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Development and Validation of High Performance Liquid Chromatography Method for Simultaneous Estimation of Nebivolol and Indapamide in Their Combined Tablet Dosage Form

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Abstract: The present study describes a new, simple, accurate and precise high performance liquid chromatography method for the simultaneous determination of Nebivolol and Indapamide in combined tablet dosage form. The chromatographic method was standardized using a Base Deactivated Silica (BDS) hypersil C_{18}, 250 mm × 4.6 mm, 5µ (particle size), Thermo scientific from Germany with isocratic conditions and mobile phase containing potassium dihydrogen orthophosphate buffer-pH 3.5 (0.05M KH_2PO_4): triethyl amine: acetonitrile (40:0.5:60) at flow rate of 1 ml/min using UV detection at 286 nm. The retention times of Nebivolol and Indapamide were 3.587 min and 5.730 min, respectively. The method was linear over the concentration range for Nebivolol 25-75µg/ml and for Indapamide 7.5-22.5µg/ml. The recoveries of Nebivolol and Indapamide were found to be in the range of 98.70-101.32% and 98.72-100.04% respectively. The validation of method was carried out utilizing ICH guidelines. The described HPLC method was successfully employed for the analysis of pharmaceutical formulations containing combined dosage form.

Key words: Nebivolol · Indapamide · Simultaneous estimation · High Performance Liquid Chromatography.

INTRODUCTION

Nebivolol is chemically designated as 1-(6-fluorochroman-2-yl)-{[2-(6-fluorochroman-2-yl)-2-hydroxy-ethyl] amino} ethanol. It is a third-generation vasodilating cardioselective â-blocking agent used in the treatment of hypertension. Its molecular formula is $C_{22}H_{25}F_2NO_4$ and it has a molecular weight of 444.90 gm/mole [1-3]. Nebivolol is a white odourless powder used for the treatment of hypertension. Its mode of action is lowering blood pressure (BP) by reducing peripheral vascular resistance and significantly increases stroke volume with preservation of cardiac output. The net hemodynamic effect of nebivolol is the result of a balance between the depressant effects of beta-blockade and an action that maintains the cardiac output [2]. Literature survey revealed that some spectrophotometric methods, HPTLC and RP-HPLC for the quantitative estimation of nebivolol have been developed [1-2, 4-13].

Indapamide is a non-thiazide sulphonamide diuretic drug generally used in the treatment of hypertension, as well as decompensated cardiac failure. Its molecule contains both a polar sulfamoyl chlorobenzamide moiety and a lipid soluble methyl-indoline moiety. It differs chemically from thiazide is that it does not possess the thiazide ring system and contains only one sulfonamide group [14]. Indapamide is chemically 3-(aminosulfonyl)-4-chloro-N-(2, 3-dihydro-2-methyl-1H-indol-1-yl). The molecular formula is $C_{16}H_{16}ClN_3O_3S$ and molecular weight is 365.8 gm/mole [15-17]. It is a white to off- white crystalline powder that is soluble in methanol, ethanol, acetic acid and ethyl acetate, very slightly soluble in ether, chloroform and benzene and practically insoluble in water. It is official drug in British Pharmacopoeia 2000 [18] and United States Pharmacopoeia 2007 [19]. Literature survey revealed that, bio-analytical methods by HPLC, LCMS were found using human serum [20-22], blood and few spectrophotometric methods and

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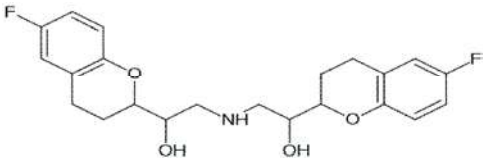

Fig. 1: Chemical structure of Nebivolol

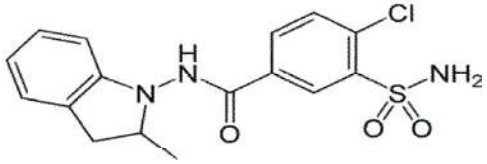

Fig. 2: Chemical structure of Indapamide

RP-HPLC for the quantitative estimation of Indapamide in bulk and pharmaceutical formulations [23-39] have been developed.

From the literature survey, it was found that many methods have been reported for estimation of Nebivolol and Indapamide individually and in combination with other drugs. Only one spectophotometric method has been reported for the quantitative estimation of Nebivolol and Indapamide in pharmaceutical dosage form and no HPLC method for simultaneous estimation of Nebivolol and Indapamide have been reported so far. Hence an attempt has been made to develop new HPLC method which is simple, rapid, reproducible and economical method for simultaneous estimation of Nebivolol and Indapamide in tablet dosage form.

MATERIALS AND METHODS

Chemicals and Reagents: The working standards of Nebivolol and Indapamide were generous gift obtained from Zydus Cadila Healthcare Ltd., Ahmedabad, India. The combination formulation of Nebivolol and Indapamide (Nebula-D marketed by Zydus Cadila Healthcare Ltd.) tablets were purchased from the local market which has Label claim: Nebivolol 5 mg, as Nebivolol and Indapamide (SR) 1.5 mg). Acetonitrile, methanol and water were used of HPLC grade make-Merck, Rankem. Potassium dihydrogen phosphate, triethyl amine and phosphoric acid used were of analytical grade.

HPLC Instrumentation: Chromatographic separation was performed with Shimadzu Prominence System (SPD-20AT, Shimadzu) having following components: LC-20AT Pump, SPD-20A Detector, BDS hypersil C_{18}, 250 mm × 4.6 mm, 5µ (particle size), Thermo scientific, Rheodyne manual Injector (20µl Capacity), Hamilton Syringe (25µl) and Chromatograms and data were recorded by means of Spinchrom CFR Software.

Preparation of Mobile Phase and Standard Solution

Mobile Phase Preparation: The mobile phase consisted of potassium dihydrogen phosphate buffer pH 3.5-triethyl amine- acetonitrile (40:0.5:60). To prepare the buffer solution, 6.8 gm potassium dihydrogen phosphate was weighed and dissolved in 900 ml HPLC grade water in 1000 ml volumetric flask and 5 ml triethyl amine is added. The pH of the buffer was adjusted to 3.5 with diluted phosphoric acid (H_3PO_4). The volume is made up to 1000 ml with HPLC grade water. Then the mixture of buffer solution and acetonitrile was prepared in the ratio of 40:60 to prepare final mobile phase. Mobile phase was filtered through a 0.45µ nylon membrane (Millipore Pvt. Ltd. Bangalore, India) and degassed in an ultrasonic bath.

Standard Preparation: Stock solution of Nebivolol: Nebivolol standard stock solution containing 500µg/ml was prepared in a 100 ml volumetric flask by dissolving 50 mg of Nebivolol in small volume of mobile phase then the volume was made up to 100 ml with mobile phase. This solution is then sonicated for 10 minutes.

Stock Solution of Indapamide: Indapamide standard stock solution containing 150µg/ml was prepared in a 100 ml volumetric flask by dissolving 15 mg of Indapamide in small volume of mobile phase then the volume was made up to 100 ml with mobile phase. This solution is then sonicated for 10 minutes.

Working Standard Preparation: Take 1ml of Nebivolol stock solution and 1ml of Indapamide stock solution and dilute with mobile phase up to 10 ml. Then solution was filtered through 0.45µ nylon syringe filter. The concentration obtained was 50µg/ml of Nebivolol and 15µg/ml of Indapamide.

Chromatographic Conditions: The mobile phase consisting of phosphate buffer-pH 3.5 (0.05M KH_2PO_4): triethyl amine: acetonitrile in the ratio (40:0.5:60) with an apparent pH adjusted to 3.5 using with diluted phosphoric acid was selected as the optimum composition of mobile phase, because it was found that this solvent system resolved both the components ideally. The mobile phase

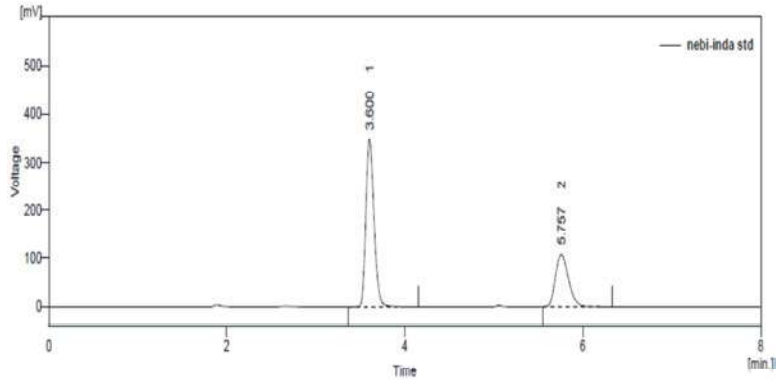

Fig. 3: HPLC chromatogram of Nebivolol and Indapamide standard solution

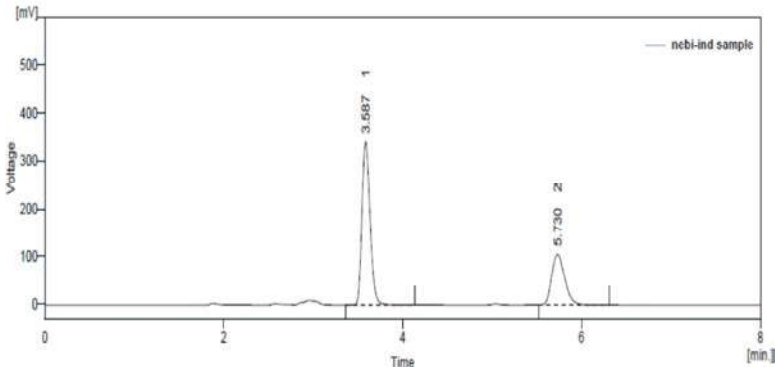

Fig. 4: HPLC chromatogram of Nebivolol and Indapamide in test solution

and samples were degassed by ultrasonication for 20 min and filtered through 0.45µ Nylon 66 (N66) 47 mm membrane filter paper. The measurements were carried out with an injection volume of 20µl, flow rate was set to 1.0 ml/min and UV detection was carried out at 286 nm. All determinations were performed at ambient column temperature (27°C). The chromatograms of the prepared standard stock solutions of Nebivolol and Indapamide were recorded under the above optimized chromatographic conditions (Fig. 3).

Test Preparation: Twenty tablets were weighed and the average weight was calculated. The tablets were crushed with a mortar and pestle for 10 min. A portion of powder equivalent to the weight of one tablet was accurately weighed and transferred to a 100 ml volumetric flask and dilute with mobile phase and sonicated of 30 minutes with normal hand-shaking. Cool the flask to room temperature. Filter this solution through 0.45µm nylon syringe filter. The concentration obtained was 50µg/ml of Nebivolol and 15µg/ml of Indapamide. The chromatogram of the prepared solution of Nebivolol and Indapamide was recorded under the above optimized chromatographic conditions (Fig. 4).

RESULT AND DISCUSSION

Method Development and Optimization: Proper selection of the methods depends upon the nature of the sample (ionic or ionisable or neutral molecule) its molecular weight and solubility. Nebivolol and Indapamide are dissolved in polar solvent hence RP-HPLC was selected for it's estimation in formulation. To develop a rugged and suitable HPLC method for the quantitative determination of Nebivolol and Indapamide, the analytical condition were selected after the consideration of different parameters such as diluent, buffer, buffer concentration, organic solvent for mobile phase and mobile phase composition and other chromatographic conditions. Preliminary trials were taken with different composition of buffer and organic phase of mobile phases with pH range of 3.5-4.5 but Nebivolol peak was appear in early eluting region even at certain composition there was double peak phenomena.

The column selection has been done on the basis of backpressure, resolution, peak shape, theoretical plates and day-to-day reproducibility of the retention time and resolution between Nebivolol and Indapamide peak. After evaluating all these factors, a BDS hypersil C_{18},

250 mm × 4.6 mm, 5µ (particle size) column was found to be giving satisfactory results. The selection of water, methanol, triethyl amine, acetonitrile and buffer was based on chemical structure of both the drugs. The acidic pH range was found suitable for solubility, resolution, stability, theoretical plates and peak shape of both components. Best results were obtained with 0.05M potassium dihydrogen orthophosphate pH 3.5 with phosphoric acid solution improved the peak shape of Nebivolol and Indapamide.

For the selection of organic constituent of mobile phase, acetonitrile was chosen to reduce the longer retention time and to attain good peak shape. Therefore, final mobile phase composition consisting of a mixture of buffer-pH 3.5 (0.05M KH_2PO_4): triethyl amine: acetonitrile (40:0.5:60), set at a flow rate of 1.0 ml/min was selected for the chromatographic analysis. Optimize mobile phase proportion was provide good resolution between Nebivolol and Indapamide.

Under above described experimental conditions, all the peaks were well defined and free from tailing. The effect of small deliberate changes in the mobile phase composition, flow rate and column temperature on results was evaluated as a part of testing for methods robustness. The peak homogeneity was expressed in terms of peak purity values and was obtained directly from the spectral analysis of the sample.

Method Validation: The developed analytical method was subjected to validation with respect to various parameters such as linearity, limit of quantification (LOQ), limit of detection (LOD), accuracy, precision, recovery studies, specificity and reproducibility and robustness / ruggedness as per the ICH guidelines [40-45].

Specificity: The specificity of the method was evaluated by assessing interference from excipients in the pharmaceutical dosage form prepared as a placebo solution. The specificity of the method for the drug was also established by checking for interference with drug quantification from degradation products formed during the forced degradation study. The peak purity of the Nebivolol and Indapamide were found satisfactory under different stress condition. There was no interference of any peak of degradation product with drug peak.

Linearity and Range: For linearity five points calibration curve were obtained in a concentration range from 25-75 µg/ml for Nebivolol and 7.5-22.5µg/ml for Indapamide. The response of the drug was found to be linear in the investigation concentration range and the linear regression equation for Nebivolol was y = 44.921x – 24.752 with correlation coefficient 0.9999 (Fig. 5) and for Indapamide was y = 75.305x - 34.106 with correlation coefficient 0.998 (Fig. 6). Where x is the concentration in µg/ml and y is the peak area in absorbance unit.

Precision (Repeatability and Reproducibility): Precision study was established by evaluating method precision and intermediate precision study. System precision was evaluated by analyzing the standard solution five times. Method precision of the analytical method was determined by analyzing three sets of sample preparation. Assay of all three replicate sample preparations was determined and mean % assay value, standard deviation; % relative standard deviation was calculated.

Data obtain from precision experiments are given in Table 1 for intraday and interday precision study for both Nebivolol and Indapamide. The RSD values for intraday precision study and interday precision study was < 2.0% for Nebivolol and Indapamide, which confirm that the method was precise.

Accuracy (% Assay): Accuracy study was assessed by determination of the % assay of Nebivolol and Indapamide in market formulation. The mean % assay of Nebivolol was 99.23% and the mean recovery of Indapamide was 99.26%, which was satisfactory.

Recovery Studies: Recovery of Nebivolol and Indapamide was done at three different concentrations (corresponding to 80, 100 and 120% of test solution concentration). Known amounts of Nebivolol (40, 50 and 60µg/ml) and Indapamide (12, 15 and 18µg/ml) were added to a diluent preparation and the amount of Nebivolol and Indapamide recovered, was calculated. For each concentration, three sets were prepared and injected in duplicate. % Recovery was calculated at each level and recorded as shown in Table 3 and 4. The mean recovery of Nebivolol was between 98.70% and 101.32% and the mean recovery of Indapamide was between 98.72% and 100.04%, which was satisfactory.

Limit of Detection (LOD)/ Limit of Quantitation (LOQ): The LOD was determined on the basis of signal to noise ratios and was determined using analytical response of three times the background noise. LOQ was determined as the lowest amount of analyte that was reproducibly quantified above the baseline noise following triplicate injections. Both LOQ and LOD were calculated on the peak area using the following equations:

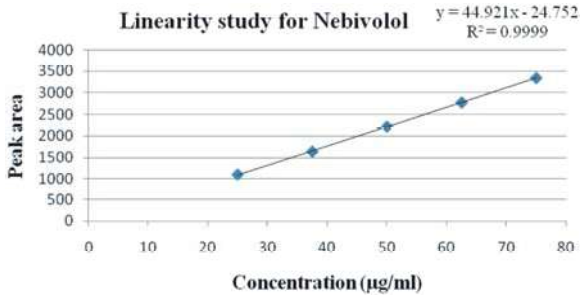

Fig. 5: Linearity study for Nebivolol

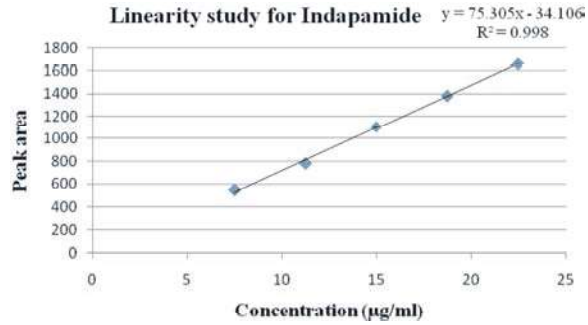

Fig. 6: Linearity study for Indapamide

Table 1: Precision data for Nebivolol and Indapamide

Drug	Concentration (µg/ml)	Intraday (n=3) Avg.±SD	C.V.	Interday (n=3) Avg.±SD	C.V.
Nebivolol	25	1117.168±8.465	0.7640	1111.273±12.230	1.1005
	50	2224.719±20.199	0.9079	2216.598±26.523	1.1965
	75	3350.476±34.863	1.0405	3351.628±38.090	1.1365
Indapamide	7.5	547.733±7.396	1.3502	549.822±3.704	0.6736
	15	1100.740±15.368	1.3961	1098.502±14.263	1.2984
	22.5	1661.298±13.614	0.8195	1663.118±17.756	1.0676

Table 2: Results of accuracy study

	Label claim in mg	Result	% Assay	Average % Assay	SD	% RSD
Nebivolol	5	4.9877	99.7544	99.2313	0.8222	0.8285
	5	4.9141	98.2836			
	5	4.9827	99.6559			
Indapamide	1.5	1.4967	99.7845	99.2608	0.8221	0.8283
	1.5	1.4746	98.3132			
	1.5	1.4952	99.6849			

Table 3: Recovery profile of Nebivolol

Recovery level	Sample concentration in µg/ml	Standard: Amount added in sample solution in µg/ml	Amount recovered in µg/ml	% Recovery
80%	50	40	39.54271539	98.85678847
80%	50	40	40.16961064	100.4240266
80%	50	40	39.96000856	99.9000214
100%	50	50	49.35084731	98.70169462
100%	50	50	50.29647352	100.592947
100%	50	50	49.84905413	99.69810825
120%	50	60	59.46037637	99.10062728
120%	50	60	60.79366414	101.3227736
120%	50	60	60.05835944	100.0972657

Table 4: Recovery profile of Indapamide

Recovery level	Sample concentration in µg/ml	Standard: Amount added in sample solution in µg/ml	Amount recovered in µg/ml	% Recovery
80%	15	12	11.94067594	99.50563283
80%	15	12	11.93953452	99.49612098
80%	15	12	11.98698507	99.89154224
100%	15	15	14.80939608	98.72930719
100%	15	15	14.9056832	99.3712213
100%	15	15	14.95909904	99.72732692
120%	15	18	17.8446008	99.13667112
120%	15	18	17.8775254	99.31958554
120%	15	18	18.00812312	100.0451285

Table 5: Statistical data for Nebivolol and Indapamide by HPLC method

Parameter	Nebivolol	Indapamide
Linear Range (µg/ml)	25-75	7.5-22.5
Slope	44.92	75.30
Limit of Detection (µg/ml)	0.855	0.101
Limit of Quantitation (µg/ml)	2.590	0.306

Table 6: Robustness data for Nebivolol

Factors		%RSD	Tailing factor (AS)	Efficiency (Theoretical plates)	Resolution
pH of mobile phase	pH 3.3	0.951892	1.435	7220	9.762
	pH 3.7	1.12492	1.391	6737	9.503
Flow rate	0.8 ml/min	0.552877	1.320	6994	9.613
	1.2 ml/min	0.579337	1.409	6989	9.650
Solvent %	-2%	1.299749	1.333	7233	9.762
	+2%	0.764669	1.313	7206	9.623

Table 7: Robustness data for Indapamide

Factors		% RSD	Tailing factor (AS)	Efficiency (Theoretical plates)	Resolution
pH of mobile phase	pH 3.3	0.469775	1.405	7196	9.762
	pH 3.7	1.473032	1.378	6882	9.503
Flow rate	0.8 ml/min	0.500934	1.385	7017	9.613
	1.2 ml/min	0.203767	1.429	7085	9.650
Solvent %	-2%	1.203667	1.405	7205	9.762
	+2%	0.609667	1.378	6890	9.623

Table 8: System Suitability Test Parameter

System Suitability Parameters	Nebivolol	Indapamide
Retention times (R_T) (min)	3.600	5.757
Theoretical plates (N)	7180	7172
Resolution (R_S)	9.762	
Tailing factor (A_S)	1.435	1.405

$LOQ = 10 \times N/B$

$LOD = 3 \times N/B$

where, N is the standard deviation (SD) of the peak areas (triplicate injections) of the drug, B is the slope of the corresponding calibration curve.

The limit of detection and limit of quantification were evaluated by serial dilutions of Nebivolol and Indapamide stock solution in order to obtain signal to noise ratio of 3:1 for LOD and 10:1 for LOQ. The LOD value for Nebivolol and Indapamide was found to be 0.855µg/ml and 0.101µg/ml, respectively and the LOQ value 2.590µg/ml and 0.306µg/ml, respectively.

Robustness: The robustness was studied by evaluating the effect of small but deliberate variations in the chromatographic conditions. The conditions studied were flow rate (altered by ± 0.2 /min), mobile phase composition (by using 38:62 and 42:58 v/v buffer pH 3.5: acetonitrile), buffer pH (altered by ± 0.2) and use of HPLC columns

from different batches. The result of robustness study of the developed assay method was established in Table 6 and Table 7. The result shown that during all variance conditions, assay value of the test preparation solution was not affected and it was in accordance with that of actual. System suitability parameters were also found satisfactory; hence the analytical method would be concluded as robust.

System Suitability: The system suitability tests represent an integral part of the method and are used to ensure adequate performance of the chromatographic system. The parameters, retention time (R_T), theoretical plates (N), peak resolution (R), peak asymmetry (T) and repeatability were evaluated using five replicate injections of the drugs. Acceptance criteria for system suitability, asymmetry should not be more than 2.0, resolution should not be more than 2.0, theoretical plate should not be less than 6800 and % RSD of peak area should not be more then 2.0, were full fill during all validation parameters. The result of system suitability study of the developed assay method was shown in Table 8.

CONCLUSION

The method described in the present investigation enables the quantification of Nebivolol and Indapamide in combined tablet dosage form. The surveillance and results obtained from each validation experiment including specificity, linearity and range, LOD and LOQ, precision, accuracy, robustness, recovery and system suitability lies well inside the acceptance criteria of ICH guideline. Since, all the results are with-in the limit, the developed analytical method is considered as validated and suitable for probable use.

Conflict of Interests: The author wishes to confirm that there is no known conflict of interests associated with this paper. The author confirms that she has given due consideration to the protection of intellectual property associated with this work and that there is no impediment to publication, including the trademarks mentioned in my paper.

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