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ISOLATION AND STRUCTURAL ELUCIDATION OF IMPURITY IN NEBIVOLOL

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Nebivolol, Preparative HPLC, Isolation, Spectroscopy, Structure elucidation

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ABSTRACT: In the synthesis of Nebivolol [a mixture of (1RS, 1' RS)-1,1'-{(2RS, 2' SR)-bis(6-fluoro 3, 4-dihydro-2H-1-benzopyran-2-yl)} -2,2'-iminodiethanol] having four chiral centers, one crucial isomeric impurity at 0.88 RRT was observed in HPLC related substance analysis in gradient HPLC method. This impurity was isolated from synthesized crude impurity substance by preparative HPLC with chiral normal phase and was elucidated as [(R)-1-[(R)-6-Fluorochroman -2-yl]-2-({(R)-2- [(R)-6-Fluorochro man-2-yl] -2-hydroxyethyl} amino) ethanol] (R, R, R, R- Nebivolol) by means of chromatographic and spectral data. Structural elucidation carried out by spectral data was reviewed.

INTRODUCTION: There are so many cardiovascular diseases reported across the world. One noticeable remedy for cardiovascular disease is β blocker (Antagonists of β-adrenergic receptors) by blocking β receptor. Nebivolol is widely used hypertensive and one of four (Nebivolol; Labetalol; Carvedilol and Celiprolol) essential and is a unique selective β1 receptor antagonist to cause enlargement of blood vessels in addition to effects on the heart having nitric oxide (NO)-potentiating, vasodilatory effect. Nebivolol reduces peripheral vascular resistance to lower blood pressure and increases remarkable stroke volume with preservation of cardiac output [1, 2].

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Worldwide clinical research proved that Nebivolol is the essential β1 blocker. Nebivolol is manufactured as hydrochloride salt of a racemate of the enantiomers (D) - and (L) -Nebivolol where D-Nebivolol is a potentially selective β1 adrenergic blocking agent, while L-Nebivolol is vasodilator effect with the L-arginine/nitric oxide as shown in **Fig. 1** and **2**. The HPLC[3-4] analysis for related substance of Nebivolol bulk drug shown the presence of impurity at 0.88 RRT was up to 0.1% level.

As per the guideline constructed by ICH and regulatory agencies, the unknown impurity level in any final API or bulk drug should < 0.1%. To meet this stringent regulatory requirements the impurity profile study has to be carried out for the final API to identify and characterize the unknown impurity that is present at > 0.1%. This paper demonstrates the isolation and structure elucidation of impurity present in the API of Nebivolol using preparative HPLC through chiral separation of Nebivolol bulk drug [5, 7].

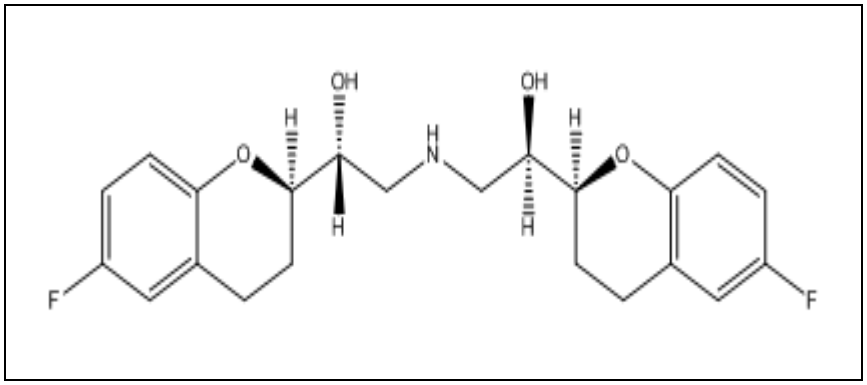


FIG. 1: D – NEBIVOLOL (RRRS – NEBIVOLOL OR (+) - NEBIVOLOL)

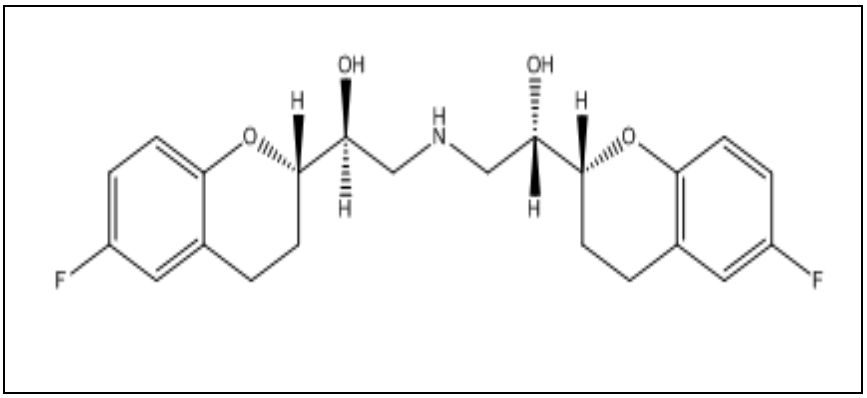


FIG. 2: L – NEBIVOLOL (SSSR-NEBIVOLOL OR (-) - NEBIVOLOL)

Experimental:

High-performance Liquid Chromatography Conventional: This impurity was analyzed by both RP-HPLC and, of course, a chiral normal phase HPLC as it is carrying a chiral center.

A Dionex Ultimate 3000 with DAD detector and chromeleon software for related substances analysis and Shimadzu 2010 integral HPLC system and LC-Solution software were used for chiral analysis.

The related substances analysis was carried out on Hypersil BDS-Phenyl, (250 × 4.6) mm, 5µ particle size column with a mobile phase consisting of buffer solution *i.e.,* 3.4 g of Tetra butyl ammonium hydrogen sulfate dissolved in 1000 mL water and added 0.3 mL diethylamine, shaked well and filtered through 0.22 µ filter and solvent acetonitrile in the ratio of 80:20. Run program isocratic. UV detection was at 220 nm at a flow rate of 1.2 ml/min.

The column oven temperature was at 25 °C. Chromeleon software was used to record data. A chiral analysis was carried out on Chiralpak AD-H, (250 × 4.6) mm column with mobile phase 0.1% Diethylamine in ethanol. Isocratic program was run with 0.8 ml/min flow rate. UV detection was at 280 nm, and column oven temperature was at ambient. LC-Solution software was used to record data.

High-performance Liquid Chromatography Preparative: A Waters quaternary 2535 Q preparative chromatography system equipped with Waters 2998 PDA detector and Rheodyne Injector Model 7725I with 5.0 ml loop was used. A Chiralpak AD-H, (250 × 20) mm, (Daicel, Japan) was employed for separation. The mobile phase consisted of n-Hexane: IPA in the ratio of 70:30 (v/v). The flow rate was set at 8.0 ml/min. Detection was carried out at 220 nm. Empower 3 software was used to record data.

NMR Spectroscopy: The 1H and ^{13}CNMR8 spectra were recorded on Bruker 400 Ultrashield spectrometer. The 1H and 13C (400 MHz) was recorded using TMS and DMSO-d6 as internal standards and diluent, respectively. Topspin 3.1 was used to record data.

Mass Spectrometry: Mass 9-11 spectrometer Waters Xevo TQD was used with ESI (75eV), and The ion spray voltage (V), Curtain energy (CE),

entrance potential and declustering potential were kept as 3600V, 39V, 15V, and 105V, respectively to record mass spectra. The sample was introduced to mass spectrometer with particle beam interface using LC.

The source manifold and quadrupole temperatures were maintained at 230 °C and 90 °C, respectively. Nitrogen was used as a reagent gas for chemical ionization (CI) mode.

The ESI mass spectra were recorded on Waters Xevo mass spectrometer with mass-lynx software.

FT-IR Spectroscopy: FT-IR 12-13 spectra were recorded on Shimadzu 8400 series FT-IR as KBr powder.

Synthesis of Nebivolol: The synthesis scheme for the synthesis of Nebivolol was shown in **Fig. 3**.

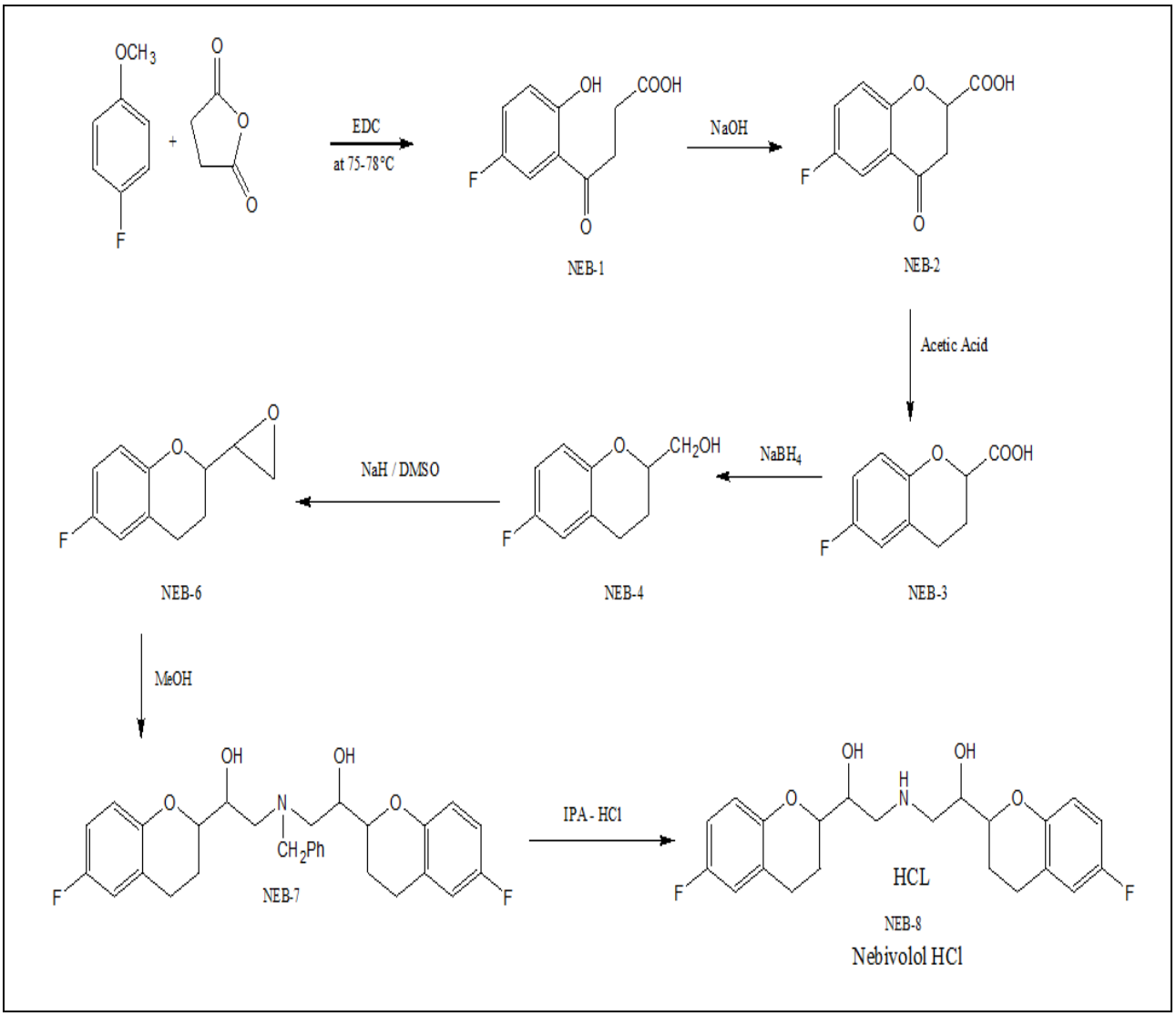
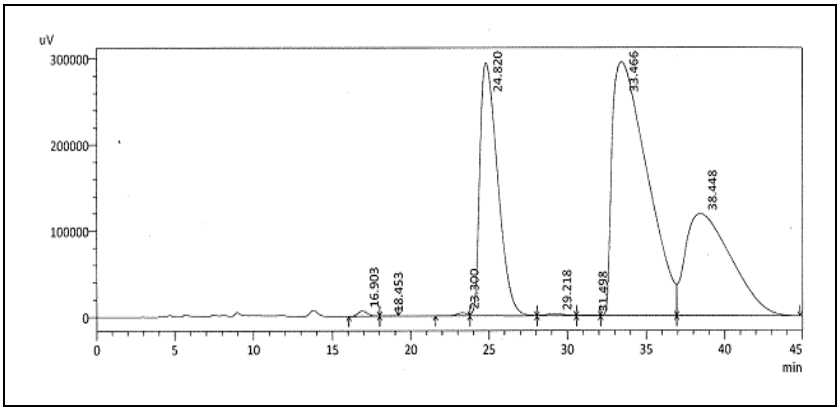


FIG. 3: REACTION SCHEME FOR NEBIVOLOL HCL BULK DRUG

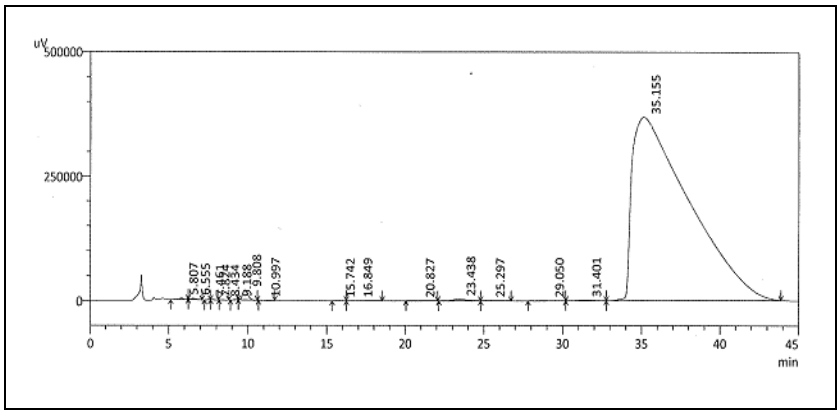
Result and Discussion:

Detection of Impurity: During a conventional related substance RP-HPLC analysis of Nebivolol API as per section 2.2, a consistent impurity at 0.88 RRT was recorded. This synthesized impurity shows two peaks that need to make pure by isolation using preparative HPLC. This objective impurity understudy was manifested as an impurity at 0.88 RRT. This impurity was also analyzed using chiral HPLC method as in section 2.2, which shows three isomeric peaks.

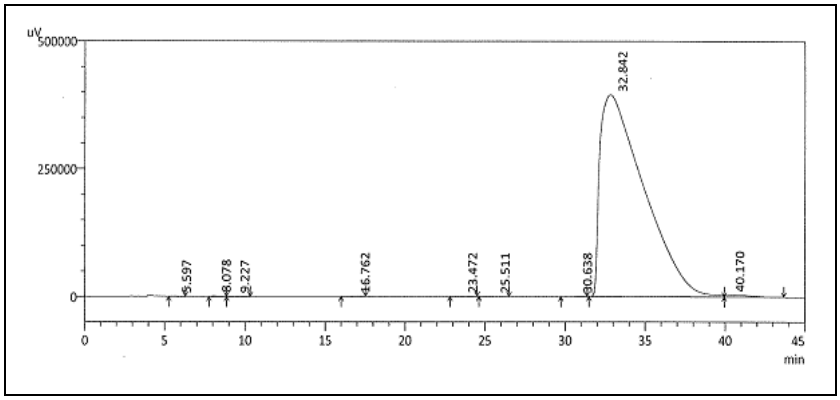
An analytical chromatogram of 0.88 RRT impurity of Nebivolol bulk was recorded and represented in **Fig. 4** (a-d) using related substance HPLC method and chiral method mentioned in section 2.2.



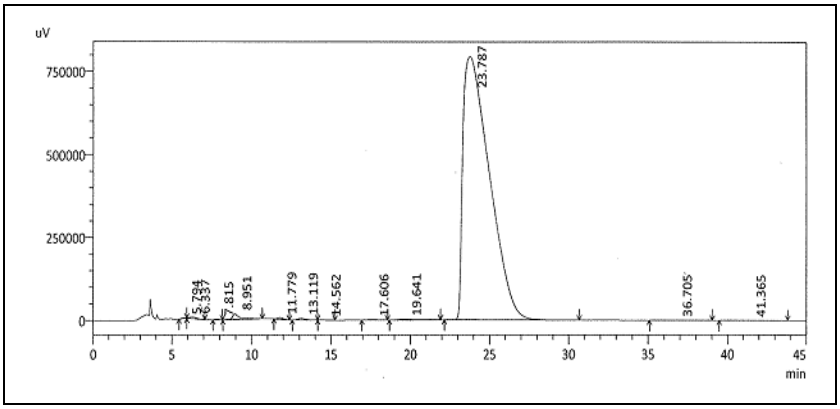
(A) CRUDE 0.88 RRT IMPURITY OF NEBIVOLOL



(B) ISOLATED ISOMER-1 FROM CRUDE 0.88 RRT IMPURITY OF NEBIVOLOL



(C) ISOLATED ISOMER-2 FROM CRUDE 0.88 RRT IMPURITY OF NEBIVOLOL



(D) ISOLATED ISOMER-3 FROM CRUDE 0.88 RRT IMPURITY OF NEBIVOLOL

FIG. 4: CONVENTIONAL CHIRAL HPLC CHROMATOGRAM OF ISOMERS OF ISOLATED IMPURITY OF NEBIVOLOL

Isolation of Impurity by Preparative HPLC: A chiral normal phase isocratic solvent delivery system mention under section 2.3 was developed and used for the isolation of this crude impurity of Nebivolol which was a mixture of three different isomers. All segments of impurity were collected separately, concentrated, and solidify by evaporation of solvent using rotavapour. These isolated solids received from different fractions were then analyzed by both chiral HPLC and related substance HPLC method (as per section 2.2) to check its purity and to use for further analytical exercise for structural elucidation. See preparative chiral HPLC chromatogram **Fig. 5**. Structural elucidation details carried out of this isomer impurity is presented in section 4.3.

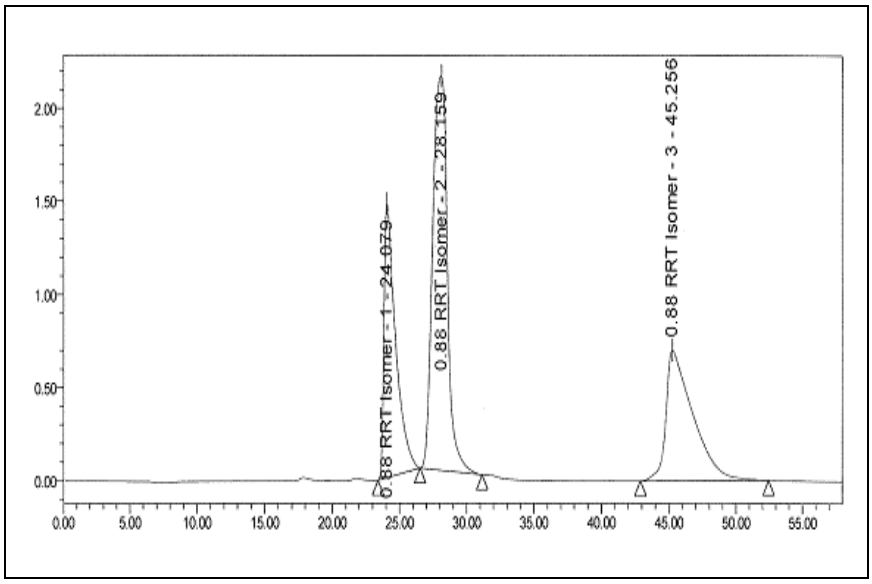


FIG. 5: PREPARATIVE CHIRAL HPLC CHROMATOGRAM OF IMPURITY OF NEBIVOLOL

Structure Elucidation: The ESI mass spectra of 0.88 RRT impurity of Nebivolol was displayed the molecular ion peak at m/z 406.45. The ES+ mass spectra further confirmed this with the presence of protonated molecular ion peak as a base peak at m/z 406.38, which is an equivalent mass unit to that of Nebivolol. This can be recognized as an isomer of the parent drug substance Nebivolol. The IR spectra of this isolated impurity show the presence of all wave numbers equal to Nebivolol a parent drug which also further confirms with 1H NMR spectra presented in **Tables 1** and **2**.

Mass and Infra-Red spectra comparison:

TABLE 1: MASS SPECTRA COMPARISON BETWEEN NEBIVOLOL AND ISOLATED IMPURITY

Mass Spectra		
	Nebivolol	**0.88 RRT Impurity**
Exact Mass	405.18	405.18
Molecular Ion (m/z)	406.35	406.38

TABLE 2: INFRA-RED COMPARISON BETWEEN NEBIVOLOL AND ISOLATED IMPURITY

Infra-Red Spectra			
Nebivolol		**0.88 RRT Impurity**	
Wave No (cm^{-1})	**Assignment**	**Wave No (cm^{-1})**	**Assignment**
1004.95	- C-F stretching	1001.06	- C-F stretching
1141.90	- C-O- alcohol stretching	1139.93	- C-O- alcohol stretching
1350.22	- O-H bending	1357.89	- O-H bending
1429.30	- C-N- stretching medium	1440.83	- C-N- stretching medium
1492.95	- C=C aromatic ring stretch	1492.90	- C=C aromatic ring stretch
1618.33	Carboxylic acid salt	1587.42	Carboxylic acid salt
2966.62	- C-H aliphatic stretching	2850.79	- C-H aliphatic stretching
3036.06	- C-H aromatic ring stretching	2927.94	- C-H aromatic ring stretching

Assignment of Proton by ^{1}H NMR:

TABLE 3: ^{1}H NMR COMPARISON BETWEEN NEBIVOLOL AND ISOLATED IMPURITY

^{1}H NMR Spectra							
Nebivolol				0.88 RRT Impurity			
Chemical Shift δ (ppm)	No. of Protons	Multiplicity	1H Assignment	Chemical Shift δ (ppm)	No. of Protons	Multiplicity	1H Assignment
1.637–1.823	2	m	-CH_2	1.70 – 1.93	4	m	-CH_2
1.922–2.143	2	d	-CH_2				
2.729–2.818	4	m	-CH_2	2.68 – 2.79	8	m	-CH_2
3.044–3.415	4	m	-CH_2				
3.885–4.142	4	m	-CH	3.68	2	m	-CH
				3.92 – 3.95	2	m	-CH
5.847–6.052	2	d	-OH	4.89	2	m	-OH
6.743–6.783	2	m	-CH	6.71 – 6.75	2	dd	-CH
6.874–6.918	4	m	-CH	6.85 – 6.92	4	m	-CH

CONCLUSION: In conclusion, this process-related impurity of Nebivolol, formed according to the Reaction Scheme for Nebivolol HCl bulk drug, was isolated and characterized as an isomer of Nebivolol API. Structural elucidations of this content were carried out by using 1H & 13C NMR, MS and IR spectral data along with HPLC analysis. The regulatory requirement was therefore fulfilled by characterizing this impurity and the prepared impurity standard. This impurity standard was further used for analytical method validation studies. This work also supported the process development optimization stage of Nebivolol and facilitated to control forming this impurity during the process.

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CONFLICTS OF INTEREST: Nil

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