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Novel RP-HPLC Method Development and Validation for the Simultaneous Estimation of Nebivolol Hydrochloride and Hydrochlorothiazide in Bulk and Pharmaceutical Dosage Form.

Srujani Ch*, and Varanasi S N Murthy.

University College of Pharmaceutical Sciences, Acharya Nagarjuna University, Guntur, Andhra Pradesh, India.

ABSTRACT

A simple, rapid, accurate, specific and sensitive reverse phase high pressure liquid chromatographic method (RP-HPLC) was developed and validated for the simultaneous estimation of Nebivolol hydrochloride and Hydrochlorothiazide in bulk and pharmaceutical dosage form. The method was developed using a C18 ODS Intersil (150mm x 4.6 mm i.d., 5µm) column with a mobile phase consisting of 0.1M Sodium phosphate buffer (pH2.5 adjusted with ortho phosphoric acid):Methanol [25:75 v/v]. The eluents were monitored at 284 nm and at a flow rate of 1 ml/min. The retention times for Nebivolol hydrochloride and Hydrochlorthiazide were found to be 3.3 and 1.7 min, respectively.The developed method was validated in terms of linearity, accuracy, precision, and specificity, limit of detection and limit of quantification in accordance with the ICH guidelines. The method was found to be linear in the range of 8-48mg/ml and 20-120mg/ml for Nebivolol hydrochloride and Hydrochlorothiazide respectively and the correlation coefficient were found to be 0.999 and 0.9994 respectively.The proposed method can be used for the estimation of Nebivolol hydrochloride and Hydrochlorothiazide in bulk and Pharmaceutical dosage forms.

Keywords: RP-HPLC, Validation, Nebivolol hydrochloride, Hydrochlorothiazide

Corresponding author

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INTRODUCTION

Nebivolol hydrochloride (figure-1) is chemically α,α'- [iminobis (methylene)] bis [6-fluoro- 3,4-dihydro-2H- 1-benzopyran-2-methonol]hydrochloride. It is benzopyran antihypertensive drug (β1 blocker) soluble in methanol. Nebivolol is a 1:1 racemic mixture of the enantiomeric pair, SRRR (d) & RSSS. The β1-antagonist action is primarily due to the d-isomer & both d - & l-isomers further contribute to the pharmacological profile of Nebivolol through vascular endothelial nitric oxide releasing capabilities. Activation of β1-receptors by epinephrine increases the heart rate and the blood pressure, and the heart consumes more oxygen [1]. Nebivolol blocks these receptors which reverses the effects of epinephrine, lowering the heart rate and blood pressure. In addition, beta blockers prevent the release of renin, which is a hormone produced by the kidneys which leads to constriction of blood vessels.

Hydrochlorothiazide (figure-2) is chemically 6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide-1,1-dioxide.It is diuretic soluble in acetone, methanol, sparingly soluble in ethanol (95%), very slightly soluble in water. Hydrochlorothiazide inhibits active chloride reabsorption at the early distal tubule via the Na-Cl co transporter, resulting in an increase in the excretion of sodium, chloride and water [2]. It also inhibits sodium ion transport across the renal tubular epithelium through binding to the thiazide sensitive sodium-chloride transporter resulting in an increase in potassium excretion via the sodium-potassium exchange mechanism.

The antihypertensive mechanism of hydrochlorothiazide is less well understood although it may be mediated through its action on carbonic anhydrases in the smooth muscle or through its action on the large-conductance calcium-activated potassium (K-Ca) channel, also found in the smooth muscle.

Some chromatographic methods were developed for the determination of these drugs alone or in combination [3-10]. So the aim of the present work is to develop and validate an simple, rapid, accurate, specific and sensitive RP-HPLC method for the simultaneous determination of Nebivolol hydrochloride and Hydrochlorothiazide in bulk and in pharmaceutical formulation by using mobile phase which was more economical when compared to the other developed methods.

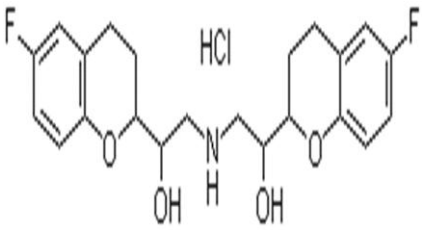

Figure 1: Structure of Nebivolol hydrochloride (MW=441.9).

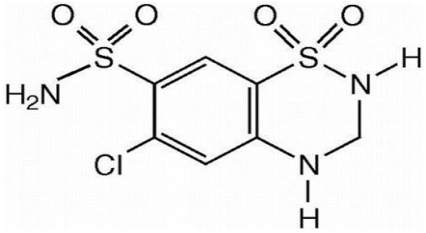

Figure 2: Structure of Hydrochlorothiazide (MW=297.73).

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MATERIALS AND METHODS

Materials

The bulk drugs of Nebivolol hydrochloride and Hydrochlorothiazide were obtained from Unichem Pharmaceuticals, Mumbai. All analytical grade chemicals and solvents were purchased from Merck Specialties Pvt. Ltd., Mumbai, India. The combination formulation of NEB and HCZ, Nebicard-H tablets (Torrent Pharmaceuticals Ltd.) were purchased from the local market. Methanol (HPLC grade) and double distilled water were obtained from Merck Ltd.

Instrumentation

The HPLC system used was binary gradient Schimadzu, equipped with UV-Visible detector (SPD-10A) , Pump (LC-10AT & LC-10ATVP) and auto-sampler controlled by Spinchrome CFR software, C18 ODS Intersil (150mm x 4.6 mm i.d., 5µm) column. EV-100 UV-Visible Spectrophotometer was also used.

Chromatographic conditions

The analysis was carried out on an HPLC system using a C18 column (250 mm x 4.6 mm ID; Particle size 3 µm) with UV detection at 284 nm. An injection volume of 20 µl was used, keeping the flow rate at 1.0 ml/min

Preparation of 0.1M Sodium phosphate Buffer:

0.1M Sodium phosphate buffer was prepared by dissolving 1.36g of Sodium di hydrogen ortho phosphate in 100ml of double distilled water and pH was adjusted to 2.5 with ortho-phosphoric acid. It was kept in sonicator for complete dissolution, filter through 0.45µm nylon membrane filter and degassed.

Preparation of Mobile Phase

The mobile phase was prepared by mixing previously ultra sonicated and filtered sodium phosphate buffer and methanol in the ratio of 25:75 (v/v).It was filtered through 0.45µm nylon membrane filter and degassed.

Preparation of standard stock solution

Weigh accurately working standards 10mg of nebivolol hydrochloride and 10mg of hydrochlorothiazide in two separate volumetric flasks containing 5ml of mobile phase.The volumetric flasks were sonicated for 5min and then the final volume was made upto 10ml with mobile phase to get a concentration of 1mg/ml in each volumetric flask.It was filtered through 0.45µ membrane filter.From the above stock solution 35µg/ml was prepared in 10ml volumetric flask containing 10µg nebivolol hydrochloride and 25µg of hydrochlorothiazide The standard calibration solutions of NEB and HCZ having concentration range 8-48 and 20-120

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µg/ml respectively were prepared by diluting appropriate aliquots of the standard stock solutions with the mobile phase.

Chromatographic conditions

The mobile phase consisting of 0.1M Sodium phosphate buffer (pH 2.5 adjusted with ortho phosphoric acid):Methanol [25:75 v/v] was selected as the optimum composition of mobile phase, as this solventsystem resolved both the components ideally. The mobile phase and samples were degassed by ultra-sonication for 20 min and filtered through 0.45 µm Nylon membrane filter paper.The measurements were carried out with an injection volume of 20µl, flow rate was set to 1 ml/min and UV detection was carried out at 284 nm. All determinations were performed at ambient column temperature (27°C). The chromatograms of the prepared standard stock solutions of NEB and HCZ were recorded under the above optimized chromatographic conditions given below in figure 3.

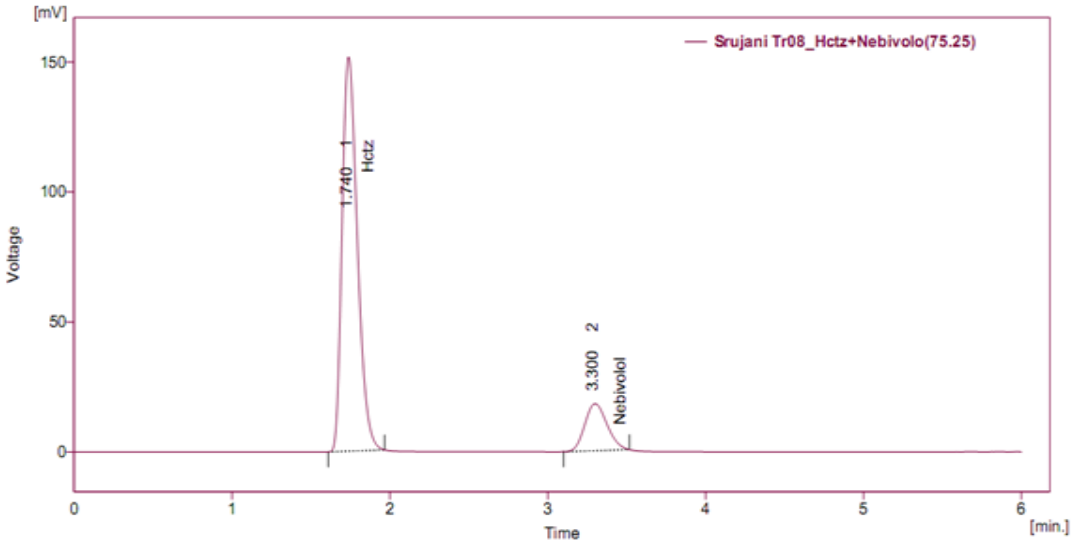

Figure 3: Optimised chromatogram of Nebivolol hydrochloride &Hydrochlorothiazide.

Analysis of formulation

Twenty tablets were accurately weighed and triturated to get fine powder.The powder formulation equivalent to 35mg was weighed and dissolved in 10ml of mobile phase and sonicated for 10min. The final volume made upto 35ml with mobile phase and filtered through 0.45µ membrane filter. The results were given in the table 1.

Table 1: Assay of Nebicard-H tablets.

Drug	Amount		% Label claim
	Labeled	Measured	
Nebivolol HCl	5mg	4.98mg	99.60
Hydrochlorothiazide	12.5mg	12.47mg	99.80

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RESULTS AND DISCUSSION

Using the above chromatographic conditions, the method developed was validated in terms of linearity, accuracy, precision, specificity, ruggedness, robustness, LOD & LOQ.

Linearity

The linear regression data for the calibration curves indicate that the response is linear over the concentration range of 8-48 µg/ml for nebivolol hydrochloride and 20-120µg/ml for hydrochlorothiazide with correlation coefficient values (r^2) of 0.999 and 0.9994 respectively. The linearity graphs were given in figures 4 & 5.

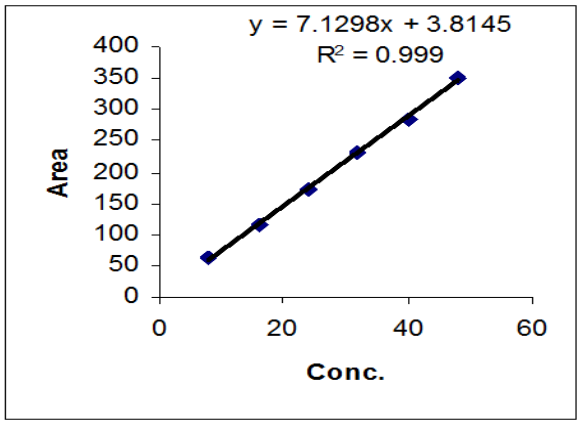

Figure 4: Linearity curve for Nebivolol hydrochloride.

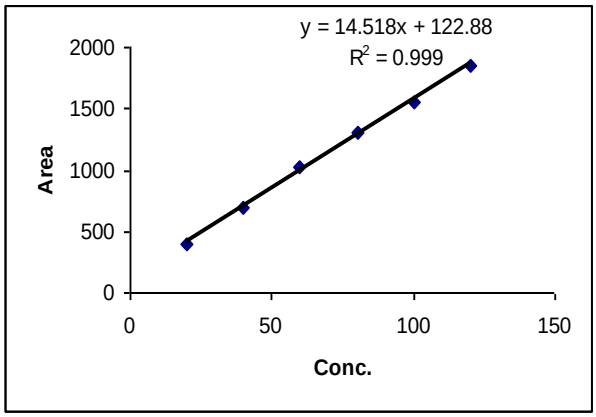

Figure 5: Linearity curve for Hydrochlorothiazide.

Precision

Method precision was performed by assaying the tablet solution at a concentration having 20 µg/ml of nebivolol hydrochloride and 50µg/ml ofhydrochlorothiazide under the same experimental conditions. System precision was also performed by six replicate injections of the freshly prepared standard solution at the same concentration. The %RSD values for nebivolol hydrochloride and hydrochlorothiazide were found to be 0.31 and 0.14 for method precision and 0.16 and 0.12 for system precision respectively.

Accuracy

The accuracy of the method was determined by use of standard additions at three different levels, i.e., at multiple level recovery studies. The sample stock solution was prepared at a concentration of 35µg/ml. This solution was spiked at 80%, 100% and 120% of the mixed standard solution at the same concentration. The mean % recoveries were found to be 100 ± 0.11for nebivolol hydrochloride and 100 ± 0.16 for hydrochlorothiazide**.** The results were given in table 2.

Table 2:Accuracy of mixed standard solution at 284 nm.

Nebivolol hydrochloride and hydrochlorothiazide			
% Spiked	**Amount added**	**Amount recovered**	**% Recovery of pure drug**
80	28	27.89	99.60
100	35	35.02	100.05
120	42	41.68	99.23

Specificity

The specificity was determined by comparing the test results from the analysis solution containing active substances. The method allows active substances to be separated and the common excipients present in the formulation did not interfere with the elution or quantification of the method. Thus the established method is suitable or specific for desired separation.

System suitability

The system suitability test was performed to check the various parameters such as column efficiency, resolution, peak tailing and retention time. The number of theoretical plates for nebivolol hydrochloride and hydrochlorothiazide were found to be 2468 and 2469 respectively. All these parameters were evaluated with the background of regulatory requirements, which also suggest the good chromatographic condition.

Robustness

Robustness were tested by introducing small deliberate variations in liquid chromatography conditions. The flow rate was changed by ±0.2ml. The %RSD values thus obtained showed that the method is robust and the results were given in the table 3.

Table 3: Effect of variation in flow rate composition.

System suitability parameters	Flow rate ml/min			Acceptance criteria
	0.8	**1.0**	**1.2**	
Tailing factor of NebivololHCl peak	1.1	1.2	1.1	NMT1.5
Tailing factor of hydrochlorothiazide peak	1.1	1.2	1.1	NMT1.5
%RSD for five replicate injections of NebivololHCl	0.0189	0.01025	0.0552	NMT2.0
%RSD for five replicate injections of hydrochlorothiazide	0.0620	0.0456	0.02533	NMT2.0

Ruggedness

Analyst to Analyst variability study was conducted under similar conditions at different times. Six samples were prepared and each were analysed as per test method.

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The percentage relative standard deviation for Nebivolol hydrochloride and Hydrochlorothiazide were found to be 0.24 and 1.34 respectively. The results were given in the table 4.

Table 4: Analyst to Analyst variability.

S. No.	Concentration 40 µg/ml	Area of Nebivolol hydrochloride	Area of Hydrochlorothiazide
1	Analyst 1	354.293	1860.958
2	Analyst 2	355.483	1904.554
% RSD		0.24	1.34

LOQ and LOD

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

$$LOQ = 10 \times N/B$$
$$LOD = 3 \times N/B$$

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 quantification and the limit of detection of NEB and HCZ were found to be 1.333µg/ml and 0.841µg/ml, 0.440µg/ml and 0.277 µg/ml respectively. The statistical data of validation parameters were shown in table 5.

Table 5: Validation of statistical data.

S. No.	Parameter	Nebivolol hydrochloride	Hydrochlorothiazide
1	Linearity	8-48µg/ml	20-120µg/ml
2	Regression equation (y= mx+c)	y=7.1298x+3.8145	y=14.518x+122.88
3	Correlation coefficient	0.999	0.9994
4	Precision(%RSD) i) Method precision ii) System precision	 0.31 0.16	 0.14 0.12
5	% Assay	99.6%	99.8%
6	Theoretical plates	2472	2380
7	Tailing factor	1.22	1.6
8	Peak asymmetry	1.33	1.56
9	Retention time	3.3min	1.74min

CONCLUSION

The proposed RP-HPLC method for the estimation of nebivolol hydrochloride and hydrochlorothiazide was validated in accordance with the ICH guidelines and the method was

found to be accurate, precise, linear, robust, simple and rapid. Hence the present RP-HPLC method is suitable for routine analysis of nebivolol hydrochloride and hydrochlorothiazide in Pharmaceutical dosage forms.

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