validation of a novel and unique analytical method for Bisoprolol & Cilnidipine estimation by RP-HPLC in accordance with ICH guidelines.[10-11]

MATERIALS AND METHODS

Chemicals and reagents

The pure analytes BISO and CILN were acquired as gift samples from Glenmark Pharmaceuticals, Mumbai. HPLC grade water, acetonitrile and trifluoro acetic acid are of Merck India made and were purchased for the preparation of mobile phase.

Instrument

The proposed simultaneous analytical estimation was established and validated on auto sampler RP-HPLC (Make: Waters) connected with e2695 model pump, 2998 PDA detector and equipped with Empower 2 software.

Preparation of 0.1% trifluoro acetic acid

The solution was freshly prepared by transferring 1ml of pure trifluoro acetic acid to 1000ml volumetric flask in combination with HPLC grade water and sonicated for 5min.

Preparation of mobile phase

Swirl 600ml of acetonitrile, 400ml of 0.1% trifluoro acetic acid in well closed & labeled 1000ml container.

Preparation of stock solution (standard)

Pure standards 25mg of BISO & 50mg of CILN were mixed and sonicated for 5min with diluent in 100ml volumetric flask before make up.

Preparation of working solution (standard)

Swirl 5ml of the above solution with 30ml of diluent in volumetric flask of capacity 50ml and adjusted to the specified standard using the same diluent, label and stored. Following each preparation step, all solutions were filtered using a 0.45µm filter.

Analytical method development

Selection of detection wavelength

Solution having BISO and CILN was prepared by using diluent and scanned at a region of 200 to 400nm using PDA detector. Both the drugs BISO and CILN have shown an acceptable peak response at 225nm and it was selected as common detection wavelength for both BISO and CILN throughout the analysis.

Streamlined chromatographic conditions

Different trials have been performed to streamline all the vital RP-HPLC parameters as per the literature required for simultaneous determination of BISO & CILN.[12-16] Finally RP C18 column, Waters X Terra RP18 (150x4.6 mm, with a 3.5µm particle size), a mobile phase made up of 60% acetonitrile and 40% 0.1% trifluoroacetic acid is utilized, at a flow rate of 1mL/min over an 8 minute were chosen as effective optimized RP-HPLC conditions for the projected estimation. The optimized conditions were tabulated in Table 1.

Analytical method validation

System suitability

For assuring system performance throughout the analysis for the projected method, system suitability test was performed. The data obtained after six replicates, it was found that the results of all the system suitability parameters have shown excellent acceptability results.

Selectivity

The developed method was selective, if BISO and CILN are absolutely distinguished from one another with high resolution & fixed retention times at streamlined RP-HPLC conditions. Selectivity of the suggested process was analyzed by six frequent injections of standard solutions (working) containing BISO and CILN.

Specificity

The justification for examine specificity was to establish interfering peaks from impurities or degradants or from excipients of formulation or

placebo at same retention times with the analytes of concern. In this method tablet extract, placebo, standard drug solutions & mobile phase (blank injection) were injected to check the specificity of method.

Linearity

A blank solution of diluent and six different standard drug solutions containing 2.52µg/ml to 37.80µg/ml of BISO and 5.0µg/ml to 75.0µg/ml of CILN were equipped with diluent exactly & injected into optimized RP-HPLC process. Using peak responses of respective concentrations calibration plots were constructed. From the calibration plots linearity, range and r^2 values were calculated.

Accuracy

To identify accuracy of the planned RP-HPLC system, standard addition technique was adopted. Solutions in 3 concentration levels containing BISO (12.5mg, 25.0mg & 37.8mg) and CILN (25.0mg, 50.0mg & 75.0mg) were prepared and the triplicate injections from each level was injected in to RP-HPLC.

Precision

System precision

It was identified through 6 times continually injecting recently equipped working standard solution containing 25ìg/ml of BISO & 50ìg/ml of CILN into RP-HPLC system and evaluated for the %RSD of peak areas.

Method precision

To compute the method precision of projected analytical method, sample solution attains with 25ìg per milli litre of BISO & 50ìg per milli litre of CILN was continuously injected for 6 times into RP-HPLC system & computed for the %RSD of drug estimated.

Intermediate precision (Ruggedness)

Six samples of same batch containing 25ìg/ml of BISO and 50ìg/ml of CILN were evaluated to prove intermediate precision of the projected analytical process by various analysts on various columns and by various instruments. From resultant chromatograms of analyte and its %RSD was tabulated.

Robustness

To verify the robustness of the anticipated analytical technique, one out of optimized chromatographic parameters was deliberately changed and the assay was performed. During the validation process rate of flow was tuned to ±0.2ml/min and organic component of the mobile phase was adjusted to ±10% & resulting changes occurred in chromatograms after injecting sample solutions in triplicates comprising 25ìg/ml of BISO and 50ìg/ml of CILN.

LOD and LOQ

The expressions LOD & LOQ are elaborated as detection of smallest amount of analyte and quantification of smallest amount of analyte, by projected analytical method. According to ICH guidelines, signal to noise ratio method is one of the recognized methods to evaluate LOD and LOQ. Triplicates of lowest concentrations of standard solutions (working) & blank solutions of BISO and CILN were analyzed to estimation LOD & LOQ of the method at optimized RP-HPLC conditions.

Assay of BISO and CILN in marketed formulation

From 20 marketed formulations (tablets), average weight of each one tablet was tabulated and grinded to fine powder. Tablet powder equivalent to 25mg and 50mg of BISO and CILN respectively was shifted to 100ml volumetric flask & swirl with 75ml diluent, sonicated for 5min, filled with the same diluent and passed through a 0.45 µm filter paper. A 5 milli litre portion of the stock filtrate was diluted to a total volume of 50 milli litre with the diluent. RP-HPLC chromatographic system was then used to inject replicates and obtain peak areas from chromatograms to determine the amounts of BISO and CILN in each tablet.

Stress degradation studies

A stability stock solution for stress degradation studies was arranged by combining 200mg of formulation powder containing BISO and CILN with 70ml of diluent in a volumetric flask of capacity 100ml. The blend underwent sonication for 15 minutes, diluted to mark using similar diluent, did go through a 0.45µm filter.

Acid induced degradation

5ml of stability stock filtrate, 1ml of 1N HCl and 30ml of diluent were mixed in volumetric flask of capacity 50ml, solution was transferred to water bath at constant temperature of 60°C for 30 minutes. After cooling, 1 mL of 1N NaOH was introduced, and solution was adjusted to final volume with diluent. It was then stored at room temperature and injected after 24 hours.

Alkali induced degradation

5ml of stability stock filtrate, 1ml of 1N NaOH and 30ml of diluent were mixed in volumetric flask of capacity 50ml, solution was transferred to water bath at constant temperature of 60°C for 30 minutes. After cooling, 1 ml of 1N HCl was introduced, and solution was adjusted to final volume with diluent. It was then stored at room temperature and injected after 24 hours.

Peroxide induced degradation

5ml of stability stock filtrate, 1ml of 30% H_2O_2 & 30ml of diluent were mixed in volumetric flask of capacity 50ml, solution was transferred to water bath at constant temperature of 60°C for 30 minutes. After cooling, solution was diluted to mark with diluent, stored at room temperature, and then injected after 24 hours.

Thermal induced degradation

The sample powder containing BISO and CILN was exposed at 105°C for 72hrs. 200mg of sample was weighed and transferred into 100ml volumetric flask with 70ml of diluent. The mixture was sonicated for 15 minutes, then diluted to mark with same diluent, mixed, and filtered through a 0.45µm filter. Next, 5ml of filtrate was diluted to 50ml with diluent, mixed, stored at room temperature and injected into RP-HPLC system after 24 hours.

Photolytic degradation

The sample powder was directly exposed under UV light for 24hrs. 200mg of this sample was weighed and transferred into 100ml volumetric flask containing 70ml of diluent and sonicated for 15min and diluted to the mark with the same diluent, mixed and was passed through 0.45µ filter. Further 5ml filtrate was diluted to 50ml with diluent, mixed, stored at room temperature and injected into RP-HPLC system after 24hrs. All the degradation samples passed through 0.45µ filter before injected in to RP-HPLC system.

Stability studies

To identify the stability of projected analytical method, test solution consisting of 25µg/ml BISO and 50µg/ml CILN was prepared freshly, passed through 0.45µ filter and the filtrate was injected into RP-HPLC system at different and standard time intervals (0hrs, 6hrs, 12hrs, 18hrs & 24hrs).

RESULTS

Analytical method validation

System suitability

Projected RP- High-Performance Liquid Chromatography method passes the system suitability test for estimation of BISO and CILN,

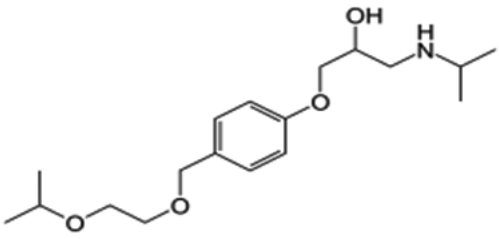

Fig. 1. Chemical structure of Bisoprolol

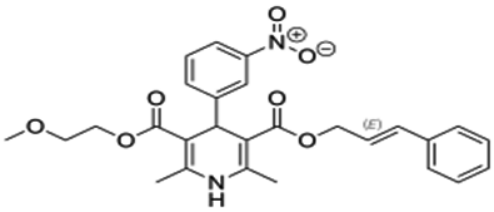

Fig. 2. Chemical structure of Cilnidipine

Table 1. Optimized HPLC conditions of BISO and CILN

Column	Waters X Terra RP_{18} (150x4.6mm, 3.5µ)
Mobile phase	Acetonitrile: 0.1% trifluoro acetic acid (60:40 v/v)
Column temperature	Ambient
Flow rate	1.0ml/min
Run time	10mins
Pumping mode	Isocratic flow
Injection volume	10µl
Wavelength	225nm
Retention times	Bisoprolol: 3.129min
	Cilnidipine: 6.925min

as the resolution (Rs) was >2, total theoretical plate count (N) was >2000, peak tailing factor (Tf) was <2 & the %RSD of peak areas was <2. The results were furnished in Table 2.

Selectivity

The chromatographic data on inspection it was establish that the retention times & resolution of analytes was fixed with adequate change, confirms selectivity of projected analytical technique. The typical chromatogram of BISO and CILN were shown in Figure 3**.**

Specificity

No interfering peaks or no additional peaks were viewed in the chromatograms except

Table 2. System suitability results of BISO and CILN

Analyte	Rt (min.)	%RSD peak areas	USP plate count	USP tailing	Resolution
BISO	3.129	0.3	4662.67	1.05	-
CILN	6.925	0.3	12144.8	0.95	16.99

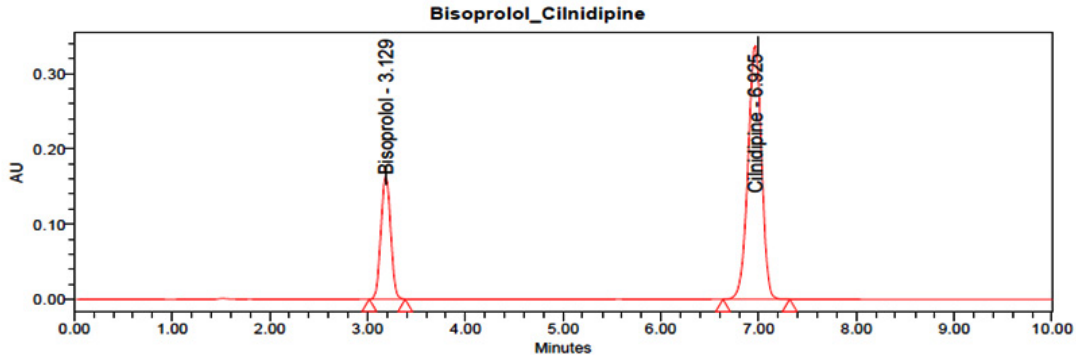

Fig. 3. Typical chromatogram of Bisoprolol and Cilnidipine

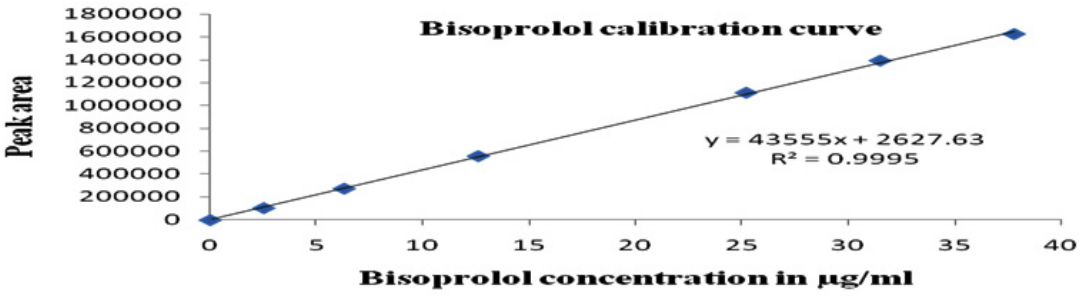

Fig. 4. Linearity curve of BISO

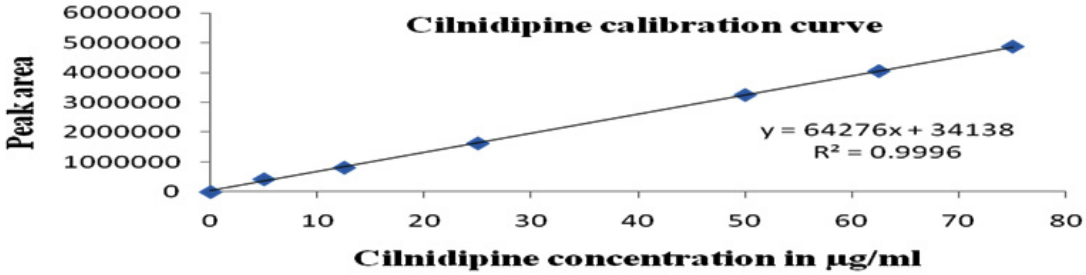

Fig. 5. Linearity curve of CILN

BISO and CILN peaks, with all the injected solutions. Thus it indicates that projected RP-HPLC system was specific to recognize BISO & CILN only.

Linearity

By the new recommended RP-HPLC method BISO has shown linearity 2.52µg/ml to 37.80µg/ml with relevant regression equation y=43555x+2627.63 and CILN has shown linearity 5.00-75.00µg/ml with relevant regression equation y=63276x+34138. The values for r^2 for BISO and CILN were 0.9995 and 0.9996 respectively. The calibration curves of BISO and CILN were shown in Figure 4 & Figure 5 and the data on linearity has been presented in Table 3.

Accuracy

Individual recoveries of all the levels of BISO and CILN were within the permitted limit. The mean %recoveries of BISO and CILN are 99.56% and 100.35% respectively and the study confirms the accuracy of the suggested RP-HPLC system. The data related to accuracy are summarized in Table 4.

Precision

System precision

The %RSD of peak areas from six replicate injections of the working standard solution was 0.41% for BISO and 0.14% for CILN. This demonstrates that the system precision is within acceptable limits.

Table 3. Linearity data of BISO and CILN

Injection	BISO		CILN	
	Concentration (micrograms per milliliter)	Peak area	Concentration (micrograms per milliliter)	Peak area
Blank	00.00	000000	00.00	000000
Linearity 1	02.52	104895	05.00	424515
Linearity 2	06.30	274526	12.50	814575
Linearity 3	12.60	558347	25.00	1627484
Linearity 4	25.20	1112478	50.00	3244563
Linearity 5	31.50	1392594	62.50	4045894
Linearity 6	37.80	1624456	75.00	4865436

Table 4. Accuracy results of BISO and CILN

Levels	BISO Found conc. (mg)	CILN Mean % recovery ±SD	Found conc. (mg)	Mean % recovery ±SD
50%	12.29	98.5±0.28	25.05	100.27±0.06
100%	25.32	101.0±0.49	50.32	100.48±0.16
150%	37.16	99.2±0.55	75.23	100.31±0.02
Mean %recovery		99.56		100.35
Mean±SD		1.18		0.13
%RSD		1.2		0.1

Table 5. Precision results of BISO and CILN

Analyte		System precision	Method precision	Intermediate precision
BISO	Mean±SD	1128268±3348.61	100.03%±0.44	99.98%±0.39
	%RSD	0.3%	0.4%	0.4%
CILN	Mean±SD	3224623±9773.93	100.08%±0.37	100.2%±0.25
	%RSD	0.3%	0.3%	0.3%

Method precision

The mean of analyte calculated and its %RSD of 6 replicate injections of sample solutions was identified as 100.03% % & 0.4% for BISO; 100.08% & 0.3% for CILN. Therefore, the projected method was reproducible.

Intermediate precision (Ruggedness)

The mean assay of analytes & their %RSD were calculated as 99.98% & 0.4% for BISO; 100.2% & 0.3% for CILN respectively. Hence, the projected RP-HPLC method was reproducible with different instrument by different analyst and on different column. The precision data was tabulated in Table 5.

Robustness

From the results it was found that the proposed analytical method was stable even after small but deliberate changes in the flow rate and the composition ratio of the mobile phase. It was pragmatic that there was slight change in system suitability parameters but net assay values of BISO and CILN were within the acceptable limits. The robustness data was reflected in Table 6.

LOD and LOQ

The estimated limit of detection (LOD) & limit of quantitation (LOQ) values for BISO were 0.025µg/ml & 0.075µg/ml respectively; for CILN the LOD & LOQ are 0.050µg/ml & 1.50µg/ml respectively. The LOD & LOQ data was tabulated in Table 7.

Assay of BISO and CILN in tablets

Assay result reveals that each tablet of BESICOR-C contains 5.03mg and 9.92mg of BISO

Table 6. Robustness results of BISO and CILN

Conditions	BISO			CILN			
	Rt (min.)	Plate count	%Drug found	Rt (min.)	Plate count	Rs	%Drug found
0.8ml/min	3.95	6444	99.73	8.85	9968	19.33	100.63%
1.2ml/min	2.65	3456	100.27	5.76	10346	15.22	99.83%
27:73v/v	3.94	6168	99.80	8.72	14256	19.04	100.67%
33:67v/v	2.73	3656	99.20	5.05	8867	11.40	100.00%

Table 7. LOD & LOQ results of BISO and CILN

Analyte	LOD	LOQ
BISO	0.025µg/ml	0.075µg/ml
CILN	0.050µg/ml	0.150µg/ml

Table 8. Assay results for marketed tablet of BISO and CILN

Brand name	Drugs	Label claim (mg)	Amount recovered (mg)	Assay
BESICOR-C	Bisoprolol	5.00	5.03	100.60%
	Cilnidipine	10.00	9.92	99.20%

Table 9. Forced degradation results of BISO and CILN

Conditions	BISO			CILN		
	% of degradation	Purity angle	Purity threshold	% of degradation	Purity angle	Purity threshold
Control	-	2.064	45.365	-	15.214	52.647
1N HCl	21.50	2.041	44.256	24.00	15.212	52.546
1N NaOH	19.20	2.042	45.268	22.10	15.142	52.461
30% H_2O_2	15.60	2.037	48.252	17.00	15.152	52.314
Thermal	17.40	2.034	45.198	16.70	15.134	52.394
Photolytic	15.70	2.026	45.367	13.90	15.291	52.471

Table 10. Stability study results of BISO and CILN

Injection No.	Time in hrs	BISO % of drug found	CILN % of drug found
1	Initial	100.2	100.6
2	6hrs	99.8	100.3
3	12hrs	99.5	100.5
4	18hrs	98.5	98.8
5	24hrs	98.3	97.6

and CILN respectively against its label claim with the projected analytical method. The assay data was furnished in Table 8.

Forced degradation studies

1N HCl originated degradation was calculated as 21.50% and 24.00%; 1N NaOH stimulated degradation was 19.20% and 22.10%; while 30% H_2O_2 stimulated degradation was 15.60% and 17.00% for BISO and CILN respectively. thermal affected degradation at 105°C was 17.40% and 16.70% and Photolytic degradation under UV light was 15.70% and 13.90% for BISO and CILN respectively. The forced degradation study data was given in Table 9.

Stability studies

The calculated %drug available after 24hrs was 98.3% for BISO and 97.6% for CILN. The stability study data was tabulated in Table 10.

DISCUSSION

In the proposed method, the retention time of Bisoprolol and Cilnidipine was found to be 3.129min and 6.925min respectively. Quantification was linear in concentration range of 2.52-37.80µg/ml for Bisoprolol and 5.0-75.0µg/ml for Cilnidipine. The regression equation of the linearity plot of concentration of Bisoprolol and Cilnidipine over its peak area was found to be y=43555x+2627.63 (r^2=0.9995) for Bisoprolol and y=63276x+34138 (r^2=0.9996) for Cilnidipine, where x is the concentration of Bisoprolol and Cilnidipine (µg/ml) and y is the corresponding peak area. The number of theoretical plates calculated was 4662.67 for Bisoprolol and 12144.8 for Cilnidipine and tailing factor was 1.05 for Bisoprolol and 0.95 for Cilnidipine, which indicates efficient performance of the column. The limit of detection and limit of quantification for Bisoprolol were found to be 0.025µg/ml and 0.075µg/ml and for Cilnidipine were found to be 0.050µg/ml and 0.150µg/ml respectively, which indicate the sensitivity of the method. The use of acetonitrile and 0.1% trifluoro acetic acid in the ratio of 60:40 v/v as mobile phase resulted in peak with good shape and resolution. The high percentage of recovery indicates that the proposed method is highly accurate. No interfering peaks were found in the chromatogram of the formulation within the run time indicating that excipients used in tablet formulations did not interfere with the estimation of the drug by the proposed HPLC method.

CONCLUSION

After extensive study by author, a novel, economical, most stable and effective reversed-phase HPLC method was designed and validated using unique optimized chromatographic conditions than previous studies for estimation of Bisoprolol and Cilnidipine in tablets. Validation report provides evidence that the system was suitable, robust, accurate, linear, rugged & precise projected analytical method even in presence of degradation products up till 24hrs. The proposed HPLC method can be reliably adopted for routine quality control analysis of Bisoprolol and Cilnidipine in its tablet dosage forms.

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Conflict of Interest

The author(s) do not have any conflict of interest.

Data Availability Statement

This statement does not apply to this article.

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials

Permission to reproduce material from other sources

Not applicable

Author's Contribution

Lakshmana Rao Atmakuri: Conceptualization, Methodology, Writing – Original Draft; Kurnool Mahammed Ismail: Project Administration, Writing – Review & Editing; Vijaya Kumar Ghanta: Data Collection, Analysis; Bhaskar Vallamkonda: Funding Acquisition, Resources; Ramesh Alluri: Supervision, Data Interpretation; Satya Venkata Sakuntala Mamidi: Visualization, Final Draft Approval.

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