www.sphinxsai.com

PharmTech

International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.1, No.4, pp 1139-1147, Oct-Dec 2009

DEVELOPMENT & VALIDATION OF STABILITY INDICATING METHOD FOR QUANTIFICATION OF NEBIVOLOL & THEIR RELATED SUBSTANCES BY HPLC-UV-PDA DETECTION IN ITS PHARMACEUTICAL DRUG PRODUCT

JIGAR MEHTA*, N.G.VYAS

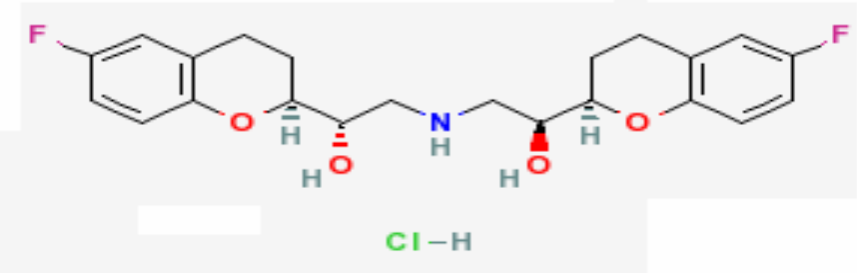
***Analytical Research & Development Laboratory, Research & Development, Unit - ICadila Pharmaceutical LTD.,1389, Trasad Road, Dholka Ahmedabad-387810, Gujarat (India), Tel: +91-02714-221481, 221483, 221484,Fax: +91-02714-221481**

***Corres.author: jigar.mehta@cadilapharma.co.in; only_jigar@rediffmail.com**

1.0 Abstract: A sensitive and precise stability indicating RP-HPLC method was developed and validated for the simultaneous determination of known related substances, isomer-I(RRT 0.85), isomer-II(RRT 1.13) and other unknown related substances of nebivolol in nebivolol tablets. The chromatographic separation was performed on Hypersil BDS Phenyl (250mm x 4.6mm) 5µm, using isocratic elution of buffer and acetonitrile (80:20 v/v) at flow rate of 1.2mL/min. UV detection was performed at 220 nm. Total run time was 70 min within which main compound and 2 other known and major unknown impurities were separated. The method was validated for accuracy from LOQ to 150 % of actual standard concentration. The system & intermediate precision was observed by analyzing the samples with two different analysts, systems, days & columns. Linearity was established at LOQ 0.036µg/mL and lowest successful determination observed at 0.012µg/mL for nebivolol and their known and unknown impurities. Stability of stock & sample aliquot was observed for 48 hrs and found satisfactory at room temperature. The method was successfully applied for the determination of nebivolol related substances in a pharmaceutical formulation of without any interference from common excipients and diluent. All the validation parameters were within the acceptance range, and concordant to ICH guidelines.
Key words: nebivolol, HPLC, related substances, degradation, validation

2.0 Introduction

Impurity profiling of active pharmaceutical ingredients (API) in both bulk material and finalized formulations is one of the most challenging tasks of pharmaceutical analytical chemists under industrial environment [1-2]. The presence of unwanted or in certain cases unknown chemicals, even in small amounts, may influence not only the therapeutic efficacy but also the safety of the pharmaceutical products [3]. For these reasons, all major international pharmacopoeias have established maximum allowed limits for related compounds for both bulk and formulated APIs. As per the requirements of various regulatory authorities, the impurity profile study of drug substances and drug products has to be carried out using a suitable analytical method in the final product [01, 02].



Nebivolol has the chemical name 1-(6-fluorochroman-2-yl)-2-[(2-(6- fluorochroman-2-yl)-2-hydroxy-ethyl] amino] ethanol. Its molecular formula is $[C_{22}][H_{25}][F_2]N[O_4]$, molecular weight 405.435 g/mole. It is the most selective [[beta]$_1$] receptor antagonist currently available for clinical use [4]. Nebivolol is a racemate of 2 enantiomers, D-nebivolol and L-nebivolol. D-nebivolol (the SRRR enantiomers represent the configuration at a particular chiral center in the nebivolol) is a potent and cardio-selective [[beta]$_1$]-adrogenic blocker, and L-nebivolol has a favorable and hemodynamic profile[5-6]. Nebivolol is a vasodilating [beta]-blocker, which can be distinguished from other [beta]-blockers by its hemodynamic profile [7]. It combines [beta]-adrenergic blocking activity with a vasodilating effect mediated by the endothelial L-arginine nitric oxide pathway [8, 9] and used in treatment of hypertension and, in Europe, also for left ventricular failure.

Literature search discovered that the method for quantification of nebivolol & its hydroxyl group in human plasma by LC-MS[10], and in human subject by online HPLC[11], HPLC & HPTLC method[12] are reported previously[13-15]. As such there is lake of a simple & precise analytical methodology for determination of related substances of nebivolol in a pharmaceutical preparation. The scope of the present study was to develop and validate for the first time, a RP liquid chromatographic method for the separation and quantification of the known & unknown related compounds of nebivolol. It's validated analytical performance in terms of major parameters such as selectivity, accuracy, precision and sensitivity is adequate for the routine quality control of the purity of nebivolol containing pharmaceutical formulations.

3.0 Experimental

3.1 Materials & Reagents:

All experiment were performed using 'A class' volumetric glassware, pharmaceutical grade nebivolol. Analytical grade tetrabutyl ammonium hydrogen sulphate(H4391A, Lancaster, UK), sodium hydroxide(2612750, Finar chem., A'bad, India), hydrochloric acid(27021128, Finar chem., A'bad, India) & hydrogen peroxide(3509242, Sdfine chem, A'bad, India) were used in the preparation of buffer solution and in forced degradation study of nebivolol. Using HPLC grade diethylamine (417452, Spectrochem, India), acetonitrile (R064B07, Rankem, India) and highly pure HPLC grade Milli Q water (Millipore, Bedford, MA, USA), mobile phase was prepared and employed for analysis. The mobile phase was filtered through 0.45µm PVDF filter (Milipore, Barcelona) and degassed under vacuum, prior to use. The pharmaceutical preparation, declaring to contain nebivolol (5mg) and excipients were obtained from M/s Cadila Pharmaceuticals LTD. for analysis.

3.2 Chromatography: (Instrumentation & analytical conditions)

The liquid chromatograph consisted of a Waters 2695 model with PDA isocratic pump, a Rheodyne injector with a 20µL loop and a model variable UV170 (DAD) detector operated at 265 nm all from Gilson (Middleton, WI). The millennium software used as data collector & processor. All pH measurements were performed with a pH-meter, model Thermo Orion, 420 Make. Separation was carried out on a Hypersil BDS phenyl column (250 mm × 4.0 mm i.d., with a particle size of 5µm).

Buffer solution: Accurately weighed 3.4gm of tetrabutyl ammonium hydrogen sulphate was dissolved in 1000mL of Milli-Q water, 0.3mL of diethylamine was added, filtered through 0.45µm membrane filter.

The separation was performed using a Hypersil BDS phenyl column (250 mm × 4.0 mm i.d., with a particle size of 5µm) as stationary phase and a mobile phase consisting of *buffer solution* and acetonitrile (80:20 v/v), which was filtered through 0.45µm membrane filter (Milipore, Barcelona) and degassed under vacuum, prior to use. The mobile phase was delivered at a flow rate of 1.2mL/min with maintaining the column temperature at 25ºC. The detection was achieved at 220nm by injecting 100µL of sample & standard aliquots, prepared using HPLC grade methanol & mobile phase, with the above chromatographic conditions and after partition equilibration, well shaped peaks were separated.

3.3 Analytical solutions: (Standard and Sample solutions):

Calibration Standard Solution (System suitability standard): Accurately weighed 32.7mg nebivolol hydrochloride of known potency is transferred to 100mL volumetric flask, added 10mL methanol & sonicated to dissolve, make up with mobile phase to volume. Further suitably diluted this solution with mobile phase to obtain the concentration of nebivolol (0.3µg/mL) and nebivolol hydrochloride (0.327µg/mL). (Factor of nebivolol HCl to nebivolol is 441.9:405.4.

Sample tablets were triturated to get fine powder, the fine powder containing about 30mg of nebivolol was transferred to 100mL volumetric flask, added 10mL methanol, sonicated for 15 min, further 30mL mobile phase was added and sonicated again for 15 min. Make up to the volume with mobile phase and filtered through 0.45µm PVDF syringe filter discarding first 5mL. The sample solution contained the nebivolol (300µg/mL)

3.4 Validation parameters:

3.4.1 Specificity / selectivity:

Specificity of an analytical method is its ability to measure accurately and specifically the concentration of analyte(s) of interest without interference from impurities, diluent, placebo (excipients omitting drug substances) and degradation products.

Specificity of the method is experience by checking following interferences.

- *Impurities Interference*: To check interference due to impurities, prepared duplicate sets of sample solution as mentioned, and injected single injections of sample solutions. Observed retention time of each impurity. Spectral homogeneity of nebivolol peak

was demonstrated by scanning chromatogram of the sample solution in the range of 210nm to 400nm with PDA detector.

- *Diluent interference:* Injected duplicate injections of diluent and observed retention time of peaks due to diluent. No peaks should be eluted at the retention time of nebivolol, and isomer I & II.
- *Placebo interference:* Performed test in triplicate on placebo equivalent present in sample solution as mentioned. Recorded retention time of peaks due to Placebo Preparation.
  Placebo preparation: Accurately weighed 1343 mg of placebo into a 100mL volumetric flask. Added 10 ml of methanol and sonicated for 15 min. further 30mL mobile phase was added and sonicated again for 15 min. Make up the volume with mobile phase and filtered through 0.45µm syringe filter discarding first 5mL.
- *Interference of Degradation products:*
  Following forced degradation study was conducted on Placebo, Drug Substance and Drug product by trial & error method with an objective to obtain 10% to 30% degradation in at least one of the stress condition.

ACID HYDROLYSIS:

Test preparation for Drug substance: Accurately weighed 32.7mg of Nebivolol hydrochloride was transferred to a 100mL volumetric flask. Added 10mL of methanol and sonicated for 15 min further added 30mL of mobile phase and sonicated again for 15 min. Into the resulting solution 5mL of 2M hydrochloric acid was added and the volumetric flask was kept at 80°C for 12 hrs in water bath. Cooled the volumetric flask to room temperature and neutralized the acid by addition of 5mL of 2M sodium hydroxide solution. Make up to volume with mobile phase and mix.

Test preparation for Drug product: Sample tablets were triturated to get fine powder, the fine powder containing about 30mg of nebivolol and treated as same mentioned into *Test preparation for Drug substance.*

BASE HYDROLYSIS:

Test preparation for Drug substance: Accurately weighed 32.7mg of Nebivolol hydrochloride was transferred to a 100mL volumetric flask. Added 10mL of methanol and sonicated for 15 min further added 30mL of mobile phase and sonicated again for 15 min. Into the resulting solution 5mL of 2M sodium hydroxide was added and the volumetric flask was kept at 80°C for 2 hrs in water bath. Cooled the volumetric flask to room temperature and neutralized the acid by addition of 5mL of 2M hydrochloric acid. Make up to volume with mobile phase and mix.

Test preparation for Drug product: Sample tablets were triturated to get fine powder, transferred the fine powder containing about 30mg of nebivolol and treated as same mentioned into *Test preparation for Drug substance.*

OXIDATIVE HYDROLYSIS:

Test preparation for Drug substance: Accurately weighed 32.7mg of Nebivolol hydrochloride was transferred to a 100mL volumetric flask. Added 10mL of methanol and sonicated for 15 min further added 30mL of mobile phase and sonicated again for 15 min. Into the resulting solution 5mL of 30% w/v solution of hydrogen peroxide was added and the volumetric flask was kept at 80°C for 12 hrs in water bath. Cooled the volumetric flask to room temperature and make up to volume with mobile phase and mix.

Test preparation for Drug product: Sample tablets were triturated to get fine powder, transferred the fine powder containing about 30mg of nebivolol and treated as same mentioned into *Test preparation for Drug substance.*

WATER HYDROLYSIS:

Test preparation for Drug substance: Accurately weighed 32.7mg of Nebivolol hydrochloride was transferred to a 100mL volumetric flask. Added 10mL of methanol and sonicated for 15 min further added 30mL of mobile phase and sonicated again for 15 min. Into the resulting solution 10mL of water was added and the volumetric flask was kept at 80°C for 24 hrs in water bath. Cooled the volumetric flask to room temperature and make up to volume with mobile phase and mix.

Test preparation for Drug product: Sample tablets were triturated to get fine powder, transferred the fine powder containing about 30mg of nebivolol and treated as same mentioned into *Test preparation for Drug substance.*

UV RADIATION:

The fine tablet powder equivalent to 65mg (3000 mg of powder) of nebivolol was weighed and transferred into a petri dish and placed it into photo stability chamber. Exposed the material for minimum of 200 watt hours/m^2 irradiation. Similarly kept about 3000 mg of placebo and 500 mg drug substance at the same irradiation level in a photo stability chamber.

Test preparation for Drug substance: Accurately weighed 32.7mg of UV light stressed Nebivolol hydrochloride was transferred to a 100mL volumetric flask. Added 10mL of methanol and sonicated for 15 min further added 30mL of mobile phase and sonicated again for 15 min and make up to volume with mobile phase and mix.

Test preparation for Drug product: Sample tablets were triturated to get fine powder, transferred the UV stressed fine powder containing about 30mg of nebivolol and treated as same mentioned into *Test preparation for Drug substance.*

HEATING:

The fine tablet powder equivalent to 65mg (3000 mg of powder) of nebivolol was weighed and transferred into a petri dish and placed it into an over at 115ºC for 24 hrs. Simultaneously kept about 3000 mg of placebo and 500 mg drug substance at the same heating condition.

Test preparation for Drug substance and *Test preparation for Drug product* for heating condition was prepared as mentioned into the UV RADIATION section.

Injected single injection of respectively prepared blank preparation, Placebo preparation, and filtered (0.45µm syringe filter) sample preparation for Drug Substance and Drug Product into the chromatograph for

all the conditions respectively. Recorded the chromatograms and calculated the %isomer-I, %isomer-II and %single unknown impurity and %total impurities by using formula.

3.4.2 Limit of Detection & Limit of Quantification:

The detection limit of individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value and quantification limit is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

Limit of detection & quantification concentrations of Nebivolol was determined based on standard deviation of response and slope method. Performed the linearity for six sets from 5.0% to 30.0% of the limit concentration of nebivolol 0.1% (limit of individual unknown impurity).

Injected duplicate injection of each linearity solution in to chromatograph and recorded chromatogram. Linearity graph of concentration in µg/ml (X-axis) versus peak area response (Y- axis) was plotted. Calculated correlation coefficient, slope of regression line and RSD of regression line. LOD and LOQ concentrations of nebivolol were determined on the basis of equation given below.

Limit of Detection = (3.3 X σ) / S &

Limit of Quantification = (10 X σ) / S

Where, σ = Residual standard deviation of regression line. S = Slope of calibration curve.

Injected six replicate injections of these LOD & LOQ concentrations and ensured the peak is detected and responses were measured.

3.4.3 Precision:

The precision is the parameter that expresses the closeness of agreement (degree of scatter) between a series of measurement obtained from multiple analysis of the same homogenous sample under the prescribed conditions. Following types of precisions were studied and performed.

- *Instrumental precision (System suitability):* System suitability shall be established to prove that the suitability and reproducibility of the chromatographic system are adequate to perform the analysis. Prepared the standard solution as mentioned (3.3) and injected six replicates of this solution. Recorded the chromatogram and measured the peak responses for analyte peak, by which instrumental precision of chromatograph was demonstrated in terms of percentage relative standard deviation, tailing factor & column efficiency (theoretical plates).
- *Method Repeatability:* The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogeneous sample. Purpose of this experiment is to prove the repeatability of the assay results obtained by this quantification methodology. For six consecutive times, a same sample solution (3.3), was injected and percentage standard deviation and relative standard deviation (R.S.D.) were observed for nebivolol, isomer I & II peaks.
- *Intermediate precision (Ruggedness):* The aim of this study to demonstrate the reliability of the test results obtain by related substances test method with day to day, analyst to analyst, system to system, and column to column variability during analysis. The repeatability was conducted on two chromatographic systems by two different analysts and on two different days.

3.4.4 Stability of analytical solutions:

This experiment was conducted to prove the stability of solutions, through out the analysis at 25ºC. Prepared standard & sample solutions were injected on to the chromatograph and recorded the chromatograms at regular interval up to 48 hours and the results were found well within the acceptance criteria of 2.0% of deviation from initial results. For acceptance of established stability of solutions the isomer-I, II and single unknown impurity of stored solution should not deviate by more than 0.05 from initial value and the Total impurities of stored solution should not deviate by more than 0.10 from initial value.

3.4.5 Filter compatibility:

The filter paper compatibility was observed for two different filters namely 0.45 µm PVDF filter and 0.45 µm Nylon filter. A single set of sample solution was prepared and some of the portion of this solution was centrifuged, Filtered and discarded 5mL of sample solution through 0.45µm Nylon filter and 0.45µm PVDF filter.

Filtered 10mL of sample solution through 0.45µm Nylon filter and 0.45µm PVDF filter, both were identified as 0.45µm Nylon test solution & 0.45µm PVDF test solution. Single injection of both these solutions and centrifuged solutions were injected and the chromatograms observed. Filter shall be considered as compatible if the isomer-I, II and single unknown impurity of stored solution not deviate by more than 0.05 from initial value and the total impurities of stored solution not deviate by more than 0.10 from initial value.

3.4.6 Linearity:

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. To demonstrate the linearity of detector response of quantification method, duplicate injections of linearity solutions with concentration ranging from limit of quantification concentration to 750.0 % (LOQ, 50.0%, 80.0%, 100.0%, 120.0%, 150.0%, 250%, 500% and 750.0%) of target concentration of Nebivolol considering limit 0.1% (limit of individual unknown impurity) and 0.5% (limit of Isomer-I and Isomer-II) were injected. Plotted a linearity graph of concentration in µg/mL (X-axis) versus peak area response (Y-axis). Correlation coefficient, slope or regression line and Y-intercept were evaluated.

Linearity stock solution preparation: Accurately weighed 32.7mg nebivolol hydrochloride of known potency is

transferred to 100mL volumetric flask, added 10mL methanol and sonicated to dissolve. Make up to the volume with mobile phase and mixed. Further diluted 10 ml to 100 ml with mobile phase and mixed.

The different concentrations were obtained by suitably diluting this stock solution

3.4.7 Accuracy (By Recovery):

Accuracy of a method is defined as the closeness of the measured value to the true value for the sample. Accuracy has been performed in the range of LOQ concentration to 750.0% (LOQ, 50.0%, 100.0%, 150.0%, 250.0%, 500% and 750.0%) of target concentration of Nebivolol considering limit 0.1% (limit of individual unknown impurity) and 0.5% of (limit of Isomer-I and Isomer-II). The stock and sample solutions were obtained as same as mentioned into the linearity section.

3.4.8 Robustness:

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate changes in method parameters and provides an indication of its reliability during normal usage. The robustness of the method was demonstrated by performing the system suitability test as per the test method in normal condition and each altered condition mentioned below.

1. Changed the temperature of column by (5°c. (i.e. 20°C and 30°C).
2. Changed the wavelength of detector by (2 nm (i.e. 218nm and 222nm).
3. Changed the flow rate of the mobile phase by (10 % (i.e. 1.0mL/min and 1.40mL/min).
4. Changed the organic solvent ratio by (5 % relative).

4.0 Results & Discussion

4.1 Method development:

A central composite design was used to analyze the influence of the chromatographic parameters and to optimize the simple & precise. In particular, a few RP-columns were compared: at the same time, several mobile phases were investigated to evaluate the effect of composition on the compound separation. As results of these experiences, using Hypersil BDS phenyl (4.6mm x 250mm), 5µm column and a mobile phase constituted from a mixture of foresaid buffer and acetonitrile (80:20 v/v) as organic modifier was found appropriate to obtain an adequate separation of all the related impurities and degradation products of the nebivolol. The optimal absorption wavelength for detection of the compounds was chosen especially with regard to absorption spectra of related compounds and degradation products which gave higher response at 220nm then any other. The aim of development and validation study was to have a simple, specific, rugged & reproducible LC chromatographic method for quantification of nebivolol and their related substances in pharmaceutical formulation of tablets.

4.2 Method validation study:

4.2.1 Specificity (Selectivity):

The observation was made from the chromatogram of interference of diluent, placebo and impurities that No peak was found at the retention time of Nebivolol peak. All impurities were well resolved from Nebivolol peak from each other. Nebivolol peak is spectrally homogeneous. The study of the purity of the peak showed that the three spectrums obtained at different times are within the established threshold for this peaks. The peak can be found improved when the peak purity angle is less than peak purity threshold, which indicated that the concern peak is pure. No peak due to unknown impurity was found at the retention time of Nebivolol, Isomer-I and Isomer-II peaks. All peaks were well resolved by R>1 from nebivolol and from each other.

Drug Substances							
Peak Name	As Such	Acid	Base	Oxi^n	Water	UV	Heat
Isomer-I (0.88)	0.031	0.035	0.027	0.021	0.028	0.043	0.028
Isomer-II (1.13)	ND	ND	ND	ND	ND	ND	ND
Single Unk.	0.041	ND	ND	2.054	0.017	0.253	0.022
Total Unk.	0.099	ND	ND	7.202	0.017	0.291	0.022
Total Imp.	0.130	0.035	0.027	7.223	0.045	0.335	0.049
% Nebivolol	99.870	99.965	99.973	92.777	99.955	99.665	99.951
Drug Product							
Condition	As Such	Acid	Base	Oxi^n	Water	UV	Heat
Isomer-I (0.88)	0.020	0.031	0.089	ND	0.027	0.011	ND
Isomer-II (1.13)	ND	ND	0.075	0.033	ND	ND	ND
Single Unk.	0.015	0.037	5.162	6.842	ND	0.237	0.389
Total Unk.	0.032	0.068	14.993	25.127	ND	0.875	0.975
Total Imp.	0.053	0.098	15.156	25.160	0.027	0.885	0.975
% Nebivolol	99.947	99.902	84.844	74.840	99.973	99.115	99.025

Table-I: Interference of Degradation Study

4.2.2 Limit of Detection & Limit of Quantification:
Performed the linearity for six sets from 5.0, 10.0, 15.0, 20.0, 25.0 & 30.0% of the limit concentration of nebivolol 0.1% (limit of individual unknown impurity). The concentration of nebivolol for each set was 0.0151, 0.0302, 0.0452, 0.0603, 0.0754 & 0.0905µg/mL and the responses was observed and a linear regression was checked which gave the LOD concentration 0.012µg/mL and LOQ concentration 0.036µg/mL. Injected six sets at LOQ & LOD concentration was observed and in each set of LOD, the peaks were detected successfully and for LOQ the RSD was below 10%, which confirmed the acceptance

.

Table-II: Data of LOD & LOQ solutions

Injection No.	**LOQ Concentration= 0.036 µg/ml**	**LOD Concentration= 0.012 µg/ml**
	Area	**Area**
1	2794	1051
2	3012	938
3	3055	1034
4	3009	970
5	3249	1036
6	3263	1048
Average	3064	1013
% RSD	5.7	4.7

4.2.3 System suitability:
Consecutive six replicate injections of standard solution revealed that, by analyzing this method, the desired suitability of HPLC instrument was achieved. The % relative standard deviations of replicate injections were 3.3% which should not exceed 5.0% for acceptance. The effective ness of selected column was observed by getting average theoretical plates above 10000 and the tailing observed was about 1.1 for analyte peak.

4.2.4 Method repeatability:
For reliability and acceptance of study, %RSD for percentage assay results of six sample preparation should be not more than 25.0 for impurities less than 0.05% and for impurities between 0.05-0.10, the %RSD should be not more than 20.0. The RSDs were well within the acceptance criteria for isomer-I & II, unknown single and total impurities and method found repeatable & precise for intended purpose.

Table-III: Data of method repeatability

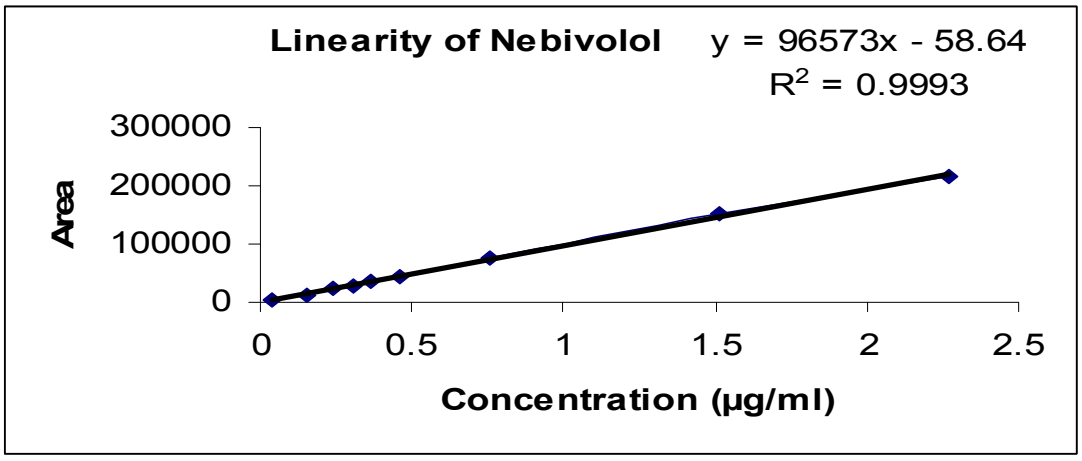
Set No.	**% Isomer-I RRT= 0.86**	**% Isomer-II RRT= 1.13**	**% Single unknown RRT=0.56**	**% Total impurities**
Set 1	0.029	ND	0.017	0.068
Set 2	0.045	ND	0.017	0.083
Set 3	0.032	ND	0.015	0.068
Set 4	0.039	ND	0.017	0.082
Set 5	0.032	ND	0.018	0.071
Set 6	0.034	ND	0.018	0.075
Average	0.035	-	0.017	0.075
% RSD	17.1	-	5.9	9.3

4.2.5 Ruggedness of Method (Intermediate precision):
The method can be found rugged if the difference between results of normal condition and altered condition is within acceptance limit. Calculated the %isomer-I & II, unknown single & total impurities for normal & altered conditions of each sample and demonstrated the precision by evaluating percentage relative standard deviation of results, for which % RSD observed was 15% and 13.3% which were less than 25.0% & 20.0% respectively. Comparison of this results complied the mentioned criteria and method found very much rugged for analysis.
4.2.6 Filter compatibility:
Centrifuged solution and the filtered solution through 0.45µm Nylon filter and 0.45µm PVDF filter, was checked for the results of isomer-I & II, unknown single and total impurities. The Isomer-I, Isomer-II, single unknown impurity and total impurities value of filtered test preparation should not deviate by more than 0.05 from centrifuge test solution value and total impurities value of filtered test solutions should not deviate by more than 0.10 from centrifuge test solution value. After study, it is found that, PVDF filter is more suitable for filtration as value of unknown single was observed higher in nylon filter than PVDF filter, so it is concluded to filter through 0.45µm PVDF filter.
4.2.7 Linearity:
The linearity was determined as linear regression with least square method on standard solution. Concentration levels were LOQ, 50, 80, 120 & 150% for target analyte concentration, while 250, 500 & 750% with respect to isomer-I & II, corresponding to the range of about 0.036–2.273µg/mL. The calibration curve obtained by plotting the nebivolol peak, area versus the concentration of standard solution was linear in the mentioned concentration range. For acceptance of linearity correlation coefficient of linearity curve can not be less than 0.990. This indicates that the method is linear up to the specified range of concentrations.

Table-IV: Linearity data (LOQ to 750%)

Linearity Level with respect to unknown impurity (limit 0.1%)	**Concentration of Nebivolol µg/ml**	**Peak area response of Nebivolol**
LOQ	0.036	2788
50.0%	0.152	13127
80.0%	0.242	23120
100.0%	0.303	29245
120.0%	0.364	34788
150.0%	0.455	44170
250.0%*	0.758	74336
500.0%*	1.516	150493
750.0%*	2.273	216405

Fig. 2: Linearity curve of nebivolol



4.2.8 Accuracy by Recovery:
The accuracy of the method was determined by measuring the drug recoveries by the study of stock recovery solution, in order to determine eventual positive or negative interferences produced by the excipients in the formulation. The results obtained for the accuracy study in the samples ranging a nebivolol concentration between LOQ to 750% indicated that the recovery percent was between 90.0 and 107.0% of recovery for nebivolol and isomer-I & II. This has found within the acceptance criteria with acceptable % RSD of not more than 10.0 at each level. The results showed the method accuracy for determination in said formulation.

Table-V: Data of Accuracy study.

Accuracy Level with respect to unknown impurity (limit 0.1%)	**Set No.**	**Practical percentage of Nebivolol**	**% Recovery**	**Average % Recovery**	**% RSD**
LOQ	Set 1	0.014	107.7	102.6	4.3
	Set 2	0.013	100.0		
	Set 3	0.013	100.0		
50.0%	Set 1	0.048	88.9	90.1	4.2
	Set 2	0.051	94.4		
	Set 3	0.047	87.0		
100.0%	Set 1	0.102	95.3	91.0	4.6
	Set 2	0.093	86.9		
	Set 3	0.097	90.7		
150.0%	Set 1	0.167	103.7	100.6	2.7
	Set 2	0.159	98.8		
	Set 3	0.160	99.4		
Accuracy Level with respect to isomer-I & II (limit 0.5%)					
250.0%	Set 1	0.271	100.7	100.2	1.9
	Set 2	0.264	98.1		
	Set 3	0.274	101.9		
500.0%	Set 1	0.575	107.1	105.3	1.5
	Set 2	0.561	104.5		
	Set 3	0.560	104.3		
750.0%	Set 1	0.846	105.0	106.1	3.0
	Set 2	0.835	103.6		
	Set 3	0.884	109.7		

4.2.9 Robustness:
The determination was observed with small but deliberate changes in the parameters i.e. detection wavelength, column temperature, change in organic solvent ratio etc. of analytical methodology, and system suitability parameters e.g. theoretical plates, tailing, %RSD were observed and found good with all the different altered conditions.

5.0 Conclusions

A precise and accurate method was successfully developed and validated for simultaneous determination of nebivolol, isomer-I & II and other related impurities and degradation products. The total run time was 70min, within which the drug and their degradation products were eventually separated. Method validation results have proved the method to be selective, precise, accurate, robust and stability indicating. Sample solution stability and filter paper compatibility was established for determination of assay as well as impurities of nebivolol. This method can be successfully applied for the routine analysis as well as stability study.

6.0 Acknowledgement

All raw data form the validation work archived at Cadila Pharmaceutical Ltd. All the validation work performed at Analytical Research Laboratory, Cadila Pharmaceutical Ltd, Dholka, India. The author is thankful to M/s Cadila Pharmaceuticals LTD, Dholka, India for providing facilities and infrastructure for the study. Author greatly acknowledges Mr. Niranjan Vyas for their constant motivation and support.

7.0 References

[01] ICH Guideline Q3A (R2), Impurities in New Drug Substances, October 25, 2006.
[02] ICH Guideline Q7A, Good Manufacturing Practice Guide for Active pharmaceutical Ingredients, November 2005.
[03] Van de Water, A., Xhonneux, R., Reneman, R., & Janssen, P. (1988) Eur. J Pharmacol. 156, 95-103
[04] Broeders, M.A., Doevendans, PA., Bekkers, B.C., Bronasaer, R., Gorsel, E., Heemaskerk, J.W, Egbrik, M.G, Breda, E., Reneman, R.S., & Zee, R. (2000) Circulation 102, 677-684
[05] Pauwels, P, Gommeren, W., Lommen-van, G., Janssen, P, & Leysen, J. (1998) J. Mol. Pharmacol. 34, 842-851
[06] Xhonneux, P., Wouters, L., Reneman, R., & Janssen, P (1990) Eur. J Pharmacol. 181, 261-265
[07] Cockcroft, J. (2004) Expert Opin. Pharmacother 5, 893-899
[08] Cockcroft, J.R., Chowienczyk, P.J., Brett, S.E., Chen, C.P, Dupont, A.G., Nueten-van, L., Wooding, S.J., & Ritter, J.M. (1997) J Pharmcol. Exp. Ther 274, 1067-1071
[09] McNeely, W., & Goa, K.L. (1999) Drugs 57,633-651
[10] J. Hendrickx, M. Bockx, C. Zwijsen, C. Borgmans, G. Mannens, W. Meuldermans, J. Heykants; Journal of Chromatography A, Volume 729, Issues 1-2, 5 April 1996, Pages 341-354
[11] N.V.S. Ramakrishna, K.N. Vishwottam, M. Koteshwara, S. Manoj, M. Santosh, D.P. Varma; Journal of Pharmaceutical and Biomedical Analysis, Volume 39, Issue 5, 4 October 2005, Pages 1006-1013
[12] LJ Patel1, BN Suhagia2, PB Shah1, Ind. Jou.phar.sci.;volumn 69, issue 4, 2007, 594-596
[13] S. Görög, M. Babják, G. Balogh, J. Brlik, A. Csehi, F. Dravecz, M. Gazdag, P. Page 16 of 26 Accepted Manuscript 16 Horváth, A. Laukó, K. Varga, Talanta 44 (1997) 1517-1526.
[14] S. Görög, TRAC-Trends Anal. Chem. 22 (2003) 407-415.
[15] Satinder Ahuja, Henrik Rasmussen, HPLC Method Development for Pharmaceuticals, 2007

*****