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Research Article

RP-HPLC VALIDATION OF RELATED SUBSTANCES OF NEBIVOLOL IN BULK & 2.5/5/10/20 MG TABLETS

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ABSTRACT

A gradient reverse phase HPLC method was developed for the determination of related substances in Nebivolol in bulk and their tablets. The known related substances are Desfuoroimpurity[1-(chroman-2-yl)-2-(2-(6fluorochroman-2-yl)-2-hydroxyethylamino)ethanol hydrochloride],Related compound-A [2S*{1R*,5R*(S*)}]-α,α`-[iminobis(methylene)]bis[6-fluoro-3,4-dihydro-2H-1-benzopyran-2-methanolhydrochloride] and benzylated impurity[2,2`-(Benzylazanediyl)bis(1-(6-fluorochroman-2yl)ethanol)oxalate]. The method was carried out on a Kinetex C_{18} column (75 x 4.6 mm; 2.6µ) using a mobile phase mixture of buffer pH 3.4, acetonitrile and water in a gradient elution at a flow rate of 1.0ml/min at wavelength of 280 nm. The impurities separated with a RRT of 0.9 for desfluoro, 1.06 for related impurity-A and 1.27 for benzylated impurity with respect to Nebivolol. The method can be used for the detection and quantitative estimation of known and unknown impurities in drug and pharmaceutical dosage form.

KEYWORDS

Nebivolol, RP-HPLC, Kinetex C18 Column, Tablets, Related substances.

INTORDUCTION

Nebivolol is α, α-[Iminobis (methylene)] bis [6-fluoro-3, 4-dihydro-2*H*-1-benzopyran-2-methanol] [1]

It is used the treatment of both hypertension and chronic heart failure[2]. Literature survey revealed that so many methods have been reported that Stability-indicating RP-HPLC[3]. RP-HPLC and HPTLC[4]. Nebivolol hydrochloride and valsartan using RP-HPLC[5]. RP-HPLC[6-9], no methods have been reported for related substances estimation in Nebivolol drug and in their formulation. So the present study by the author describes a rapid, accurate and precise RP–HPLC method for the determination of known and unknown related substances from bulk sample and pharmaceutical dosage form. The method was validated as per ICH guidelines.

EXPERIMENTAL

Chromatographic Conditions

Agilent 1200 series with high pressure liquid chromatographic instrument provided with Auto sampler, and VWD & photo diode array detector, thermostatted column compartment connected with EZ Chrom software connected with a Kinetx C_{18} column (75 x 4.6 mm ; 2.6µ). HPLC grade methanol, acetonitrile, water were purchased from E. Merck Co; Mumbai, India, and tetra butyl ammonium hydrogen sulphate was purchased from E. Merck Co; Mumbai. India were used in the study.

Drug Samples

The reference sample and impurities supplied by Bio-Leo Analytical Labs India (P) Ltd, Prasanthinagar, Hyderabad. Branded formulation of Nebivolol was purchased from local market.

Mobile phase

Accurately weigh and dissolve 3.4g of tetra butyl ammonium hydrogen sulphate in 1000ml of HPLC grade water and mixed. A mixture of buffer and acetonitrile in the ration 95:5 v/v was used as mobile phase preparation A, a mixture of acetonitrile and water in the ratio of 95:5 v/v used as mobile preparation B, a mixture of acetonitrile and water in the ratio of 50:50 v/v used as diluent, the solutions were filtered through 0.45µ membrane filter and was degassed and sonicated. The gradient program given in **Table 1**.

Standard Préparation

Weigh accurately about 21.74 mg of Nebivolol hydrochloride (équivalent to 20mg of Nebivolol) in to a 200ml volumetric flask, add 120 ml of diluent and sonicate dissolve, further make up the volume with diluent. Further dilute 5 ml to 100 ml with diluent.

For 2.5mg

Weigh and crush the tablets in to powder not fewer than 30 tablets. Transfer the tablet powder equivalent to 50 mg in 50 mL volumetric flask; add 30 mL of diluent sonicate to dissolve for about 20 min, further

make up the volume with diluent. Filter through 0.45 micron filter.

For 5/10/20mg

Weigh and crush the tablets in to powder not fewer than 20 tablets. Transfer the tablet powder equivalent to 50 mg in 50 mL volumetric flask; add 30 mL of diluent sonicate to dissolve for about 20 min, further make up the volume with diluent. Filter through 0.45 micron filter.

Placebo preparation:

Transfer placebo powder equivalent to 50 mg into 50 mL volumetric flask, add 30 mL of diluent and sonicate to dissolve for about 20 min, further make up the volume with diluent. Filter through 0.45 micron filter.

Table 1: Gradient Programme

Time(Min)	Sol-A(%)	Sol-B(%)
0	85	15
3	85	15
8	76	24
10	76	24
18.5	0	100
20	85	15
24	85	15

VALIDATION PARAMETERS

System suitability was conducted by injected 10 µL of diluent as a blank, placebo preparation, standard preparation in three replicate and sample preparation into the chromatographic system. Disregarded peaks due to blank and placebo preparation, the asymmetry and theoretical plates determined. Linearity of the detector response was determined by taking measurement at Six concentration prints (6 replicates at each point) working dilution of Nebivolol, desfluoro, related impurity-A and benzylated impurity.The specificity of the method was performed by injected samples of related substances on placebo equivalent to the amount present in test preparation, and spiked known impurities with blend mixture of Nebivolol tablets. The precision was performed by prepared six sample preparations representing a single batch and the % of impurities was determined, the intermediate precision also performed by prepared six preparations of a single batch by different analysts, different columns, different day and different instruments. The accuracy of the test method was performed by prepared known quantities of impurities at the level of LOQ, 50%, 100%, 150% and 200% of target concentration. The percentage recovery of the amount added was estimated at each level. The robustness of the test method was performed as such condition and each of altered conditions such as column temperature, extraction and flow variation, and also bench top stability initial and 24hours and 48 hours was performed, and filter paper variation was studied. The LOD & LOQ of were determined by injected solutions of Nebivolol and impurities by S/N ratio method. The RRT's and RF values are calculated from the linearity levels of 50%, 100% and 200%.

RESULTS AND DISCUSSION

The present study was aimed at developing a sensitive precise and accurate HPLC method for the separation of Nebivolol and impurities in bulk drug and in pharmaceutical dosage. the system suitability solution parameters given in **Table 2**. The diluted concentrations of Nebivolol with impurities given in **Fig.1** and the impurities with Nebivolol in Tablets preparation given in **Fig.2**.

The peak areas of Nebivolol were reproducible as indicated by low coefficient of variation. A good linear relationship (r^2 = 0.9992) was observed for Nebivolol, (r^2 = 0.9998) was observed for desfluoro impurity, (r^2 = 0.9999) was observed for related compound-A and (r^2 = 0.9999) was observed for benzylated impurity the regression characteristics are given in **Table 3**.

Table 2: System suitability study

System suitability Parameters &Acceptance criteria	Theoretical plates (NLT 2500)	Asymmetry (NMT 2.0%)
Nebivolol	54397	0.98

Table 3: Linearity of Nebivolol and impurities

S. No	Linearity Level	Concentration (ppm)	Average area of Nebivolol	Average area of Desfluoro impurity	Average area of Related compound-A	Average area of Benzylated impurity
2	25.0%	1.41	227449	114989	119340	107285
3	50.0%	2.82	371894	230904	243108	207780
4	100.0%	5.63	750794	467109	489656	415464
5	150.0%	8.45	1128334	694620	725734	610422
6	200.0%	11.26	1561769	954198	987816	827097

	Nebivolol	Desfluoro impurity	Related compound-A	Benzylated impurity
Correlation coefficient	0.9992	0.9998	0.9999	0.9999
Slope	136058	98870	98443	84649
Y-Intercept	2895.02	5190.9	3244.09	1985.59
Residual sum square	2.9295×10^{9}	2.7300×10^{8}	1.3705×10^{8}	9.8463×10^{7}
Residual standard deviation	27063	8261	5854	4961

Table 4: Specificity study

S. No.	Compound Name	Peak Purity
1	Nebivolol	1.00000
2	Desfluoro impurity	1.00000
3	Related compound-A	1.00000
4	Benzylated impurity	1.00000

Table 5: Precision study

Nebivolol Tablets 2.5 mg Tablets:

S. No	Desfluoro impurity	Related compound-A	Benzylated impurity
1	0.51	0.49	0.50
2	0.50	0.51	0.49
3	0.51	0.49	0.51
4	0.50	0.51	0.51
5	0.52	0.52	0.51
6	0.52	0.51	0.52
Avg:	**0.51**	**0.50**	**0.51**
SD:	**0.01**	**0.01**	**0.01**
% RSD:	**1.66**	**2.12**	**2.13**

Nebivolol Tablets 5 mg Tablets:

S. No	Desfluoro impurity	Related compound-A	Benzylated impurity
1	0.51	0.50	0.51
2	0.52	0.51	0.52
3	0.51	0.51	0.52
4	0.52	0.52	0.52
5	0.52	0.52	0.52
6	0.52	0.52	0.52
Avg:	**0.51**	**0.51**	**0.52**
SD:	**0.00**	**0.01**	**0.01**
% RSD:	**0.76**	**1.53**	**0.99**

Nebivolol Tablets 10 mg Tablets:

S. No	Desfluoro impurity	Related compound-A	Benzylated impurity
1	0.52	0.52	0.52
2	0.52	0.51	0.52
3	0.52	0.52	0.52
4	0.51	0.51	0.51
5	0.50	0.51	0.52
6	0.50	0.52	0.50
Avg:	**0.51**	**0.52**	**0.52**
SD:	**0.01**	**0.00**	**0.01**
% RSD:	**2.09**	**0.91**	**1.49**

Nebivolol Tablets 20 mg Tablets:

S. No	Desfluoro impurity	Related compound-A	Benzylated impurity
1	0.50	0.50	0.52
2	0.50	0.51	0.52
3	0.52	0.52	0.51
4	0.51	0.50	0.50
5	0.52	0.51	0.51
6	0.51	0.51	0.52
Avg:	**0.51**	**0.51**	**0.51**
SD:	**0.01**	**0.01**	**0.01**
% RSD:	**1.58**	**1.61**	**1.55**

Table 6 : Intermediate Precision (ruggedness) study

Nebivolol Tablets 2.5 mg Tablets:

S. No	Desfluoro impurity	Related compound-A	Benzylated impurity
1	0.48	0.49	0.51
2	0.50	0.50	0.51
3	0.48	0.48	0.50
4	0.49	0.51	0.53
5	0.49	0.50	0.52
6	0.52	0.51	0.50
Avg:	**0.49**	**0.50**	**0.51**
SD:	**0.02**	**0.01**	**0.01**
% RSD:	**3.15**	**2.32**	**1.94**

Nebivolol Tablets 5 mg Tablets:

S. No	Desfluoro impurity	Related compound-A	Benzylated impurity
1	0.49	0.49	0.51
2	0.52	0.52	0.50
3	0.52	0.51	0.52
4	0.50	0.52	0.51
5	0.51	0.50	0.52
6	0.49	0.50	0.52
Avg:	**0.51**	**0.51**	**0.51**
SD:	**0.02**	**0.01**	**0.01**
% RSD:	**3.01**	**2.29**	**1.55**

Nebivolol Tablets 10 mg Tablets:

S. No	Desfluoro impurity	Related compound-A	Benzylated impurity
1	0.51	0.52	0.49
2	0.50	0.52	0.52
3	0.50	0.51	0.52
4	0.50	0.51	0.51
5	0.51	0.51	0.52
6	0.50	0.51	0.52
Avg:	**0.50**	**0.52**	**0.51**
SD:	**0.00**	**0.01**	**0.01**
% RSD:	**0.92**	**1.17**	**2.52**

Nebivolol Tablets 20 mg Tablets:

S. No	Desfluoro impurity	Related compound-A	Benzylated impurity
1	0.52	0.51	0.48
2	0.52	0.52	0.52
3	0.49	0.48	0.50
4	0.52	0.51	0.51
5	0.52	0.52	0.51
6	0.51	0.52	0.54
Avg:	**0.51**	**0.51**	**0.51**
SD:	**0.01**	**0.01**	**0.02**
% RSD:	**2.92**	**2.80**	**3.49**

Note: For Analyst - 1, Column -1 and System -1 results refer Repeatability

Table 7 : Recovery studies
Nebivolol Tablets 2.5/5/10/20 mg Tablets
Recovery of Desfluoro impurity :

Spike level	Amount Added (ppm)	Amount Recovered (ppm)	% Recovery	% Mean Recovery
LOQ level	0.10	0.10	101.50	101.01
LOQ level	0.10	0.10	101.61	
LOQ level	0.10	0.10	99.91	
50%	2.53	2.51	99.58	99.34
50%	2.53	2.50	99.11	
50%	2.53	2.53	100.09	
100%	5.05	5.13	101.52	101.89
100%	5.05	5.16	102.26	
100%	5.05	5.17	102.39	
150%	7.58	7.77	102.61	103.63
150%	7.58	7.93	104.65	
150%	7.58	7.84	103.51	
200%	10.10	10.62	105.17	105.40
200%	10.10	10.67	105.64	
200%	10.10	10.46	103.61	

Recovery of Related compound-A:

Spike level	Amount Added (ppm)	Amount Recovered (ppm)	% Recovery	% Mean Recovery
LOQ level	0.11	0.11	97.99	99.32
LOQ level	0.11	0.11	99.46	
LOQ level	0.11	0.11	100.52	
50%	2.50	2.33	93.01	92.87
50%	2.50	2.32	92.74	
50%	2.50	2.32	92.57	
100%	5.01	4.94	98.74	99.92
100%	5.01	5.06	101.10	
100%	5.01	5.09	101.69	
150%	7.51	7.34	97.68	98.24
150%	7.51	7.42	98.80	
150%	7.51	7.57	100.79	
200%	10.02	10.47	104.57	104.14
200%	10.02	10.39	103.71	
200%	10.02	10.26	102.42	

Recovery of Benzylated impurity:

Spike level	Amount Added (ppm)	Amount Recovered (ppm)	% Recovery	% Mean Recovery
LOQ level	0.06	0.06	99.80	99.85
LOQ level	0.06	0.06	101.43	
LOQ level	0.06	0.06	98.32	
50%	2.53	2.52	99.55	99.82
50%	2.53	2.53	100.10	
50%	2.53	2.50	98.82	
100%	5.06	5.02	99.10	99.97
100%	5.06	5.11	100.84	
100%	5.06	5.13	101.34	
150%	7.59	7.73	101.84	102.14
150%	7.59	7.78	102.45	
150%	7.59	7.82	103.02	
200%	10.13	10.47	103.45	102.88
200%	10.13	10.36	102.32	
200%	10.13	10.16	100.36	

Table 8: Robustness study

Condition	Theoretical Plates	symmetry
Normal Condition (i.e as such condition)	54397	0.98
Flow changed to 0.9 mL/min	45407	0.96
Flow changed to 1.1mL/min	53205	0.97
Column Temperature changed to 20°C	53664	1.03
Column Temperature changed to 30°C	54747	1.02

Table 9: Solution stability

Bench top solution stability Results:

Time (hours)	% of Desfluoro impurity	% of difference	% of Related compound-A	% of difference	% of Enzylated impurity	% of difference
Initial	0.00	NA	0.13	NA	0.00	NA
After 24 hours	0.00	Nil	0.12	0.01	0.00	Nil
After 48 hours	0.00	Nil	0.14	0.01	0.00	Nil

Table 10: LOD Study

S. No	Name of the Component	S/N Ratio	% level of component w.r.t to sample concentration
1	Nebivolol	2.56	0.0024
2	Desfluoro impurity	3.01	0.0029
3	Related compound-A	2.98	0.0032
4	Benzylated impurity	2.73	0.0017

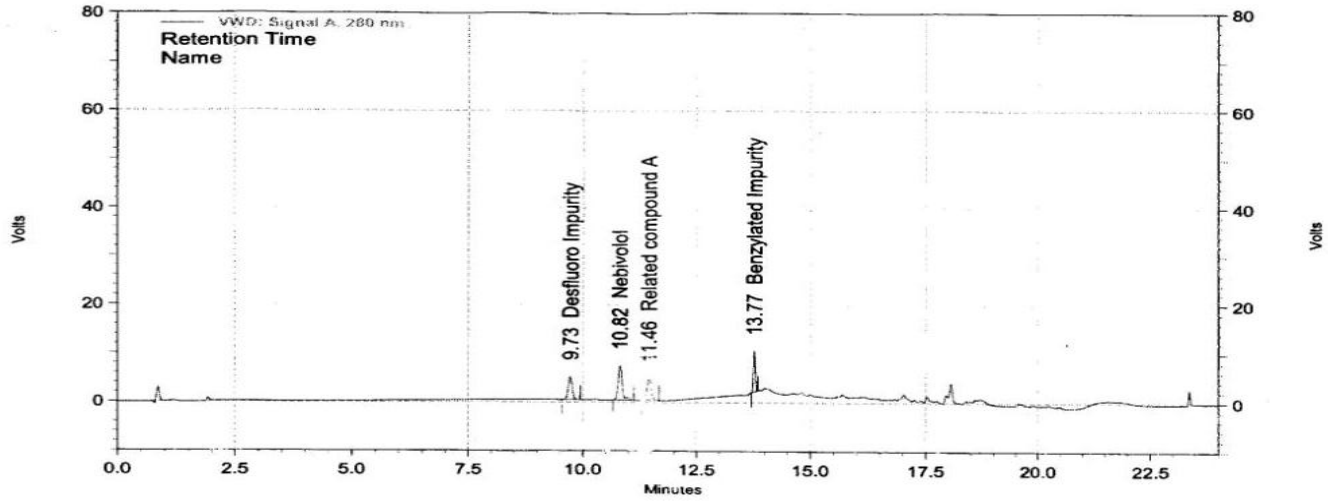
LOQ Study

S. No	Name of the Component	S/N Ratio	% level of component w.r.t to sample concentration
1	Nebivolol	10.09	0.0079
2	Desfluoro impurity	10.14	0.0096
3	Related compound-A	9.70	0.0108
4	Benzylated impurity	10.18	0.0058

Table 11: Filter Variation Study

	Centrifuged	Nylon Filter	PVDF Filter
% of Isomer-1 impurity	0.02	0.02	0.02
% Difference	NA	Nil	Nil
% of Isomer-2impurity	0.13	0.13	0.12
% Difference	NA	Nil	0.01
% of Total impurities	0.48	0.50	0.46
% Difference	NA	0.02	0.02

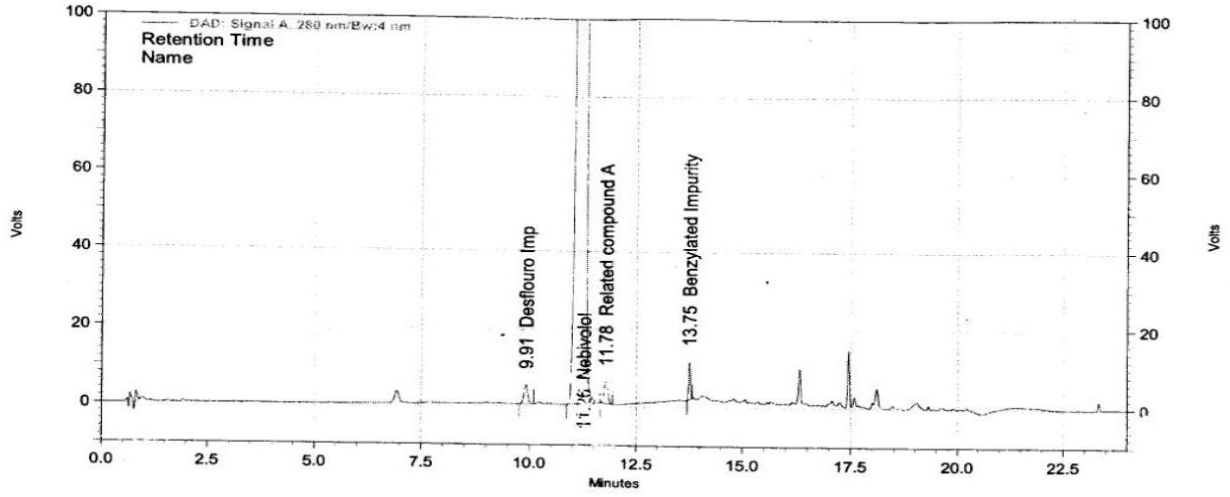
Fig 1 : Chromatogram of Nebivolol and impurities



VWD: Signal A, 280 nm Results

Name	Retention Time	Area
Desfluoro impurity	9.73	468303
Nebivolol	10.82	747044
Related compound A	11.46	491174
Benzylated Impurity	13.77	415340
Totals		2121861

Fig 2. Chromatogram of Nebivolol with impurities in tablet preparation



DAD: Signal A, 280 nm/Bw:4 nm Results

Name	Retention Time	Area	Peak purity
Desflouro Imp	9.91	62162	1.00000
Nebivolol	11.26	16240568	1.00000
Related compound A	11.78	74258	1.00000
Benzylated Impurity	13.75	56579	1.00000
Totals		16433567	

The specificity of the proposed method was observed that there was no interference of blank and placebo at the retention time of analyte and impurity peaks. Peak purity of analyte and individual impurities is not less than 0.99 indicates the method is specific. The results of specificity data for degradation study are given in **Table 4**.

The precision was established by six replicate injections at LOQ level of the test preparation containing impurities of interest. The values of relative standard deviation were found to be within the acceptance limit, indicating the injection repeatability of the method. The results are presented in **Table 5**. The intermediate precision (ruggedness) of the method was by carried out was found to be within the acceptance limit, which shows that the method is rugged. The results are presented in **Table 6**.

The recovery values obtained from the different dosage form by the proposed method indicates the method is accurate. The impurity content in tablets was quantified using the proposed analytical method are given in **Table 7**.

The percentage of individual and total impurities observed were deliberate changes in the method proves that the method is robust. The robustness study results are presented in **Table 8**. The difference between initial and bench top stability sample for % of individual impurities and total impurities were found within the acceptance criteria which indicates the solution were stable up to 48 hours. The results are presented in **Table 9**. The lowest value of LOD and LOQ as obtained by the proposed method indicates the sensitivity of the method. The results are presented in **Table 10**.

The filter paper variation of the method was carried out by injected filtered through different 0.45 µ membrane filters, the difference between % of individual and total impurities were found within the acceptance limit. The results are presented in **Table 11**.

Hence the proposed HPLC method is sensitive, specific and reproducible for the determination of known and unknown related substances in Nebivolol and in pharmaceutical dosage form.

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